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Silage Fermentation: A Potential Microbial Approach for the Forage Utilization of *Cyperus esculentus* L. By-Product

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Abstract: *Cyperus esculentus* L. leaves (CLL) are agricultural by-products produced from *Cyperus esculentus* L. harvesting, and can be used as livestock feed despite their low economic value for human consumption. This study aims to develop a favorable approach to processing *Cyperus esculentus* L. by-product as coarse fodder. The chopped CLL was pretreated by (1) mixing with canola straw at a 4:1 ratio, or (2) wilting it for 8 h, then it ensiling with or without compounded lactic acid bacteria (LAB) additives for 60 days. Our results demonstrated that compounded LAB additives: improved CLL silage fermentation quality by increasing acetic acid and lactic acid contents and decreasing ethanol and ammonia-N contents; preserved nutrients by raising the level of crude protein and water soluble carbohydrates; modified the bacterial community by increasing the relative abundance of *Lactobacillus* while decreasing the relative abundance of undesirable *Enterococcus*; and also might improve animal health by increasing the relative concentrations of antioxidant substances (such as 7-galloylcatechin) and antibacterial compounds (such as ferulic acid). This study provides strong evidence that *Cyperus esculentus* L. by-product can be a potential livestock feed after being ensiled with compounded LAB additives.

Keywords: *Cyperus esculentus* L.; silage fermentation; microbial community; forage resource; metabolomics

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1. Introduction

The perennial herb *Cyperus esculentus* L. comes from the family Cyperaceae, and is also known as tiger nuts [1–3]. After it was first introduced to China in 1952, it was widely planted due to its resistance to cold temperatures, drought, waterlogging, barrenness, salt, and alkaline conditions; the productivity of *Cyperus esculentus* L. tubers can reach 15–18 t/hm^2 [4]. *Cyperus esculentus* L. is comprised of above-ground leaves, underground tubers and roots [5]. The nutritional value of these plant parts means that *Cyperus esculentus* L. can serve as a beneficial crop for both humans and animals [6]. Tubers with rich flavor and fats have historically been melted into "Horchata de Chufa," a kind of drink with a nutty taste, and squeezed oil, leaving by-products such as leaves, which are typically burned or discarded [1,7].

However, *Cyperus esculentus* L. leaves (CLL) is a source of polysaccharides, fiber, and antioxidant compounds (such as vitamin E and polyphenols). CLL is comprised of 9.8% crude protein (CP) and 19.3% crude fiber, meaning its leaves can also be used as functional silage materials [4]. The CP content of CLL is similar to that of oats (*Avena sativa*), which are known for their high protein content and digestible fraction [8,9]. This suggests that CLL could be used as livestock feed, especially in the absence of high-quality forage. If mixed with forage and fed to cows, it can increase milk yield while decreasing cost [4]. Jing et al. have reported the neuroprotective capacity of *Cyperus esculentus* L. orientin obtained from CLL [5]. The antioxidant capability of silage can increase the accumulation of desirable

fatty acids in the milk and meat of livestock, and improve the antioxidant capabilities of meat, as well as aid in fighting off rumen bloat [10]. Despite the potential benefits of CLL, it is still underutilized and there is little information about how it can be used as a coarse fodder.

Ensiling is typically the most effective and economical method to preserve fresh forage grass and it can improve palatability and prolong the maximum storage duration via anaerobic fermentation [11,12]. For silage fermentation, the low dry matter (DM) content (19.9%), the low water-soluble carbohydrates (WSC) content (2.52% DM), and the low level of epiphytic lactic acid bacteria (LAB, $<10^5$ colony-forming units (CFU)/g fresh matter (FM)) of fresh CLL make it difficult to ensile [12]. The DM content can be adjusted by adding absorbents (such as crops straw) and wilting. The common additives used to obtain high-quality silage are molasses and LAB. Molasses can be used as a soluble carbohydrate for LAB metabolism, and is subsequently converted into lactic acid to decrease silage pH value during the ensiling process [13,14]. LAB is divided into homofermentative and heterofermentative strains. Homofermentative LAB strains, such as *Lactobacillus plantarum*, quickly produce lactic acid, which helps to preserve nutrition by lowering the silage pH [15]; heterofermentative LAB strains, such as *Lactobacillus buchneri*, could improve aerobic stability by suppressing the growth of yeast and mold through producing acetic acid. Thus, compounded LAB additives (mainly consisting of *L.plantarum* CGMCC No.13318, *L. buchneri* CGMCC No.16534 and molasses) were applied to ensile the agricultural by-product CLL under proper DM content (36–37%).

This study aims to provide a suitable approach to forage utilization of *Cyperus esculentus* L. by-product. The effects of compounded LAB additives on CLL silage, pretreated with canola straw or wilted, were evaluated. In particular, the bacteria community and metabolomics were studied, which could provide vital information for further regulation of fermentation and the exploitation of bioactive ingredients.

2. Materials and Methods

2.1. CLL Harvest and Silage Making

The CLL was harvested from an experimental field of the Yellow River Delta Modern Agricultural Technology Innovation Center (37°67′ N; 118°90′ E) on 19 September 2020. Before ensiling, the harvested CLL were divided into two groups: the Sample 1 (S1) group was mixed with canola straw at a 4:1 ratio, while the Sample 2 (S2) group wilted for 8 h. Following these pretreatments, the DM contents of both groups adjusted to 36–37%.

The CLL were chopped into theoretically 2 cm-long pieces with a crop chopper. Then, they were randomly ensiled with the compound LAB additives, which were comprised of *L. plantarum* CGMCC No.13318, *L. buchneri* CGMCC No.16534, and molasses. The inoculants were added at a rate of 10^6 CFU/g FM, and the molasses were added at a level of 0.5% (*w*/*w*) FM in treatments (LM in the S1 group and WLM in the S2 group). An equal amount of distilled water was sprayed for the controls without additives (CK in the S1 group and WCK in the S2 group). They were then homogenously mixed with additives or distilled water, after which 300 g of fresh forage was packed into vacuum-sealing polyethylene plastic bags. A total of 24 silos (2 groups × 2 treatments × 6 replicates) were maintained at room temperature (21–30 °C). Samples were obtained after 60 days of ensiling.

2.2. Analysis of Culture-Based Microbial

To calculate the microbial counts, samples of 10 g CLL silage were mixed with 90 mL of a sterile saline solution, after which they were diluted from 10^{-1} to 10^{-7}. One hundred µL were spread on the various agar plates. The amount of LAB was calculated on de Man, Rogosa, Sharpe agar (MRS), which was enumerated at 37 °C for 48 h under anaerobic conditions. The yeast and molds were counted on a potato dextrose agar (PDA) and were incubated at 30 °C for 24 h under aerobic conditions. Aerobes were calculated on Nutrient Agar (NA) at 37 °C for 24 h. All culture media were purchased from Beijing Solarbio Science

& Technology Co., Ltd. Yeast and molds were distinguished from bacteria according to the appearance and cell morphology of the colony.

