The Journal of Agricultural Science

cambridge.org/ags

Animal Research Paper

Cite this article: Li X, Liu H, Dai T, Dong D, Xu G, Jia Y, Shao T (2023). Effects of natamycin, hexanoic acid and *Lactobacillus plantarum* on fermentation, aerobic stability and *in vitro* digestibility of an ensiled total mixed ration containing water bamboo sheath leaves. *The Journal of Agricultural Science* **161**, 877–886. https://doi.org/10.1017/S0021859624000054

Received: 29 August 2022
Revised: 19 December 2023
Accepted: 31 December 2023
First published online: 9 January 2024

Keywords:
aerobic stability; fermentation quality; *in vitro* digestibility; silage additives; total mixed ration silage

Corresponding author:
Tao Shao;
Email: taoshaolan@163.com

Effects of natamycin, hexanoic acid and *Lactobacillus plantarum* on fermentation, aerobic stability and *in vitro* digestibility of an ensiled total mixed ration containing water bamboo sheath leaves

Xinbao Li[1], Haopeng Liu[1], Tongtong Dai[1], Dong Dong[1], Guofeng Xu[1], Yushan Jia[2] and Tao Shao[1]

[1]Institute of Ensiling and Processing of Grass, College of Agro-grassland Science, Nanjing Agricultural University, Nanjing 210095, China and [2]Key Laboratory of Forage Cultivation, Processing and High Efficient Utilization, Ministry of Agriculture, Inner Mongolia Agricultural University, Hohhot Inner Mongolia 010018, China

Abstract

The work aimed to evaluate the effect of lactic acid bacteria, natamycin and hexanoic acid on the fermentation quality, aerobic stability and *in vitro* digestibility of total mixed ration (TMR) silages containing water bamboo sheath leaves. The treatments were as follows: (1) no additives (control, C), (2) lactic acid bacteria (L), (3) natamycin (N), (4) hexanoic acid (H), (5) lactic acid bacteria + natamycin (LN), (6) lactic acid bacteria + hexanoic acid (LH). Silos were opened and silages were evaluated to assess fermentation quality, aerobic stability and *in vitro* digestibility. All TMR silages were well-preserved as indicated by dominant lactic acid content, low ammonia nitrogen/total nitrogen, trace propionic acid and negligible butyric acid contents. All additives improved the fermentation quality and *in vitro* digestibility, evidenced by higher lactic acid/acetic acid ratio, lactic acid, acetic acid, water-soluble carbohydrate contents and lactic acid bacteria count, and lower pH, aerobic bacteria and yeast counts. The LN had the highest lactic acid, acetic acid, lactic acid/acetic acid ratio, *in vitro* gas production and *in vitro* digestibility. During aerobic exposure, the LN silage had the highest lactic acid and acetic acid contents, and the lowest pH among all TMR silages. The LN and LH prolonged aerobic stability, reaching 85.5 and 81.5 h, respectively. The LN is recommended to improve the fermentation quality, aerobic stability and *in vitro* digestibility of TMR prepared with water bamboo sheath leaves.

Introduction

Water bamboo shoot (WBS, *Zizania aquatica* L.) is a perennial aquatic vegetable with high nutritional values, which has been cultivated for at least 2000 years in China (Zhang *et al.*, 2021). The outer sheath leaves from WBSs are usually removed, and discarded into the river or open incinerated directly, which would result in resource waste and environmental pollution. Guo *et al.* (2007) reported that the outer sheath leaves have abundant protein, and essential amino acids along with trace elements. The utilization of the outer sheath leaves in the form of total mixed ration (TMR) silages is regarded as an efficient method for recycling of resources, and are increasingly applied in animal husbandry in recent years. Nevertheless, it is a challenge for ensiling the outer sheath leaves without mixing with other forages or additives, because of their high moisture and low WSC contents which may not sufficiently limit the activity of undesirable microorganisms during ensiling, leading to poor silage quality.

TMR is a type of complete diet formula that can meet the nutritional requirements of ruminants (Zhao *et al.*, 2020). However, TMR is only suitable to be fed for a short time owing to its high deterioration after being prepared, restricting its use on some farms with labour shortage (Yuan *et al.*, 2018). Ensiling TMR incorporated with concentrate, by-products and roughage can stabilize rumen function, avoid self-selection by animals that improves palatability and reduce labour and machinery requirement (Wang *et al.*, 2023). Hence, blending by-products of outer sheath leaves from WBSs with concentrate as TMR silage provides year-round nutritionally balanced quality feed, improves palatability by altering odours and flavours from by-products through silage fermentation and offers a potential approach for waste recycling and reducing feed costs (Han *et al.*, 2022).

The additives were widely used to improve fermentation quality and prolong aerobic stability (Wuisman *et al.*, 2006). Lactic acid bacteria (LAB) as fermentation stimulators were extensively used to improve the fermentation quality. However, one downside of silages with

LAB-based additives is to be prone to poor aerobic stability (McEniry *et al.*, 2007) due to the limited synthetic amounts of antifungal compounds during ensiling (Guo *et al.*, 2020). The additives with bacteriostatic efficacies could prevent yeast and mould growth and lower risks in silage making. Natamycin is an effective antifungal agent produced by *Streptomyces natalensis* (te Welscher *et al.*, 2008). Pinto *et al.* (2020) reported that the addition of low-dose natamycin combined with heterofermentative lactic bacteria (*Lactobacillus buchneri*) reduced yeast count and improved the aerobic stability of maize silage. Aranega-Bou *et al.* (2014) expounded on the importance of the antifungal effect in hexanoic acid by inhibiting spore germination and mycelia growth in target fungi. The antimycotic effect of hexanoic acid was confirmed by Mugabe *et al.* (2020) who suggested that the addition of hexanoic acid or combination with *Lactobacillus plantarum* reduced the yeast counts and improved aerobic stability of Napier grass silages. However, there are few works evaluating the effect of natamycin and hexanoic acid either alone or in combination with *L. plantarum* on the fermentation quality and aerobic stability of TMR silage.

