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Influences of Organic Acid Salts and Bacterial Additives on Fermentation Profile, Aerobic Stability, and In Vitro Digestibility of Total Mixed Ration Silage Prepared with Wet Hulless Barley Distillers' Grains

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Abstract: This study aimed to investigate the impacts of three organic acid salts and two bacterial additives on the fermentation profile, in vitro digestibility and gas production, and aerobic stability of total mixed ration (TMR) silage prepared with 10% fresh weight (FW) of wet hulless barley distillers' grains (WHDG), 35% FW of common vetch, 15% FW of whole-crop oat, 10% FW of hulless barley straw, and 30% FW of mixed concentrate. The total mixed ration (555 g kg⁻¹ FW) was ensiled with six treatments: (1) no additives (control); (2) calcium propionate (CAP; 0.5% FW); (3) potassium sorbate (POS; 0.1% FW); (4) sodium diacetate (SDA; 0.5% FW); (5) *Lactobacillus buchneri* (LB; 1 × 10⁶ cfu g⁻¹ FW); and (6) *Lactobacillus casei* (LAC; 1 × 10⁶ cfu g⁻¹ FW). All silos (20 L) were opened for the fermentation profile and in vitro digestibility analysis after 95 days of fermentation, and then subjected to an aerobic stability experiment for 14 days. All the TMR silage was well preserved with a low pH (4.32~4.51), acceptable levels of butyric acid (1.34~1.56 g kg⁻¹ dry matter), and ammonia nitrogen (69.1~87.1 g kg⁻¹ total nitrogen). All the groups were steady during aerobic exposure, while the SDA treatment was more stable with lower populations of undesirable microorganisms on day 14. The CAP, POS, and SDA treatments evidently ($p < 0.05$) enhanced the in vitro digestibility of dry matter (54.8~57.5 vs. 48.4%) and neutral detergent fiber (48.4~51.6 vs. 41.1%) compared to the control. By comprehensive consideration, SDA is recommended as additive to enhance fermentation quality, aerobic stability, and in vitro digestibility of TMR silage containing 10% FW of WHDG.

Keywords: aerobic stability; bacterial inoculants; fermentation profile; organic acid salts; total mixed ration silage



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1. Introduction

The Tibetan Plateau is one of the five most important livestock production districts in China, and most Tibetan herders feed their cattle with agro-industrial byproducts because of the harsh environment and insufficient feed supply during the long cold season [1]. Hence, the efficient and rational exploitation and utilization of agro-industrial byproducts is very important for the development of local animal husbandry in Tibet. The Tibetan people have a history of making and drinking hulless barley wine over thousands of years, so plenty of wet hulless barley distillers' grains (WHDG) are produced every year. The WHDG are characterized by high digestibility and crude protein (>25% based on dry matter (DM)), while high moisture (>85%) restricts their storage time [2]. Moreover, artificial drying can greatly increase the cost of WHDG. Thus, most WHDG are discarded or fresh-fed only for limited periods of time in Tibet. Recently, Wang et al. [3] reported that making total mixed ration (TMR) silage has the potential to utilize wet brewers' grains (a by-product very similar to WHDG), making it is possible to utilize the WHDG as TMR silage.

The total mixed ration has been widely utilized for dairy cattle because it includes concentrates, roughage, minerals, and vitamins in ratios that are well balanced to satisfy the animal's demands [4]. The TMR materials are ensiled in a sealed and anaerobic environment and fermented for a long period, which is known as TMR silage [5]. Several advantages are related to TMR silage, such as the potential for incorporating unpalatable byproducts, uniform composition during storage, and a decreased demand for machinery and labor [6]. However, TMR silage prepared with high-moisture byproducts may face a higher risk of aerobic deterioration; hence, different kinds of additives should be applied to ensure the silage's quality [7]. *Lactobacillus buchneri* was utilized to improve the aerobic stability of maize silage [8]. Inoculating with *Lactobacillus casei* could decrease the biogenic amine production of silage regardless of the silage type [9]. However, biological additives rely on the characteristics of forage and environmental conditions [10]. Consequently, chemical additives might be more steady than bacterial inoculants [11]. Calcium propionate, potassium sorbate, and sodium diacetate are organic acid salts with antifungal properties. Calcium propionate has been widely used as the main component of preservatives in forage and food due to its ability to restrict populations of molds and other microorganisms [12]. Potassium sorbate was used in maize silage to avoid aerobic deterioration through its antimicrobial activity [13]. Knický and Spörndly [14] reported that potassium sorbate was effective in inhibiting yeasts, molds, and spore-forming bacteria. Sodium diacetate can ionize into acetic acid easily, suppressing the activity and growth of harmful microorganisms during ensiling [15]. Okur et al. [16] also found the aerobic stability of maize silage treated with sodium diacetate was much improved after exposure to air. However, limited research has simultaneously compared bacterial inoculants and organic acid salts for improving the TMR silage prepared with WHDG.

Therefore, this study aimed to evaluate the impacts of three organic acid salts and two bacterial additives on the fermentation profile, aerobic stability, and in vitro gas production and digestibility of TMR silage containing WHDG.