2.3. Fermentation Quality Analysis

Samples of 10 g CLL silage were blended with 90 mL sterilized water, after which they were incubated overnight at 4 °C. They were then filtered through a 0.22 μm filter [13]. The resulting extract was used to measure pH value (pH 213; HANNA; Italy), the contents of ammonia-N, and organic acid. Levels of ammonia-N were analyzed using the ninhydrin colorimetric and phenol-hypochlorite procedures outlined by Broderick and Kang [16]. The contents of organic acid were assessed by High-Performance Liquid Chromatography (HPLC, 1200, Agilent, California, America) equipped with a 210 nm UV detector and an ICSep COREGEL-87H column (temperature of column: 55 °C). The mobile phase was 0.004 mol/L H_2SO_4 with a flow rate of 0.6 mL/min and an injection volume of 10 μL. The buffering capacity (BC) was measured according to the methods used by Playne and McDonald [17].

2.4. Chemical Composition Analysis

Approximately 150 g samples (pre-ensiled material and silage sample) were collected. They were then placed in an oven at 65 °C for 48 h, and the temperature was increased to 105 °C until they reached a constant weight. The DM content was then calculated as a former record [18]. The dried sample was ground to a particle diameter of 1.0 mm to analyze the chemical composition. The WSC was determined according to the methods used by Murphy [19] and the CP was examined according to the procedures established by the AOAC [20]. The content of the neutral detergent fiber (NDF), the acid detergent fiber (ADF), and the acid detergent lignin (ADL) were all measured according to the procedures used by VanSoest et al. [21].

2.5. Bacterial Diversity Sequenced by High-Throughput Sequencing

Samples of 10 g CLL silage were randomly selected and vigorously blended with 40 mL sterilized water at 4 °C and shaken at 120 rpm for 30 min. The resulting mix was then filtered through a filter (0.22 mm, Cat. B-CYD400G1), after which it was obtained by centrifugation at 4 °C at 5000 rpm for 2 min, which removed the impurities and transported the supernatant to obtain microbial pellets. It was then centrifuged at 4 °C and 12,000 rpm for 10 min. The total DNA was extracted from the samples using a MolPure Soil DNA Kit (Yeasen, Beijing, China), according to the manufacturer's instructions.

Universal primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGG GTWTCTAAT) were used to amplify the V3–V4 variable regions of the bacterial 16S rDNA. The PCR reactions were conducted according to the methods used by Su et al. [14]. To minimize PCR deviation, a triple PCR reaction was performed for each sample. Three PCR products were mixed and sequenced using paired-end reads (2 × 300 bp) with an Illumina MiSeq platform (Shanghai Majorbio Bio-pharm Technology Co. Ltd.) according to standard protocols. The sequenced reads were processed according to the methods used by Ni et al. [22]. The raw tags were quality-filtered and merged using Trimmomatic (Version 3.29) and FLASH (Version 1.2.11). ACE, Simpson, Chao, Shannon, and Good's coverage were all calculated to assess the alpha diversity.

2.6. Metabolomics Analysis Using LC-MS

A non-targeted metabolome method was used to evaluate the metabolomics of CLL ensiling with the LC-MS ABSciex Triple TOF 5600TM (ABSCIEX, USA) in Majorbio Bio-pharm Technology (Shanghai, China) according to the published procedures of a previous study [23]. A partial extract (75 μL) from each sample was used for the quality control (QC) samples. The raw data were imported to ProgenesisQI (WatersCorporation, Milford, CT, USA) to normalize the retention times, MZ, observations and peak intensities. According to the characteristic peaks and the MS mass spectra information, the metabolites were matched

with the metabolic databases such as: http://www.hmdb.ca/ (accessed on 23 March 2021) https://metlin.scripps.edu/ (accessed on 13 April 2021) and self-built databases on the on the Majorbio I-Sanger platform (www.i-sanger.com, accessed on 15 April 2021). The MS mass error was set to less than 10 ppm. Relative quantifications of metabolites were carried out as a ratio of their peak areas to the peal area of internal standard substance (0.02 mg/mL 2-Chloro-L-Phenylalanine, CAS. 103616-89-3, Adamas-beta). The data were then filtered the missing value, missed value recoding, normalized and logarithmic transformed. Principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were carried out to show the metabolism changes among the S1 and S2 groups after mean centering and unit variance scaling. The differential metabolites were defined at the fold change $\geq$1 or $\leq$0.5 as well as variable importance in the projection (VIP) of the OPLS-DA model greater than 1.

2.7. Calculation of Relative Feed Value and Fermentation Coefficient

Relative feed value (RFV) is a forage evaluation index typically used for assessing intake and energy value and was published by the American Forage and Grassland Council [24]. RFV is calculated by ADF and NDF according to the following formula [24]:

$$DDM\ (\%DM) = 88.90 - 0.779 \times ADF\ (\%DM)$$

$$DMI\ (\%Body\ weight) = 120/NDF\ (\%DM)$$

$$RFV = (DDM \times DMI)/1.29$$

DDM: digestible dry matter; *DMI*: dry matter intake.

To evaluate the ensilability of CLL, a fermentation coefficient (*FC*) was calculated from *DM*% content, *WSC* content, and *BC* of CLL according to the following formula [25]:

$$FC = DM\% + 8 \times WSC\ (\%DM)/BC$$

2.8. Statistical Analysis

Unless otherwise stated, the statistical analyses were conducted using the SAS (version 9.0, 2002; SAS Institute, Cary, NC, USA). The independent sample t-tests of variance were applied to compare the data in S1 and S2 groups, respectively. The significance was declared if $p < 0.05$. The data presented are the means $\pm$ standard deviation (SD). Microbial counts were assessed via log 10 and analyzed based on their FM. Correlations between differentially presented metabolites and main bacteria species in the S1 and S2 groups were plotted as Spearman correlations, respectively. p values < 0.05 were considered statistically significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. Characteristics of Fresh CLL

Table 1 displays the chemical composition and the number of microorganisms in the raw materials prior to ensiling. In harvested CLL, the DM content was approximately 20%; the CP content was 11.56% DM; the NDF, ADF, and ADL concentrations were 56.08% DM, 28.92% DM and 9.54% DM, respectively; and the WSC content was approximately 2.52% DM. After it was mixed with canola straw at a 4:1 ratio in the S1 group, the DM content was 37.20%; the CP content was 5.17% DM; the NDF, ADF, and ADL concentrations were 61.40% DM, 40.55% DM, and 4.54% DM, respectively; and the WSC contents was 6.46% DM. After wilting for 8 h in the S2 group, the DM content was 36.40%; the CP content was 11.96% DM and the NDF, ADF, and ADL concentrations were 55.10% DM, 29.40% DM, and 8.40% DM, respectively; and the WSC content was 3.20% DM. Otherwise, the amount of epiphytic LAB, yeast, and aerobes in CLL was close to that of canola straws (which were obtained via plate culturing). The amount of LAB was lower than 10^5 CFU/g FM, while the number of yeast and aerobes was approximately 10^6 CFU/g FM.

