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Effect of Ammonium Chloride on Fermentation Quality and Bacterial Community of Distillers Grains Silage

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Key words
Ammonium chloride, Distiller grains, Ammonium-N, Bacterial composition dynamics, Volatile fatty acids

ABSTRACT

The distillers grains (DGS) have high fiber, protein, and vitamins and are primarily fed to ruminants for maintenance and production. This study was conducted to investigate the effects of ammonium chloride on the fermentation quality and microbial dynamics of two different distiller grains (DGS), originating from Wuliangye and Moutai. The two kinds of DGS were treated with 0.3% N ammonium chloride and sampled on d 3, 7, 14, 30, and 60 after ensiling. HPLC and 16s rRNA platform were used to determine the volatile fatty acid (VFA) content and microbial composition. The results of the current study showed that ammonium chloride increased the yield of lactic acid and reduced the level of ammonium-N at d 14 and 30 in Wuliangye and Moutai DGS, respectively. Acetic and propionic acids increased with time in the two kinds of DGS. In addition, ammonium chloride decreased microbial α adversity, such as the observed species and Shannon index; the abundance of *Lactobacillus* was increased, whereas the abundance of *Acetobacter* was reduced. Ammonium chloride could be used as a useful DGS preservative, however, different DGS reaches its stable period is different.

INTRODUCTION

In China, the Chinese Baijiu distillers grains (DGS) production is 1,000,000t annually (Liu *et al*., 2022). There are numerous utilization strategies for DGS, including feeding, high-value component extraction, biogas generation, and composting (Liu *et al*., 2022). The DGS have high fiber content (neutral detergent fiber content of 50%-65%) and have been fed primarily to ruminants (Beretta *et al*., 2021). In addition, DGS also have high protein and vitamin content because of which the DGS have a very high price-performance ratio, and the market price is rising yearly. However, because of 60%-65% moisture content in DGS, it is most often used locally. DGS are now being used as the main dietary ingredient of beef cattle, especially in the southwest of China. The production of Baijiu is seasonal, particularly in the summer, because of the high temperature and humidity, which usually cannot produce Baijiu. DGS with high moisture content is vulnerable to rapid spoilage by the action of bacteria, yeasts, and molds. Therefore, the DGS must be properly stored and used in the absence of Baijiu production.

Numerous feed and chemical additives are used for the fermentation of the silage (Chen *et al*., 2021). Nofsinger *et al*. (1983) reported that the addition of sorbic acid, potassium propionate, calcium hydroxide, and ammonium hydroxide could improve DGS silage quality. In high moisture content corns, ammonia has been used to reduce the moisture content and also in barley silage (Song and Kennelly, 1989), and moderate concentrations of

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ammonia upsurge levels of acetic and lactic acids (Muck and Kung, 1997), reduced proteolysis (Huber *et al*., 1979), and enriched the aerobic corn silage stability. Reports also showed that silage prepared from DGS with Napier grass could promote and improve fermentation (Chiou *et al*., 2000). In addition, ammonium chloride can inhibit the growth of molds and yeasts (Pernak and Chwała, 2003; Ito *et al*., 2019). Ammonium chloride, as an anionic salt, can improve the calcium metabolism of cows during the perinatal period (Wang and Beede, 1992). Ammonia treatment in cottonseed meal has been approved by FDA to use in specific quantities in feed additives. However, till now, no research focusing on the effect of adding ammonium chloride on DGS dynamics of microbial population and fermentation quality exists.

It is hypothesized that the addition of ammonium chloride in the process of DGS storage would result in improved feed quality, though the reaction of different DGS to ammonium chloride treatment would be different. Therefore, the two most representative Baijiu DGS in China viz. W (Wuliangye) and M (Moutai) to examine the effects of ammonium chloride on the fermentation quality and microbial flora variation dynamics of DGS silage, which can provide technical and theoretical support for DGS storage.

MATERIALS AND METHODS

Raw materials and silage preparation

Fresh W and M DGS were manually collected on January 12, 2018, from the Wuliangye Yibin Co., Ltd. (Yibin City, Sichuan Province) and Kweichow Moutai Co., Ltd. (Huairen City, Kweichou Province). Immediately, these fresh materials were then transported to the Chengdu campus of Sichuan Agricultural University. DGS were distributed to the following ensiling treatments, i.e., without or with ammonium chloride (0.3% N), which were mentioned as -A and +A. In particular, a plastic silo bag measuring 10 cm by 30 cm was filled with about 300 g of fresh materials and vacuum sealed. This amount was determined by the small silo bag's capacity and the sample required. A total of 80 bags (2 kinds of DGS × 2 treatments × 4 replicates × 5-time points) were prepared and stored at ambient temperature (17–28°C). Bacterial populations, fermentation traits, and protein fractions were examined after 3, 7, 14, 30, and 60 d of fermentation.

