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Characteristics of Isolated Lactic Acid Bacteria and Their Application in High-Moisture Broccoli Waste Silage

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Abstract: Four lactic acid bacteria (LAB) strains isolated from naturally ensiled broccoli waste were characterized, and their effects on the fermentation quality of high-moisture broccoli waste silage were studied. The four isolated strains were assessed using the morphological, physiological and biochemical tests. The four strains were added to broccoli waste at three moisture contents (90%, W0; 87%, W1; 80%, W2) and ensiled for 60 days. All strains (CB89, CB94, CB112, and CB120) grew at 15–45 °C, pH 3.0–7.0, and in 3.0–6.5% NaCl and were identified as *Lactiplantibacillus plantarum* by 16S rDNA sequencing. Inoculation of CB120 significantly ($p < 0.05$) increased lactic acid, starch, and non-structural carbohydrate content, and significantly ($p < 0.05$) decreased pH values and aerobic bacteria count compared with control (CK) at all three moisture contents. In conclusion, CB120 improved the fermentation quality and nutritional value of broccoli waste silage at three moisture contents and could be applied as a promising additive for high-moisture material.

Keywords: lactic acid bacteria; broccoli waste; identification; high-moisture silage; fermentation quality

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1. Introduction

In China, out of the 800 million tons of vegetables produced annually, over 30% of the vegetables are wasted during the harvesting, transportation, marketing, and processing stages, causing resource waste and environmental pollution [1]. Broccoli (*Brassica oleracea* L.var. *italic* Planch.) is widely cultivated around the world, and under the stimulus of dietary changes and urbanization of the population, its annual production (up to 25.5 million tons [2]) and cultivation area are continuously increasing. Consequently, broccoli waste is produced in large quantities, and its preservation and utilization have become a major issue due to its high moisture and perishable characteristics. In addition to green forage, ensilage technique also serves as a cost-effective solution for the long-term conservation of perishable biomass [3,4]. During the ensiling process, the metabolism of lactic acid bacteria (LAB) attached to the substrate can convert water-soluble carbohydrates into organic acids such as lactic acid and acetic acid, rapidly reducing the pH of the silage [5]. The acidic and anaerobic conditions help suppress the activity of undesirable microbes, reduce the risk of raw material spoilage, and preserve nutrients [3,6,7].

However, it is well known that the desirable dry matter (DM) content for ensiling should above 20% [5]. Ensiling high-moisture materials poses significant challenge because the whole fermentation process is complicated due to their high-water content and buffering capacity, prolonging the time to reach a stable fermentation state, thus facilitating the activity of *Clostridium* spp., leading to butyric fermentation and unsuccessful silage [8]. The higher the water content of the material, the lower the critical pH value required for well preservation [9]. For feedstuffs with moisture content as high as 85%, even a pH reduction to 4.0 cannot inhibit the proliferation of clostridia; however, at the same pH level, the activity of clostridia is suppressed in materials with 80% moisture content, and when the moisture content is further reduced to below 70%, the activity of clostridia is almost completely inhibited [5,10]. Thus, the

performance of broccoli waste silage depends on the acidification efficacy of LAB. Insufficient quantities of epiphytic LAB fail to dominate the microbial population, unable to effectively reduce pH to suppress undesirable microorganisms, resulting in suboptimal fermentation quality [11]. *Lentilactobacillus buchneri*, *Lacticaseibacillus casei*, and *Lactiplantibacillus plantarum* are commonly used commercial LAB strains to enhance the fermentation performance of conventional silage [12]. While, under certain extreme conditions, such as high temperatures, low temperature, or high osmotic pressure, the efficacy of these LAB may not remain consistently stable. Moreover, Yahaya et al. [13] and Bureenok et al. [14] stated that forages ensiled with epiphytic LAB inoculant resulting in better fermentation quality compared to commercial inoculant due to its host specificity. Wang et al. [15] also found that the optimal isolates for specific crops may originate from those very crops. Based on this, we speculate that the LAB strains isolated from high-moisture broccoli waste can also improve the quality of high-moisture broccoli waste silage.

To make full use of the vegetable waste, it imperative to isolate LAB strains suitable for high-moisture silage. However, there is little information available. Therefore, this study investigates the characteristics of isolated LABs and their impact on the fermentation quality and nutritional values of broccoli waste silage, which may provide new insight for the development of functional LAB inoculants.