2. Materials and Methods

2.1. Total Mixed Ration Silage Making

The total mixed ration included WHDG (acquired from a private brewery in Shigatse, Tibet), whole-crop oat (milk-ripe stage), mixed concentrate, common vetch (pod-bearing stage), and hulless barley straw (the remainder after grain harvest). All the crops were planted in Shigatse, Tibet (N 29°270', E 88°880'), and harvested on 25 September 2016. Mixed concentrate (purchased from New Hope Feed Co., Ltd., Lasa, China) contained 27.5% distillers dried grains with solubles, 20% rape cake meal, 7.5% crack corn, 20% cotton seed, 5% vitamin-mineral, and 20% wheat bran. The harvested crops were cut to lengths of about 20 mm. The chemical components of the ingredients for TMR are presented in Table 1. The ingredient proportions, microbial and chemical compositions of pre-ensiled TMR are presented in Table 2.

Table 1. Chemical components (g kg^{−1} DM, unless otherwise stated) of ingredients.

Items ¹	Wet Hulless Barley Distillers' Grain	Common Vetch	Whole-Crop Oat	Hulless Barley Straw	Mixed Concentrate
Dry matter (g kg ^{−1} of FW)	147	283	485	968	904
Crude protein	288	188	79.9	42.5	159
aNDFom	499	447	417	576	161
ADFom	165	78.1	253	151	148
Ether extract	140	54.7	74.3	46.8	60.7
Ash	79.9	74.2	50.7	63.1	114
Water soluble carbohydrate	71.6	85.4	139	46.3	110
Buffering capacity (mEq kg ^{−1} DM)	168	320	209	43.9	171

¹ FW: fresh weight; DM: dry matter; mEq: milligram equivalent; aNDFom: neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash; ADFom: acid detergent fiber expressed exclusive of residual ash.

Table 2. Ingredient proportions, microbial and chemical compositions of pre-ensiled total mixed ration.

Items ¹	Total Mixed Ration
Ingredient proportions (%FW)	
Wet hulless barley distillers' grain	10.00
Common vetch	35.00
Whole-crop oat	15.00
Hulless barley straw	10.00
Mixed concentrate	30.00
Chemical components (g kg ⁻¹ DM)	
Dry matter (g kg ⁻¹ FW)	555
Crude protein	158
Water soluble carbohydrate	95.6
aNDFom	375
ADFom	141
Ether extract	67.2
Ash	82.1
Buffering capacity (mEq kg ⁻¹ DM)	216
Microbial populations (Log ₁₀ cfu g ⁻¹ FW)	
Lactic acid bacteria	4.62
Aerobic bacteria	6.29
Yeasts	6.64
Molds	5.81

¹ DM: dry matter; FW: fresh weight; aNDFom: neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash; ADFom: acid detergent fiber expressed exclusive of residual ash; mEq: milligram equivalent; Log₁₀: decimal logarithm; cfu: colony-forming units.

Six treatments were individually prepared as follows: (1) deionized water alone (Control); (2) calcium propionate (CAP; food-grade additive, purity 99%, purchased from Aokai Biotechnology Development Co., Ltd., Nantong, China) applied at 0.5% fresh weight (FW); (3) potassium sorbate (POS; food-grade additive, purity 98%, purchased from Wanglong Science and Technology Co., Ltd., Ningbo, China) applied at 0.1% FW; (4) sodium diacetate (SDA; food-grade additive, purity 99%, purchased from Jirong Amino Acid Co., Ltd., Jinzhou, China) applied at 0.5% FW; (5) *Lactobacillus buchneri* (LB) applied at 1×10^6 colony-forming units (cfu) g⁻¹ FW; and (6) *Lactobacillus casei* (LAC) applied at 1×10^6 cfu g⁻¹ FW. Six replicated silos (20 L capacity, 292 mm diameter, and 300 mm tall for each silo) were performed for each treatment. *Lactobacillus casei* and *L. buchneri* strains were both provided by the Institute of Ensiling and Processing of Grass in Nanjing Agricultural University of China. Inoculants were made and enumerated in de Man, Rogosa and Sharp (MRS) broth (Shanghai Bio-way Technology Co., Ltd., Shanghai, China) to determine the amount of inoculant required to meet the targeted inoculation rate of 1×10^6 cfu g⁻¹ FW for *L. buchneri* (applied at 1×10^6 cfu g⁻¹ FW) and *L. casei* (applied at 1×10^6 cfu g⁻¹ FW). Oliveira et al. [17] demonstrated that the most effective rates for improving the fermentation quality were 10^5 and 10^6 cfu g⁻¹ FW, and the latter rate was more effective. Hence, 1×10^6 cfu g⁻¹ FW was used as the inoculant application rate in this experiment. Inoculants were mixed with deionized water and uniformly sprayed onto the raw material with a manual sprayer, followed by constant mixing. The chemical additives were diluted in deionized water, and 10 mL of solution per kg of raw material was applied with a manual sprayer. The application rates of chemical additives were according to the report of Dai et al. [5]. The control TMR silage was sprayed with an equivalent volume of deionized water.

Before packing, all the pre-ensiled TMR materials (about 330 kg) were thoroughly mixed by the traction feed mixer (RH-9 JGW-3, Runhua Machinery Co., Ltd., Qufu, China) for 20 min according to the ingredient proportions presented in Table 2. Then, the mixed materials were randomly divided into 6 piles (55 kg for each pile) according to 6 treatments. After adding the additives into each pile according to the experimental design, each pile

was randomly divided into 6 parts (about 9 kg for each part) due to the 6 replicates for each treatment. Finally, each silo was made by the pre-ensiled TMR materials. All the above steps were finished on a large plastic film on the ground. Each silo was compacted by human pressure with approximately 9 kg of raw material to achieve a packing density of about 250 kg of DM per m³. The silos in the control group were made first to avoid contamination from additives. A total of 36 silos (6 treatments × 6 replicates) were sealed tightly and maintained in a barn at room temperature (20~24 °C) for 95 days. The 95-day ensiling period was chosen because of the harsh environment, insufficient feed supply, and long cold season on the Tibetan Plateau, which requires the ensiling time to be longer.

