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Screening of High 1,2-Propanediol Production by *Lactobacillus buchneri* Strains and Their Effects on Fermentation Characteristics and Aerobic Stability of Whole-Plant Corn Silage

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Abstract: The study was conducted to screen high 1,2-propanediol produced by *Lactobacillus buchneri* strains, isolated from baled silages stored for 1 or 2 years, and to evaluate their effects on fermentation quality and aerobic stability of whole-plant corn silage. In total, 31 *L. buchneri strains* were isolated from alfalfa, whole-plant corn and oat silages. Based on growth performance and 1,2-propanediol and acetic acid production, two strains, *L. buchneri* 9-2 and *L. buchneri* 10-1, from alfalfa silage, were further assessed in an ensiling trial on whole-plant corn. The corn silage inoculated with *L. buchneri* 9-2 or *L. buchneri* 10-1 had a higher concentration of 1,2-propanediol (34.7 or 34.6 g/kg dry matter (DM)) and acetic acid (47.2 or 45.9 g/kg DM) in comparison with *L. buchneri* 40788 (reference strain) treated silage (19.5 and 35.9 g/kg DM) after 90 d of fermentation. In addition, these two strains performed better in improving silage aerobic stability relative to control and *L. buchneri* 40788. The results above indicated that *L. buchneri* 9-2 and *L. buchneri* 10-1 could be candidate strains to increase 1,2-propanediol and acetic acid concentrations and improve the aerobic stability of whole-plant corn silage.

Keywords: acetic acid; aerobic stability; corn silage; *Lactobacillus buchneri*; 1,2-propanediol

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1. Introduction

The ensiling process is characterized by the fermentation of fresh forage initiated by lactic acid bacteria (LAB) under anaerobic conditions. During this process, water-soluble carbohydrates (WSC) are converted into organic acids through LAB activity to promote forage conservation and produce distinctive fragrance and acid flavor that are attractive to ruminant animals [1–3]. Therefore, ensiling is a feasible alternative for overcoming seasonality problems regarding feed supply. Corn (*Zea mays* L.) is characterized by high and stable-yield crop and it is well known all over the world [4]. Ensiling is the most effective technique for fresh corn preservation as high-quality forage [5,6]. At present, whole-plant corn silage has become more popular because it has great nutritional value, high concentration in WSC, which makes it suitable for the LAB fermentation, and it has good palatability [7]. However, corn silage is vulnerable to aerobic deterioration during the feedout stage [8]. Therefore, improvement in aerobic stability of corn silage is a conventional but constant concern for making high-quality and safe silage.

L. buchneri is one of the most common types of heterofermentative lactic acid bacteria that are used to improve the aerobic stability of silage due to its production of acetic acid in an anaerobic environment [9]. As a heterofermentative lactic acid bacterium, *L. buchneri* not only plays an important role in improving the aerobic stability of ensiled forage but also can enhance the quality and nutritional value of silage [10–12]. A considerable number of investigations have been conducted on the effects of *L. buchneri* on whole-plant corn or

alfalfa silage quality, and the results confirmed that *L. buchneri* could effectively enhance the aerobic stability of silage by inhibiting spoilage microbes such as yeasts and molds, thereby prolonging the contact of silage with air [13–18]. Additionally, the inoculation of *L. buchneri* could lower the amino-peptidic nitrogen and butyric acid in ensiled forages [14,19]. Existing literature also shows that *L. buchneri* inoculation stimulates the production of 1,2-propanediol in silage [20], which is often fed to animals to prevent ketosis and has been recognized as playing an important role in dairy cow health [10,21]. Therefore, screening lactic acid bacteria strains with the capacity of producing a high amount of 1,2-propanediol is of great significance.

It was reported that *L. buchneri* is characterized by its high acid tolerance and this often works in the later stages of silage fermentation [22–24]. Therefore, the long storage period silage might provide good material for screening *L. buchneri* strains with good performance in acid tolerance and production of 1,2-propanediol. To our best knowledge, however, little information is available on describing the isolation of *L. buchneri* strains from long-term fermented silage and on investigating their application in ensiled forage. Thus, the aims of the present study were to screen high 1,2-propanediol production *L. buchneri* strains isolated from silages stored for 1 or 2 years, and to investigate their effects on the aerobic stability and fermentation quality of whole-plant corn silage.

2. Materials and Methods

2.1. LAB Strains Activation

A total of 31 *L. buchneri* strains were isolated from alfalfa, whole crop corn and oat silages that were ensiled for 1 year or 2 years and were used in this study. Thirteen silage samples that were used to isolate LAB strains were collected from commercial baled silages made by Minxiang Forage Ltd., Dingxi City, Gansu Province, China. The isolated and identified *L. buchneri* strains were stored in a De Man, Rogosa and Sharpe (MRS) medium at −80 °C with 10% (*v*/*v*) dimethyl sulfoxide (DMSO). Prior to the experiment, the strains were grown at least twice at 37 °C for 18 h using 1% (*v*/*v*) inoculum in MRS broth to increase the vitality of the bacteria. Then, the strains were purified with the streak plate method and cultured at least two consecutive times using MRS broth.