Therefore, the experiment evaluated the effect of natamycin, hexanoic acid, as well as their combination with *L. plantarum* on the fermentation quality, aerobic stability and *in vitro* digestibility of TMR silages prepared with WBS leaves.

Materials and methods

Total mixed ration preparation and treatments

TMR consisted of the outer sheath leaves, alfalfa, rice straw and concentrate. The outer sheath leaves were collected from a WBS processing factory in Zhejiang, China. The alfalfa, rice straw and concentrate were obtained from Wushan Dairy Farm (29.43 °N, 121.48 °E, Zhejiang, China). The concentrate contained 0.075 crack corn, 0.20 rape cake meal, 0.20 cotton seed meal, 0.275 distillers' dried grain with soluble, 0.20 wheat bran, 0.05 vitamin-mineral. The outer sheath leaves, alfalfa and rice straw were chopped to 2–3 cm length with a forage cutter. The chopped raw materials, concentrate and TMR were sampled at random for chemical composition and microbial population analyses (Table 1). The outer sheath leaves, alfalfa, rice straw and concentrate were mixed at a ratio of 12:2:21:65 on DM basis. After being mixed thoroughly, the TMR mixture (6 kg) was packed into 10 litre laboratory silos (27.5 cm diameter × 31.6 cm height, Lantian Biological Experimental Instrument Co., Ltd., Jiangsu, China), and sealed by two screw tops and plastic tape. The treatments were as follows: (i) control (C, sterile water alone); (ii) LAB (*L. plantarum*) inoculant applied at 1×10^6 colony-forming units (cfu)/g fresh weight (FW); (iii) natamycin (N; Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) added at 20 mg/kg FW; (iv) hexanoic acid (H; Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) added at 20 mg/kg FW; (v) LAB (applied at 1×10^6 cfu/g FW) + natamycin (added at 20 mg/kg FW) (LN); (vi) LAB (applied at 1×10^6 cfu/g FW) + hexanoic acid (added at 20 mg/kg FW) (LH). *L. plantarum*, natamycin and hexanoic acid were diluted to the intended rate with deionized water, and uniformly applied to TMR at 3 ml/kg FW before ensiling. Meanwhile, an equal volume of distilled water but with no additive was added to the control. Each treatment was applied to each of five silos (replicates). A total of 120 silos were stored at ambient temperature (25–28 ℃). All silos were opened after 120 days of ensiling, and samples were taken for analysis at 0, 2, 4 and 6 days after opening the silo. In practical TMR silage production, it is common that not all silage is fed immediately after silo opening, and thus the quality of the silage remaining at different days after opening the silo was evaluated. The exposure days reflect real-world scenarios in pasture management, considering the conditions encountered in actual practices of farm.

Table 1. Chemical compositions and microbial counts of raw materials and TMR[a]

Item[b]	Outer sheath leaves	Alfalfa	Rice straw	Concentrate[c]	TMR[a]
Ingredient proportions (g/kg FW)	250	50	300	400	
Chemical compositions					
DM (g/kg FW)	260	251	591	909	618
CP (g/kg DM)	188	225	51	155	136
WSC (g/kg DM)	43.1	75.3	32.2	110	70.6
aNDF (g/kg DM)	613	331	641	381	492
ADF (g/kg DM)	309	244	478	152	293
Ash (g/kg DM)	63.2	75.7	82.1	116	90.7
EE (g/kg DM)	43.9	51.8	35.9	72.2	53.2
BC (mEq/kg DM)	38.7	368	123	145	123
Microbial counts					
LAB (log_{10} cfu/g FW)	5.5	4.8	3.4	4.2	6.2
Aerobic bacteria (log_{10} cfu/g FW)	8.6	7.7	4.8	6.3	6.3
Yeast (log_{10} cfu/g FW)	< 2.0	6.4	4.3	4.8	5.1

[a]Total mixed ration (TMR) consists of 0.25 water bamboo sheath leaves, 0.05 alfalfa, 0.30 rice straw and 0.40 concentrate.
[b]DM, dry matter; FW, fresh weight; CP, crude protein; WSC, water-soluble carbohydrate; aNDF, neutral detergent fibre (with a heat-stable amylase and expressed inclusive of residue ash); ADF, acid detergent fibre; EE, ether extract; BC, buffer capacity; LAB, lactic acid bacteria; Log_{10}, decimal logarithm; cfu, colony-forming unit.
[c]Concentrate: 0.075 crack corn, 0.20 rape cake meal, 0.20 cotton seed meal, 0.275 distillers' dried grain with soluble, 0.20 wheat bran, 0.05 vitamin-mineral.

https://doi.org/10.1017/S0021859624000054 Published online by Cambridge University Press

Chemical composition and fermentation quality analysis

The contents of each silo were homogenized by mixing, and sampled for chemical compositions and fermentation quality analysis. The buffering capacity of raw materials and TMR was titrated by the hydrochloric acid and sodium hydroxide method of Playne and McDonald (1966). The raw materials, TMR and TMR silage samples were oven-dried at 65 ℃ for at least 60 h to constant weight to determine dry matter (DM) content, and then were grounded by using a laboratory mill (FW100, Taisite Instrument Co., Ltd., Tianjin, China) to pass a 1 mm screen for later chemical composition analysis. Total nitrogen (TN) was measured by the Kjeldahl nitrogen analyser (Kjeldahl TM2300 analyser, FOSS Ltd., Sweden) and the crude protein (CP) content was determined as TN × 6.25. Water-soluble carbohydrate (WSC) content was determined by sulphuric acid-anthrone colorimetric reaction (Wang *et al.*, 2019). The contents of ether extract (EE) and ash were determined according to the Soxhlet extraction method 963.15 and 924.05 of the Association of Official Analytical Chemists (AOAC 1990), respectively. The heat-stable α-amylase-treated neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined according to the method of Van Soest *et al.* (1991), and the NDF and ADF were expressed without residual ash (Table 2).