Table 1. Chemical composition and cultivable microbial population of raw materials before ensiling.

Item	CLL	Canola Straw	S1	S2
Chemical composition				
DM%	19.90	90.78	37.20	36.40
CP (%DM)	11.56	5.21	5.17	11.96
NDF (%DM)	56.08	62.40	61.40	55.10
ADF (%DM)	28.92	50.51	40.55	29.40
ADL (%DM)	9.54	3.53	4.54	8.40
WSC (%DM)	2.52	8.45	6.46	3.20
Cultivable microbial population (log_{10} CFU/g FM)				
Yeast	6.71	5.16	/	/
Aerobes	6.92	6.14	/	/
LAB	4.00	4.70	/	/

DM, dry matter; FM, fresh matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; WSC, water soluble carbohydrates; LAB, lactic acid bacteria; CFU, colony forming units; "/", the detection was not performed; CLL, *Cyperus esculentus* L. leaves; S1, Sample 1 (mixed with canola straw at a ratio of 4:1); S2, Sample 2 (wilted for 8 h).

3.2. Effect of Additives on Silage Quality and Microbial Population of CLL Silages

As listed in Table 2, the additives significantly affected fermentation quality, chemical composition, and microbial population in both the S1 and S2 groups after 60 days of ensiling. In the S1 group, the pH value was significantly lower in the LM than in the CK (4.09 vs. 5.66, $p < 0.01$). A similar phenomenon was observed in the S2 group (3.91 vs. 5.84 of WLM vs. WCK, $p < 0.01$). Acetic acid and lactic acid were the predominant fermentation end products of CLL silage after 60 days of ensiling, followed by ethanol. The contents of lactic acid were 97.78 g/kg DM and 146.33 g/kg DM in the LM and WLM, respectively, and none was detected in CK and WCK. Neither butyric acid nor propanoic acid were detected. Additionally, the contents of ammonia-N significantly decreased by 42.28% and 71.2% in the S1 and S2 groups, respectively ($p < 0.01$). In the S1 group, LM had a higher FC value (36.83 vs. 36.01); in the S2 group, WLM also had a higher FC value (37.61 vs. 31.94). This indicated that the additives had promoted the fermentation quality of CLL silage.

Table 2. Effects of additives on CLL silage quality and microbial population after 60 days of storage.

Item	S1 Group			S2 Group		
	CK	LM	*p* Value	WCK	WLM	*p* Value
The fermentation quality						
pH value	5.66 ± 0.18 ^{a}	4.09 ± 0.06 ^{b}	<0.01	5.84 ± 0.02 ^{a}	3.91 ± 0.01 ^{b}	<0.01
Lactic acid (g/kg DM)	ND	97.78 ± 24.05	<0.01	ND	146.33 ± 8.98	<0.01
Acetic acid (g/kg DM)	14.00 ± 2.10 ^{b}	146.34 ± 13.74 ^{a}	<0.01	18.67 ± 1.97 ^{b}	215.00 ± 2.83 ^{a}	<0.01
Ethanol (g/kg DM)	17.00 ± 1.63 ^{a}	10.17 ± 1.94 ^{b}	<0.01	19.67 ± 5.35	ND	<0.01
Ammonia-N (g/kg DM)	2.26 ± 0.12 ^{a}	1.30 ± 0.20 ^{b}	<0.01	5.66 ± 0.49 ^{a}	1.63 ± 0.32 ^{b}	<0.01
BC (meq/100 g DM)	19.79 ± 1.29	22.83 ± 1.84	0.19	21.55 ± 0.06	18.45 ± 0.56	0.02
FC	36.01 ± 1.30	36.83 ± 0.27	0.48	31.94 ± 2.74	37.61 ± 2.61	0.17
Chemical composition						
DM%	36.88 ± 0.02	37.89 ± 0.02	0.31	32.74 ± 0.01 ^{b}	37.20 ± 0.03 ^{a}	<0.01
CP (%DM)	8.21 ± 0.38 ^{b}	9.60 ± 0.10 ^{a}	<0.01	12.17 ± 0.57 ^{b}	13.35 ± 0.17 ^{a}	<0.01
NDF (%DM)	63.68 ± 1.89	61.78 ± 1.93	0.21	55.75 ± 1.06 ^{a}	51.70 ± 1.88 ^{b}	0.01
ADF (%DM)	43.95 ± 1.06	41.82 ± 2.51	0.17	30.68 ± 0.48 ^{a}	28.69 ± 0.32 ^{b}	<0.01
ADL (%DM)	8.95 ± 0.75	8.62 ± 0.84	0.57	3.89 ± 0.37	3.63 ± 0.28	0.30
WSC (%DM)	0.39 ± 0.05 ^{b}	0.71 ± 0.04 ^{a}	<0.01	0.63 ± 0.20 ^{b}	1.01 ± 0.08 ^{a}	0.01
RFV	79.93 ± 3.61	84.92 ± 5.39	0.17	108.5 ± 2.51 ^{b}	119.87 ± 4.94 ^{a}	<0.01
Cultivable microbial population (log_{10} CFU/g FM)						
LAB	3.95 ± 2.21 ^{b}	5.54 ± 0.15 ^{a}	<0.01	2.04 ± 2.36 ^{b}	5.38 ± 0.14 ^{a}	<0.01
Aerobes	4.39 ± 0.12 ^{a}	2.37 ± 1.84 ^{b}	<0.01	4.22 ± 0.03 ^{a}	1.17 ± 1.61 ^{b}	<0.01

CK, mixed with canola straw without additives; LM, ensiled with additives; WCK, wilted for 8 h without additives; WLM, wilted for 8 h and ensiled with additives; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; WSC, water soluble carbohydrates; RFV, relative feed value; BC, buffering capacity; FC, fermentation coefficient; LAB, lactic acid bacteria; CFU, colony forming units; ND, not detected. S1, Sample 1 (mixed with canola straw at a ratio of 4:1); S2, Sample 2 (wilted for 8 h). Means ± SD within the same row with different letters is significantly different ($p < 0.05$).

The additives significantly improved the nutritive quality of CLL silage. The DM content after ensiling significantly increased in the WLM than that in the WCK (32.74% vs. 37.20%, $p < 0.01$), a similar trend was also found in the LM than that in the CK (36.88% vs. 37.89%, $p = 0.31$). Compared to the WCK, the NDF, ADF and ADL contents were obviously decreased in the WLM. Thus, the WLM had higher RFV than the WCK ($p < 0.01$). The similar results were found in S1 group although there is no significance. The CP contents increased by 16.93% and 9.70% in the S1 group and S2 group, respectively ($p < 0.01$), the WSC content also increased by 82.05% and 60.32% in the S1 and S2 groups, respectively ($p < 0.01$).

Table 2 also displays the culturable microorganism detected in the S1 and S2 groups. The LAB levels both significantly increased to over 10^5 CFU/g FM and the aerobe numbers decreased to below 10^3 CFU/g FM ($p < 0.01$) due to the additives. Mold and yeast were not detected in any samples.