2.2. Screening of Lactic Acid Bacteria Strains

The strains were screened according to their acid production ability, growth performance, acid tolerance and production of 1,2-propanediol and acetic acid. Determinations of acid production, growth rates and acid tolerance of isolates were performed according to the method described by Silva et al. [25] with some modifications. Briefly, in order to measure the growth and acid production rates, all activated isolates (freshly prepared bacterial cultures in MRS broth) were cultured in MRS broth at a 3% inoculation amount, incubated at 37 °C for 24 h with two replicates, and then sampled every 3 h from 0 to 18 h of culturing. The pH of the sampled fermentation broth was measured by using a glass electrode pH meter (Hanna Instruments Italia Srl, Padova, Italy). A spectrophotometer (Hitachi U-2900, Tokyo, Japan) was used to evaluate the growth of the isolates by quantifying the optical density of the sample at 620 nm (OD_{620}). For the measurement of acid tolerance of the isolates, the isolates were cultured in MRS broth with two different pH levels (3 and 4, respectively) and incubated at 37 °C for 24 h. The appointed pH values of the MRS liquid medium were adjusted by using 7.14 M sulfuric acid. After incubation for 24 h, the absorbance of the cultured samples was measured by spectrophotometer (Hitachi U-2900, Tokyo, Japan) at 620 nm.

2.3. Determination of Acetic Acid and 1,2-Propanediol

After incubating the test strains at 37 °C for 24 h, the fermentation broth was acidified with 7.14 M H_2SO_4, followed by filtration with the 0.45 μm filter (Huaou, Qianhe Lab, Jiangsu, China). Acetic acid and 1,2-propanediol concentrations in the filtered solutions were measured by high-performance liquid chromatography (HPLC, Carbomix

H-NP10 column, Shodex; Shimadzu, Japan; oven temperature 55 °C; flow rate 0.6 mL/min; refractive index detector) according to the method described by Muck and Dickerson [26].

2.4. Mini-Silos Preparation

Whole-plant corn (long silage 2, Gansu Academy of Agricultural Sciences) obtained from Dingxi, Gansu, China was harvested using a pull-type forage harvester, equipped with a mechanical processor and chopped to 1–2 cm by a conventional forage harvester. Fresh forage was taken right away to the laboratory and the laboratory replicates for mini-silos research were arranged by following the method of editorial note by Robinson et al. [27]. Briefly, the fresh forage was randomly divided into 20 subsamples (about 500 g for each subsample) for experimental treatments and for chemical and microbial composition analysis. Four subsamples were frozen at −20 °C for subsequent analysis. The rest of the 16 subsamples were then assigned to one of the following treatments: (1) control (untreated, distilled water), (2) *L. buchneri* 40788 (Lallemand Animal Nutrition, Milwaukee, WI, USA), (3) *L. buchneri* 9-2 or (4) *L. buchneri* 10-1. The two strains of *L. buchneri* 9-2 (GenBank accession number: MN386234) and *L. buchneri* 10-1 (GenBank accession number: MN386235) were screened from baled alfalfa silage stored for 2 years based on their growth performance, production of acetic acid and 1,2-propanediol in the present study. The commercial *L. buchneri* strain was isolated from maize silage ensiled about 3 months and is deposited in the National Collection of Industrial and Marine Bacteria (NCIMB) with the accession number NCIMB 40788. The application rate of each inoculant was 1×10^6 cfu/g forage of fresh weight (FW) basis. In order to evenly mix the inoculum in the chopped corn, each LAB culture was centrifuged and resuspended in sterile distilled water (10 mL/kg of FW) to achieve an application rate of 1×10^8 cfu of viable cells/mL. These inoculants were manually and uniformly sprayed onto random piles of 500 g forage using a mini sprayer (50 mL; spray bottle; Hejian Shengkun Plastic Product Factory, Cangzhou, China), which were subsequently thoroughly mixed in a plastic container disinfected with ethanol. For the control group, the same volume of distilled water was applied under the same conditions. To avoid possible cross-contamination, four different containers were used for the treatments. After being thoroughly mixed, inoculated or untreated forages were packed into polyethylene plastic bags with 300 mm × 300 mm dimensions (Embossed Food saver bag) provided by Tiancheng Soft-Packing Color-Printing Co. Ltd. (Jiangsu, China). Bags were tightly vacuum-sealed using a vacuum sealer (DZ-260PD, Wenchuan Vacuum Packaging Machinery Co. Ltd., Wuxi, China). Silos were stored at room temperature for 90 d.

2.5. Analytical Methods

After 90 d of ensiling, silo bags were then opened and a sample of silage was immediately frozen (−20 °C) for subsequent analysis. For each silo, 20 g of silage sample was taken, added to a juice extractor (BA-828, Mannengda Plasthetics Co. Ltd., Wuhan, China) and diluted with 180 mL of distilled water. The juice was thoroughly mixed for 60 s using a blender (WBL1021S, Midea, Tianshu, Wuhan, China) at high speed. The juice was filtered with four layers of cheesecloth (Oyeah Health, Fujian, China). After this process, the filtrate was subdivided into two portions. The pH was immediately measured and one portion of the filtrate was acidified with 7.14 M H_2SO_4 and filtered with a 0.45 µm filter (Huaou, Qianhe Lab, Jinhua, China). Lactic, acetic, propionic and butyric acids, ethanol and 1,2-propanediol were determined by HPLC (Carbomix H-NP10 column, Shodex; Shimadzu, Japan; oven temperature 50 °C; SPD 210 nm; flow rate 0.6 mL/min) following the methodology described by Muck and Dickerson [26]. An aliquot of 10 mL trichloroacetic acid (TCA; 250 g/L, wt/vol) was added to 40 mL of the second portion of the filtrate. This solution was reserved overnight at 4 °C in order to precipitate the protein, and centrifuged at 18,000× *g* for 15 min at 4 °C. The supernatant was collected and subsequently analyzed for the concentrations of amino acid nitrogen (AA-N) and ammonia nitrogen (NH_3-N), according to the method described by Broderick and Kang [28]. Non-protein nitrogen (NPN) and water-soluble carbohydrates (WSC) were measured according to the procedure

described by Licitra et al. [29,30], respectively. Neutral detergent fiber (NDF) concentration was determined following the method described by Van Soest et al. [31] and acid detergent fiber (ADF) concentration was determined following the method described by Robertson and Van Soest [32] using an Ankom 200 fiber analyzer (Ankom Technology Corp., Fairport, NY, USA). The NDF was analyzed using heat-stable α-amylase, and then NDF and ADF concentrations were expressed inclusive of residual ash. Dry matter (DM) concentration of fresh forage and silage was determined after drying the samples in an oven with forced-air at 65 °C for 72 h. After drying, samples were milled (1 mm screen; DQ-280, Shengtian, Quanzhou, Fujian, China) and analyzed for Kjeldahl N [33]. Crude protein (CP) content was calculated as Kjeldahl N × 6.25. Starch was determined by using the total starch assay kit (Megazyme, Bray, Ireland) [33]. Samples were also combusted in a muffle furnace for 5 h at 550 °C for ash content determination [34].