The TMR silage (35 g) of each silo was blended with 70 ml of distilled water and stored at 4 ℃ for 24 h. Then, the extract was filtered through double-layered cheesecloth and a Whatman filter paper (pore size of 11 µm, Xinhua Co., Hangzhou, China). The filtrate was used for determining pH, ammonia nitrogen (AN) and organic acid contents. The pH was measured with a glass electrode pH meter (HANNA pH211, Hanna Instruments Co. Ltd., Italy). The AN was determined as described by Chen *et al.* (2016). The organic acids (lactic acid, acetic acid, propionic acid, butyric acid) and ethanol contents were determined by Agilent 1260 HPLC system (Agilent Technologies Inc., California, USA) equipped with a refractive index detector (Carbomix® H-NP5 column, 2.5 mM H_2SO_4, 0.5 ml/min) as described by Li *et al.* (2021).

For determination of microbial counts, 10 g of each parent TMR and TMR silages were blended with 90 ml sterilized saline solution, and then the solution was serially diluted in sterilized saline water. The LAB were counted on deMan, Rogosa and Sharp (MRS) agar medium (Shanghai Bio-way Technology Co., Ltd., Shanghai, China) after incubation for 48 h at 37 °C under anaerobic condition. Aerobic bacteria (AB) were counted on nutrient agar medium (Qingdao Hope Bio-technology Co., Ltd.) after incubation for 24 h at 37 °C. Yeast was counted on Potato Dextrose Agar medium (Shanghai Bio-way Technology Co., Ltd.) incubation for 48 h at 28 °C.

Aerobic stability

Aerobic stability was defined by the time (hours) before the temperature in the silage was at least 2°C above the ambient temperature during aerobic exposure. The TMR silage was taken out from each silo, mixed thoroughly and loosely placed into a bigger open-tap and sterile polyethylene bottle (15 l capacity), which was covered with double-layered cheesecloth to prevent dust pollution and water loss. The probes of multi-channel temperature recorder (MDL-1048A high precision temperature recorder, Shanghai Tianhe Automation Instrument Co., Ltd.) were placed in the centre of the bottle for measuring temperature variation. Six probes were placed in the environment as blanks. The pH, the contents of WSC, AN and organic acid, and the counts of LAB, AB and yeast were determined 0, 2, 4 and 6 days after opening the silo.

In vitro *ruminal incubation*

The experiment was approved by the Ethics Committee of the Nanjing Agricultural University (Jiangsu, China) on 27 September 2021. The rumen fluid was obtained from four rumen-cannulated Boer male goats (50.00 ± 4.00 kg of live weight) before the morning feeding. The Boer goats were fed a diet containing 0.59 Guinea grass, 0.35 concentrate and 0.06 alfalfa hay at 1.3 times the maintenance level. The rumen fluid was filtered through four layers of cheesecloth and mixed with buffer solution (1:2, v/v). *In vitro* incubation was performed in serum bottles following the procedure described by Contreras-Govea *et al.* (2011). The nylon bag (8 × 12 cm, 42 µm pore size, F57; ANKOM Technology, Macedon, NY, USA) was previously washed with acetone and dried at 65 °C to a constant weight. A dried ground silage sample (1 g) was placed into a nylon bag, and each nylon bag was heat-sealed and placed into a pre-heated serum bottle (120 ml capacity).

Table 2. Chemical compositions of TMR[a] silages

	Treatments[c]							
Item[b]	C	L	N	H	LN	LH	SEM[d]	*P* value
DM (g/kg FW)	603	581	578	580	589	584	3.9	0.083
CP (g/kg DM)	118	124	127	124	132	129	4.8	0.510
aNDF (g/kg DM)	345	348	349	367	347	350	2.7	0.094
ADF (g/kg DM)	225	221	229	223	225	215	2.4	0.354
Ash (g/kg DM)	85.7	86.0	85.5	85.3	85.2	85.8	0.10	0.116
EE (g/kg DM)	37.6	38.7	39.4	39.7	40.7	37.9	0.34	0.052
Ethanol (g/kg DM)	14.3	12.0	11.6	12.3	12.1	12.3	0.41	0.345

[a]Total mixed ration (TMR) consists of 0.25 water bamboo sheath leaves, 0.05 alfalfa, 0.30 rice straw and 0.40 concentrate.
[b]DM, dry matter; FW, fresh weight; DM; dry matter; CP, crude protein; WSC, water-soluble carbohydrate; aNDF, neutral detergent fibre (with a heat-stable amylase and expressed inclusive of residue ash); ADF, acid detergent fibre; EE, ether extract.
[c]C, control; L, lactic acid bacteria; N, natamycin; LN, lactic acid bacteria and natamycin; H, hexanoic acid; LH, lactic acid bacteria and hexanoic acid.
[d]SEM, standard error of the mean.

https://doi.org/10.1017/S0021859624000054 Published online by Cambridge University Press

Sixty millilitres of the mixed inoculum was assigned to each serum bottle and then incubated in a water bath at 39 °C with continuous flushing with CO_2. The serum bottles containing only mixed inoculum were added as blank. Gas production (GP) was recorded at 4, 8, 12, 24, 36, 48 and 72 h using a pressure transducer according to the method of Dai *et al.* (2022) and corrected by the blank. After 72 h of incubation, all nylon bags were gently rinsed with cold tap water to be clean and then dried at 65 °C for 48 h to a constant weight.