After 95 days of fermentation, the silos were opened. At silo opening, approximately the top 5 cm of TMR silage from the surface of each experimental unit was discarded. Then, all the residual TMR silage in each silo was emptied onto a plastic film and thoroughly mixed. After mixing, a portion of the silage mass (about 1 kg FW) from each silo was collected by hand for fermentation quality and microbiological analyses. Moreover, about 3-kg allotments were taken for the aerobic stability experiment.

2.2. Aerobic Stability

Approximately 3 kg of TMR silage was transferred into sterile plastic bottles (10 L capacity, 254 mm diameter, and 200 mm tall for each silo) without compaction and allowed air exposure for 14 days at ambient temperature (18~22 °C). The TMR silage collected from each silo was thoroughly mixed before transferring, so that it can maintain an equally loose state in each new bottle after transferring. The TMR silage surface was covered with gauze to avoid contamination and reduce evaporation while allowing the ingress of air. Six replicated TMR silages of each treatment were sampled to test pH, ammonia nitrogen (NH₃-N), organic acid, and water-soluble carbohydrate (WSC) content as well as microbial populations after 0, 6, 9, and 14 days of aerobic exposure. The monitoring-temperature method [3] was not used to judge the aerobic deterioration due to the low environmental temperature (<20 °C) in practical production in Tibet. Aerobic deterioration was identified as a rise in the pH value of silage by 0.5 above the initial pH value (on day 0 of the aerobic stability test), as described by Wilkinson and Davies et al. [18].

2.3. Chemical and Microbiological Analyses

At sampling, a portion of fresh ingredients, pre-ensiled TMR mixture, and TMR silage were mixed, respectively, and collected by hand. The first subsample (20.0 g) was homogenized with deionized water (100 mL) and filtered through a double layer of sterile gauze. The filtrate (conserved at −20 °C in the fridge) was utilized for testing the NH₃-N, ethanol, and organic acid content, buffering capacity, and pH values. The second subsample (150 g) was oven-dried to a constant weight (at 65 °C, at least for 48 h). After crushing and filtering the screen (1-mm), the dry powder of the samples was utilized to determine DM, total nitrogen (N), WSC, fiber, ether extracts (EE), and ash content. The third subsample (10.0 g) was utilized to test microbial numbers according to the report by Wang et al. [3].

The pH was determined using a pH meter (Mettler Toledo, Zurich, Switzerland). The buffering capacity was measured according to the report by Jasaitis et al. [19]. The ethanol and organic acid (propionic acid, acetic acid, butyric acid, and lactic acid) concentrations were tested by HPLC method as described by Wang et al. [3]. The NH₃-N content was measured using the phenol-hypochlorite method [20]. The WSC content were measured using the methods by Owens et al. [21]. The acid detergent fiber (ADF, method 973.18), DM (method 930.15), EE (method 920.39), ash (method 942.05), and crude protein (CP, method 984.13) content were analyzed based on the standard procedures of the AOAC [22]. Amylase-treated neutral detergent fiber (aNDFom) content was measured based on the report by Van Soest et al. [23].

2.4. In Vitro Digestibility and Gas Production

The following experiments in this study were approved by the Ethics Committee of Nanjing Agricultural University (Jiangsu, Nanjing). The TMR silage samples were used to measure the in vitro digestibility and gas production according to the report by Wang et al. [3]. The calculations on the in vitro digestibility of DM (IVDMD) and NDF (IVNDFD) were according to the report by Liu et al. [24]. The short-chain fatty acid content (SCFA), net energy for lactation (NE_L), metabolizable energy (ME), and microbial crude protein (MCP) biomass production were predicted based on the report of Wang et al. [3]. Their specific equations were as follows:

$$\text{ME (MJ kg}^{-1} \text{ of DM)} = 2.20 + 0.1357 \text{ GP}_{24} + 0.0057 \text{ CP} + 0.0002859 \text{ EE}^2 \quad (1)$$

$$\text{NE}_L \text{ (MJ kg}^{-1} \text{ of DM)} = 0.54 + 0.0959 \text{ GP}_{24} + 0.0038 \text{ CP} + 0.0001733 \text{ EE}^2 \quad (2)$$

$$\text{SCFA (mmol 200 mg}^{-1} \text{ DM)} = 0.0222 \text{ GP}_{24} - 0.00425 \quad (3)$$

$$\text{MCP (mg g}^{-1} \text{ DM)} = \text{IVDMD (mg)} - \text{GP}_{24} \text{ (mL)} \times 2.2 \text{ mg mL}^{-1} \quad (4)$$

where EE is ether extract (%DM), CP is crude protein (%DM), and GP₂₄ is 24 h net gas production (mL 200 mg^{−1} DM).

The kinetic parameters of gas production (GP) were measured according to the following equation:

$$Y = b \times (1 - e^{-ct}) \quad (5)$$

where b is the potential gas production (mL), Y is the volume of gas produced at time t, and c is the gas production rate constant. Parameters c and b were calculated with the iterative least squares method by SAS [25].

2.5. Statistical Analysis

A completely randomized experimental design was prepared with 6 treatments and 6 replicates per treatment. The statistical procedures were conducted by SPSS (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL, USA). Data on fermentation characteristics and in vitro characteristics of TMR silage were analyzed by one-way analysis of variance (ANOVA):

$$Y_i = \mu + T_i + e_{ij} \quad (6)$$

where μ is the least square mean; Y_i is the dependent variable; e_{ij} is the residual error term; and T_i is the effect of additives.