3.3. Effects of the Additives on the Microbial Communities of CLL Silages

High-throughput sequencing of 16S rDNA gene amplicons demonstrates how bacterial communities are affected after systematic ensiling. The alpha diversity of all samples is exhibited in Table 3. The coverage values of all samples exceeded 0.99, suggesting that the sequencing depth was adequate to detect most bacteria. Compared with the controls in both groups, the treatment groups had both lower Chao and Shannon indexes, reflecting decreases in bacterial diversity due to dominant microorganisms that could have been replaced through cluster analysis. The variance in the microbial population in the S1 and S2 groups can be illustrated by principal component analysis (PCA). CK and LM can both be separated, while a similar discrepancy was also found in the S2 group (Figure S1). These results indicate that the additives significantly affected the bacterial community of CLL silage.

Table 3. Alpha diversity of the bacterial community in the S1 and S2 groups after 60 days of ensiling.

Item	S1 Group			S2 Group		
	CK	LM	*p* Value	WCK	WLM	*p* Value
Shannon	1.99 ± 0.08 [a]	0.58 ± 0.03 [b]	<0.01	2.00 ± 0.24 [a]	0.83 ± 0.08 [b]	<0.01
Simpson	0.25 ± 0.02 [b]	0.69 ± 0.02 [a]	<0.01	0.29 ± 0.06 [b]	0.55 ± 0.05 [a]	0.02
ACE	240.46 ± 11.46	209.68 ± 16.93	0.13	207.95 ± 21.69	159.66 ± 10.51	0.13
Chao 1	234.94 ± 11.42	177.19 ± 18.94	0.03	190.38 ± 17.50	136.69 ± 10.24	0.07
Coverage	0.99	0.99	0.18	0.99	0.99	0.24

CK, mixed with canola straw without additives; LM, ensiled with additives; WCK, wilted for 8 h without additives; WLM, wilted for 8 h and ensiled with additives. S1, Sample 1 (mixed with canola straw at a ratio of 4:1); S2, Sample 2 (wilted for 8 h). Means ± SD within the same row with different letters is significantly different ($p < 0.05$).

Figure 1 displays the bacterial communities at the genus and species level of the S1 and S2 groups after 60 days of ensiling. In the control groups (CK and WCK), *Enterobacter* was the most abundant genus, comprising approximately 77.51% and 50.16% of the entire communities, respectively. *Kosakonia* and *Enterococcus* were also present in the materials before ensiling. However, the relative abundance of *Enterobacter* significantly decreased or even disappeared in treatments (LM and WLM). *Lactobacillus* is a desirable genus that became the most prevalent genus; its abundance reached nearly 90% of the total population. Additionally, several spore-forming bacteria, such as *Clostridium* and *Bacillus*, were also detected in raw materials. These are always associated with aerobic deterioration. These results implied that the additives have obviously modified the fermented microbiota of CLL silage.

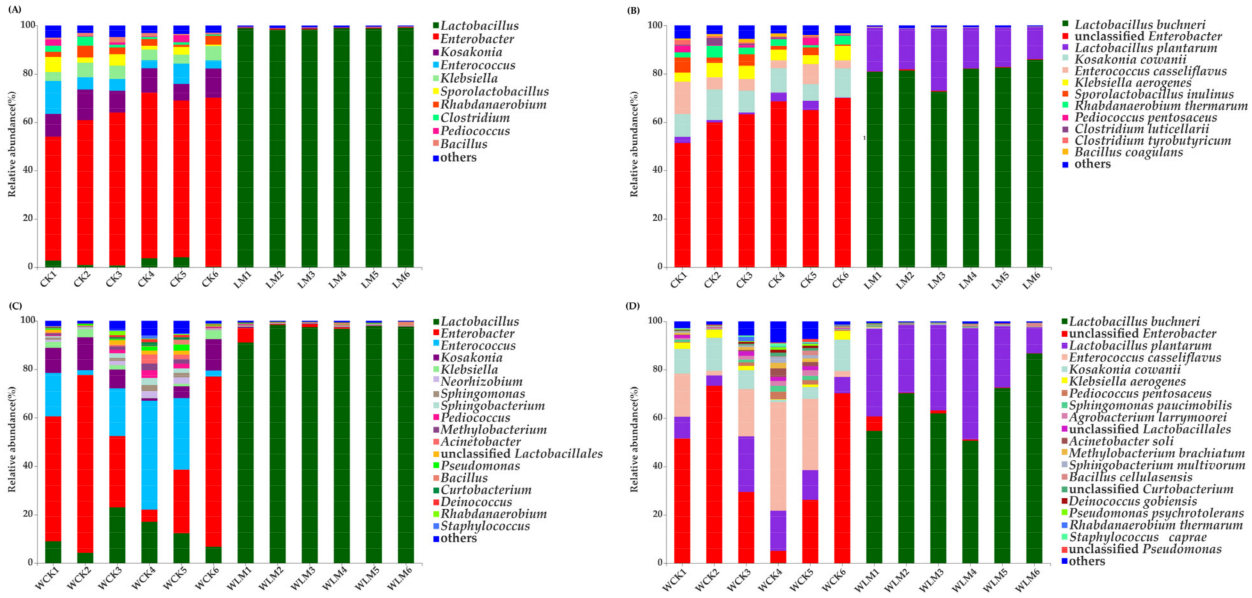

Figure 1. Relative abundances of bacteria communities in CLL silage. (**A**): the genus level of the S1 group; (**B**): the species level of the S1 group; (**C**): the genus level of the S2 group; (**D**): the species level of the S2 group. S1, Sample 1 (mixed with canola straw at a ratio of 4:1); S2, Sample 2 (wilted for 8 h). CK, mixed with rapeseed straw without additives; LM, ensiled with additives; WCK, wilted for 8 h without additives; WLM, wilted for 8 h and ensiled with additives.

3.4. Metabolomics Profiles of CLL Silages Ensiling for 60 Days

Total 891 substances were detected by analyzing the retention time and mass:charge ratio of all ions found in the chromatograms of the CLL silage samples. Of these, 361 metabolites in the S1 group and 406 substances in the S2 group and their relative concentrations were identified and annotated based on the Human Metabolome Database (HMDB). PCA of positive and negative (Figure S2) demonstrated that there were clear differences between CK, LM, and QC (Quality Control) in the S1 group. Similarly, WCK, WLM, and QC were also clearly separated in the S2 group, indicating that there are differences between samples within treatments.

Table 4 displays the relative concentration of certain metabolites with biological functions in the S1 and S2 groups. In the S1 group, LM has more cinnamic acids and derivatives (such as sinapic acid, caffeic acid, and 2-hydroxycinnamic acid), flavonoids (such as the quercetin, theaflavic acid, and isorhamnetin), ferulic acid, and zeranol compared to CK. All of these have antioxidant properties and antibacterial activities. Toxic compounds such as hypoxanthine, 7a-hydroxy-5b-cholanic acid, and 7-epi-Jasmonic acid decreased in the LM compared to the CK. In the S2 group, higher contents of carboxylic acids and derivatives (such as valyl-isoleucine, threoninyl-isoleucine, glycyl-isoleucine, asparaginyl-isoleucine), flavonoids (such as kaempferol 3-(2″-rhamnosylrutinoside) and 7-galloylcatechin), organooxygen compounds (such as 1,3-dicaffeoylquinic acid, chlorogenic acid, and 3-O-feruloylquinic acid), ferulic acid, and D-(+)-3-phenyllactic acid were seen in WLM than in WCK, while the contents of compounds with a foul smell (such as grandisine III and piperidine) decreased.