The method described by Reich and Kung [35] was used to enumerate the number of yeasts, molds and lactic acid bacteria in both fresh forage and ensiled corn. Briefly, a 10 g sample was homogenized in 100 mL of sterile Ringer's solution (Oxoid BR52, Basingstoke, UK) for 1 min and serially diluted (10-fold). The Rogosa agar was used for the enumeration of lactic acid bacteria employing the spread plate method (Oxoid CM627) after 48–72 h of incubation at 37 °C. Pour plating serial method was employed for the determination of yeast and molds on malt extract agar (Oxoid CM0059) that had been acidified with lactic acid (concentration of 85%, added at 5%, vol/vol). Plates were incubated at 32 °C for 48–72 h. Plates, which yielded 30 to 300 colonies, were used for counting the number of colonies. The results of microbial numbers were transformed into $\log_{10}$.

Aerobic stability was carried out by adding about 200 g of silage samples to polyethylene bottles with 250 mL volume. Bottles were then placed inside a Styrofoam block and the silage samples were exposed to air at a constant temperature (25 ± 1 °C). Thermocouple wires were inserted into the geometric center of the silage mass for every insulated bottle. Wires were connected to an online multichannel data logger (MDL-1048A, Shanghai Tianhe Automation Meters Co. Ltd., Shanghai, China) provided with 24 data transfer channels. The temperature was automatically evaluated and recorded at 30 min intervals. In order to protect silos from environmental contamination and drying, a double layer of sterile cheesecloth was placed on it, which still allowed air ingress into the silage. Aerobic stability was defined as the time taken to increase the temperature of the sample for 2 °C above room temperature after the silo was opened.

2.6. Statistical Methods and Analysis

Data regarding composition and fermentation quality were presented on a DM basis, except DM (fresh weight basis) and pH. Microbial data were converted into $\log_{10}$ and presented on a fresh weight basis. The data set was analyzed by one-way ANOVA using the Statistical Package for Social Science (SPSS 18.0, SPSS, Inc., Chicago, IL, USA). A pairwise comparison among treatment means was performed using a Tukey's test when the overall effect of treatment was found to be significant. Least squares means and standard error of the means were presented per treatment and differences among means were declared to be significant at $p < 0.05$.

3. Results

3.1. Screening of Lactic Acid Bacteria Strains

Performances in acid production, growth rate and acid tolerance of the tested 31 *L. buchneri* strains are shown in Table 1. According to Table 1, it can be clearly observed that two strains, *L. buchneri* 9-2 and *L. buchneri* 10-1, exhibited overall better performance in acid production, growth rate and acid tolerance in comparison with other strains.

Table 1. Growth performance, acid production and acid tolerance of *L. buchneri* isolates from different silages fermented for 1 or 2 years (mean ± standard deviation).