Data on the aerobic stability of TMR silage were analyzed by two-way ANOVA as follows:

$$Y_{ij} = \mu + T_i + D_j + (T \times D)_{ij} + e_{ij} \quad (7)$$

where μ is overall mean; Y_{ij} is the dependent variable; D_j is the effect of exposure days; T_i is the effect of additives; $(T \times D)_{ij}$ is the effect of interactions between exposure days and additives; and e_{ij} is the residual error term.

Statistical differences among means were determined using Tukey's multiple comparison. Values of $p < 0.05$ were regarded as significant.

3. Results and Discussion

3.1. Chemical Compositions and Microbial Populations of Ingredients and Pre-Ensiled TMR

High CP (~288 g kg^{−1} DM) and EE (~140 g kg^{−1} DM) content were observed in WHDG (Table 1). Common vetch had high CP content (~188 g kg^{−1} DM) and buffering capacity (~320 mEq kg^{−1} DM). The high buffering capacity of common vetch could be attributed to the high content of crude protein and potassium, calcium, and magnesium salts of organic acids [26]. High WSC (~139 g kg^{−1} DM) and ADFom content (~253 g kg^{−1} DM) were found in whole-crop oats. Hulless barley straw had high DM (~968 g kg^{−1} FW) and aNDFom

(~576 g kg⁻¹ DM) content. High DM (~904 g kg⁻¹ FW) and WSC (~110 g kg⁻¹ DM) content were observed in the mixed concentrate.

Quality silage could be obtained from raw material that have proper DM content (250~400 g kg⁻¹ DM), low buffering capacity, a high content of WSC, and an adequate LAB population to compete against the undesirable microorganisms before fermentation [27]. Therefore, a high DM content (~555 g kg⁻¹ FW) was found in pre-ensiled TMR (Table 2), which may inhibit the lactic acid fermentation. The moisture of raw material plays a critical role in affecting fermentation products because moisture is demanded by LAB for metabolic reactions and has a great influence on the initial level and transport of O₂ during ensiling [28]. The high DM content of silage may limit the multiplication and metabolic activity of the indigenous LAB strains [29]. Hence, it is necessary to apply some bacterial additives to accelerate the lactic acid fermentation and enhance the silage quality. In addition, a sufficient part of raw material should be WSC content because it is a critical factor influencing the fermentation quality of silage. In this study, the pre-ensiled TMR had a WSC content of 95.6 g kg⁻¹ DM, and this was higher than 60~70 g kg⁻¹ DM (considered as a theoretical requirement for making good silage), which may benefit the growth of desirable microbes, promote lactic acid fermentation, and ensure the successful fermentation [1].

It is worth noting that high populations of yeasts (~6.64 log₁₀ cfu g⁻¹ FW) and molds (~5.81 log₁₀ cfu g⁻¹ FW) were found in pre-ensiled TMR. During ensiling, the growth of yeasts induces a large loss of DM and energy, which are principally fermented to ethanol, CO₂, and water under anaerobic condition [30]. After exposure to air, yeasts are the main initiators of aerobic spoilage by consuming lactic acid and WSC, thus raising the pH and inner temperature of silage [31]. Finally, molds completely deteriorate the quality of silage [32]. For quality silage, the populations of yeasts and molds should not exceed 3~4 log₁₀ cfu g⁻¹ FW [33], and higher numbers of yeasts and molds in raw material indicate a higher risk of aerobic deterioration after exposure to air. Hence, it is necessary to use some organic acid salts to improve the fermentation quality and aerobic stability of TMR silage. Consequently, we hypothesized the pre-ensiled TMR prepared with WHDG could be utilized to produce quality TMR silage with the help of organic acid salts and bacterial additives.

3.2. Fermentation Profile, Microbial and Chemical Compositions of TMR Silage

In the current study, the pH in all groups was higher than the critical value of 4.2 [26]. However, silage with the higher DM content stabilized at a higher pH [34]. Hence, after 95 days of ensiling, all the TMR silage was well preserved in the range of the pH values (4.32~4.51) (Table 3). Greater ($p < 0.05$) lactic acid (76.5~82.4 vs. 62.5~66.6 g kg⁻¹ DM) content and lower ($p < 0.05$) pH values (4.32~4.33 vs. 4.42~4.51) were found in the LB and LAC groups compared to other groups. This study demonstrated the efficiency of LAB inoculants in accelerating lactic acid fermentation and converting WSC into organic acids, thus lowering pH. Moreover, the LAC group had higher ($p < 0.05$) lactic acid content (82.4 vs. 76.5 g kg⁻¹ DM) than the LB group, which can be attributed to the different metabolic pathways of LAB strains. Holzer et al. [35] reported that *L. casei* belongs to facultative hetero-fermentative LAB and usually ferments hexoses homo-fermentatively into lactic acid, but, under special conditions, ferments hexoses hetero-fermentatively into lactic acid, CO₂, and ethanol (or acetic acid). *L. buchneri* belongs to the obligate hetero-fermentative LAB and ferments hexoses to lactic acid, CO₂, and ethanol (or acetic acid), and has a lower efficiency than *L. casei* in producing lactic acid. Hence, in the LAB inoculants of silage, *L. casei* performed better than *L. buchneri* in accumulating lactic acid during ensilage. Moreover, the SDA group had the highest ($p < 0.05$) acetic acid (~31.3 g kg⁻¹ DM) content and the CAP group had the highest ($p < 0.05$) propionic acid (~7.41 g kg⁻¹ DM) content among all groups, which may have resulted from the ionization of sodium diacetate and calcium propionate to acetic and propionic acids.