In summary, the contents of metabolites with beneficial biofunctional activities (such as flavonoids, cinnamic acids and derivatives, steroids and steroid derivatives, and ferulic acid) increased in LM and WLM in the S1 and S2 groups, compared to the control groups (CK and WCK). The type of flavonoids in the S1 group increased more than in the S2 group, while more dipeptide and organooxygen compounds were observed in the S2 group.

Table 4. Relative concentration and fold-changes in differential metabolites in S1 and S2 groups after 60 days of ensiling.

Metabolite Name		S1 Group			S2 Group		
		CK	LM	Fold Change	WCK	WLM	Fold Change
Benzene and substituted derivatives	Syringic acid	12.42 ± 0.18 ^{b}	14.29 ± 0.55 ^{a}	1.87 ± 0.53	13.28 ± 0.30 ^{b}	15.3 ± 0.13 ^{a}	2.02 ± 0.31
Carboxylic acids and derivatives	L-Tyrosine	6.52 ± 0.83 ^{b}	9.89 ± 0.14 ^{a}	3.37 ± 0.88	ND	ND	ND
	Valyl-Isoleucine	ND	ND	ND	2.92 ± 0.65 ^{b}	9.87 ± 0.15 ^{a}	3.50 ± 0.61
	Threoninyl-Isoleucine	ND	ND	ND	1.55 ± 2.33 ^{b}	9.63 ± 0.15 ^{a}	8.08 ± 2.34
	Glycyl-Isoleucine	ND	ND	ND	5.18 ± 2.18 ^{b}	10.85 ± 0.11 ^{a}	5.66 ± 2.14
	Asparaginyl-Isoleucine	ND	ND	ND	6.29 ± 0.65 ^{b}	10.50 ± 0.12 ^{a}	4.20 ± 0.74
Cinnamic acids and derivatives	Sinapic acid	9.53 ± 1.29	11.09 ± 0.70	1.56 ± 1.86	2.92 ± 0.65 ^{b}	9.87 ± 0.15 ^{a}	6.96 ± 0.77
	Caffeic acid	10.04 ± 0.11	11.81 ± 0.79	1.78 ± 0.82	10.60 ± 0.49 ^{b}	13.53 ± 0.12 ^{a}	2.93 ± 0.48
	2-hydroxycinnamic acid	12.25 ± 0.90 ^{b}	14.76 ± 0.12 ^{a}	2.51 ± 0.90	ND	ND	ND
	M-Coumaric acid	8.18 ± 0.80 ^{b}	11.61 ± 0.29 ^{a}	3.42 ± 0.94	9.27 ± 0.76 ^{b}	12.64 ± 0.08 ^{a}	3.38 ± 0.73
Punicic acid	Punicic acid	14.93 ± 0.28	15.25 ± 0.11	0.32 ± 0.27	ND	ND	ND
Flavonoids	Quercetin	12.21 ± 1.40	14.14 ± 0.63	1.94 ± 1.68	ND	ND	ND
	Theaflavic acid	14.73 ± 0.29	15.27 ± 0.20	0.54 ± 0.38	ND	ND	ND
	Isorhamnetin	14.21 ± 1.13	15.52 ± 0.31	1.31 ± 1.21	ND	ND	ND
	Isovitexin	14.27 ± 0.33	14.76 ± 0.13	0.49 ± 0.40	ND	ND	ND
	7-galloylcatechin	ND	ND	ND	2.96 ± 1.54 ^{b}	10.24 ± 0.41 ^{a}	7.27 ± 1.76
	Kaempferol 3-(2″-rhamnosylrutinoside)	ND	ND	ND	9.31 ± 0.79 ^{b}	13.64 ± 0.29 ^{a}	4.32 ± 0.67
Imidazopyrimidines	Hypoxanthine	12.34 ± 0.28	10.95 ± 0.15	−1.39 ± 0.37	12.50 ± 0.39 ^{a}	10.09 ± 0.20 ^{b}	−2.40 ± 0.49
Macrolides and analogues	Zeranol	8.84 ± 0.53 ^{b}	10.48 ± 0.78 ^{a}	1.64 ± 0.84	ND	ND	ND
Organooxygen compounds	1,3-dicaffeoylquinic acid	ND	ND	ND	11.39 ± 0.94	12.35 ± 0.36	0.96 ± 0.92
	3-O-*p*-Coumaroylquinic acid	ND	ND	ND	10.07 ± 0.24 ^{b}	12.43 ± 0.54 ^{a}	2.36 ± 0.43
	Chlorogenic acid	ND	ND	ND	11.73 ± 0.54 ^{b}	14.67 ± 0.57 ^{a}	2.95 ± 0.72
	N-di-Demethyl roxithromycin	ND	ND	ND	9.52 ± 1.47 ^{b}	11.59 ± 0.58 ^{a}	2.07 ± 1.87
	3-O-feruloylquinic acid	ND	ND	ND	7.17 ± 1.38 ^{b}	10.71 ± 0.75 ^{a}	3.54 ± 1.51
Phenylpropanoic acids	Hydroxyphenyllactic acid	ND	ND	ND	10.34 ± 0.42 ^{b}	12.18 ± 0.14 ^{a}	1.84 ± 0.45
Prenol lipids	Annoglabasin E	8.02 ± 0.94	9.96 ± 0.33	1.94 ± 1.12	6.36 ± 0.38 ^{b}	10.56 ± 0.55 ^{a}	4.20 ± 0.40
	Betulin	ND	ND	ND	7.08 ± 1.61 ^{b}	11.02 ± 0.92 ^{a}	4.02 ± 1.82
Piperidine	Piperidine	ND	ND	ND	14.64 ± 0.21	14.39 ± 0.10	−0.25 ± 0.18
Steroids and steroid derivatives	7a-hydroxy-5b-cholanic acid	13.19 ± 0.66	11.87 ± 0.97	−1.32 ± 1.41	3.97 ± 0.20	3.57 ± 0.30	−0.40 ± 0.10
Quinolines and derivatives	Grandisine III	ND	ND	ND	11.94 ± 0.74	10.05 ± 0.54	−1.88 ± 0.91
Others	Ferulic acid	11.47 ± 0.69	13.31 ± 0.20	1.84 ± 0.63	12.26 ± 0.63	13.09 ± 0.14	0.83 ± 0.71
	D-(+)-3-phenyllactic acid	13.66 ± 0.21	14.71 ± 0.06	1.05 ± 0.26	12.67 ± 0.52 ^{b}	14.86 ± 0.06 ^{a}	2.19 ± 0.53
	7-epi-Jasmonic acid	12.15 ± 1.06 ^{a}	8.46 ± 0.48 ^{b}	−3.69 ± 1.42	12.77 ± 3.24 ^{a}	2.42 ± 1.23 ^{b}	−10.36 ± 3.24

The relative concentration of each metabolite is an average of the data from six biological replicates. FC, fold-changes, positive value means upregulation; negative value means downregulation. CK, mixed with canola straw without additives; LM, ensiled with additives; WCK, wilted for 8 h without additives; WLM, wilted for 8 h and ensiled with additives; "ND", not detected. "Others" means there is no corresponding classification in the Human Metabolome Database (HMDB). Means ± SD within the same row with different letters is significantly different ($p < 0.05$).