Strains	Strain Resource	Growth Rate [1]				Acid Production Rate [2]				Acid Tolerance Test [3]	
		OD_{620} Value				pH Value				OD_{620} at pH = 3	OD_{620} at pH = 4
		3 h	6 h	12 h	18 h	3 h	6 h	12 h	18 h	24 h	24 h
10-1	2 years alfalfa silage	0.82 ± 0.01	1.72 ± 0.02	2.31 ± 0.01	2.80 ± 0.01	5.36 ± 0.02	4.78 ± 0.01	4.03 ± 0.01	3.87 ± 0.01	0.06 ± 0.01	2.29 ± 0.01
9-2	2 years alfalfa silage	0.80 ± 0.01	1.71 ± 0.01	2.30 ± 0.02	2.69 ± 0.01	5.40 ± 0.01	4.85 ± 0.01	4.16 ± 0.01	3.95 ± 0.01	0.07 ± 0.01	2.30 ± 0.01
1-2	1 year oat silage	0.71 ± 0.02	1.54 ± 0.01	2.39 ± 0.01	2.55 ± 0.01	5.47 ± 0.01	4.89 ± 0.02	4.21 ± 0.02	4.08 ± 0.01	0.07 ± 0.01	2.30 ± 0.02
6-2	1 year corn silage	0.56 ± 0.01	1.52 ± 0.01	2.19 ± 0.02	2.32 ± 0.02	5.57 ± 0.02	5.32 ± 0.01	5.16 ± 0.01	4.32 ± 0.01	0.05 ± 0.01	1.87 ± 0.01
2-5	1 year corn silage	0.60 ± 0.01	1.39 ± 0.02	2.04 ± 0.02	2.28 ± 0.01	5.48 ± 0.02	4.95 ± 0.01	4.26 ± 0.01	4.24 ± 0.02	0.06 ± 0.01	2.24 ± 0.01
4-1	1 year oat silage	0.20 ± 0.02	0.53 ± 0.01	1.01 ± 0.01	1.25 ± 0.01	5.59 ± 0.01	5.42 ± 0.01	5.18 ± 0.01	5.11 ± 0.01	0.06 ± 0.01	1.75 ± 0.01
1-1	1 year oat silage	0.20 ± 0.01	0.52 ± 0.02	0.88 ± 0.01	1.30 ± 0.01	5.57 ± 0.03	5.59 ± 0.01	5.31 ± 0.01	5.06 ± 0.01	0.05 ± 0.02	1.62 ± 0.01
4-2	1 year oat silage	0.21 ± 0.01	0.51 ± 0.01	0.96 ± 0.01	1.31 ± 0.02	5.69 ± 0.01	5.61 ± 0.01	5.26 ± 0.02	5.01 ± 0.02	0.06 ± 0.01	1.61 ± 0.01
11-1	1 year alfalfa silage	0.20 ± 0.01	0.47 ± 0.01	0.93 ± 0.02	1.22 ± 0.01	5.72 ± 0.01	5.62 ± 0.01	5.35 ± 0.01	5.11 ± 0.01	0.05 ± 0.01	1.62 ± 0.01
5-3	1 year alfalfa silage	0.17 ± 0.02	0.43 ± 0.02	0.93 ± 0.01	1.20 ± 0.01	5.65 ± 0.01	5.57 ± 0.02	5.33 ± 0.01	5.11 ± 0.01	0.05 ± 0.01	1.62 ± 0.01
11-3	1 year alfalfa silage	0.20 ± 0.02	0.49 ± 0.01	0.98 ± 0.01	1.27 ± 0.01	5.63 ± 0.02	5.55 ± 0.01	5.26 ± 0.01	5.08 ± 0.02	0.05 ± 0.01	1.62 ± 0.02
11-4	1 year alfalfa silage	0.20 ± 0.01	0.46 ± 0.01	1.08 ± 0.01	1.28 ± 0.02	5.62 ± 0.01	5.58 ± 0.01	5.25 ± 0.02	5.07 ± 0.01	0.05 ± 0.01	1.65 ± 0.01
12-1	1 year alfalfa silage	0.25 ± 0.01	0.57 ± 0.01	1.21 ± 0.01	1.44 ± 0.01	5.61 ± 0.01	5.51 ± 0.02	5.26 ± 0.02	5.06 ± 0.01	0.06 ± 0.01	1.63 ± 0.01
6-3	1 year corn silage	0.20 ± 0.01	0.53 ± 0.02	1.06 ± 0.02	1.29 ± 0.02	5.6 ± 0.02	5.53 ± 0.01	5.27 ± 0.01	5.07 ± 0.01	0.05 ± 0.01	1.65 ± 0.01
5-1	1 year alfalfa silage	0.23 ± 0.02	0.52 ± 0.01	1.13 ± 0.01	1.30 ± 0.01	5.63 ± 0.01	5.58 ± 0.01	5.26 ± 0.03	5.07 ± 0.01	0.05 ± 0.01	1.65 ± 0.01
3-2	2 years corn silage	0.22 ± 0.01	0.51 ± 0.02	1.03 ± 0.03	1.30 ± 0.01	5.61 ± 0.01	5.52 ± 0.01	5.25 ± 0.01	5.07 ± 0.01	0.05 ± 0.01	1.64 ± 0.02
13-5	1 year alfalfa silage	0.21 ± 0.01	0.51 ± 0.01	1.07 ± 0.02	1.29 ± 0.01	5.62 ± 0.03	5.53 ± 0.02	5.29 ± 0.01	5.09 ± 0.01	0.05 ± 0.01	1.60 ± 0.01
7-1	2 years corn silage	0.20 ± 0.02	0.52 ± 0.02	1.04 ± 0.01	1.28 ± 0.01	5.68 ± 0.02	5.6 ± 0.01	5.32 ± 0.01	5.08 ± 0.01	0.07 ± 0.01	1.54 ± 0.01
1-8	1 year alfalfa silage	0.22 ± 0.01	0.52 ± 0.01	1.11 ± 0.02	1.30 ± 0.01	5.62 ± 0.02	5.52 ± 0.01	5.3 ± 0.02	5.06 ± 0.02	0.05 ± 0.01	1.65 ± 0.01
3-1	2 years corn silage	0.23 ± 0.01	0.53 ± 0.01	1.11 ± 0.01	1.31 ± 0.03	5.65 ± 0.01	5.53 ± 0.01	5.27 ± 0.01	5.05 ± 0.01	0.05 ± 0.01	1.68 ± 0.01
8-2	1 year alfalfa silage	0.21 ± 0.02	0.53 ± 0.02	1.02 ± 0.02	1.30 ± 0.01	5.63 ± 0.01	5.52 ± 0.03	5.26 ± 0.01	5.07 ± 0.01	0.06 ± 0.02	1.65 ± 0.01
4-3	1 year oat silage	0.23 ± 0.01	0.53 ± 0.01	1.12 ± 0.01	1.31 ± 0.02	5.61 ± 0.01	5.51 ± 0.01	5.28 ± 0.01	5.04 ± 0.01	0.06 ± 0.01	1.65 ± 0.01
13-2	1 year alfalfa silage	0.22 ± 0.02	0.53 ± 0.01	1.06 ± 0.01	1.30 ± 0.01	5.66 ± 0.01	5.56 ± 0.02	5.32 ± 0.01	5.07 ± 0.01	0.06 ± 0.01	1.65 ± 0.01
10-3	2 years alfalfa silage	0.18 ± 0.01	0.49 ± 0.02	1.02 ± 0.02	1.21 ± 0.02	5.59 ± 0.02	5.48 ± 0.01	5.15 ± 0.01	5.1 ± 0.01	0.06 ± 0.01	1.74 ± 0.02
13-1	1 year alfalfa silage	0.21 ± 0.01	0.51 ± 0.01	1.10 ± 0.01	1.30 ± 0.01	5.62 ± 0.01	5.5 ± 0.01	5.23 ± 0.02	5.08 ± 0.02	0.05 ± 0.01	1.65 ± 0.01
14-1	1 year alfalfa silage	0.37 ± 0.01	0.66 ± 0.01	1.52 ± 0.03	1.73 ± 0.01	5.53 ± 0.02	5.49 ± 0.01	5.21 ± 0.01	4.86 ± 0.01	0.06 ± 0.01	1.69 ± 0.01
11-2	1 year alfalfa silage	0.19 ± 0.02	0.50 ± 0.02	1.07 ± 0.01	1.23 ± 0.02	5.58 ± 0.01	5.52 ± 0.01	5.33 ± 0.01	5.09 ± 0.01	0.05 ± 0.01	1.59 ± 0.01
7-3	2 years corn silage	0.22 ± 0.01	0.51 ± 0.01	1.07 ± 0.01	1.30 ± 0.01	5.62 ± 0.02	5.56 ± 0.02	5.35 ± 0.02	5.06 ± 0.03	0.06 ± 0.01	1.63 ± 0.01
9-1	2 years alfalfa silage	0.33 ± 0.01	0.62 ± 0.02	1.12 ± 0.02	1.36 ± 0.02	5.61 ± 0.01	5.48 ± 0.01	5.25 ± 0.01	5.05 ± 0.01	0.06 ± 0.01	1.70 ± 0.01
5-2	1 year alfalfa silage	0.20 ± 0.02	0.47 ± 0.01	1.03 ± 0.01	1.26 ± 0.01	5.65 ± 0.02	5.51 ± 0.01	5.23 ± 0.01	5.09 ± 0.02	0.05 ± 0.01	1.62 ± 0.01
9-3	2 years alfalfa silage	0.19 ± 0.01	0.47 ± 0.02	1.03 ± 0.01	1.24 ± 0.01	5.66 ± 0.01	5.57 ± 0.01	5.25 ± 0.01	5.09 ± 0.01	0.05 ± 0.01	1.60 ± 0.01
L. buchneri 40788		0.78 ± 0.01	1.64 ± 0.01	2.30 ± 0.02	2.56 ± 0.01	5.52 ± 0.01	4.98 ± 0.01	4.32 ± 0.02	4.12 ± 0.01	0.06 ± 0.01	2.25 ± 0.01