Table 3. Fermentation profile, microbial and chemical compositions of total mixed ration silage after 95 days of ensiling.

Items ¹	Treatments ²						SEM	<i>p</i> -Value ³
	Control	CAP	POS	SDA	LB	LAC		
Fermentation profile (g kg ⁻¹ DM)								
pH	4.49 a	4.47 ab	4.42 b	4.51 a	4.32 c	4.33 c	0.018	<0.001
Lactic acid	63.1 cd	66.6 c	62.5 d	63.5 cd	76.5 b	82.4 a	1.87	<0.001
Acetic acid	28.2 b	21.2 cd	25.0 bc	31.3 a	19.7 d	22.4 cd	1.02	<0.001
Lactic acid/acetic acid	2.25 c	3.15 b	2.50 c	2.03 c	3.89 a	3.67 a	0.174	<0.001
Propionic acid	3.22 b	7.41 a	1.33 d	1.96 c	1.82 c	1.88 c	0.505	<0.001
Butyric acid	1.44 bc	1.40 bc	1.38 bc	1.34 c	1.44 b	1.56 a	0.019	<0.001
VFAs	32.8 ab	30.0 bc	27.7 c	34.6 a	23.0 d	25.9 cd	1.01	<0.001
Ethanol	26.3 a	10.9 d	15.8 bc	16.9 b	12.3 cd	14.1 bcd	1.25	<0.001
Water soluble carbohydrates	25.0 b	29.8 a	23.1 bc	21.0 c	22.4 bc	21.6 c	0.76	<0.001
NH ₃ -N (g kg ⁻¹ total nitrogen)	87.1 a	69.1 c	75.4 b	73.5 bc	77.4 b	74.1 b	1.38	<0.001
Chemical compositions (g kg ⁻¹ DM)								
Dry matter (g kg ⁻¹ FW)	503 d	530 a	525 ab	512 c	524 b	497 e	2.9	<0.001
Crude protein	151 a	152 a	145 b	143 b	142 b	138 c	1.3	<0.001
Ash	83.9 a	85.3 a	75.9 b	84.8 a	76.9 b	83.9 a	0.99	<0.001
Ether extract	83.2 a	73.9 c	75.2 bc	75.5 bc	74.9 c	79.5 ab	0.86	<0.001
aNDFom	443 b	428 c	426 c	385 e	407 d	462 a	5.9	<0.001
ADFom	151 c	150 c	169 b	114 d	158 bc	276 a	5.2	<0.001
Microbial numbers (Log ₁₀ cfu g ⁻¹ FW)								
Lactic acid bacteria	7.91	7.71	7.85	7.69	7.48	7.47	0.058	0.119
Aerobic bacteria	7.37 a	5.62 c	6.28 b	5.56 c	5.51 c	5.57 c	0.170	<0.001
Yeasts	5.67 b	6.52 a	6.49 a	5.80 b	5.66 b	5.75 b	0.096	<0.001
Molds	4.63 c	5.26 b	5.74 a	4.59 c	4.62 c	4.57 c	0.062	<0.001

Values in the same row with different superscript letters (a–e) are significantly different ($p < 0.05$). ¹ DM: dry matter; FW: fresh weight; VFAs: volatile fatty acids; NH₃-N, ammonia nitrogen; aNDFom: neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash; ADFom: acid detergent fiber expressed exclusive of residual ash; mEq: milligram equivalent; Log₁₀: decimal logarithm; cfu: colony-forming units. ² Control: untreated silage; CAP: calcium propionate at 0.5% of fresh weight; POS: potassium sorbate at 0.1% of fresh weight; SDA: sodium diacetate at 0.5% of fresh weight; LB: *Lactobacillus buchneri* at 1×10^6 cfu g⁻¹ fresh weight; LAC: *Lactobacillus casei* at 1×10^6 cfu g⁻¹ fresh weight. ³ SEM: standard error of the mean.

In silage, a good fermentation requires butyric acid less than 5 g kg⁻¹ DM [36]. Herein, all the groups had less than 2.00 g kg⁻¹ DM of butyric acid, suggesting good fermentation. The NH₃-N in silage is an indicator of the degree of protein degradation, which impairs the nutritive value of forage. In well-preserved silage, the NH₃-N content should not exceed 100 g kg⁻¹ total N [37]. In the present study, all the groups had less than 90.0 g kg⁻¹ total N of NH₃-N, indicating a good fermentation quality. The high DM could restrict the growth of enterobacteria and clostridia [38], which might be responsible for the low butyric acid and NH₃-N content in all groups.

Compared to the DM content (555 g kg⁻¹ FW) of pre-ensiled TMR, the DM content (497–530 g kg⁻¹ FW) in all TMR silages consistently declined after ensiling. This decrease could be attributed to the fact that the easily degradable constituents (WSC) of silage were transformed into organic acids, ethanol, and CO₂ by microorganisms during fermentation; as a consequence, the WSC content declined in all TMR silages. In addition, the EE content is beneficial to the growth of undesirable bacteria during ensiling, which is an important factor affecting the silage quality. The National Research Council (NRC) recommends that the maximum EE level in the diet be 60–70 g kg⁻¹ DM because too much fat decreases the digestibility and passage rate of fiber, thereby accelerating the growth of undesirable bacteria and reducing rumen fermentation [39,40]. Thus, the EE content (73.9–75.5 vs. 83.2 g kg⁻¹ DM) in the CAP, POS, SDA, and LB groups were lower ($p < 0.05$) than that of the control, indicating that these additives are effective in reducing EE content during ensiling.