Chemical composition and microbial population analysis

To determine the DGS's dry matter (DM) content, a dried air oven at 65°C for 48 h was used and ground to pass 1.0 mm screen for chemical analysis (AOAC, 1990). The methods of AOAC (1990) were used to determine the protein fractions, such as crude protein, true protein, non-protein nitrogen, while Van Soet *et al*. (1991) procedure was adopted to analyze the neutral detergent fiber, acid detergent fiber, and Murphy (1958) protocol was used to determine the water-soluble carbohydrates.

After ensiling, the DGS sample (20 g) was added to 180 mL of sterile water, suspended at 4 °C overnight, and filtered through four layers of cheesecloth to measure fermentation parameters. The pH, ammonia-N, and organic acids were measured in the filtrate. The Broderick and Kang (1980) technique was used to determine the ammonia-N content. High-performance liquid chromatography was used to analyze the organic acids (lactic acid, acetic acid, propionic acid, and butyric acid) in the conditions specified by Wang *et al*. (2019). Approximately 20 g of DGS samples were combined with 180 mL of sterilized saline water for the microbial population analysis, and the mixture was serially diluted. After two d of anaerobic incubation at 30 °C, lactic acid bacteria (LAB) was counted on de Man, Rogosa, Sharpe (MRS) agar. After two d of aerobic incubation at 28 °C, yeasts and molds were counted on Rose Bengal Agar. After being cultured at 30 °C for two d, coliforms were counted on Violet Red Bile Agar. Colonies were measured as the number of colony-forming units (CFU) per gram of fresh matter (FM) that were viable.

Microbial diversity analysis

Total genomic DNA was extracted from DGS samples in accordance with the directions on the DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA) packaging. After extraction, DNA concentration, and purity were determined using a NanoDrop ND-1000 spectrophotometer (Nyxor Pharmacia, Paris, France), and DNA integrity was confirmed using 0.8% agglutinate gel electrophoresis. Before being employed as templates for real-time PCR and Illumina sequencing analysis, all extracted DNA samples were stored at -20°C. The V4 variable of the 16s rRNA genes amplified a template, the entire DNA of the distillers grains. For the bacteria PCR (Caporaso *et al*., 2011), the universal primer set 515F and 806R was used. The PCR product was then purified using an OMEGA Gel Extraction Kit from Omega Bio-Tek in the United States. The library quality was evaluated using a Qubit@ 2.0 Fluorometer from Thermo Scientific and an Agilent Bioanalyzer 2100 instrument. The Hiseq Illumina Sequencing Platform (Rhonin Biosciences Co., Ltd., Chengdu, China) was then used to pair-end sequence the pooled amplicons (2250 bp). Using FLASH, paired-end readings from the original DNA fragments were combined, and each sample was allocated according to its barcode. Using Uchime, the sequence was checked for chimmeras (Edgar *et al*., 2011). The UPARSE method was

used to cluster the sequences into OTUs at a 97% identity criterion. Silva database was used to assign taxonomies, and PyNAST was used to align the sample sequences. Vegan (Version 2.0-2.R CRAN packet) was used to study alpha, which includes computing the observed species, Chao 1, Shannon, and Simpson indices (Kembel *et al*., 2010). The significance of differences between samples was evaluated using principal component analysis (PCoA).

Statistical analysis

The data were analyzed using a two-way analysis of variance, the effects of ensiling days, ammonium chloride addition, and the relationship between them were examined. The significance threshold was set at $P < 0.05$. SAS 9.3 software (SAS Institute Inc., Cary, NC, USA) was used for all statistical operations. The DNA sequencing data were analyzed on a free online platform called OmicShare tools (http://www.omicshare.com/tools).

RESULTS AND DISCUSSION

Chemical and microbial composition of distillers grains before ensiling

The chemical and microbial compositions of the two kinds of DGS before ensiling are presented in Table I, and W and M are the two most famous Baijiu in China, however, their brewing processes are entirely different (Xu *et al*., 2021). The raw materials used for the two kinds of Baijiu production are also different (Liu *et al*., 2022). In W preparation, rice, wheat, corn, sorghum, and glutinous rice are fermentation substances, and rice husk is added later to distill ethanol, whereas in the M preparation, sorghum is used only.

Both M and W DGS have low pH, with 3.58 and 3.92, respectively, resulting from the Baijiu fermentation products. This low pH further decreases dry matter intake (DMI) and deepens rumen subacute acidosis when DGS is supplemented in fresh conditions (Watson *et al*., 2014; McDaniel *et al*., 2021). The two kinds of DGS have similar dry matter (DM) content (913 g/kg DM vs. 899 g/kg DM). The crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents of the two DGS were 135~224 g/kg DM, 434~535 g/kg DM, and 268~366 g/kg DM, close to the data summarized by Liu *et al*. (2022). The CP content of W DGS was lower, whereas the NDF and ADF were higher than that of M DGS, because rice husk was added in its later brewing process (Liu *et al*., 2022). The non-protein-N proportion of W DGS was lower than that of M (33.9 g/kg DM vs. 75.1 g/kg DM); this might occur due to its higher CP content of M DGS. In terms of ether extract (EE), because in the brewing process, starch was fermented to produce ethanol, the content of EE in DGS (33.9~61.1 g/kg DM) was higher than its raw grains. The water soluble carbohydrate (WSC) content of both DGS was above 50 g/kg DM, exceeding the minimum requirement for successful silage fermentation quality (Ni *et al*., 2018). This means that the two kinds of DGS can be preserved well, though it was reported that the DGS could be stored better in a mixed condition with other by-products (Mjoun *et al*., 2011; Gunn *et al*., 2013).