3.5. Correlations between Main Microorganisms and Metabolites in CLL Silage

A Spearman correlation heat map was generated at the species level to display the association between differential metabolites and dominant bacteria in S1 and S2 groups respectively (Figures 2 and 3). Metabolites positively correlated with LAB were negatively correlated with undesirable bacteria during the ensiling process. In the S1 group, ferulic acid, *m*-Coumaric acid, and *p*-Coumaric acid were positively correlated with *L. plantarum* and *L. buchneri*, and were negatively correlated with the unclassified *Enterobacter*, *Pediococcus pentosaceus*, unclassified *Pantoea*, *Streptomyces albidoflavus*, and unclassified *Curtobacterium*. In the S2 group, 7-galloylcatechin, chlorogenic acid, and 3-O-*p*-Coumaroylquinic acid were positively correlated with *L. plantarum* and *L. buchneri*, and were negatively

correlated with the unclassified *Lactobacillales*, *Enterococcus casseliflavus*, *Kosakonia cowanii* and unclassified *Enterobacter*, *Klebsiella aerogenes*, and *Agrobacterium larrymoorei*.

In summary, metabolites with biological functions tended to positively correlated with LAB and negatively correlated with undesirable bacteria.

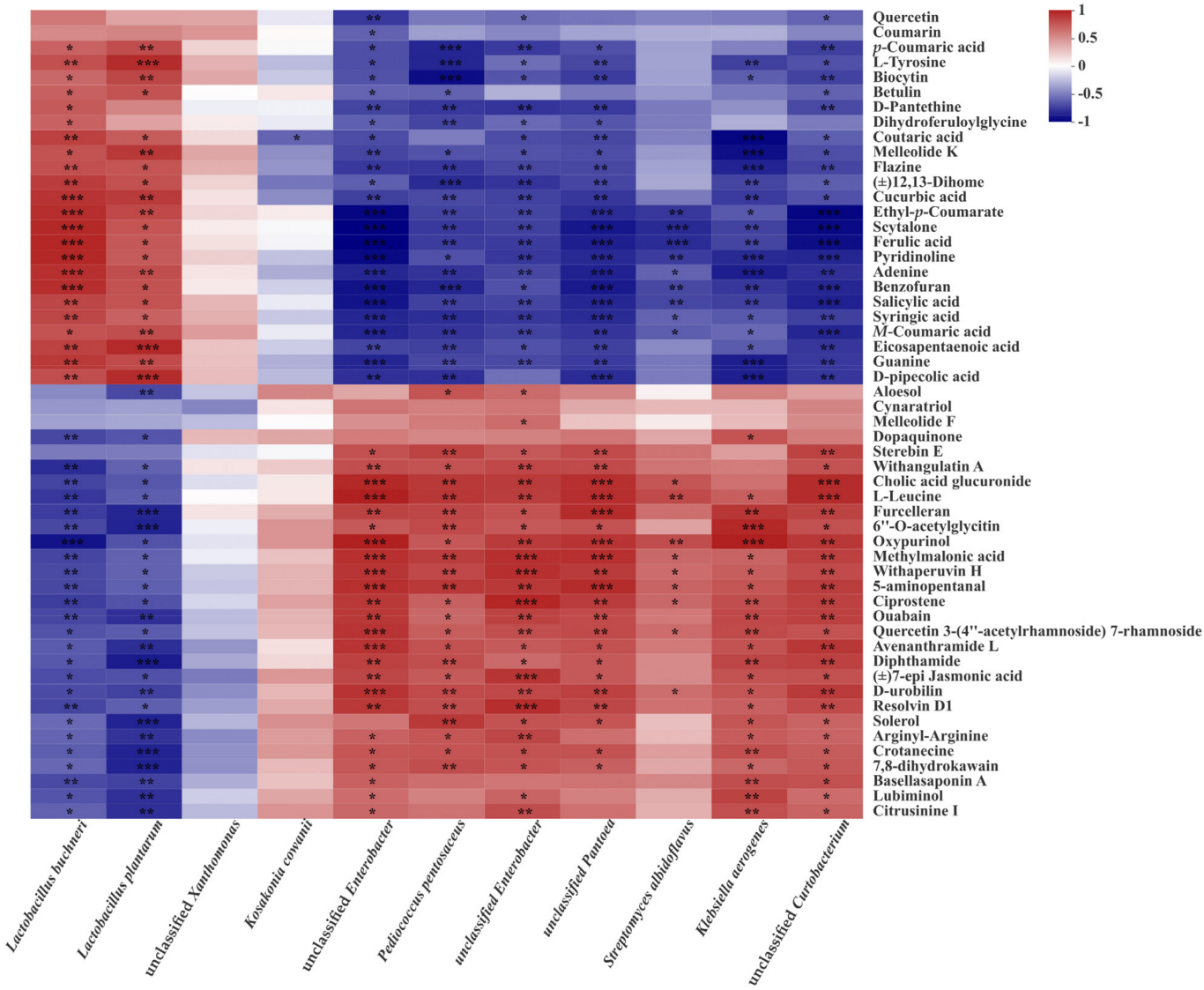

Figure 2. Spearman correlations between main bacteria species and differentially presented metabolites in S1 group. The differentially presented metabolites during fermentation were screened by OPLS-DA; *p*-values are shown as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