In vitro dry matter digestibility (IVDMD), *in vitro* neutral detergent fibre digestibility (IVNDFD) and *in vitro* acid detergent fibre digestibility (IVADFD) were calculated according to the differences in their weight before and after incubation.

Cumulative GP data were fitted to the exponential equation:

$$y = b \times (1 - e^{-ct})$$

where y is the volume of gas produced at time t, b is the asymptotic GP (ml), c is the fractional fermentation rate and t is the incubation time (h). The metabolizable energy (ME) was calculated following the method of Elahi *et al.* (2016).

Statistical analyses

All statistical procedures were performed with SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Data on fermentation quality and *in vitro* parameters of silage at silo opening were analysed using the general linear model procedure according to the model for a factorial treatment design as follows:

$$Yij = \mu + Ti + \varepsilon ij$$

where Yij is the dependent variable; μ is the least square mean; Ti is the effect of additives; εij is the residual error term.

In the aerobic stability test, silage pH, LA, AA, WSC, AN/TN, LAB, AB and yeasts data were analysed as completely randomized design with repeated measurement over time. Data were evaluated using simple main effects tests and Bartlett's *spherical* test. Bonferroni correction showed that treatment by time interaction was significant. Therefore, data were subjected to separate one-way ANOVAs for the analysis of treatment (silage additive) effects within each time, and for the time effects of day after opening the silage within each treatment, in all cases considering a completely randomized design. Linear and quadratic polynomial effects of time (days) after opening the silo were assessed. Tukey's multiple comparisons were used for determination of the statistical difference among means and considered as significant at the level of $P < 0.05$.

Results

Chemical compositions and microbial counts of raw materials and total mixed ration

The chemical compositions and microbial counts of raw materials are shown in Table 1. Rice straw and concentrate had higher DM contents (591 and 909 g/kg FW), and alfalfa had the highest CP content and BC. The outer sheath leaves were similar to the rice straw in aNDF and ADF contents. Concentrate had the highest WSC, EE and ash contents among all raw materials. The outer sheath leaves had the highest LAB count (more than 10^5 cfu/g FW) and the lowest yeast count (below 10^2 cfu/g FW). Rice straw had the lowest AB count (below 10^5 cfu/g FW) among all raw materials.

The DM content of TMR was 618 g/kg FW, the contents of CP and WSC were 136 and 70.6 g/kg DM, respectively. The LAB, AB and yeast counts were 6.2, 6.3 and 5.1 cfu/g FW, respectively.

Fermentation quality of total mixed ration

Compared with the control, all additives significantly ($P < 0.05$) increased lactic acid, acetic acid contents and LA/AA, and significantly ($P < 0.05$) decreased pH. TMR silages treated with *Lactobacillus* (L, LN, LH) significantly ($P < 0.05$) increased LA, and significantly ($P < 0.05$) decreased pH as compared with N and H, and the LN had the highest LA content and LA/AA, and the lowest pH among all TMR silages. The acetic acid content was detected at a low level in all TMR silages. The ethanol and PA contents were detected in only small amounts in all TMR silages. The AN/TN was below 100 g/kg in all TMR silages, and all additives showed numerically ($P > 0.05$) lower AN/TN as compared with control.

The additives significantly ($P < 0.05$) increased WSC contents (Table 3), whereas insignificant on the DM, NDF, ADF, ash and EE contents ($P > 0.05$) as compared with control. The LN and LH had higher WSC contents than that in the other treatments. The LN had numerically ($P > 0.05$) the highest CP content among all TMR silages.

The L, LN and LH had higher ($P < 0.05$) LAB counts than the other treatments. The counts of AB and yeast in all additives decreased below 10^5 cfu/g FW, and are the lowest counts in LN silages (Table 4).

Aerobic stability of total mixed ration silages

With the increase of days after opening the silo, the LA, AA and WSC contents were linearly ($P < 0.05$) decreased, while pH contents were linearly ($P < 0.05$) increased. The AA were quadratically ($P < 0.05$) affected by the increased days. All TMR silages decreased ($P < 0.05$) LA, AA and WSC contents and increased ($P < 0.05$) pH and AN/TN during aerobic exposure. Compared with control, additives significantly ($P < 0.05$) increased LA, AA and WSC contents, while significantly ($P < 0.05$) decreased pH and AN/TN. The LN silages had the highest LA and AA contents and the lowest pH (6.8) on the day 6 of aerobic exposure. The L, LN and LH had significantly ($P < 0.05$) higher LA content than the N and H. The LN and LH had higher WSC contents than that of the other silages, and the AN/TN was below 100 g/kg in additive-treated silages.

With the increase of days after opening the silo, the counts of yeast and AB were linearly ($P < 0.05$) increased of all TMR silages. Additives significantly ($P < 0.05$) increased LAB count, and significantly ($P < 0.05$) decreased the counts of yeast and AB as compared to the control. The LN and LH had significantly ($P < 0.05$) higher LAB counts, and significantly ($P < 0.05$) lower AB and yeast counts in comparison with the other treatments.