Table I. Chemical and microbial composition of W and M DGS before ensiling.

Item	W	M
Chemical composition		
pH	3.58	3.92
DM (%)	913	899
Crude protein (g/kg DM)	135	224
Nonprotein-N (g/kg DM)	33.9	75.1
True protein (g/kg DM)	101	149
Ether extract (g/kg DM)	33.9	61.1
Neutral detergent fiber (g/kg DM)	535	434
Acid detergent fiber (g/kg DM)	366	268
Acid detergent lignin (g/kg DM)	152	99
Water soluable carbohydrate (g/kg DM)	87.5	55.8
Microorganism (CFU/g of FM)		
Lactic acid bacteria (log_{10} CFU/g FM)	4.87	4.33
Molds (log_{10} CFU/g FM)	<2.00	<2.00
Yeasts (log_{10} CFU/g FM)	4.28	4.74
Coliform bacteria (log_{10} CFU/g FM)	4.64	5.35

DM, dry matter; FM, fresh matter; W, Wuliangye; M, Moutai; CFU, colony-forming unit. Data are means of samples determined in triplicate.

The minimum lactic acid bacteria (LAB) requirement for good fermentation is 5 log_{10} CFU/g FM (Cai *et al*., 1998); however, the LAB of the two kinds of DGS used in our experiment were 4.87 and 4.33 log_{10} CFU/g FM, which was lower than the minimum requirement. Therefore, additives are essential for the storage of DGS. It was reported that the low pH of the DGS was not suitable for the growth of LAB (Saarisalo *et al*., 2007); thus, the weak acidic ammonium chloride buffer solution was added, trying to create an environment suitable for the growth of LAB. The undesirable microorganism yeasts and coliforms were detected in the fresh material of DGS, and ranged from 4.28-5.35 log_{10} CFU/g FM. The brewing process is high-temperature sterilization, and the two kinds of DGS were relatively fresh, so the molds belowed the detectable limitation. After processing, the distiller's grains are

Table II. Organic acids content, pH, protein fractions and microbial population of W DGS silage.

Item	Treat-ment	Ensiling days					Mean	SEM	P-value		
		3	7	14	30	60			A	T	A×T
Organic acids (g/kg DM)											
pH	-A	3.59aB	3.52abB	3.43b	3.40b	3.68aA	3.52ab	0.150	0.094	0.035	0.225
	+A	3.98aA	3.79abA	3.32b	3.30b	3.33bB	3.54ab				
Lactic acid	-A	45.44c	51.22b	72.57aB	60.44abB	46.86bcB	55.31ab	7.695	<0.001	<0.001	<0.001
	+A	46.77c	53.13bc	79.69aA	67.53abA	64.35bA	62.29b				
Acetic acid	-A	18.33d	22.56c	35.34abA	37.28abA	40.17aA	30.74b	3.763	<0.001	<0.001	<0.001
	+A	17.67c	21.89bc	28.77bB	30.43abB	34.32aB	26.81b				
Propionic acid	-A	ND	ND	ND	ND	ND	-	-	-	-	-
	+A	ND	ND	ND	ND	ND	-	-	-	-	-
Butyric acid	-A	ND	ND	ND	ND	ND	-	-	-	-	-
	+A	ND	ND	ND	ND	ND	-	-	-	-	-
Protein fractions											
Crude protein (g/kg DM)	-A	135	139	135	134	138	136	1.941	0.669	0.769	0.685
	+A	138	137	134	141	140	138				
True protein (g/kg TN)	-A	692a	656ab	613b	571bcB	533cB	613bB	50.024	0.024	0.032	0.043
	+A	695a	678a	632ab	610bA	591cA	641abA				
Nonprotein-N (g/kg TN)	-A	308c	344bcA	387b	429abA	467aA	387bA	51.226	0.015	0.033	0.158
	+A	305c	322bcB	368ab	390abB	409aB	347bB				
Ammonia-N (g/kg TN)	-A	48.4c	51.1bc	57.4b	66.6aA	69.4aA	58.6b	3.786	<0.001	0.022	0.031
	+A	50.2c	52.4bc	55.8b	62.2aB	64.6aB	57.0b				
WSC (g/kg DM)	-A	82.2a	74.3ab	55.8bA	42.5cA	40.6cA	57.2b	7.454	0.023	<0.001	0.244
	+A	81.5a	70.4ab	49.5bB	37.5cB	34.8cB	53.2b				
Microbial population (log_{10} CFU/g FM)											
Lactic acid bacteria	-A	4.35c	6.56b	7.68aB	6.32bB	6.44bB	6.44b	0.954	<0.001	<0.001	<0.001
	+A	4.54d	6.88c	9.57aA	7.67bA	7.85bA	7.32b				
Molds	-A	<2.00	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-
	+A	<2.00	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-
Yeasts	-A	4.11	3.58	<2.00	<2.00	<2.00	-	-	-	-	-
	+A	4.02	3.14	<2.00	<2.00	<2.00	-	-	-	-	-
Coliform bacteria	-A	3.14	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-
	+A	2.69	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-