[1] Bacterial growth rate was monitored by measuring the OD_{620} (optical density of sample at 620 nm) with a spectrophotometer at 3 h intervals; [2] bacterial acid production rate was monitored by measuring the OD_{620} with a spectrophotometer at 3 h intervals; [3] bacterial acid tolerance test was monitored by measuring the OD_{620} with a spectrophotometer after 24 h of cultivation.

These two strains, *L. buchneri* 9-2 and *L. buchneri* 10-1, performed well in the production of acetic acid and 1,2-propanediol according to Table 2. Therefore, the two strains were finally selected based on overall consideration of their performance in acid production, growth rate, acid tolerance, acetic acid and 1,2-propanediol production.

Table 2. Acetic acid and 1,2-propanediol production of *L. buchneri* isolates from different silages fermented for 1 or 2 years.

Strains	Strain Resource	Acetic Acid [1] (mg/mL; Mean ± Standard Deviation)	1,2-Propanediol [1] (mg/mL; Mean ± Standard Deviation)
10-1	2 years alfalfa silage	4.67 ± 0.02	3.25 ± 0.03
9-2	2 years alfalfa silage	4.97 ± 0.03	3.36 ± 0.02
1-2	1 year oat silage	4.80 ± 0.02	3.20 ± 0.01
6-2	1 year corn silage	4.54 ± 0.01	3.06 ± 0.02
2-5	1 year corn silage	4.90 ± 0.02	3.27 ± 0.03
4-1	1 year oat silage	4.75 ± 0.02	3.17 ± 0.02
1-1	1 year oat silage	3.88 ± 0.04	2.59 ± 0.01
4-2	1 year oat silage	4.23 ± 0.03	3.01 ± 0.03
11-1	1 year alfalfa silage	3.94 ± 0.02	2.79 ± 0.02
5-3	1 year alfalfa silage	4.13 ± 0.01	2.99 ± 0.02
11-3	1 year alfalfa silage	3.97 ± 0.02	2.76 ± 0.03
11-4	1 year alfalfa silage	4.19 ± 0.02	2.88 ± 0.04
12-1	1 year alfalfa silage	4.15 ± 0.01	3.00 ± 0.03
6-3	1 year corn silage	3.94 ± 0.03	2.75 ± 0.02
5-1	1 year alfalfa silage	4.04 ± 0.03	2.87 ± 0.02
3-2	2 years corn silage	4.06 ± 0.03	2.85 ± 0.01
13-5	1 year alfalfa silage	4.14 ± 0.02	2.96 ± 0.03
7-1	2 years corn silage	4.00 ± 0.04	2.89 ± 0.04
1-8	1 year alfalfa silage	4.27 ± 0.05	3.06 ± 0.03
3-1	2 years corn silage	3.98 ± 0.03	2.76 ± 0.02
8-2	1 year alfalfa silage	4.21 ± 0.01	2.84 ± 0.02
4-3	1 year oat silage	4.36 ± 0.04	3.11 ± 0.03
13-2	1 year alfalfa silage	4.19 ± 0.02	2.98 ± 0.03
10-3	2 years alfalfa silage	4.42 ± 0.03	3.08 ± 0.01
13-1	1 year alfalfa silage	4.19 ± 0.02	2.86 ± 0.03
14-1	1 year alfalfa silage	4.14 ± 0.01	2.88 ± 0.02
11-2	1 year alfalfa silage	4.05 ± 0.02	2.78 ± 0.02
7-3	2 years corn silage	4.26 ± 0.03	3.02 ± 0.03
9-1	2 years alfalfa silage	4.14 ± 0.02	2.95 ± 0.01
5-2	1 year alfalfa silage	4.25 ± 0.01	2.99 ± 0.02
9-3	2 years alfalfa silage	4.10 ± 0.03	2.76 ± 0.03
L. Buchneri 40788		4.54 ± 0.02	3.18 ± 0.01

[1] The acetic acid and 1,2-propanediol production of *L. buchneri* isolates were determined by measuring the concentration of these chemicals in the MRS liquid medium after incubating the isolates for 24 h at 37 °C.