2. Materials and Methods

2.1. LAB Strains

A total of 160 LAB strains were isolated from high-moisture broccoli waste silage (90.1% moisture content) based on the methods of Cai et al. [16]. In brief, 10.0 g of broccoli waste silage was put in a sealed Erlenmeyer flask containing 90.0 mL of sterile saline (0.85% NaCl). The mixture was agitated at 120 rpm, 30 °C for 1 h, then filtered through a layer of sterile gauze. The filtrate was subjected to gradient dilution (10^{-5}, 10^{-6}, and 10^{-7}) and 100 μL of each dilution was plated on de Man, Rogosa, and Sharpe (MRS) agar medium (10 g peptone, 8 g beef extract powder, 4 g yeast extract powder, 20 g glucose, 2 g potassium hydrogen phosphate, 2 g diammonium hydrogen citrate, 5 g sodium acetate, 0.2 g magnesium sulfate, 0.04 g manganese sulfate, 14 g AGAR, 1 g Tween80, dissolved in 1000 mL distilled water, 121 °C high pressure steam sterilization for 15 min) and incubated at 37 °C for 48 h. Prominent colonies with good growth and large diameter on MRS agar medium were picked, streaked for isolation and purified twice. Colony morphology on the plates was observed, and typical colonies were candidate LABs via Gram staining, showing single purple cells and a negative catalase reaction. All 160 strains were coded as CB1 to CB160 and preserved at −80 °C in glycerol.

Then, these strains were activated and inoculated at a 1% concentration in MRS broth medium. The cultures were incubated statically at 37 °C for 24 h. The optical density at 600 nm (OD600) of the cultures was measured using a spectrophotometer (Multiskan Sky, Thermo Scientific, Waltham, MA, USA), and the pH of the fermentation supernatant was directly measured using a pH meter (S600, Mettler Toledo LLC, Zurich, Switzerland). Among these, 37 LAB strains, which had OD600 values > 1.50 and pH < 3.80, were identified as promising strains during the initial screening.

2.2. Substrate Fermentation Test of LAB Strains

Fresh broccoli waste was enzyme-inactivated at 105 °C for 30 min and then dried at 65 °C until constant weight was achieved. The dried materials were then ground and passed through a 0.63 mm sieve to obtain the powder. About 1.0 g of the above powder was placed in a zip-lock bag, to which 2 mL of sterile water was added and mixed thoroughly. Subsequently, the initially screened 37 strains (300 μL suspension) were respectively added to each mixture at the inoculation level of 10^6 colony forming units per gram of dry matter (CFU g^{-1} DM). For the control, an equivalent volume of sterile water was used. Triplicates per treatment was prepared. After air expulsion and thorough mixing, the bags were sealed and incubated statically at 30 °C for 48 h. Post-incubation, 27 mL of sterile water was

added and mixed well, and the pH was measured. A lower pH value indicates a stronger acidification capability of the LAB strain on the broccoli waste substrate. Based on this, 4 LAB strains (CB89, CB94, CB112, and CB120) with rapid fermentation ability were obtained (Table S1).

2.3. Growth Curve and Acid Production Performance of LAB Strains

The above 4 LAB strains were reactivated and inoculated into MRS broth medium at a 1% concentration. Before inoculation, the initial OD600 and pH values of the fermentation broth were measured after thorough mixing. During the incubation at 37 °C without agitation, both OD600 nm and pH of fermentation broth were measured every 2 h within a 32 h period. Growth curves and acid production curves were plotted with incubation time on the x-axis and OD600 and pH values on the y-axis, respectively. After 36 h of fermentation, the broth was filtered through a 0.22 μm aqueous phase filter membrane, and the filtrate was analyzed using HPLC system (1260 Infinity, Agilent Technologies, Inc., Waldbronn, Germany) to determine the types and concentrations of organic acids present (Running column: Carbomix®H-NP5; Sepax Technologies, Inc., Newark, DE, USA; eluent: 2.5 mmol/L H_2SO_4, 0.5 mL/min; temperature: 55 °C).

2.4. Physiological and Biochemical Characterization of LAB Strains

Gram stain, catalase reaction, and gas production from glucose were determined according to Kozaki et al. [17]. Growth temperature range of LAB strains was assessed by the OD600 value in MRS broth at various temperatures including 5 °C, 15 °C, 25 °C, 35 °C, and 45 °C for 24 h. At 37 °C, growth pH range of LAB strains was assessed by the OD600 value in MRS broth at various pH levels (7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, and 2.5) for 24 h, and salinity tolerance range of LAB strains were assessed by the OD600 value in MRS broth at various NaCl concentrations of 3.0%, 6.5%, 10.0%, and 20.0% (*w*/*v*) for 24 h [18,19].