-A, without ammonium chloride; +A, with ammonium chloride; FM, fresh matter; DM, dry matter; CFU: colony-forming unit; ND, not detected; –, default; D, ensiling days; T, treatment; D×T, interaction of ensiling days and treatment; SEM, standard error of means. Means with different letters in the same row (a-c) or column (A-B) differ ($P < 0.05$).

essentially sterile, although they may still contain some residual yeast. If DGS were contaminated with 2 log_{10} CFU/g FM, when DGS cools down, the yeast population could increase to more than 5 log_{10} CFU/g FM in less than 10 h (Kung *et al.*, 2000). The transportation of the two kinds of DGS from the winery to our laboratory was about 6 h, and this might account for the yeasts observed in the present experiment.

The fermentation parameters of W and M DGS

The dynamics of organic acids, pH, protein fractions, and microbial populations of W and M DGS during ensiling are presented in Tables II and III. Ammonium chloride addition and time significantly affected the lactic acid, acetic acid, ammonium-N, and LAB of the two kinds of DGS. An interaction effect between ammonium chloride and time was observed. The pH of W DGS of both

Table III. Organic acids content, pH, protein fractions, and microbial population of M DGS silage.

Item	Treat-ment	Ensiling days					Mean	SEM	P-value		
		3	7	14	30	60			A	T	A×T
Organic acids (g/kg DM)											
pH	-A	3.86aB	3.72abB	3.62abB	3.45b	3.64bA	3.60ab	0.167	0.432	0.046	0.324
	+A	4.19aA	3.94abA	3.78abA	3.44b	3.39bB	3.58ab				
Lactic acid	-A	37.82c	43.54b	52.28ab	59.46aB	42.83bB	47.19b	8.796	<0.001	<0.001	<0.001
	+A	39.23c	41.42c	53.72b	66.68aA	63.56abA	52.92b				
Acetic acid	-A	13.97c	19.02bA	29.64abA	35.31aA	37.69aA	27.13abA	5.204	<0.001	<0.001	<0.001
	+A	12.78b	14.95bB	20.57abB	24.32aB	26.25aB	19.76abB				
Propionic acid	-A	ND	ND	ND	ND	ND	-	-	-	-	-
	+A	ND	ND	ND	ND	ND	-	-	-	-	-
Butyric acid	-A	ND	ND	ND	ND	ND	-	-	-	-	-
	+A	ND	ND	ND	ND	ND	-	-	-	-	-
Protein fractions											
Crude protein (g/kg DM)	-A	227	229	234	226	221	227	5.667	0.648	0.742	0.844
	+A	230	232	226	223	222	226				
True protein (g/kg TN)	-A	648a	627ab	586bB	553bcB	547cB	592bB	40.243	0.221	<0.001	0.332
	+A	651a	644a	638abA	617bA	615bA	634abA				
Nonprotein-N (g/kg TN)	-A	352c	373b	414abA	447aA	453aA	400abA	32.457	0.436	<0.001	0.463
	+A	349b	356b	362abB	383aB	385aB	367ab				
Ammonia-N (g/kg TN)	-A	76.5b	78.2b	83.2ab	95.6a	102.6aA	87.2ab	6.476	<0.001	<0.001	<0.001
	+A	79.8b	83.5ab	86.4ab	94.8a	97.5aB	88.4ab				
WSC (g/kg DM)	-A	53.3a	48.7ab	32.3b	26.2bc	23.8c	36.9b	11.246	0.335	<0.001	0.442
	+A	52.4a	45.6ab	30.2b	25.5bc	23.2c	35.4ab				
Microbial population (log_{10} CFU/g FM)											
Lactic acid bacteria	-A	4.89b	6.56ab	7.33a	7.56aB	7.12abB	6.69b	1.362	<0.001	<0.001	<0.001
	+A	5.12c	6.65b	7.53ab	9.62aA	8.34aA	7.25ab				
Molds	-A	<2.00	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-
	+A	<2.00	<2.00	<2.00	<2.00	<2.00	-	-	-	-	-
Yeasts	-A	4.65	4.03	3.02	<2.00	<2.00	-	-	-	-	-
	+A	4.15	3.67	2.56	<2.00	<2.00	-	-	-	-	-
Coliform bacteria	-A	5.02	3.78	<2.00	<2.00	<2.00	-	-	-	-	-
	+A	4.84	2.66	<2.00	<2.00	<2.00	-	-	-	-	-