3.2. Chemical Composition and Epiphytic Microflora of Fresh Whole-Plant Corn before Ensiling

The chemical and microbial compositions of fresh whole-plant corn are shown in Table 3. Fresh corn forage had 237 g/kg DM while the pH value was 5.24. Counts of epiphytic LAB, molds and yeasts were 6.41, 4.93 and 5.67 $\log_{10}$ cfu/g of fresh forage, respectively. The NH_3-N in corn forage prior to ensiling was 44 g/kg of total N (TN). Additionally, NPN and AA-N were 90 g/kg and 7 g/kg of TN, respectively. The NDF and ADF were 445 g/kg and 254 g/kg DM, respectively.

3.3. Chemical Composition of Corn Silages after 90 Days of Fermentation

The DM contents in the inoculated silages were higher ($p < 0.05$) than those in the control silage (Table 4). Higher DM recovery was found ($p < 0.05$) in all inoculated silages when compared with the control silage but no statistical ($p > 0.10$) difference was noticed among the inoculated silages. The control silage had higher ($p < 0.05$) WSC concentration when compared with the inoculated silages but there was no difference in WSC concentra-

tions among the inoculated treatments. Inoculation with *L. buchneri* decreased ($p < 0.05$) the NPN concentration in ensiled whole-plant corn and the lowest NPN was observed in *L. buchneri* 9-2 inoculated silage. Although the NH_3-N concentrations were similar among the control, *L. buchneri* 40788 and *L. buchneri* 10-1 inoculated corn silages, the lower NH_3-N concentration was observed in the *L. buchneri* 9-2 inoculated silage. The NDF and ADF in the control silage were higher ($p < 0.05$) than *L. buchneri* inoculated silages; however, there was no difference ($p > 0.1$) among the inoculated silages.

Table 3. Chemical composition and microbial populations of fresh whole-plant corn prior to ensiling.

Item [1]	Value (Mean ± Standard Deviation)
DM, g/kg	237 ± 5.3
pH	5.24 ± 0.03
WSC, g/kg DM	88 ± 0.6
NH_3-N, g/kg TN	44 ± 0.7
NDF, g/kg DM	445 ± 1.7
ADF, g/kg DM	254 ± 0.9
Ash, g/kg DM	58 ± 0.02
CP, g/kg DM	71 ± 1.2
NPN, g/kg DM	90 ± 0.4
AA-N, g/kg TN	7 ± 0.02
Starch, g/kg DM	238 ± 3.86
LAB, $\log_{10}$ cfu/g of fresh weight	6.41 ± 0.03
Yeasts, $\log_{10}$ cfu/g of fresh weight	5.67 ± 0.03
Molds, $\log_{10}$ cfu/g of fresh weight	4.93 ± 0.02

[1] DM, dry matter; WSC, water soluble carbohydrates; NH_3-N, ammonia N; NDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; CP, crude protein; NPN, non-protein N; AA-N, free AA nitrogen; LAB, lactic acid bacteria; cfu, colony-forming units.

Table 4. Chemical composition of whole-plant corn silage after 90 d of ensiling period.

Item [1]	Control	*L. buchneri* 40788	*L. buchneri* 9-2	*L. buchneri* 10-1	SEM [2]	*p*-Value
DM g/kg	228 [b]	239 [a]	242 [a]	240 [a]	2.2	0.057
DM recovery g/kg	941 [b]	972 [a]	973 [a]	974 [a]	4.7	0.005
NH_3-N, g/kg TN	76 [a]	70 [a]	62 [b]	71 [a]	1.7	0.014
WSC, g/kg DM	21 [a]	16 [b]	16 [b]	16 [b]	0.7	0.001
CP, g/kg DM	86 [a]	84 [a,b]	86 [a]	81 [b]	0.8	0.021
NPN, g/kg TN	476 [a]	452 [a,b]	420[c]	448 [b]	7	0.009
AA-N, g/kg TN	154	141	146	134	6.2	0.503
NDF, g/kg DM	488 [a]	463 [b]	459 [b]	444 [b]	5.5	0.006
ADF, g/kg DM	288 [a]	264 [b]	266 [b]	262 [b]	3.8	0.021
Starch, g/kg DM	247	252	251	249	1.3	0.591
Ash, g/kg DM	70	68	68	66	0.6	0.264

[a, b] Means within a row with different superscripts differ ($p < 0.05$).[1] DM, dry matter; DM recovery, dry matter recovery; NH_3-N, ammonia N; WSC, water soluble carbohydrates; CP, crude protein; NPN, non-protein N; AA-N, free AA nitrogen; NDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; [2] SEM, standard error of the means.