Yeasts could metabolize WSC to produce CO₂ and ethanol during ensiling [41]. The activity of yeasts was inhibited by organic acid salts and bacterial additives, as indicated

by the lower ($p < 0.05$) ethanol (10.9~16.9 vs. 26.3 g kg⁻¹ DM) content than the control. This finding may be because the acidic condition and ionized organic acids restrict the activity of yeasts [5]. The calcium propionate, potassium sorbate, and sodium diacetate additives could ionize to produce these organic acids (primarily propionic, sorbic, and acetic acids) and salt ions, which have antibacterial properties and acidic characteristics [42]. The undissociated molecules of these organic acids could pass through the plasma membrane and liberate protons to acidify the cytoplasm, thus limiting the growth and activity of microorganisms [43].

It is interesting to note that similar ($p > 0.05$) LAB populations were found among the LB (7.48 log₁₀ cfu g⁻¹ FW), LAC (7.47 log₁₀ cfu g⁻¹ FW), and control (7.91 log₁₀ cfu g⁻¹ FW) groups. Muck [44] reasoned that the absence of an inoculant impacts the LAB number after fermentation through its effect on related factors that restrict its growth, such as nutrient shortage, or the accumulation of excretory products of the organism, resulting in insufficient conditions to cause a rapid decline in pH and to overcome the epiphytic population. Similar results in silage have been described by Ni et al. [45], who found no significant difference in LAB populations between LAB-inoculated and control silage. Hence, in the selection process of inoculant strains, it is critical to consider the survival of the selected strains until the end of the ensiling process.

3.3. Aerobic Stability

The microorganisms linked with aerobic deterioration can increase pH and cause nutrient loss [26]. In the experiment, the pH increased by 0.05, 0.11, 0.04, 0.05, 0.06, and 0.12 for the control, CAP, POS, SDA, LB, and LAC groups, respectively, during 14 days of aerobic exposure (Table 4). This finding indicated that all the groups were steady during aerobic exposure, probably due to the sufficient acetic acid (13.0~31.3 g kg⁻¹ DM) content in TMR silage after air exposure. Studies have demonstrated that acetic acid could effectively restrict the growth and activity of undesirable microorganisms, thus enhancing the aerobic stability of silage [46]. Moreover, the lactic acid content in all groups decreased to varying degrees, which may be correlated with the consumption of lactate-assimilating yeasts [47].

Table 4. Changes in the pH and fermentation products of total mixed ration silage after exposure to air.

Items ¹	Treatments ²	Days of Air Exposure				SEM ³	p-Value ⁴		
		0	6	9	14		T	D	T × D
pH	Control	4.49 Bb	4.54 Aa	4.53 ABa	4.53 ABb	0.002	<0.001	<0.001	<0.001
	CAP	4.47 Dab	4.54 Ba	4.51 Cab	4.58 Ab				
	POS	4.42 Cb	4.46 Ab	4.46 ABb	4.43 BCc				
	SDA	4.51 a	4.54 a	4.51 ab	4.56 b				
	LB	4.32 Bc	4.38 Ac	4.37 ABc	4.38 Ac				
	LAC	4.33 Cc	4.40 BCc	4.45 Bb	5.30 Aa				
Lactic acid (g kg ⁻¹ DM)	Control	63.1 Bcd	58.7 BCb	68.2 Aab	56.7 Cc	0.19	<0.001	<0.001	<0.001
	CAP	66.6 Bc	60.4 Cb	70.8 Aa	66.7 Bb				
	POS	62.5 Bd	57.8 Cb	59.3 BCc	69.4 Aab				
	SDA	63.5 ABcd	57.9 BCb	64.9 Ab	56.6 Cc				
	LB	76.5 Ab	66.2 Ca	70.8 Ba	72.1 Ba				
	LAC	82.4 Aa	69.6 Ba	56.7 Cc	67.0 Bb				
Acetic acid (g kg ⁻¹ DM)	Control	28.2 Aab	25.3 ABa	26.2 ABa	22.4 Bab	0.18	<0.001	<0.001	<0.001
	CAP	21.2 cd	19.4 bc	22.2 ab	21.9 abc				
	POS	25.0 bc	22.4 ab	20.9 b	24.2 a				
	SDA	31.3 Aa	24.8 Ba	26.3 Ba	25.8 Ba				
	LB	19.7 Ad	17.3 ABc	16.0 Bc	17.5 ABbc				
	LAC	22.4 Acd	19.1 Bbc	13.0 Dc	16.8 Cc				
Propionic acid (g kg ⁻¹ DM)	Control	3.22 Ab	2.54 ABb	2.53 ABb	2.15 Bb	0.022	<0.001	<0.001	<0.001
	CAP	7.41 Aa	6.46 Ba	7.27 Aa	7.45 Aa				
	POS	1.33 ABd	1.21 Bc	1.28 ABc	1.34 Ac				
	SDA	1.96 c	1.42 c	1.50 c	1.53 c				
	LB	1.82 Ac	1.38 Bc	1.36 Bc	1.47 Bc				
	LAC	1.88 Ac	1.56 Bc	1.45 Bc	1.56 Bc				
Butyric acid (g kg ⁻¹ DM)	Control	1.44 Ab	1.21 AB	1.25 AB	1.11 B	0.009	0.087	<0.001	0.002
	CAP	1.40 Abc	1.16 C	1.27 B	1.08 C				
	POS	1.38 Abc	1.17 B	1.12 B	1.15 B				
	SDA	1.34 c	1.37	1.28	1.18				
	LB	1.44 Abc	1.12 B	1.14 B	1.29 AB				
	LAC	1.56 Aa	1.21 B	1.21 B	1.14 B				