-A, without ammonium chloride; +A, with ammonium chloride; FM, fresh matter; DM, dry matter; CFU, colony-forming unit; ND, not detected; –, default; D, ensiling days; T, treatment; D×T, interaction of ensiling days and treatment; SEM, standard error of means. Means with different letters in the same row (a-c) or column (A-B) differ ($P < 0.05$).

ammonium chloride treated and not treated decreased, the lowest pH was observed on d 30 after ensiling ($P = 0.035$). On d 3 and 7, the pH of ammonium chloride-treated DGS was higher than the control group ($P < 0.05$). However, on d 60, the pH of ammonium chloride-treated DGS was lower than the control group ($P < 0.05$). As for the M DGS, the pH of ammonium chloride treated decreased all the time after ensiling, whereas the control decreased first and then increased. The pH of ammonium chloride treated group was higher on d 3, 7, and 14, but was lower than the control group on d 60 ($P < 0.05$). pH is the simplest and most direct parameter to evaluate silage fermentation extent and quality. Usually, a pH lower than 4.2 was necessary for the silage to be well preserved (Webster, 2008). In the

present study, the pH value of the two kinds of DGS at the beginning of the experiment was very low (3.58 vs. 3.92). Therefore, the method to preserve DGS should be different from that of making other silages. Making corn or Italian ryegrass silages requires a rapid reduction in pH by adding LAB (Wang *et al*., 2019; Yan *et al*., 2019). In contrast, to create a better growth condition for LAB, a weak acidic buffer solution was adopted to promote the pH value of the DGS.

Furthermore, the lactic acid content of both ammonium chloride treated and not treated W and M DGS increased first and then decreased, and the highest values were observed on d 14 and 30 after ensiling, respectively. In addition, the lactic acid content of ammonium chloride treated W and DGS was higher than that of control on d 14, 30 and 60 ($P < 0.05$), whereas it was higher than the control on d 30 and 60 in M DGS ($P < 0.05$). The acetic acid content increased all the time during the 60 d of ensiling in both W and M DGS, and the acetic acid content of ammonium chloride treated W DGS was lower than that of the control group on d 14, 30, and 60 ($P < 0.05$), but it was lower than the control group on d 7, 14, 30 and 60 in M DGS after ensiling ($P < 0.05$). With the extension of ensiling time, the lactic acid and acetic acid contents were increased in alfalfa (Dong *et al*., 2020b) and whole-plant corn with bamboo shoot shell silage (Zhao *et al*., 2020). It has been reported that heterotypic fermentation lactic acid bacteria can further ferment lactic acid as a substrate to produce acetic acid with the progress of silage time (Parvin and Nishino, 2009). This might lead to the accumulation of acetic acid. The propionic acid and butyric acid were undetectable in both W and M DGS in the present experiment. Propionic and butyric acids are mainly the products of *Clostridium* (Ferrero *et al*., 2019; Chen *et al*., 2020), and *Clostridium* are less acid-tolerant, whose suitable growth conditions are at pH 5-6 (Webster, 2008). In the present study, the pH never matched this condition, therefore, no propionic acid and butyric acid were detected. This also showed that the silage quality of the two kinds of DGS was good under the present experimental condition.

The true protein fraction decreased, whereas the non-protein-N fraction and the ammonium-N increased in both W and M DGS with the progress of ensiling. In addition, the ammonium-N content of the ammonium chloride treated group was lower than the control on d 30 and 60 in W DGS ($P < 0.05$) and on d 60 in M DGS ($P < 0.05$). In almost all silage processes, some crude protein can be converted into non-protein-N, which has less bioavailability for animals (Webster, 2008). In the transformation process, crude protein is usually degraded into peptides and amino acids first, then further degraded into ammonia, amine, and other end products under the action of some undesirable microorganisms such as *Enterobacter* (Yuan *et al*., 2017). The ammonium-N in the ammonium chloride treated group in the two kinds of DGS was lower than that of the control group at 60 d after ensiling ($P < 0.05$), indicating that *Enterobacter* may be better inhibited because it was reported that *Enterbacter* could produce ammonium-N by fermentation with amino acids as substrate (Kung *et al*., 2018; Chen *et al*., 2021). Moreover, it has been reported that the growth of *Enterobacter* will be inhibited when the pH is lower than 4.35 (Dong *et al*., 2020a). The above results implied that adding ammonium chloride could help preserve feedstuff protein fraction better.

The WSC content of both ammonium chloride treated and not treated decreased with prolonged ensiling time in both W and M DGS and stopped decreasing after 30 d of ensiling. This phenomenon was similar to the reported high-moisture corn stover silage (He *et al*., 2020). The WSC is the fermentation substrate of LAB and *Enterobacter*, whose main end products are lactic acid and acetic acid. The WSC content of both W and M DGS is over 5% and is enough to support the growth of LAB (Ni *et al*., 2018), therefore, the WSC decreased first. However, when the pH continued to decrease, the LAB and *Enterobacter* stopped growing, and then the WSC content kept stable. The present study observed increased lactic acid and acetic acid with decreased WSC.