Additives improved the aerobic stability of TMR silages compared with the control (Fig. 1). The H, L and N silages kept the aerobic stability for 62.5, 72 and 73.5 h, respectively. The LH and LN had better aerobic stability for 81.5 and 85.5 h.

In vitro *parameters of total mixed ration silages*

The GP parameters and *in vitro* digestibility of TMR silages are presented in Fig. 2 and Table 5.

https://doi.org/10.1017/S0021859624000054 Published online by Cambridge University Press

Table 3. Fermentation parameter of TMR[a] silage after opening the silo

Items[b]	Treatments[c]	Days after unloading (d)				SEM[d]	Model construction P^e	
		0	2	4	6		L	Q
pH	C	5.99	6.21	7.09	7.35	0.048	<0.001	0.714
	L	5.37	5.41	6.62	7.17	0.071	<0.001	<0.001
	N	5.69	5.90	7.07	7.28	0.092	<0.001	0.980
	H	5.84	5.96	6.99	7.28	0.076	<0.001	0.154
	LN	5.12	5.24	5.54	6.80	0.055	<0.001	0.002
	LH	5.31	5.36	5.87	7.04	0.039	<0.001	<0.001
	SEM[d]	0.119	0.050	0.065	0.199			
	P value	<0.001	<0.001	<0.001	0.114			
LA (g/kg DM)	C	26	19	9	4	1.0	<0.001	0.301
	L	42	38	32	21	1.5	<0.001	0.010
	N	32	25	17	8	2.2	<0.001	0.504
	H	29	25	10	7	1.5	<0.001	0.715
	LN	45	41	36	27	1.8	<0.001	0.011
	LH	39	37	30	19	1.4	<0.001	0.026
	SEM[d]	2.8	1.3	1.2	1.0			
	P value	<0.001	<0.001	<0.001	<0.001			
AA (g/kg DM)	C	7.2	6.8	6.7	4.1	0.13	<0.001	0.001
	L	8.9	8.0	6.9	6.4	0.20	0.001	<0.001
	N	7.5	7.4	6.7	6.6	0.17	<0.001	<0.001
	H	7.3	7.1	6.6	6.3	0.10	<0.001	<0.001
	LN	9.0	8.2	7.4	7.1	0.18	<0.001	<0.001
	LH	8.3	8.1	6.8	6.6	0.17	0.001	<0.001
	SEM[d]	0.22	0.15	0.17	0.13			
	P value	0.001	<0.001	0.005	0.180			
WSC (g/kg DM)	C	39	33	25	15	0.9	<0.001	0.027
	L	43	30	25	21	1.3	<0.001	0.122
	N	46	42	36	30	1.3	<0.001	0.110
	H	43	38	33	28	1.7	<0.001	0.898
	LN	47	46	40	33	1.7	<0.001	<0.001
	LH	47	42	37	31	1.6	<0.001	0.565
	SEM[d]	1.6	1.0	1.0	1.5			
	P value	<0.001	<0.001	<0.001	<0.001			
AN/TN (g/kg TN)	C	75	78	87	101	12.0	0.372	0.035
	L	66	69	76	85	14.0	0.550	0.636
	N	64	64	69	76	16.6	0.746	0.288
	H	62	65	71	85	12.3	0.597	0.592
	LN	62	64	68	74	12.2	0.172	0.060
	LH	66	70	73	82	12.0	0.160	0.196
	SEM[d]	12.7	9.7	9.7	17.3			
	P value	0.657	0.250	0.260	0.750			

[a]Total mixed ration (TMR) consists of 0.25 water bamboo sheath leaves, 0.05 alfalfa, 0.30 rice straw and 0.40 concentrate of fresh weight.
[b]LA, lactic acid; AA, acetic acid; WSC, water-soluble carbohydrate, AN, ammonia nitrogen; TN, total nitrogen.
[c]C, control; L, lactic acid bacteria; N, natamycin; LN, lactic acid bacteria and natamycin; H, hexanoic acid; LH, lactic acid bacteria hexanoic acid.
[d]SEM, standard error of the mean.
[e]L and Q, linear and quadratic effect of days after unloading.

https://doi.org/10.1017/S0021859624000054 Published online by Cambridge University Press

Table 4. Lactic acid bacteria, aerobic bacteria and yeast counts of TMR[a] silages after opening the silo

Items[b]	Treatments[c]	Day after unloading (d)				SEM[d]	Model construction *P*[e]	
		0	2	4	6		L	Q
LAB (log_{10} cfu/g FW)	C	7.1	6.8	5.6	4.8	0.77	0.012	<0.001
	L	8.4	8.0	7.3	6.3	0.19	0.230	0.015
	N	7.2	6.9	6.1	5.0	0.63	<0.001	<0.001
	H	7.0	6.9	5.7	5.1	0.16	0.004	<0.001
	LN	8.5	8.1	7.6	6.6	0.25	0.031	0.123
	LH	8.4	8.0	7.0	6.5	0.14	0.362	0.467
	SEM[d]	0.21	0.92	0.81	0.20			
	P value	<0.001	<0.001	0.130	<0.001			
Aerobic bacteria (log_{10} cfu/g FW)	C	5.7	6.1	6.7	7.3	0.39	<0.001	<0.001
	L	4.3	4.5	6.1	7.7	0.45	<0.001	0.452
	N	4.4	4.7	6.3	7.6	0.66	0.003	<0.001
	H	4.5	4.8	6.1	6.9	0.45	<0.001	<0.001
	LN	4.2	4.7	5.3	5.9	0.54	0.001	0.038
	LH	4.2	4.4	5.6	6.7	0.42	0.001	0.533
	SEM[d]	0.84	0.39	0.36	0.30			
	P value	<0.001	<0.001	<0.001	<0.001			
Yeasts (log_{10} cfu/g FW)	C	4.6	5.9	6.5	7.3	0.67	<0.001	<0.001
	L	4.2	4.3	5.1	6.1	0.27	0.001	0.473
	N	4.1	4.2	4.8	6.0	0.23	0.002	0.353
	H	4.0	4.2	5.1	6.5	0.43	<0.001	0.142
	LN	3.9	4.1	4.7	5.3	0.24	0.001	0.401
	LH	4.0	4.4	5.0	5.9	0.53	0.002	0.503
	SEM[d]	0.51	0.35	0.40	0.33			
	P value	<0.001	<0.001	<0.001	<0.001			