2.5. Genomic DNA Extraction and Species Identification of LAB Strains

Genomic DNA was extracted from the 4 isolated LAB strains and subjected to polymerase chain reaction (PCR) amplification using the universal prokaryotic 16S rRNA primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), following protocols described by Assareh et al. [20]. The amplified products were sent to Qingke Xinye Biotechnology Co., Ltd. (Beijing, China) for sequencing. The phylogenetic tree of the final 4 isolated strain was constructed using MEGA software (version 11.0). The obtained 16S rDNA sequences were submitted to the NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast; accessed on 20 March 2024) for BLAST homology comparison analysis to determine the taxonomic classification of the strains.

2.6. High-Moisture Broccoli Waste Silage Making

Broccoli waste was collected at Lishui planting base, Jiangsu Academy of Agricultural Sciences, and chopped into 2–3 cm lengths, then subjected to different wilting durations of 0 h, 2.5 h, and 5 h (designated as W0, W1, and W2, respectively). Broccoli waste was ensiled as follows: (1) distilled water (CK); (2) CB89; (3) CB94; (4) CB112; (5) CB120, packed into bag-type silos (40 cm × 30 cm polyethylene bag), vacuum sealed, and stored at room temperature for 60 days. The volume of distilled water or inoculant was 3 mL, and the inoculation concentration of all 4 LAB strains was 5×10^6 CFU g^{-1} fresh weight (FW). For each treatment, three replicates were prepared. After 60 days of fermentation, silos were opened and sampled for the following parameter analyses.

2.7. Parameter Analyses

About 150 g of broccoli waste or its silage was enzyme-inactivated at 105 °C for 30 min, followed by drying at 65 °C to a constant weight to measure the DM content [21]. After milling through a 0.38 mm sieve, the crude protein (CP) content was determined by distillation using a FOSS 8400 automatic Kjeldahl nitrogen analyzer. The determination of

water-soluble carbohydrates (WSC) and starch content was carried out according to the method described by Yoshida [22]. The content of non-structural carbohydrates (NSC) was the sum of WSC and starch content. The content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined using the procedures described by Van Soest [23]. The hemicellulose content was calculated based on the content of NDF and ADF.

Another 20.0 g sample of broccoli waste or its silage was homogenized with 180 mL of distilled water and extracted at 4 °C for 24 h. After filtering through four layers of cheesecloth, the pH of filtrate was measured immediately. The filtrate was then filtered through a 0.22 μm aqueous phase filter membrane, and the contents of lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA), and isobutyric acid (ISOBA) were analyzed under HPLC conditions as specified by Zhao et al. [24]. The concentration of ammonia nitrogen (NH_3-N) in the filtrate was determined using the phenol-sodium hypochlorite colorimetric method [25].

For microbial analysis, 10.0 g of broccoli waste or its silage was added to 90.0 mL of sterile physiological saline (0.9%) and agitated at 120 rpm, 37 °C for 1 h. The suspension was obtained by filtering through a layer of sterile cheesecloth. Then, gradient dilutions were prepared to achieve 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions [8]. Plate counting methods were used to count aerobic bacteria, and LAB. A volume of 100 μL from each dilution gradient was spread onto Nutrient Agar (10 g peptone, 3 g beef extract powder, 5 g NaCl, 15 g AGAR, dissolved in 1000 mL distilled water, 121 °C high pressure steam sterilization for 15 min), and MRS Agar medium [26]. Aerobic bacteria were conducted after incubating the Nutrient Agar medium at 37 °C for 1 day; LABs were counted after incubating the MRS Agar medium at 37 °C for 2 days. The microbial counts were expressed in CFU g^{-1} FW.

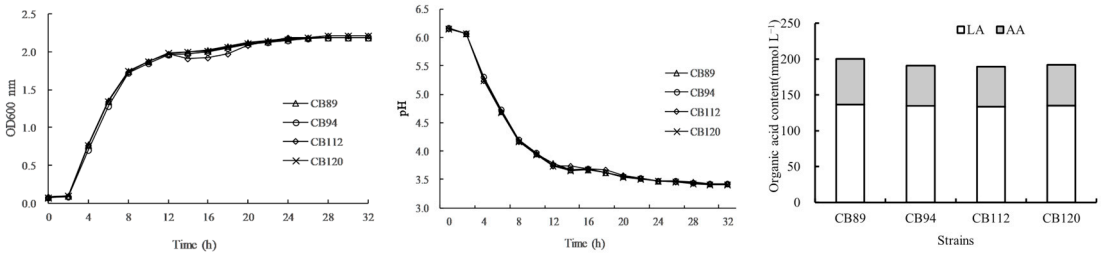
2.8. Data Statistics and Analysis

Statistical analyses were conducted using SPSS software (version 13.0, IBM, Chicago, IL, USA). The Least Significant Difference (LSD) test was applied with a significance level of $p < 0.05$. Figure processing was performed using Origin software (version 8.6) and Photoshop software (version CS6).