Microbial composition, pH value, aerobic stability and fermentation end products of whole-crop corn silages after 90 d of the ensiling period are presented in Table 5. All inoculated silages resulted in lower ($p < 0.05$) pH when compared with the control silage. Silage inoculated with *L. buchneri* 40788 resulted in a lower ($p < 0.05$) lactic acid concentration than the control silage but the difference was quite small. As expected, *L. buchneri* treated silages resulted in considerable increases ($p < 0.05$) in concentrations of acetic acid and 1,2-propanediol in silage. Silages inoculated with the selected strains of *L. buchneri* 9-2 and *L. buchneri* 10-1 had higher ($p < 0.05$) acetic acid (47.2 and 45.9 g/kg DM, respectively) and 1,2-propanediol (34.7 and 34.6 g/kg DM, respectively) than the *L. buchneri* 40788 treated

silage (35.9 g/kg DM in acetic acid, 19.5 g/kg DM in 1,2-propanediol). The inoculation of *L. buchneri* reduced ($p < 0.05$) ethanol concentration in whole-plant corn silage, and the lowest ethanol concentrations were observed in silages inoculated with *L. buchneri* 9-2 and *L. buchneri* 10-1. Inoculation of *L. buchneri* also increased ($p < 0.05$) aerobic stability of the present silage, and greater values were observed in *L. buchneri* 9-2 (458 h) and *L. buchneri* 10-1 (448 h) treated silages in comparison with silage inoculated with *L. buchneri* 40788 (353 h). The total number of LAB was greater ($p < 0.05$) while the number of yeasts was lower ($p < 0.05$) in inoculated silages than those in the control silage; no yeasts were observed in *L. buchneri* 9-2 or *L. buchneri* 10-1 inoculated silages.

Table 5. Fermentation quality (g/kg DM basis), aerobic stability and microbial content (wet basis) of corn silage after 90 d of ensiling period.

Item [1]	Control	*L. buchneri* 40788	*L. buchneri* 9-2	*L. buchneri* 10-1	SEM [2]	*p*-Value
pH	3.95 [a]	3.89 [b]	3.86 [b]	3.87 [b]	0.01	0.018
Lactic acid, g/kg DM	60.6 [a]	58.7 [b]	61.6 [a]	60.1 [a,b]	0.4	0.025
Acetic acid, g/kg DM	9.6 [c]	35.9 [b]	47.2 [a]	45.9 [a]	4.6	<0.001
1,2-propanediol, g/kg DM	0 [c]	19.5 [b]	34.7 [a]	34.6 [a]	4.3	<0.001
Ethanol, g/kg DM	20.6 [a]	17.0 [b]	13.4 [c]	13.3 [c]	0.9	<0.001
Aerobic stability, h	97 [c]	353 [b]	458 [a]	448 [a]	43.97	<0.001
LAB, $\log_{10}$ cfu/g	6.45 [c]	7.91 [b]	8.70 [a]	8.61 [a]	0.27	<0.001
Yeasts, $\log_{10}$ cfu/g	4.18 [a]	1.60 [b]	0 [c]	0 [c]	0.54	<0.001
Molds, $\log_{10}$ cfu/g	0.72	0	0	0	0.18	0.441

[a–c] Means within a row with different superscripts differ ($p < 0.05$). [1] DM, dry matter; LAB, lactic acid bacteria; cfu, colony-forming units; [2] SEM, standard error of the means.

4. Discussion

The fermentation process of forage silages is basically ruled by the activity of lactic acid bacteria. Heterofermentative lactic acid bacteria, such as *L. buchneri*, have been studied for many years as a silage additive due to its ability to enhance aerobic stability [36,37] and fermentation quality [3,38]. McDonald et al. [39] and Xu et al. [24] suggested that microorganisms used for silage preparations should have a uniform fermentation route, which can not only rapidly ferment to produce the maximum amount of lactic acid and quickly lower the pH to inhibit other microorganisms, but also has the ability to withstand acid. In this study, the greater acid-producing ability was numerically observed in the two strains of *L. buchneri* 10-1 and 9-2 when compared with other isolates and the widely used inoculant of *L. buchneri* 40788. After fermentation for 18 h, the pH values of the culturing mediums inoculated by the two strains were numerically the lowest (3.87 and 3.95, respectively) and the OD_{620} values were numerically the greatest (2.80 and 2.69, respectively) among the tested strains including *L. buchneri* 40788. In addition, the strains *L. buchneri* 10-1 and *L. buchneri* 9-2 also had good performance in producing acetic acid and 1,2-propanediol relative to other strains. Therefore, *L. buchneri* 10-1 and 9-2 could be good candidates suitable for silage preparation. Based on the productions of acetic acid and 1,2-propanediol of the tested strains isolated from the baled silages with 1 or 2 years storage time, it seems that the acetic acid or 1,2-propanediol production capacity of the tested strains was not related to the silage storage time. However, previous studies showed that the species of *L. buchneri* is characterized by its high acid tolerance and often works in the later stages of silage fermentation [22–24]. Therefore, the long-term fermented silage still has great potential in screening *L. buchneri* strains with high acetic acid and 1,2-propanediol production capacity. In addition, the positive correlations between the acid tolerance and acetic acid or 1,2-propanediol productions of the tested strains indicated that the acid tolerance of *L. buchneri* strains might be a key indicator of their acetic acid or 1,2-propanediol producing capacity.

A meta-analysis study showed that DM recovery was lower in silage treated with *L. buchneri* than in non-inoculated silage although the difference was very small [10].