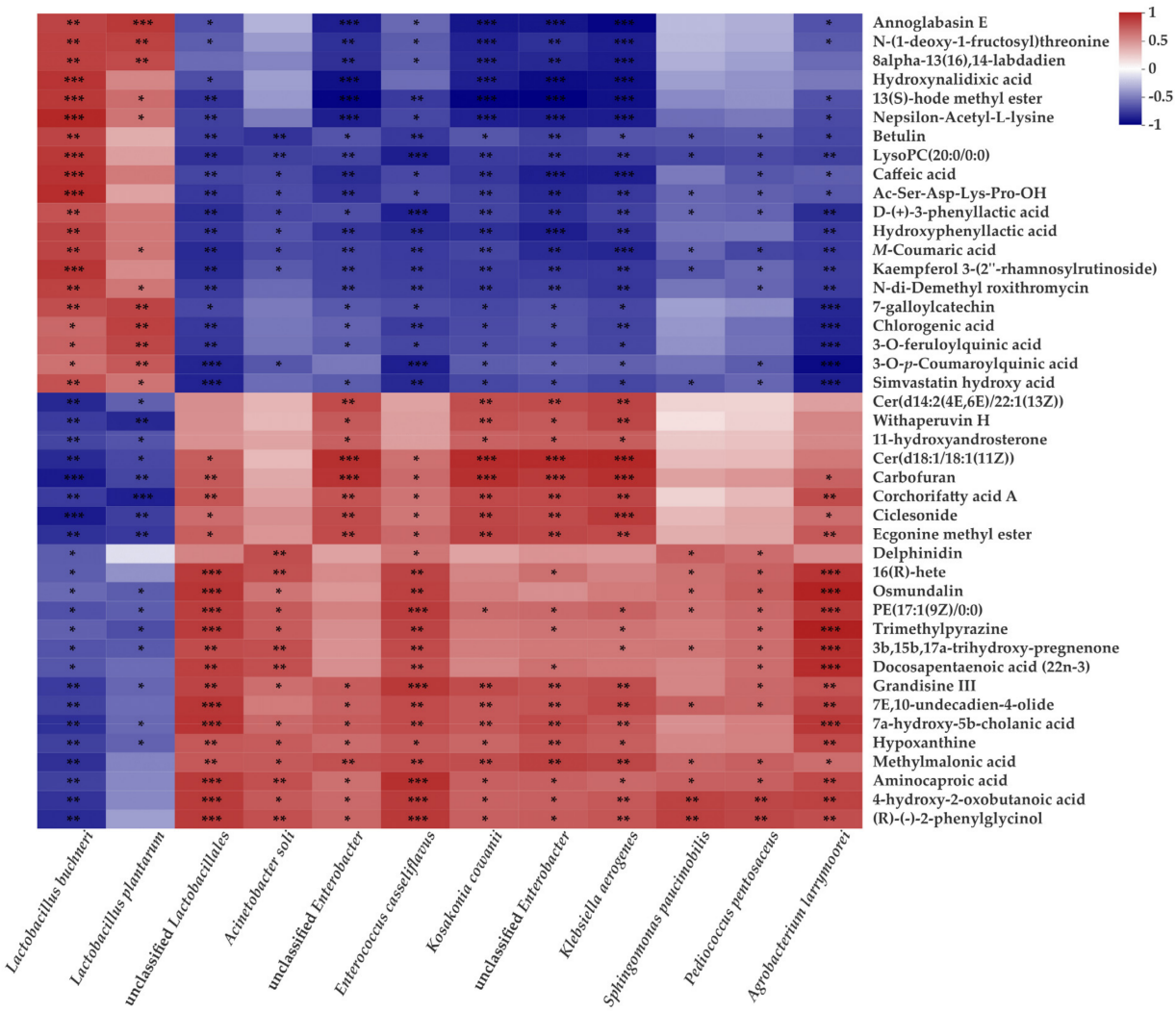

Figure 3. The differentially presented metabolites during fermentation were screened by OPLS-DA; *p*-values are shown as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

4. Discussion

Agricultural by-products are important sources of feed for livestock, especially when livestock cannot acces high-quality roughage. Ensiling is a method typically used to prolong the storage time of forage by using LAB fermentation under anaerobic conditions [13]. As such, a better understanding of the metabolomics and bacterial community during the fermentation process could provide powerful evidence for making high-quality silages [26]. Ensiling is a complex process that is affected by several factors, such as the DM content of materials, WSC content and epiphytic microbiota of materials. Low moisture content helps condense WSC content, promoting lactic acid fermentation and suppressing the production of ammonia-N [27]. In addition, abundant WSC content affects the quality of fermentation, while WSC contents exceeding 5% DM typically produce silage of sufficient quality [13]. Molasses is considered the most effective and economically fermentable substrate [13]. Concerning epiphytic microbiota, both Cai et al. [28] and Oliveira et al. [29] stressed that the forage would only be well-preserved if the level of LAB exceeded 10^5 CFU/g FM. During the ensiling process, LAB can produce lactic acid, hydrogen peroxide, and antimicrobial substances to inhibit undesirable microorganisms to reduce nutritional loss [14]. Otherwise, DM recovery decreases while ammonia-N concentration increases [28–30]. While aerobes are undesirable, they typically appear when the silo opens and are usually associated with high DM loss and decreased aerobic stability [30]. The high moisture content, low WSC

content, low levels of epiphytic LAB, and presence of harmful microorganisms indicates that the ensiling process of fresh CLL must be controlled only if mixed with absorbent or wilted to increase DM content and inoculated with LAB and extra WSC.

Fermentation quality significantly improved after 60 days of ensiling with compound LAB additives. The pH values of additives treated CLL silage were below 4.2, which could be attributed to higher organic acid content produced by LAB. Increased levels of LAB resulted in significant increases in lactic acid and acetic acid contents. Lactic acid is the main product of LAB and has a *pKa* 3.86, which is the main organic acid affecting pH value and promotes the reductions in pH value observed in ensiled material [13]. However, acetic acid was the dominant fermentation product in both the S1 and S2 groups and likely resulted from the abundant heterofermentative pattern favored by *L. buchneri*, which aligns with the findings of Kung et al. [31], Zhang et al. [32] and Wang et al. [18] reported that acetic acid has significant antifungal properties, which are crucial for improving the aerobic stability of silage. Mold and yeast were not detected in all samples, which could be due to low pH values. As Li et al. [33] reported, anaerobic conditions and organic acid can inhibit yeast growth, especially after long storage periods. Additionally, ammonia-N contents decreased in both LM and WLM, which typically occurs during the ensiling process and is a result of the fermentation of *Clostridium* or the activity of plant protease [34]. In this study, *Clostridium* was found in CK/WCK instead of LM/WLM; as such, decreased ammonia-N could be attributed to the inhibited growth of *Clostridium*. Herrmann et al. [35] demonstrates that a raw material is suited for ensiling if it contains sufficient WSC as fermentable substrate, a low BC to allow rapid for acidification, and sufficient DM content. Therefore, the ensilability of silage raw material could be assessed by FC [35]. Weissbach et al. [25] proposed that if the FC value reaches 35, it means that high-quality silage is produced. In this study, the FC value in LM and WLM both exceed 35 and were higher than WCK and CK. The FC value in WLM was higher than in LM, suggesting that wilting may be a better way to promote fermentation than absorbents (canola straw).