3. Results

3.1. Analyses of Growth Curve and Acid Production Performance of LAB Strains

Figure 1A demonstrates that, based on OD600 values, strains CB89, CB94, CB112, and CB120 initiated rapid growth between 2 to 4 h, reached maximum growth rates between 4 to 12 h, and showed a slowdown in growth rate around 14 h, entering a stable phase thereafter with minimal changes over 32 h, with no decline in cell growth observed. The OD600 values of the fermentation broth at 12 h for strains CB89, CB94, CB112, and CB120 were 1.967, 1.955, 1.976, and 1.988, respectively, indicating similar growth rates.



(**A**) (**B**) (**C**)

Figure 1. Growth curve (**A**), acid production curve (**B**), and acid production capability (**C**) of the isolated LAB strains.

As depicted in Figure 1B, the four strains exhibited a rapid acid production rate between 2 to 8 h, which gradually decreased from 8 to 12 h, and then stabilized from 14 to

32 h, with the final pH stabilizing around 3.42. The pH values after 12 h of fermentation for strains CB89, CB94, CB112, and CB120 were 3.78, 3.76, 3.74, and 3.74, respectively, showing similar rates of acid production.

Further analysis by HPLC was conducted to determine the concentrations of LA, AA, PA, BA, and ISOBA in the fermentation broths of the four strains. As shown in Figure 1C, all strains predominantly produced LA. CB89 produced the highest amount of AA, while CB112 produced the least. The production of acetic acid by other two strains showed no significant variation, indicating differential organic acid production capacities among the strains.

3.2. Analyses of Physiological and Biochemical Characterization of LAB Strains

As illustrated in Table 1, strains CB89, CB94, CB112, and CB120 are all Gram-positive and catalase-negative bacilli. They do not produce gas in glucose fermentation tests, identifying them as homofermentative LABs. These four LAB strains can grow well (+++) in MRS broth at a 3.0% NaCl concentration and within a temperature range of 15 to 35 °C and a pH range of 3.5 to 7.0. They exhibit moderate growth (++) in MRS medium with a 6.5% NaCl concentration and at pH 3.0. However, they show weak growth (+) in MRS medium with a 10.0% NaCl concentration and at temperatures of 5 °C and 45 °C. They are unable to grow (—) in MRS broth containing 20.0% NaCl or at pH 2.5.

Table 1. Physiological and biochemical characterization of the isolated LAB strains.

Characteristics	Strains			
	CB89	**CB94**	**CB112**	**CB120**
Shape	Rod	Rod	Rod	Rod
Gram stain	Positive	Positive	Positive	Positive
Catalase reaction	Negative	Negative	Negative	Negative
Gas from glucose	Negative	Negative	Negative	Negative
Fermentation type	Facultative hetero	Facultative hetero	Facultative hetero	Facultative hetero
Growth pH				
pH 2.5	—	—	—	—
pH 3.0	++	++	++	++
pH 3.5	+++	+++	+++	+++
pH 4.0	+++	+++	+++	+++
pH 4.5	+++	+++	+++	+++
pH 5.0	+++	+++	+++	+++
pH 5.5	+++	+++	+++	+++
pH 6.0	+++	+++	+++	+++
pH 6.5	+++	+++	+++	+++
PH 7.0	+++	+++	+++	+++
Growth in NaCl				
0.00% NaCL	+++	+++	+++	+++
3.00% NaCL	+++	+++	+++	+++
6.50% NaCL	++	++	++	++
10.0% NaCL	+	+	+	+
20.0% NaCL	—	—	—	—
Growth temperature				
5 °C	+	+	+	+
15 °C	+++	+++	+++	+++
25 °C	+++	+++	+++	+++
35 °C	+++	+++	+++	+++
45 °C	+	+	+	+

"—" represents OD600 value < 0.05, indicating no growth; "+" represents 0.05 < OD600 value < 0.1, indicating weak growth; "++" represents 0.1 < OD600 value < 0.5, indicating moderate growth; "+++" represents OD600 value > 0.5, indicating good growth.

3.3. Species Identification Analysis of LAB Strains

Based on bootstrap analysis of the phylogenetic tree (Figure 2), strains CB89, CB94, CB112, and CB120 were all most closely related to *L. plantarum* with the 100% bootstrap

values. The sequencing results were submitted to the NCBI for BLAST comparison analysis to identify the closest relatives of these four LAB strains. As shown in Table 2, all four LAB strains exhibited a similarity greater than 99% with *L. plantarum*.