A–D: Means in the same row with different capital letters differed ($p < 0.05$); a–d: Means in the same column with different lowercases differed ($p < 0.05$). ¹ DM: dry matter; FW: fresh weight. ² Control: untreated silage; CAP: calcium propionate at 0.5% of fresh weight; POS: potassium sorbate at 0.1% of fresh weight; SDA: sodium diacetate at 0.5% of fresh weight; LB: *Lactobacillus buchneri* at 1×10^6 cfu g⁻¹ fresh weight; LAC: *Lactobacillus casei* at 1×10^6 cfu g⁻¹ fresh weight. ³ SEM: standard error of the mean. ⁴ T: effect of treatment; D: effect of day of aerobic exposure; T × D: effect of treatment and day of aerobic exposure interactions.

On day 14, the SDA group had evidently ($p < 0.05$) or numerically ($p > 0.05$) lower aerobic bacteria (5.54 vs. 5.82~5.97 $\log_{10}$ cfu g^{-1} FW), yeast (4.55 vs. 4.59~6.46 $\log_{10}$ cfu g^{-1} FW) and mold (3.45 vs. 3.87~4.61 $\log_{10}$ cfu g^{-1} FW) populations than other groups (Table 5). It indicated that SDA had a superior inhibitory effect on the aerobic bacteria, mold, and yeast populations than other treatments after air exposure. It was probably because sodium diacetate can ionize to a large amount of acetic acid (25.8~31.3 g kg^{-1} DM) with antimicrobial property during aerobic exposure, and the un-dissociated acetic acid could get through the plasma membrane and affect the microbial metabolism [48]. Hence, the growth and activity of harmful microbes were greatly limited in SDA treatment.

Table 5. Changes in microbial and chemical compositions of total mixed ration silage after exposure to air.

Items ¹	Treatments ²	Days of Air Exposure				SEM ³	p-Value ⁴		
		0	6	9	14		T	D	T × D
Water soluble carbohydrates (g kg^{-1} DM)	Control	25.0 Ab	25.0 Ab	14.6 Bbc	16.4 Bde	0.19	<0.001	<0.001	<0.001
	CAP	29.8 Aa	29.8 Aa	26.6 Aa	19.5 Bcd				
	POS	23.1 Abc	23.1 Ab	18.6 Bb	23.6 Aab				
	SDA	21.0 Bc	21.7 Bb	13.2 Cbc	26.9 Aa				
	LB	22.4 Abc	23.4 Ab	12.5 Bc	22.4 Abc				
	LAC	21.6 Ac	23.3 Ab	11.2 Cc	15.6 Be				
Ammonia nitrogen (g kg^{-1} total nitrogen)	Control	87.1 Aa	63.2 Ba	55.7 Cab	33.7 Db	0.24	<0.001	<0.001	<0.001
	CAP	69.1 Ac	43.7 Cd	57.9 Ba	45.0 Ca				
	POS	75.4 Ab	55.0 Bb	56.8 Bab	46.4 Ca				
	SDA	73.5 Abc	47.2 Ccd	58.0 Ba	46.3 Ca				
	LB	77.4 Ab	55.3 Bb	53.2 Bbc	36.2 Cb				
	LAC	74.1 Ab	52.6 Bbc	49.1 Bc	47.0 Ba				
Lactic acid bacteria ($\log_{10}$ cfu g^{-1} FW)	Control	7.91 B	7.42 Ca	8.31 Aa	8.29 A	0.019	<0.001	<0.001	<0.001
	CAP	7.71 B	7.33 Ba	7.33 Bbc	8.35 A				
	POS	7.85 B	6.48 Db	6.99 Cc	8.33 A				
	SDA	7.69 B	7.22 Ba	7.30 Bbc	8.49 A				
	LB	7.48 B	6.70 Cb	7.65 Bb	8.13 A				
	LAC	7.47 B	7.39 Ba	7.65 Bb	8.24 A				
Aerobic bacteria ($\log_{10}$ cfu g^{-1} FW)	Control	7.37 Aa	5.61 Bb	5.66 Bb	5.97 Ba	0.021	<0.001	0.014	<0.001
	CAP	5.62 Bc	6.41 Aa	5.66 Bb	5.91 ABab				
	POS	6.28 Ab	5.64 Bb	5.70 Bb	5.85 Bab				
	SDA	5.56 Bc	5.83 ABb	6.48 Aa	5.54 Bb				
	LB	5.51 c	5.58 b	5.61 b	5.82 ab				
	LAC	5.57 Bc	5.70 Bb	6.18 Aa	5.85 ABab				
Yeasts ($\log_{10}$ cfu g^{-1} FW)	Control	5.67 Ab	4.63 Cc	5.70 Ac	5.13 Bb	0.020	<0.001	<0.001	<0.001
	CAP	6.52 a	6.22 a	6.65 a	6.46 a				
	POS	6.49 Aa	6.19 Bab	6.20 Bb	5.54 Cb				
	SDA	5.80 Bb	6.45 Aa	6.24 Ab	4.55 Cc				
	LB	5.66 Ab	4.71 Bc	5.35 Ad	4.59 Bc				
	LAC	5.75 Ab	5.67 ABb	5.22 Bd	5.52 ABb				
Molds ($\log_{10}$ cfu g^{-1} FW)	Control	4.36 Bc	4.57 Aa	4.63 Aa	4.25 Bb	0.024	<0.001	<0.001	<0.001
	CAP	4.56 Ab	4.28 Cc	4.68 Aa	4.42 Bab				
	POS	4.52 Ab	4.21 Bc	4.23 Bb	3.87 Cc				
	SDA	4.75 Aa	4.49 Bb	4.21 Cb	3.45 Dd				
	LB	4.67 Aa	4.68 Aa	4.32 Bb	4.61 Aa				
	LAC	4.76 Aa	4.62 Aa	4.26 Cb	4.54 Ba				