[a]Total mixed ration (TMR) consists of 0.25 water bamboo sheath leaves, 0.05 alfalfa, 0.30 rice straw and 0.40 concentrate of fresh weight.
[b]LAB, lactic acid bacteria; cfu: colony-forming unit.
[c]C, control; L, lactic acid bacteria; N, natamycin; LN, lactic acid bacteria + natamycin; H, hexanoic acid; LH, lactic acid bacteria + hexanoic acid.
[d]SEM, standard error of the mean.
[e]L and Q, linear and quadratic effect of days after unloading.

Compared with control, additives significantly ($P < 0.05$) increased GP_{24}, rate constant of GP, IVDMD, IVNDFD, IVADFD and ME, while only numerically ($P > 0.05$) increased asymptotic GP. On average, the GP_{24}, rate constant of GP, IVDMD, IVNDFD, IVADFD and asymptotic GP were followed by the LN, LH, H, N and L, except for ME.

Discussion

Chemical compositions and microbial populations of total mixed ration

A successful ensiling requested a low BC, adequate DM (300–400 g/kg FW) and WSC > 60 g/kg DM, and the epiphytic LAB count more than 1×10^5 cfu/g FW (Nkosi *et al.*, 2009). In this experiment, the WSC was 70.6 g/kg DM, and the LAB count was more than 1×10^5 cfu/g FW. However, the DM was 618 g/kg FW and the AB and yeast counts were more than 1×10^5 cfu/g FW, which implies the risk of poor fermentation quality and aerobic stability (Chen *et al.*, 2021). Therefore, the TMR would be difficult to obtain a high-quality TMR silage without the additives

Fermentation quality of total mixed ration

The important indicators of well-fermented silages included the high lactic acid content (>30 g/kg DM), the low pH (<4.2) and AN/TN (<100 g/kg DM) (Guo *et al.*, 2020). The pH of all TMR silages in this experiment was above 4.2; however, they were generally well-preserved as indicated by dominant lactic acid content, low AN/TN and trace propionic acid contents. The high pH might be related to the high DM content in this experiment.

When TMR had high DM content, the epiphytic LAB had insufficient water activity, and the fermentation is curtailed due to the lack of metabolic moisture for LAB growth (Wang *et al.*, 2019). Similar to the previous study, Qiu *et al.* (2014) reported

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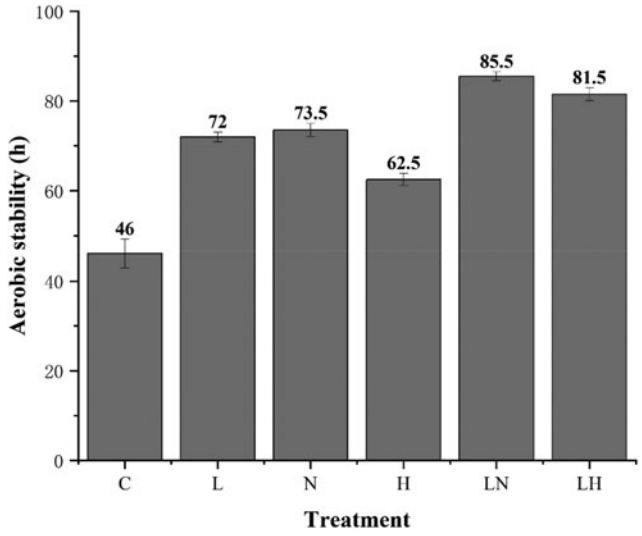

Figure 1. Effect of additives on the aerobic stability of TMR silages.C, control; L, lactic acid bacteria; N, natamycin; LN, lactic acid bacteria + natamycin; H, hexanoic acid; LH, lactic acid bacteria + hexanoic acid.

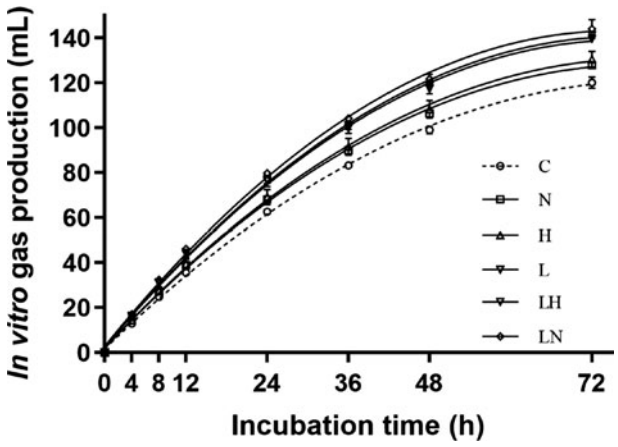

Figure 2. Gas production profiles (ml/g DM) from *in vitro* fermentation of TMR silages for 72 h (bars indicate standard errors of the means).C, control; L, lactic acid bacteria; N, natamycin; LN, lactic acid bacteria + natamycin; H, hexanoic acid; LH, lactic acid bacteria + hexanoic acid.

that the TMR with high DM content (545 g/kg FW) could be well-preserved (pH 4.76).