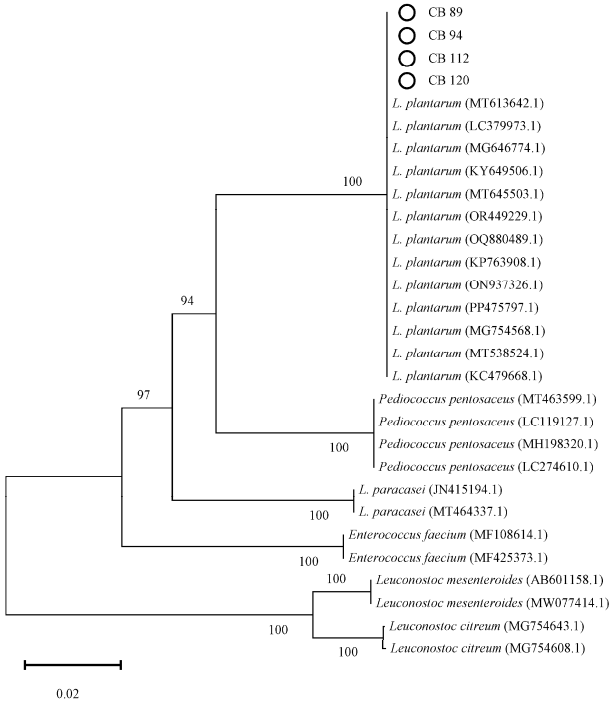


Figure 2. Phylogenetic tree of the four isolated strains and related species in the nucleotide database of GenBank. The bar indicates 2% sequence divergence. Bootstrap values based on 1000 replications are given at the nodes.

Table 2. NCBI comparison results based on 16S rRNA sequences.

Strain	Accession Number	Similar Strain	Homology
CB89	PP814960	*L. plantarum* MT613642.1	100%
CB94	PP814961	*L. plantarum* MT645503.1	100%
CB112	PP814962	*L. plantarum* MT538524.1	100%
CB120	PP814963	*L. plantarum* MG754568.1	99.8%

3.4. Characteristic of Broccoli Waste before Ensiling

The broccoli waste material exhibited a DM content of 12.08%, a WSC content of 14.28%, a starch content of 4.58%, a CP content of 17.21%, a NDF content of 37.59%, and an acid detergent fiber content of 22.97%. Meanwhile, the counts of LAB, aerobic bacteria, yeasts, and molds amounted to 4.98, 7.97, 5.02, and 3.45 $\log_{10}$ CFU g^{-1}, respectively (Table 3).

Table 3. Chemical and microbial characteristics of fresh broccoli waste.

Items	Broccoli Waste
DM (%FW)	12.1
WSC (%DM)	14.3
Starch (%DM)	4.58
CP (%DM)	17.2
NDF (%DM)	37.6
ADF (%DM)	23.0
LAB ($\log_{10}$ CFU g^{-1} FW)	4.98
Aerobic bacteria ($\log_{10}$ CFU g^{-1} FW)	7.97
Yeast ($\log_{10}$ CFU g^{-1} FW)	5.02
Mold ($\log_{10}$ CFU g^{-1} FW)	3.45

DM, dry matter; WSC, water-soluble carbohydrate; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; LAB, lactic acid bacteria.

3.5. Effects of LAB Strains on the Fermentation Quality of Broccoli Waste Silage

As presented in Table 4, treatment and moisture content had significant ($p < 0.05$) effects on the values of pH and LA/AA ratio, the contents of LA, AA, and NH_3-N, and the counts of LAB and aerobic bacteria. The moisture content of the broccoli waste was determined to be 90.1%, 86.6%, and 80.0% for W0, W1, and W2 treatments, respectively. Regardless of moisture content, inoculation with CB120 had significantly ($p < 0.05$) lower silage pH value compared to CK. And regardless of treatment, the highest pH value of 4.77 was observed at 90% moisture content, significantly ($p < 0.05$) higher than the pH values at 87% and 80% moisture contents. Lactic acid content was highest in CB120, followed by CB94, both significantly ($p < 0.05$) higher than CB89, CB112, and CK. NH_3-N content for CB120 was not significantly different from CK ($p > 0.05$), while other strains showed higher levels than CK ($p < 0.05$). The LA/AA ratio was lowest at 90% moisture content, with no significant differences between 87% and 80% moisture contents ($p > 0.05$). NH_3-N content was highest at 90% moisture content, significantly ($p < 0.05$) higher than the other two moisture contents, with the lowest at 80% moisture content. Regardless of moisture content, except for CB120, the inoculation of CB89, CB94, and CB112 resulted in significantly ($p < 0.05$) higher LAB counts compared to CK, with CB89 exhibiting the highest counts, and no significant ($p > 0.05$) differences between strains CB94 and CB112. Aerobic bacterial counts for all four strains were significantly ($p < 0.05$) lower than CK, with CB120 showing the lowest counts, followed by CB94 and CB112.