A–D: Means in the same row with different capital letters differed ($p < 0.05$); a–e: Means in the same column with different lowercases differed ($p < 0.05$). ¹ DM: dry matter; FW: fresh weight; $\log_{10}$: decimal logarithm; cfu: colony-forming units. ² Control: untreated silage; CAP: calcium propionate at 0.5% of fresh weight; POS: potassium sorbate at 0.1% of fresh weight; SDA: sodium diacetate at 0.5% of fresh weight; LB: *Lactobacillus buchneri* at 1×10^6 cfu g^{-1} fresh weight; LAC: *Lactobacillus casei* at 1×10^6 cfu g^{-1} fresh weight. ³ SEM: standard error of the mean. ⁴ T: effect of treatment; D: effect of day of aerobic exposure; T × D: effect of treatment and day of aerobic exposure interactions.

3.4. In Vitro Digestibility and Gas Production

The IVDMD is a critical parameter that reflects the rate of feed utilization in the rumen, and the digestion of DM principally contains WSC, protein, fiber content, and other substances [49]. Herein, CAP, POS, and SDA treatments increased ($p < 0.05$) IVDMD (54.8~57.5 vs. 48.4%) and IVNDFD (48.4~51.6 vs. 41.1%) compared to control (Table 6), which may be attributed to the lower ($p < 0.05$) aNDFom (385~428 vs. 443 g kg⁻¹ DM) content in CAP, POS, and SDA treatments. Lema et al. [50] reported that a negative correlation existed between IVDMD and NDF, and similar results were also found by Xu et al. [51]. There is no significant ($p > 0.05$) difference in SCFA, ME, and NE_L among all groups. All the treated groups had evidently ($p < 0.05$) or numerically ($p > 0.05$) higher cumulative gas production at incubation time 72 h (GP₇₂) (173~223 vs. 168 mL) and potential gas production (174~227 vs. 169 mL) than the control, which may be attributed to the inhibition impacts on the growth and activity of harmful microorganisms, reducing nutrient loss and supplying adequate substrates and energy for microbial metabolism [5].

Table 6. In vitro gas production kinetics and digestibility, and available energy of total mixed ration silage after 95 days of fermentation.

Items ¹	Treatments ²						SEM ³	<i>p</i> -Value
	Control	CAP	POS	SDA	LB	LAC		
In vitro digestibility								
IVDMD (%)	48.4 c	57.5 a	54.8 ab	56.0 a	53.1 abc	51.1 bc	0.82	<0.001
IVNDFD (%)	41.1 c	51.6 a	48.4 ab	49.8 a	47.2 ab	45.2 bc	0.88	<0.001
SCFA (mmol g ⁻¹ DM)	13.2	17.2	13.5	13.7	14.5	15.2	0.57	0.371
MCP (mg g ⁻¹ DM)	290 cd	291 cd	347 ab	319 bc	273 d	351 a	7.4	<0.001
Available energy (MJ kg ⁻¹ DM)								
Metabolizable energy (ME)	18.5	23.4	18.9	19.1	20.1	21.0	0.70	0.367
Net energy for lactation (NE _L)	12.1	15.5	12.3	12.5	13.2	13.8	0.49	0.373
Gas production kinetics								
GP ₇₂ (mL)	168 c	223 a	173 bc	174 bc	180 bc	193 b	4.8	<0.001
Potential gas production (mL)	169 d	227 a	174 d	180 c	182 c	193 b	1.4	0.007
Gas production rate constant (mL h ⁻¹)	0.048 b	0.049 b	0.047 b	0.041 c	0.049 b	0.053 a	0.0015	0.026

Values in the same row with different superscript letters (a–d) are significantly different ($p < 0.05$). ¹ DM: dry matter; FW: fresh weight; MCP: microbial crude protein; IVDMD: in vitro dry matter digestibility; SCFA: short chain fatty acid; IVNDFD: in vitro neutral detergent fiber digestibility; GP₇₂: cumulative gas production at incubation time 72 h. ² Control: untreated silage; CAP: calcium propionate at 0.5% of fresh weight; POS: potassium sorbate at 0.1% of fresh weight; SDA: sodium diacetate at 0.5% of fresh weight; LB: *Lactobacillus buchneri* at 1×10^6 cfu g⁻¹ fresh weight; LAC: *Lactobacillus casei* at 1×10^6 cfu g⁻¹ fresh weight. ³ SEM: standard error of the mean.

4. Conclusions

All the TMR silage was well preserved with a low pH and acceptable levels of NH₃-N and butyric acid. During the 14 days of aerobic exposure, all the groups were steady, while the SDA group was more stable with lower populations of undesirable microorganisms on day 14. The CAP, POS, and SDA treatments evidently enhanced the IVDMD and IVNDFD of TMR silage. Therefore, after comprehensive consideration, SDA is recommended as an additive to improve the fermentation quality, in vitro digestibility, and aerobic stability of TMR silage prepared with 10% FW of WHDG.

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