Additives improved the fermentation quality, indicated by the higher LA and AA content, and lower pH compared with control. This might be attributed to adding LAB, natamycin and hexanoic acid had positive effects on increasing LA contents and reducing pH. The purpose of inoculating LAB is to make exogenous LAB predominant in epiphytic microbes, and to stimulate the efficient conversion of WSC into LA (Chen *et al.*, 2017). It has been found that natamycin and hexanoic acid have the potential for antimicrobial properties (Chen *et al.*, 2017; Mugabe *et al.*, 2020; Shah *et al.*, 2020), and Yuan *et al.* (2022) confirmed that they had a strong inhibiting effect on undesirable microorganisms, and the application of natamycin and hexanoic acid is favourable in improving fermentation quality.

The L, LN and LH had significantly ($P < 0.05$) higher LA contents and LA/AA than that in other treatments, which may result in stimulating homolactic fermentation and producing more LA resulting from the exogenous *L. plantarum*; this was in agreement with the finding of Guo *et al.* (2020) who reported that the LAB inoculums accelerated the accumulation of LA. The fact that LH and LN had higher LA content and LA/AA than other treatments might be related to the beneficial synergistic effect between the *L. plantarum* with hexanoic acid and natamycin; it has been demonstrated that a combination of *L. plantarum* with natamycin and hexanoic acid is more effective in improving fermentation than an inoculum used on its own (Mugabe *et al.*, 2019; Wang *et al.*, 2022).

In the experiment, propionic acid was detected at trace level, and no significant ($P > 0.05$) difference was observed among all TMR silages. This was probably that the activity of undesirable microorganisms might be inhibited due to the high DM. Konig *et al.* (2019) reported that the high DM silage (>300 g/kg FW) was negligible of propionic acid.

The NH_3-N was identified as the degree of protein degradation during the ensiling (Fijałkowska *et al.*, 2015). The protein hydrolysis is mainly caused by plant enzymes and undesirable microorganism (clostridia and enterobacteria) before the pH drop to below 5 (Muck *et al.*, 2018). In the experiment, the AN/TN in all TMR silages was less than 100 g/kg TN, which indicated less proteolysis occurred during ensiling. This was probably

Table 5. *In vitro* gas production parameters, *in vitro* digestibility of TMR[a] silages

	Treatments[c]							
Items[b]	C	L	N	H	LN	LH	SEM[d]	*P* value
In vitro gas production kinetics								
GP_{24} (mL/g DM)	64	68	70	77	87	86	2.1	<0.001
Asymptotic GP (mL/g DM)	150	156	160	166	169	168	5.1	0.093
Fractional fermentation rate (mL/h)	0.02	0.02	0.02	0.03	0.03	0.03	0.028	0.004
In vitro rumen digestibility								
IVDMD	0.5	0.5	0.6	0.6	0.6	0.6	0.10	<0.001
IVNDFD	0.47	0.48	0.50	0.51	0.55	0.56	0.091	<0.001
IVADFD	0.26	0.27	0.31	0.33	0.48	0.45	0.023	<0.001
ME (MJ/kg DM)	12.2	12.9	13.2	14.0	14.5	13.9	0.20	<0.001

[a]Total mixed ration (TMR) consists of 0.25 water bamboo sheath leaves, 0.05 alfalfa, 0.30 rice straw and 0.40 concentrate.
[b]GP_{24}, 24 h cumulative gas production; asymptotic GP, asymptotic gas production; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fibre digestibility; IVADFD, *in vitro* neutral detergent digestibility; ME, metabolizable energy.
[c]C, control; L, lactic acid bacteria; N, natamycin; LN, lactic acid bacteria + natamycin; H, hexanoic acid; LH, lactic acid bacteria + hexanoic acid.
[d]SEM, standard error of the mean.

https://doi.org/10.1017/S0021859624000054 Published online by Cambridge University Press

because the high DM suppressed the activity of undesirable microorganisms, resulting in inhibition of the deamination of amino acids.

TMR silages had high residual WSC content, and might be attributed to the fact that high DM condition (or low water activity) of TMR inhibited the activities of undesirable microbes as well LAB in silage (Zhao *et al*., 2021a, 2021b). Compared to the control, additives increased WSC content and reduced ethanol content. This may be related to the antifungal properties of natamycin and hexanoic acid, inhibiting the WSC consumption by undesirable microorganisms, which is proved by the lower AB and yeast counts. te Welscher *et al*. (2008) demonstrated that natamycin obstructed the ergosterol synthesis, blocking the growth and the function of the cell membranes of AB and yeast. Hexanoic acid can inhibit bacteria by altering the permeability of the membrane, which results that the bacteria can no longer regulate intracellular pH followed by cell lysis. Additionally, the hexanoic acid may exert its bacteriostatic activity by stimulating ATPase activity as found with mitochondria (Galbraith and Miller, 1973).

As expected, the addition of LAB (L, LN, LH) resulted in higher LAB counts, indicating a well adaption and establishment of exogenous *L. plantarum* in TMR silages (Zhao *et al*., 2021a, 2021b). The higher LAB counts and lower AB and yeast counts of LN and LH silages than that of other treatments demonstrated that the LAB combined with natamycin and hexanoic acid had a synergistic effect on reducing undesirable microorganism activity (Pinto *et al*., 2020).