The nutritional quality also improved in compound LAB additive-treated CLL silage. DM is a vital measure of the organic matter accumulation of silage, and increasing DM content maximizes the efficiency of the ensiling process [36]. The DM contents increased in both the S1 and S2 groups. The NDF, ADF, and ADL contents represent the digestibility of cellulose and hemicellulose by ruminant animals, which affects digestibility and feed intake [37]. Compared with CK and WCK, relatively low contents of NDF, ADF, and ADL were observed in LM and WLM. This indicates that LM and WLM had low lignocellulosic structures and became highly digestible. The contents of NDF and ADF were not significantly affected in the S1 group, which could be attributed to the addition of canola straw in the S1 group, making the fermentation effect inferior to that in the S2 group. RFV is widely acknowledged to predict forage quality [38]. The RFV of WCK and WLM exceeded that of CK and WCK, which could account for the lower content of ADF, NDF, and the higher content of WSC, which was used to calculate and parameterize the RFV. Alfalfa is a high-quality forage plant with a high protein content; it is cultivated and used for animal feed all over the world [14]. Ertekin et al. [37] reported that the RFV of alfalfa silage (harvested at 50% flowering period) could reach 107.09. The RFV of WCK and WLM exceeded that of alfalfa (108.5 and 119.87, respectively), suggesting that wilted CLL has great potential as a high-quality forage material. In addition, the WSC and CP contents were higher in the wilted group (S2 group), especially in WLM (compound LAB additives treated), indicating that the CLL silage were well-preserved and digestible for additives. Thus, accounting for chemical composition, the S2 group performed better than the S1 group.

High throughput analyses produce data sufficient for taxonomically classifying microbiota identified during ensiling [13]. However, no studies have assessed the bacterial community in CLL silage. The alpha diversity values obtained by this study demonstrate that the bacterial community saw decreases in richness and diversity compared to the con-

trol groups, which was similar to the results of Xu et al. [39]. The main genus in the control groups (CK and WCK) was *Enterobacter*, followed by *Kosakonia*, *Enterococcus*, *Klebsiella*, *Sporolactobacillus,* and *Sphingomonas*. These were defined as undesirable bacteria and were not expected found in silage, for they always competed with LAB and negatively affected silage [30]. After ensiling for 60 days, they were replaced by *Lactobacillus*, which occupied over 98% in the S1 group as well as 95.4% in the S2 group. *Lactobacillus* can inhibit undesirable microorganisms by producing organic acids and metabolites with antimicrobial activity [40]. Additionally, differences in the microbial community could significantly affect silage quality [22]. In this study, *L. buchneri* and *L. plantarum* were the main species found in the CLL silage after ensiling for 60 days. Furthermore, the proportion of *L. buchneri* exceeded that of *L. plantarum*, which is similar to what Xu et al. [40] reported in whole crop corn: that inoculation with *L. plantarum* can promote the growth of *L. buchneri* and result in more acetic acid production than lactic acid production. Heinl et al. [41] reported that the lactic acid produced by homofermentative LAB could be utilized as the substrate of heterofermentative LAB, which could account for this phenomenon.

The fermentation process entails many types of microorganisms, produces various metabolites, and is extremely complex. As such, a better understanding of the metabolomics and bacterial community during the fermentation process could provide powerful evidence for making high-quality roughage [26]. The Spearman correlation analyzed the main bacteria and the main differential metabolites in CLL silage. Our results demonstrate that the correlation between metabolites and *Lactobacillus* was opposite from that in the undesirable microorganisms, indicating that *Lactobacillus* and the undesirable microorganisms competed during CLL ensiling. Furthermore, metabolites with biological activities were always positively associated with *Lactobacillus* and negatively associated with undesirable microorganisms. In the S1 group, *p*-Coumaric acid, which is a precursor of various phenolic compounds and exerts antioxidant and antimicrobial activities, was positively associated with *L. plantarum* and *L. buchneri* [42]. As the major derivatives of *p*-Coumaric acid detected in natural plants with antifungal properties [43], ethyl−*p*−Coumarate was positively correlated with *L. plantarum* and *L. buchneri*. Solerol was negatively associated with *L. plantarum* and *L. buchneri* and is typically a marker for succinic semialdehyde dehydrogenase, which can cause neurological and cognitive disorders [44]. In the S2 group, betulin is positively associated with *L. buchneri*, which is an active natural triterpenoid alcohol with antioxidant potential [45]; 7-galloylcatechin is the key precursor that forms gallic acid during anaerobic fermentation [46] and is positively correlated with *L. plantarum* and *L. buchneri*. Carbofuran is extremely lethal to mammals due to its anticholinesterase activity [47], it has a significantly negative correlation with *L. plantarum* and *L. buchneri*. Additionally, the ferulic acid content increased in LM and WLM. This indicates that the fiber digestibility in the additives treatments is better than in the control since it is typically linked to polysaccharides by ferulic acid ester in plant cell walls, forming a solid protective layer [14]. The up-accumulation of nucleobase and dipeptide was detected, which was consistent with the findings of Guo et al. [26] and Xu et al. [40] that *L. buchneri* can produce certain amino acids during fermentation. Metabolites with biofunctions (such as corchorifatty acid, osmundain, and furelleran, et al.) were negatively correlated with LAB species, which could be due to sample concentrations that were too low to sufficiently detect, which was similar to the findings of Xu et al. [40]. In summary, samples treated with additives (LM/WLM) resulted in the accumulation of certain metabolites with antioxidant properties and antimicrobial activity, including *m*-Coumaric acid, theaflavic acid, quercetin, isorhamnetin, ferulic acid, and 7-galloylcatechin. This caused a reduction in toxic and hazardous substances, including carbofuran and solerol. Admittedly, the Spearman correlation is based on statistics and correlates parameters to obtain a result that represents nothing more than speculation. Even so, the correlations between LAB and the metabolites with biological activities in CLL silage provided clues on screening targeted functional LAB strains for modulating silage fermentation with the aim of obtaining high quality silage.

5. Conclusions

Cyperus esculentus L. by-product could be processed into silage with additives after being mixed with canola straw or wilted. The compound LAB additives produced positive fermentative properties, increased the nutritional value of CLL silage. Additives enhanced the fermentation quality, resulting in low pH values, high contents of lactic acid and acetic acid, and low ammonia-N concentrations. The improved nutritional values are reflected in the increased CP and WSC contents in additives treated groups. In the perspective of FC and RFV, the wilting approach is superior to mixing with canola straw. The compound LAB additives also altered the microbiota of CLL silage. The relative abundance of *Lactobacillus* was enhanced and the relative abundance of undesirable microorganisms, including *Clostridium* and *Enterobacter*, was reduced. In CLL silage, substances with antioxidant properties, bacteriostatic activity, and other flavoring agents were detected, all of which typically have a positive relationship with *L. buchneri* and *L. plantarum*. As the major species in CLL silage, *L. buchneri* and *L. plantarum* might regulate silage fermentation through modifying microbiota and metabolomics. In conclusion, *Cyperus esculentus* L. by-product could be used as a potential roughage after it is ensiled with compounded LAB additives.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7040273/s1, Figure S1: Principal component analysis (PCA) of metabolic profiles in S1 group (S1 A) and S2 group (S1 B) silage inoculated without (red circle) or with additives (blue triangle) (n = 6). S2: Principal component analysis (PCA) of metabolic profiles in S1 (pos: S2.A, neg: S2.B) and S2 (pos: S2 C, neg: S2 D) silage without or with additives. CK, mixed with canola straw without additives; LM ensiled with additives; WCK, wilted for 8 h without additives; WLM, wilted for 8 h and ensiled with additives.

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