The LAB increased first and peaked on d 14 after ensiling and then decreased in W DGS, but it peaked on d 30 in M DGS and then decreased. This might be due to the difference in the chemical and bacterial composition of the two DGS (Liu *et al*., 2022). The WSC content of W DGS was higher than that of M (88.5 vs 55.8 g/kg DM). Thus, LAB grew faster in W than in M DGS. On d 14, 30, and 60, the LAB of the ammonium chloride treated group was higher than that of the control group in W DGS ($P < 0.05$), but it was higher than the control group in M DGS only on d 30 and 60 ($P < 0.05$). This suggested that adding ammonium chloride was more conducive to the growth of LAB. Chen *et al*. (2020) reported that *Clostridium* could degrade lactic acid into acetic acid. This might account for the decrease of lactic acid and increase of acetic acid after 30 d of ensiling. Different from bacteria, yeast has very strong acid resistance. Therefore, yeast could be detected even if the pH was always below 4.0. Some yeast could survive under a pH value below 2.0 (Kung *et al*., 2018). In the present study, yeasts were detected on d 3 and 7 in W DGS, and on d 3, 7, and 14 in M DGS after ensiling. Molds were not detected completely after ensiling in both DGS. This might be attributed to the short transportation time (6 h) from the winery to the university and the low pH of the two DGS. Previous studies have shown that adding ammonium hydroxide could inhibit mold growth in corn

silage (Kung *et al*., 2000). After ensiling, coliform bacteria were only detected on d 3 in W DGS and d 3 and 7 in M DGS. The decrease of pH with prolonged ensiling time might be responsible for this.

Table IV. Alpha diversity of bacterial community after 3, 7, 14, 30 and 60 days of ensiling.

Item	Groups	Ensiling days				
		3	7	14	30	60
OTUs	WC	417	489	425	387	382
	WN	302	407	370	350	348
Observed species	WC	190	249	233	208	184
	WN	144	190	185	170	170
Chao1	WC	219	248	210	224	228
	WN	284	278	243	220	229
Shannon	WC	3.71	3.92	3.47	3.27	2.75
	WN	3.14	3.50	3.80	3.60	2.94
Simpson	WC	0.94	0.91	0.84	0.93	0.89
	WN	0.85	0.94	0.95	0.94	0.85
OTUs	MC	442	464	481	492	455
	MN	419	439	377	453	412
Observed species	MC	243	244	253	224	251
	MN	214	217	178	222	203
Chao1	MC	265	277	286	255	295
	MN	239	270	232	278	255
Shannon	MC	3.81	3.49	3.32	3.29	2.92
	MN	3.54	3.46	3.20	3.19	2.68
Simpson	MC	0.93	0.91	0.95	0.84	0.89
	MN	0.94	0.87	0.91	0.94	0.90

WC and WN stand for Wuliangye distiller's grains without and with ammonium chloride respectively; MC and MN stand for Moutai distiller's grains without and with ammonium chloride.

The bacterial composition dynamics of W and M DGS during ensiling

Alpha diversity of bacterial community after 3, 7, 14, 30, and 60 d of ensiling is presented in Table IV. As for the W DGS, the OTUs, observed species, and Shannon index of control and ammonium chloride treated group increased first with prolonged ensiling time and decreased after that, this was in agreement with Liu *et al*. (2022), who reported decreased bacterial richness of barley silage with the addition of LAB after 60 d of ensiling. In the first few d of silage fermentation, there was aerobic respiration, so the abundance of microorganisms increased, and anaerobic respiration in the later stage reduced the abundance of microorganisms. Regarding M DGS, the shannon index of the control and ammonium chloride treated group decreased linearly with prolonged silage time. The OTUs and Observed species of the ammonium chloride-treated M DGS were lower than that of the control group. The response of microorganisms in the two kinds of DGS to time and ammonium chloride treatment was different, indicating that the microbial flora of the two DGS was different (Yang *et al*., 2021). Thus, the microbial composition and fermentation quality parameters varied between DGS to time and ammonium chloride treatment. However, no matter what kind of DGS or other silages, it is a consensus that the bacterial diversity decreases with the extension of ensiling time with good treatment.

To clarify the effect of ammonium chloride addition and time on the microbial composition of DGS, the principal coordinate analysis was performed, and the results are presented in Figure 1. The principal coordinate 1 (PCo1) and 2 (PCo2) accounted for 33.4% and 13.1% of the total variance, respectively. The ammonium chloride treated group and the control group of W and M DGS at several time points (WC14, WN14, MC14, and MN14) could be clearly distinguished. This meant that time and ammonium chloride addition significantly affected microbial composition.