Table 4. Effects of the isolated LAB strains on the fermentation quality of broccoli waste silage after 60 days of ensiling.

Treatments	Moisture Content	pH	LA (g·kg^{-1} DM)	AA (g·kg^{-1} DM)	LA/AA	NH_3-N (mmol/kg DM)	LAB (log_{10} CFU g^{-1} FW)	Aerobic Bacteria (lg CFU g^{-1} FW)
CK	W0	4.50	4.67	1.47	3.23	275.81	6.20	3.34
	W1	4.34	3.25	0.69	4.72	250.57	7.04	3.36
	W2	4.31	3.55	0.77	4.70	187.63	6.60	2.71
CB89	W0	4.93	4.59	3.99	1.15	500.24	7.33	2.76
	W1	4.32	3.08	0.66	4.76	304.00	7.78	2.97
	W2	4.21	4.55	0.81	5.61	189.15	6.67	3.09
CB94	W0	5.01	3.50	2.01	1.78	453.46	7.34	2.33
	W1	4.32	5.49	1.27	4.38	278.02	6.81	2.66
	W2	4.16	4.40	1.04	4.26	159.22	6.88	2.93
CB112	W0	5.14	3.29	2.55	1.31	465.43	7.50	3.36
	W1	4.25	4.02	0.84	4.99	220.97	6.69	2.16
	W2	4.29	4.03	0.88	4.56	220.86	6.64	2.34
CB120	W0	4.28	6.24	1.28	4.97	346.89	6.72	2.63
	W1	4.28	6.46	1.49	4.32	228.65	6.58	2.22
	W2	4.12	3.72	0.84	4.47	190.22	5.99	2.74
CK		4.38 ^{b}	3.82 ^{c}	0.98 ^{d}	4.22 ^{ab}	238.00 ^{d}	6.61 ^{c}	3.14 ^{a}
CB89		4.49 ^{ab}	4.08 ^{bc}	1.82 ^{a}	3.84 ^{bc}	331.13 ^{ab}	7.26 ^{a}	2.94 ^{b}
CB94		4.50 ^{ab}	4.46 ^{b}	1.44 ^{bc}	3.47 ^{c}	296.90 ^{bc}	7.01 ^{b}	2.64 ^{c}
CB112		4.56 ^{a}	3.78 ^{c}	1.43 ^{bc}	3.62 ^{c}	302.42 ^{bc}	6.95 ^{b}	2.62 ^{cd}
CB120		4.23 ^{c}	5.47 ^{a}	1.20 ^{cd}	4.59 ^{a}	255.25 ^{cd}	6.43 ^{d}	2.53 ^{d}
W0		4.77 ^{a}	4.46 ^{a}	2.26 ^{a}	2.49 ^{b}	408.37 ^{a}	7.02 ^{a}	2.89 ^{a}
W1		4.30 ^{b}	4.46 ^{a}	0.99 ^{b}	4.64 ^{a}	256.44 ^{b}	6.98 ^{a}	2.63 ^{c}
W2		4.22 ^{b}	4.05 ^{b}	0.87 ^{b}	4.72 ^{a}	189.42 ^{c}	6.56 ^{b}	2.76 ^{b}
T		**	**	**	**	**	**	**
M		**	*	**	**	**	**	**
T × M		**	**	**	**	*	**	**

LA, lactic acid; AA, acetic acid; LA/AA, lactic acid/acetic acid; NH_3-N, ammonia nitrogen; LAB, lactic acid bacteria. T, the effect of treatment; M, the effect of moisture content; T × M, the interaction effects of treatment and moisture content. *, $p < 0.05$; **, $p < 0.01$. Different letters in the same column indicated significant difference at $p < 0.05$.

3.6. Effects of LAB Strains on the Nutritional Value of Broccoli Waste Silage

Treatment and moisture content significantly ($p < 0.05$) affects the contents of WSC, starch, NSC, CP, NDF, and ADF (Table 5). No significant ($p > 0.05$) differences in DM content were observed among the four strains after ensiling. NSC content in CB94 was not

significantly ($p > 0.05$) different from CK, and significantly ($p < 0.05$) lower than other strains, with CB112 having the highest content followed by CB89. CB94 and CB112 had significantly ($p < 0.05$) higher CP contents compared to CK, CB89, and CB120. The content of NDF was significantly ($p < 0.05$) higher than CK for all strains, with CB112 and CB120 significantly ($p < 0.05$) higher than CB89 and CB94. CB120 had significantly ($p < 0.05$) higher ADF content than other treatments, followed by CB112 and CB89. The moisture content of 80% showed superior parameters in DM, NSC, and CP compared to other two moisture contents.