Reports of lower DM recovery in silage inoculated with *L. buchneri* are variable by some researchers [8], but not others [11]. Generally, the increase in DM losses is mainly caused by extensive heterolactic fermentation during ensiling. However, in the present study, all the inoculated silages resulted in greater DM recovery than untreated silages, which suggested good fermentation for *L. buchneri* inoculated silages. McDonald et al. [39] reported that the additional losses (51 g/kg^{-1} DM) caused by inoculation would be less than those anticipated in aerobic deterioration. The disadvantage regarding DM recovery when *L. buchneri* is applied can be compensated for by many other benefits, for instance the inhibition of aerobic deterioration in silage. Kleinschmit and Kung [10] reported that application of *L. buchneri* in whole-plant corn and small grain cereal crop silages did not affect NH_3-N concentration of silages, while the present study indicated that inoculation of *L. buchneri* 9-2 resulted in lower NH_3-N in comparison with control silage, which was probably due to the lower pH in *L. buchneri* 9-2 treated silage. Similarly, a decrease in NPN was also observed in silage inoculated with *L. buchneri*, which might be caused by the accelerated fermentation of corn silages after inoculation of *L. buchneri* [40]. Generally, inoculating silages with *L. buchneri* has resulted in lower WSC concentrations [41], which agreed well with the current study. Inoculation did not affect the CP concentration of corn silage when compared to untreated corn silage, and the result is in line with previous findings [15,42], except for *L. buchneri* 10-1, which resulted in lower CP concentration than other treatments. After the inoculation of *L. buchneri*, lower concentrations of NDF and ADF were found in inoculant treated silages as compared with the control silage. Although the reason is unclear, similar results were also observed in a prior study [43]. However, Kleinschmit et al. [15] reported that inoculation with *L. buchneri* did not affect the NDF and ADF in silage.

Inoculation with homolactic acid bacteria sometimes makes the aerobic stability worse [19,44,45]. As reported by Danner et al. [13] and Muck [46], when using heterofermentative lactic acid bacteria, such as *L. buchneri*, aerobic stability was improved in all treated silages due to the low number of yeasts in these silages. Numerous studies have proved that inoculation with *L. buchneri* inhibited the growth of molds and yeast and, consequently, improved the aerobic stability of silage [8,17,18,47–53]. The improved aerobic stability is mainly due to the formation of acetic acid and 1,2-propanediol during degradation of lactic acid by *L. buchneri* under anaerobic conditions [45,47]. This process would be particularly accelerated along with the ensiling process [47], which would lead to more 1,2-propanediol production in the later stages of ensiling. The acetic acid in silage has been declared to be a strong inhibitor of fungi [54] and a good precursor of aerobic deterioration [39]. It has been reported that 1,2-propanediol may be partially converted into propionic acid by *L. diolivorans* [55]. Propionic acid is also a potential antimycotic agent but it was not detected in the present study. In this study, the inoculation of all three *L. buchneri* strains resulted in remarkable improvement in aerobic stability when compared with untreated silage. Moreover, silages inoculated with *L. buchneri* 9-2 and *L. buchneri* 10-1 were more stable than *L. buchneri* 40788 treated silage, which is probably due to the greater concentrations of acetic acid and 1,2-propanediol in *L. buchneri* 9-2 and *L. buchneri* 10-1 treated silages versus *L. buchneri* 40788 inoculated silage. The 1,2-propanediol concentrations in *L. buchneri* 9-2 and *L. buchneri* 10-1 treated silages were almost two folds of that in the *L. buchneri* 40788 inoculated silage. The concentration of acetic acid in the *L. buchneri* 9-2 and *L. buchneri* 10-1 treated silages was also considerably higher than the *L. buchneri* 40788 inoculated silage. In addition, the untreated silages had greater concentrations of ethanol than the inoculated silages in the present study. Spoelstra et al. [56] and Wambacq et al. [23] indicated that some microorganisms, such as acetic acid bacteria, may initiate spoilage because these bacteria have the ability to oxidize acetic acid to ethanol. Therefore, the lower concentration of ethanol in the inoculated silages may be another contributor to improving aerobic stability.

Generally, *L. buchneri* is stimulated by the low pH of silages after the active phase of fermentation, and under anaerobic conditions, it is able to transform a moderate quan-

tity of lactic acid into equal parts of 1,2-propanediol and acetic acid, and also traces of ethanol [45]. The present results indicated that the screened strains of *L. buchneri* 9-2 and *L. buchneri* 10-1 were more active in converting lactic acid into 1,2-propanediol and acetic acid during the ensiling of the whole crop corn as compared with the commercial strain *L. buchneri* 40788. Based on the previous study of Nishino et al. [20], silages inoculated with *L. buchneri* produced a high quantity of 1,2-propanediol (>40 g/kg in DM) after 120 d fermentation, which indicates that great amounts of 1,2-propanediol might be consumed by ruminant animals when fed with silages inoculated with *L. buchneri*. Wilkinson and Rinne [21] indicated that 1,2-propanediol in ensiled forages has potential benefits to animal health. As reported by Lau et al. [57], feeding dairy cows with *L. buchneri* inoculated grass silage, which contained a high level of propylene glycol, led to a considerably lower beta-hydroxybutyrate concentration and reduced the ketone bodies in the blood of dairy cows after calving. Therefore, screening strains with the capacity of producing a high amount of 1,2-propanediol is of great significance, which will not only improve the aerobic stability of ensiled forage but also can be beneficial to animal health.

5. Conclusions

In summary, strains *L. buchneri* 9-2 and *L. buchneri* 10-1 were selected based on their great potential in total acid production, fermentation rate, acid tolerance, acetic acid and 1,2-propanediol formation among the 31 strains isolated from alfalfa, oat and corn silages ensiled for 1 or 2 years. Silages inoculated with the *L. buchneri* 9-2 and *L. buchneri* 10-1 had considerably greater amounts of acetic acid and 1,2-propanediol as well as higher aerobic stability compared with the corn silage inoculated with the widely used strain of *L. buchneri* 40788 or control silage. Because 1,2-propanediol is often fed to ruminants to prevent ketosis and plays an important role in livestock health, *L. buchneri* 9-2 and *L. buchneri* 10-1 could be candidate strains to further increase 1,2-propanediol concentration and aerobic stability of ensiled whole-plant corn with great health beneficial potential to animals.

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