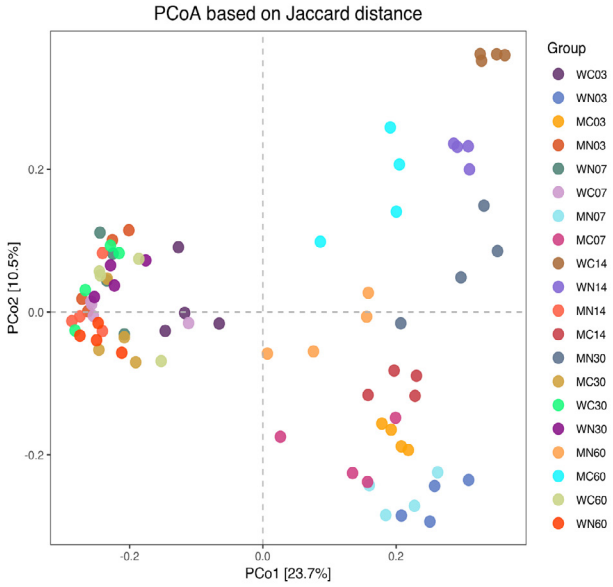

Fig. 1. The principal coordinate analysis of bacterial community for W and M DGS silages treated without and with ammonium chloride. WC and WN stand for Wuliangye distiller's grains without and with ammonium chloride at day 3, 7, 14, 30 and 60, respectively; MC and MN stand for Moutai distiller's grains without and with ammonia chloride on days 3, 7, 14, 30 and 60, respectively.

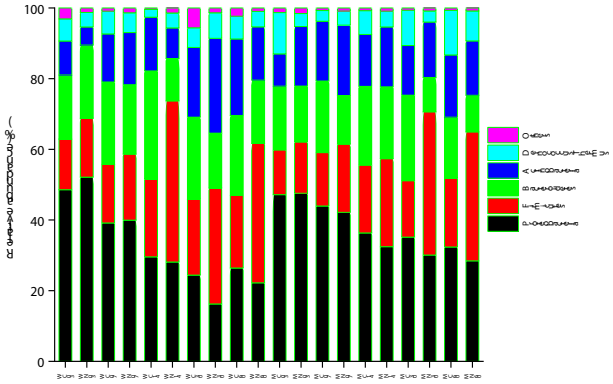

Fig. 2. The bacterial community and relative abundance by phylum for W and M DGS silages treated without and with ammonium chloride. WC and WN stand for Wuliangye distiller's grains without and with ammonium chloride on days 3, 7, 14, 30 and 60, respectively; MC and MN stand for Moutai distiller's grains without and with ammonium chloride on days 3, 7, 14, 30 and 60, respectively.

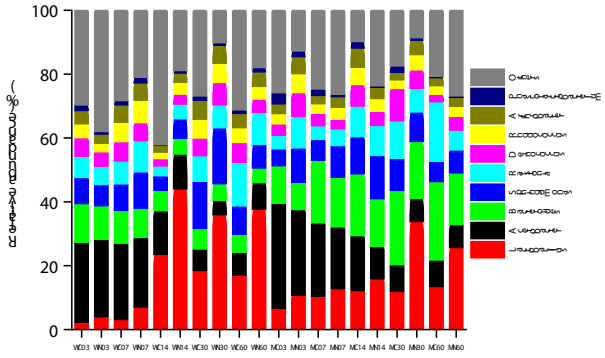

Fig. 3. The bacterial community and relative abundance by genus for W and M DGS silages treated without and with ammonium chloride. WC and WN stand for Wuliangye distiller's grains without and with ammonium chloride at day 3, 7, 14, 30 and 60, respectively; MC and MN stand for Moutai distiller's grains without and with ammonium chloride on days 3, 7, 14, 30 and 60, respectively.

The bacterial community dynamics by phylum for two DGS treated without and with ammonia chloride are presented in Figure 2. In both DGS, the most abundant phylum is Proteobacteria, which accounted for approximately 50% of the relative abundance, followed by Firmicutes, Bacteroidetes, Actinobacteria, and Deinococcus-Thermus. It differed from Mulberry leaf and barley silage (Wang *et al*., 2019) in which Firmicutes was the most abundant, followed by Proteobacteria. This difference may be attributed to different materials, and *Daqu* used for Baijiu production (Yang *et al*., 2021). During the 60 d of ensiling, in both DGS, the relative abundance of Firmicutes increased at the cost of Proteobacteria and Bacterioidetes and remained stable. However, different from M DGS, the relative abundance of Firmicutes peaked on d 14 in W DGS, but it peaked on d 30 in M DGS. This again showed that the microbial flora composition of the two DGS was different; therefore, the variation trend of microorganisms phylum was different during storage.

The bacterial population and relative abundance by genus for W and M DGS treated without and with ammonium chloride are presented in Figure 3. The top 5 abundant microorganisms in the two DGS were *Lactobacillus, Acetobacter*, *Bacteroides*, *Sphingomounas,* and *Ralstonia*. This was similar to the bacterial composition of high-moisture and rehydrated corn grain silages, in which *Lactobacillus*, *Acetobacter*, *Enterococcus*, *Leuconostoc*, *Ralstonia*, *Klebsiella* and *Clostridium* were the most dominant bacterial genera (Carvalho-Estrada *et al*., 2020).