Table 5. Effects of the isolated LAB strains on the nutritional value of broccoli waste silage after 60 days of ensiling.

Treatments	Moisture Content	DM (%FW)	WSC (%DM)	Starch (%DM)	NSC (%DM)	CP (%DM)	NDF (%DM)	ADF (%DM)
	W0	9.82	1.03	4.13	5.16	22.87	24.09	18.28
CK	W1	13.75	0.97	5.02	6.00	24.58	22.31	17.74
	W2	16.20	0.98	4.76	5.75	24.10	22.01	17.31
	W0	10.24	0.32	8.96	9.27	21.18	31.93	23.65
CB89	W1	11.56	1.05	5.02	6.07	24.28	27.43	20.94
	W2	15.53	1.08	4.78	5.86	24.45	20.92	16.34
	W0	10.36	0.82	5.75	6.58	23.07	29.43	21.64
CB94	W1	12.30	0.94	4.89	5.83	25.44	28.50	20.90
	W2	16.73	1.10	4.43	5.52	26.36	18.23	14.09
	W0	9.70	0.65	5.31	5.96	23.44	35.84	23.16
CB112	W1	12.23	0.71	6.74	7.45	25.88	28.44	20.32
	W2	16.01	0.59	7.35	7.95	25.74	28.44	18.71
	W0	9.70	0.98	5.58	6.56	24.78	30.14	22.37
CB120	W1	11.07	0.87	4.88	5.75	22.17	33.65	25.58
	W2	16.75	0.65	6.55	7.19	24.49	29.42	19.76
CK		13.26	1.00 ^{a}	4.64 ^{c}	5.63 ^{c}	23.85 ^{b}	22.81 ^{c}	17.78 ^{c}
CB89		12.44	0.82 ^{b}	6.25 ^{a}	7.07 ^{a}	23.30 ^{c}	26.76 ^{b}	20.31 ^{b}
CB94		13.13	0.96 ^{a}	5.02 ^{c}	5.98 ^{c}	24.96 ^{a}	25.39 ^{b}	18.88 ^{c}
CB112		12.65	0.65 ^{c}	6.47 ^{a}	7.12 ^{a}	25.02 ^{a}	30.90 ^{a}	20.73 ^{b}
CB120		12.51	0.83 ^{b}	5.67 ^{b}	6.50 ^{b}	23.81 ^{b}	31.07 ^{a}	22.57 ^{a}
W0		9.96 ^{c}	0.76 ^{b}	5.95 ^{a}	6.71 ^{a}	23.07 ^{c}	30.29 ^{a}	21.82 ^{a}
W1		12.18 ^{b}	0.91 ^{a}	5.31 ^{b}	6.22 ^{b}	24.47 ^{b}	28.07 ^{b}	21.10 ^{a}
W2		16.25 ^{a}	0.88 ^{a}	5.57 ^{ab}	6.45 ^{ab}	25.03 ^{a}	23.80 ^{c}	17.24 ^{b}
T		NS	**	**	**	**	**	**
M		**	**	*	*	**	**	**
T × M		NS	**	**	**	**	**	**

DM, dry matter; WSC, water-soluble carbohydrate; NSC, nonstructural carbohydrate; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber. T, the effect of treatment; M, the effect of moisture content; T × M, the interaction effects of treatment and moisture content. NS, not significant; *, $p < 0.05$; **, $p < 0.01$. Different letters in the same column indicated significant difference at $p < 0.05$.

Thus, strain CB120 significantly improved the fermentation quality of broccoli waste silage even at 90% moisture content.