Aerobic stability

When silages are exposed to the air, the WSC, organic acids and other substrates would be oxidized by AB into carbon dioxide and water, and release excessive heat, resulting in an increase in temperature and a deterioration of the silage (Wilkinson and Davies, 2013). Herein, aerobic deterioration was defined as the time (h) elapsed when the silage temperature is 2°C above the ambient temperature.

The pH gradually increased and LA and AA contents gradually decreased in all TMR silages during aerobic exposure, which mainly attributed to the catabolism of lactic acid and acetic acid by aerobic microbes, which would contribute to a rise in pH. When the air infiltrates, the aerobic microbes (lactic acid assimilating yeasts) begin to proliferate, which can metabolize lactic acid that leads to rise in pH and temperature of silage, providing better conditions for multiplication of AB and moulds begin to grow and further aggravate the spoilage (Kleinschmit *et al*., 2005).

The AN/TN in all TMR silages increased gradually; it is mainly produced by microbial metabolism rather than plant protease activity (Mugabe *et al*., 2020; Wang *et al*., 2022).

The silages treated with additives resulted in more stability than the control during aerobic exposure, and this was confirmed by the higher LA, AA and WSC contents and LAB count, and the lower pH, AN/TN, the counts of AB and yeast. The antimicrobial activity of the bacteriostatic action of added natamycin and hexanoic acid, which mechanism of action involves the formation of ethyl esters and hydroperoxides, cell membrane disruption by lipid peroxidation results in oxidative stress of cell (Mugabe *et al*., 2019). The highest acetic acid, lactic acid contents as well as lowest pH in LN silage were attributed to a positive synergetic effect in bacteriostatic and antimicrobial activity of *L. plantarum* and natamycin (Chen *et al*., 2017; Mugabe *et al*., 2020). LN and LH silages had higher WSC content and lower AN/TN was attributed to the positive synergistic effect of combining natamycin and hexanoic acid with *L. plantarum* in restricting proteolysis and reducing WSC metabolism by undesirable microorganisms.

MacDonald *et al*. (1991) reported that yeasts are always primarily responsible for aerobic deterioration, and silages are highly prone to deterioration when yeast counts are more than 10^5 cfu/g FW. The yeast counts increased with time after silo opening, and grew much faster because it could obtain energy via the glycolysis and citric acid cycle and electron transport chain under aerobic respiration (Reddy *et al*., 2015). Additionally, the growth of yeasts occurs mainly at a pH higher than 4.5, and the high pH may have favoured the accelerated development of yeasts, detrimental to silage preservation (Junior *et al*., 2021).

The lower AB and yeast count in LN and LH was observed than that in other treatments, and it was attributed to the positive synergetic effect of *L. plantarum* with natamycin and hexanoic acid in inhibiting undesirable microorganisms.

LN and LH prolonged aerobic stability by about 86% (up to 85.5 h) and 77% (up to 81.5 h) compared with C (46 h), respectively, and both showed higher aerobic stability than that in other treatments. These can be explained by the combination of *L. plantarum* with natamycin and hexanoic acid led to a synergistic effect, ensuring benefits when compared to the usage of a single additive. This is an important result because it demonstrates the feasibility of prolonged aerobic stability time with a combination of additives (Pinto *et al*., 2020).

In vitro *digestibility of total mixed ration silages*

In vitro GP is a valuable indicator to evaluate the rumen degradability and the ME of animal feed (Wang *et al*., 2019; Guo *et al*., 2020). Additives increased GP_{24}, asymptotic GP and fractional fermentation rate of TMR silages; this results from the reduction in losses of TMR silages, providing sufficient energy and substrates for microbial degradation in the rumen (Chen *et al*., 2019; Dai *et al*., 2022). *In vitro* DM digestibility is widely used to evaluate feed nutritional value and intake (Dai *et al*., 2022). Additives had higher IVDMD, IVNDFD, IVADFD and ME as compared with the control; this might be related to the additives beneficial to DM and nutritional substrates preservation, especially CP and WSC, which were essential substrates for rumen microbial degradation (Guo *et al*., 2020). Furthermore, the GP_{24}, rate constant of GP, IVDMD, IVNDFD, IVADFD and asymptotic GP in LH and LN were higher than that in other treatments, which suggested the positive synergistic effect of combining *L. plantarum* with natamycin and hexanoic acid in inhibiting DM and nutritional substrates loss, and consequently providing more nutritional residual for rumen microbial during the *in vitro* incubation.

Conclusion

All TMR silages prepared with water bamboo sheath leaves were well-preserved. Additives improved fermentation quality, aerobic stability and *in vitro* digestibility of TMR silages. *Lactobacillus plantarum* is compatible with natamycin and hexanoic acid, and the combinations of lactobacilli with chemical additives seem to result in a synergistic effect. In particular, mix of *L. plantarum* and natamycin is recommended for silage making of TMR containing water bamboo sheath leaves.

https://doi.org/10.1017/S0021859624000054 Published online by Cambridge University Press

Author contributions. Tao Shao conceived and designed the study. Xinbao Li, Haopeng Liu, Tongtong Dai and Dong Dong conducted data gathering. Xinbao Li, Guofeng Xu, Haopeng Liu and Yushan Jia performed statistical analyses. Xinbao Li wrote the article.

Funding support. Research on Key Technologies of Digestion of Anti-nutrient Factors and Palatability Improvement of Forage, Grant/Award Number: 2021YFD1300302; Major Science and Technology Special Project of Tibet-Technology Mode Innovation and Demonstration of Spatio-temporal Expansion of Tibet Prataculture, Grant/Award Number XZ202101ZD0003N

Competing interests. None.

Ethical standards. The procedures of animal experiment were approved by the Ethics Committee of Nanjing Agricultural University.

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