The relative abundance of *Bacteroides* and *Ralstonia* in M DGS was greater than in W DGS. This might also have originated from different materials' bacterial differences. Similar to the phenomenon observed at phylum level, in W DGS, the relative abundance of *Lactobacillus* increased first, then decreased and kept stable with the compensation of *Acetobacter* and *Bacteroides*. Different from W DGS, the relative abundance of *Lactobacillus* increased first and then decreased and kept stable with the compensation of *Acetobacter*, and *Bacteroides* increased with prolonged ensiling time. *Acetobacter* is an obligate aerobic gram-negative bacterium. With the extension of ensiling time, oxygen decreased, therefore, its abundance decreased. The relative abundance of *Lactobacillus* increased sharply after 14 d of ensiling in W DGS. In contrast, the relative abundance of *Lactobacillus* in M DGS peaked at 30 d after ensiling and kept decreasing and stable. This suggested that the anaerobic fermentation in M DGS was slower than in W DGS. Surprisingly, the relative abundance of *Bacteroides* decreased in W DGS, while increasing with prolonged ensiling time in M DGS. This might result from different species of *Bacteroides* genus in different DGS.

In addition, the ammonium chloride treated group disregarded W or M, and the relative abundance of Lactobacillus was higher than that of the control group and occupied a significant position after 30 d of ensiling. The variation of microorganisms was also reflected in the fermentation quality. In W DGS, the lactic acid content was the highest, and pH was the lowest after 14 d of fermentation, whereas, in M DGS, the lactic acid content was the highest, and pH was the lowest after 30 d of fermentation. This implied that ammonium chloride could

be used as a preservative for both W and M DGS, however, W DGS only needs two weeks to be well preserved, whereas the M needs a month to be well preserved.

Microbial composition and fermentation quality were analyzed for correlation, and the results indicated that the *Bacterides* ($P < 0.046$; r = 0.476) and *Deinococcus* ($P < 0.030$; r = 0.543) had a significant positive correlation with ammonium-N content (Fig. 4). The output of enzymes varied significantly amongst strains of the same species. It was discovered that cultures of *B. gingivalis*, *B. asaccharolyticus*, *B. endodontalis*, *B. intermedius*, and *B. corporis* had general proteolytic activity on gelatin and Azocoll (Van Steenbergen *et al*., 1986). Including ammonium hydroxide could decrease ammonium-N levels in whole-plant corn silage (Kung *et al*., 2000). However, in W DGS, the relative abundance of *Bacterides* decreased, while in Moutai DGS, the abundance of *Bacterides* increased. This was also shown in the ammonium-N content after ensiling.

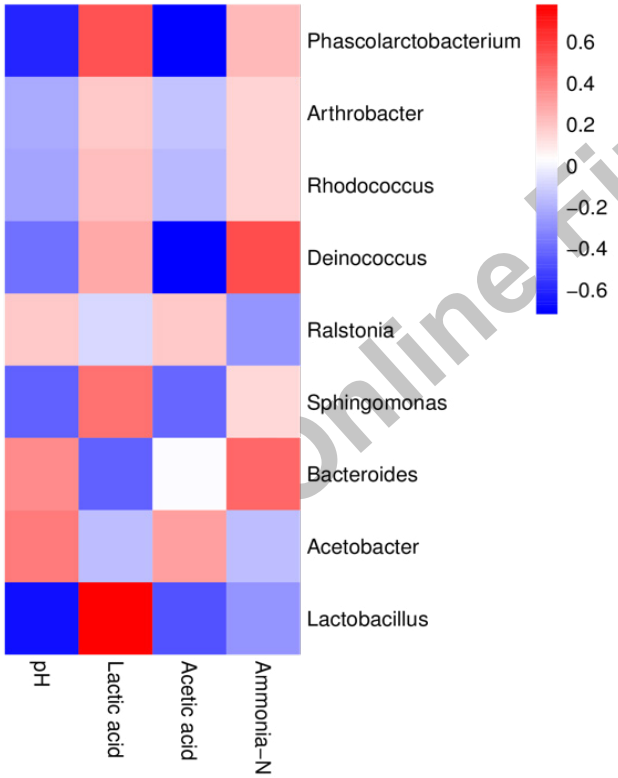

Fig. 4. Correlation analysis of the bacterial community composition and silage fermentation parameters.

CONCLUSIONS

It is concluded that adding 0.3% N ammonium chloride could increase the relative abundance of *Lactobaccilus*, and production of lactic acid, reduce the number of coliform bacteria, and the relative abundance of *Acetobacter* and content of ammonium-N. The addition of ammonium chloride could inhibit the excessive fermentation of *Acetobacter* and preserve the protein components of DGS well. However, DGS is a fermentation product; different producers use different grains and different bacterial strains; hence, the microbial composition of fresh DGS is different. Therefore, the time for different DGS to reach the stable period is different after the addition of ammonium chloride. Ammonium chloride can be used as a practical preservative for DGS storage.

DECLARATIONS

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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