4. Discussion

The content of soluble carbohydrate and the number of lactic acid bacteria in silage materials are the key indicators to determine the success of silage. According to McDonald et al. [5], the level of WSC and lactic acid bacteria required for well storage of silage should exceed 50 g/kg DM and $\log_{10}$ CFU g^{-1} FW, respectively. In this study, although the WSC content is sufficient, the LAB count is difficult to satisfy the requirement of desirable silage fermentation, which explained the unacceptable silage quality of CK. The uninoculated CK showed signs of spoilage after 60 days of ensiling, with a pungent acidic odor upon opening, likely related to BA and high levels of ammoniacal NH_3-N produced during natural fermentation. Moreover, material moisture content is also a major constraint to determine the fermentation pattern of silage. Even after 5 h of wilting, broccoli waste

still can be classified as high-moisture material due to the moisture up to 80%. Vegetable residues, due to their high water activity and buffering capacity, take longer to reach a stable state of fermentation, making them susceptible to the activity of clostridia, which can lead to butyric acid-type fermentation and cause extensive protein degradation [27]. Similarly, highest content of NH_3-N was found in broccoli waste silage at 90% moisture content. During the ensilage of vegetable residues, LAB must grow rapidly and have a strong acid-producing capability to quickly reduce the pH of the ensiled vegetable residues, thereby preventing spoilage fermentation and thus producing high-quality silage [28]. Thus, the addition of exogenous LAB to the high-moisture materials broccoli waste is necessary and required.

According to their morphology, LAB can be classified into three major categories: cocci, rod-shaped, and branched [29]. These bacteria are diverse and widely distributed; to date, at least 27 genera of LAB have been identified, with *Lactobacillus* being the largest genus, encompassing over 150 species [30]. Due to their acid-producing characteristics and probiotic roles in the intestines of humans and animals, they are classified as Generally Recognized as Safe (GRAS) microorganisms [31], and are frequently used in fermented foods and feeds. The research focusing on the selection of superior LAB strains for artificial inoculation in silage fermentation is increasingly becoming a focal point in silage studies [32]. LAB fermentation can be categorized into homolactic and heterolactic types [5]. Homolactic fermentation produces only lactic acid, consuming less energy; heterolactic fermentation produces a mix of lactic acid, ethanol, acetic acid, and other products, requiring more energy. Thus, when producing silage, considering the fermentation products, it is advisable to use microbial preparations capable of homolactic fermentation. Among all LABs, *Lactobacillus* species can produce lactic acid in large amounts over an extended period, dominating the fermentation process. In this study, four rapid-growing and high acid-producing LAB strains (CB89, CB94, CB112, and CB120) were isolated from naturally ensiled broccoli waste samples and identified *L. plantarum*. All four strains improved the fermentation quality and nutritional value of broccoli waste silage in different aspects, with CB120 doing so the best. These results are expected because numerous studies have indicated that inoculating *L. plantarum* can enhance LA production and improve the silage quality [33]. And for high-moisture material silages, the effectiveness of *L. plantarum* were also observed in several studies [34–36]. Zhang [37] isolated strains from cabbage waste material (60% moisture content) source from various locations in Sichuan and identified an excellent strain, C20, with 100% similarity to *L. plantarum* MH544641. Wang [38] utilized various vegetable residues, including Chinese cabbage, sweet potato leaves, water spinach, lettuce celtuce, baby bok choy, and lettuce, and subsequently isolating a superior LAB strain, SCR10, with 99.6% similarity to *L. plantarum* NRRL B-14768T. These strains isolated in prior studies are identical to this study, suggesting that the species *L. plantarum* may possess potential adaptability to high-moisture conditions.

The broccoli waste silage with added LABs showed no signs of spoilage, and the silage emitted a pleasant acidic aroma upon opening. This result aligns with findings by Zhou et al. (2015), who noted that naturally ensiled high-moisture alfalfa had low nutritional value and poor fermentation quality, with high levels of NH_3-N and BA, whereas the addition of *L. plantarum* promoted lactic acid fermentation and inhibited BA fermentation. Additionally, ensiling broccoli waste with CB120 at different moisture contents above 80% achieved good results reflected by the low pH, NH3-N, and aerobic bacteria levels, as well as the high LA content, indicating that this LAB strain has excellent development potential. Therefore, in the current study, we found that selecting superior LAB strains from high-moisture silage and then applying them in high-moisture silage can better adapt to the fermentation process of high-moisture materials, which is consistent with the finding of Wang et al. [15] that the optimal isolates for specific crops may originate from those very crops, making the selection of superior LAB strains from vegetable residue silage an effective strategy for developing additives for high-moisture material ensilage.

5. Conclusions

In this study, four LAB strains, CB89, CB94, CB112, and CB120, were isolated and identified from ensiled broccoli waste samples as *L. plantarum*. These strains exhibited characteristics such as rapid fermentation rate, strong acid production capability, considerable acid tolerance, and a wide range of carbohydrate utilization. Among them, the strain CB120 effectively reduced the aerobic bacteria counts and ultimately enhanced the fermentation and nutritional quality of high-moisture broccoli waste silage (moisture content above 80%), which may provide excellent bacterial strains for the production of high-moisture silage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060282/s1, Table S1: Substrate fermentation test of LAB strains.

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