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Effects of *Lactiplantibacillus plantarum* and Fermentation Time on the Quality, Bacterial Community, and Functional Prediction of Silage from *Lotus corniculatus* L. in Karst Regions

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Abstract: Abstract: To improve the silage quality of *Lotus corniculatus* L. and expedite the promotion of cultivated varieties, this study investigates the impact of *Lactiplantibacillus plantarum* on the fermentation characteristics, bacterial community, and functional aspects of silage. The experiment included a control (CK) and a *Lactiplantibacillus plantarum* (LP) treatment, with sampling conducted at 3, 7, 15, and 45 days of fermentation to monitor nutritional value and fermentation quality, as well as changes in the bacterial community at 3 and 45 days. The results indicated that compared to the CK, the addition of LP significantly increased the lactic acid, dry matter, and crude protein content ($p < 0.05$) while substantially decreasing the water-soluble carbohydrates, pH, NH_3-N, and acetic acid levels ($p < 0.05$). And the effect of adding LP was the most significant after 45 days of fermentation. LP promoted the growth of beneficial bacteria and inhibited harmful bacteria, with LP becoming the predominant genus and species after 45 days of fermentation. The metabolic pathway analysis revealed that the addition of LP enhanced carbohydrate metabolism and improved the replication and repair, translation, transcription, and membrane transport functions of the bacterial community. In summary, the addition of LP significantly enhances the silage quality of *L. corniculatus* and may serve as an effective method for promoting the application of *L. corniculatus* in karst regions.

Keywords: *Lotus corniculatus* L.; silage; *Lactiplantibacillus plantarum*; fermentation quality; bacterial community

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1. Introduction

Southwest China is home to one of the largest contiguous karst regions in the world [1,2]. The ecological environment of karst stony desertification areas is fragile, with severe soil erosion significantly hindering local agricultural development [3]. The predominant subtropical and tropical monsoon climates in Southwest China foster humid conditions, which are conducive to forage growth and livestock development [4]. Cultivating perennial forage grasses not only enhances agricultural income but also plays a crucial role in conserving soil and water in karst regions while preventing desertification. However, extreme environmental factors, such as desertification and soil erosion, lead to food shortages for herbivores during the winter and early spring, severely limiting livestock development in these areas [5]. The preparation of hay and silage serves as an effective means to mitigate seasonal forage shortages. However, hay production is often compromised by rainfall, fungal contamination, and leaf drop, resulting in losses of dry

matter (DM) and nutritional value [5]. Silage is a microbiologically driven process that utilizes lactic acid bacteria (LAB) to ferment the soluble carbohydrates in forages under anaerobic conditions, converting them into organic acids, primarily lactic acid (LA). This process lowers the pH of the silage; when the pH reaches a critical level (pH = 4.2), the growth of various harmful microorganisms is inhibited, thus preserving the nutritional characteristics of the silage over the long term [6,7]. Silage not only maximizes the retention of nutrients from raw materials but also offers better palatability, making it well suited for livestock development in karst regions.

Microorganisms play a crucial role in improving the quality of silage. Aerobic microorganisms, such as clostridia and actinomycetes, remain active until the oxygen is depleted, thereby inhibiting microbial fermentation. During the later stages of silage production, facultative anaerobic microorganisms reduce the pH and promote the growth of acid-resistant LAB, which ultimately dominate the fermentation process [8]. The addition of LAB increases lactic acid production, thereby lowering pH levels. *Lactiplantibacillus plantarum*, a homofermentative LAB, ferments soluble sugars through glycolysis, enhancing the fermentation quality of silage [7]. Given that the fermentation process is highly dependent on interactions among various bacterial species and that these communities are closely linked to fermentation quality, it is crucial to analyze changes in fermentation characteristics and microbial composition during silage production [6]. The development of third-generation full-length amplicon sequencing technology facilitates a better understanding of the silage process and improvements in silage quality [9].

Lotus corniculatus L., also known as horned flower, five-leaf clover, or birds foot trefoil, is an herbaceous plant belonging to the Fabaceae family. It is highly palatable and tolerant to poor soil conditions, making it one of the world's most renowned perennial legumes for forage [10,11]. As the demand for high-quality leguminous forages in karst regions has increased, the cultivation area of *Lotus corniculatus* has expanded annually. Although it is rich in crude protein (17.1% DM), *Lotus corniculatus* has low soluble sugar content (5.89% DM) and a low abundance of lactic acid bacteria, making successful silage production challenging [12]. The research indicates that LAB genera are a minor component in alfalfa silage but become dominant when commercial LAB is added [13,14]. Therefore, we hypothesize that (1) the addition of *Lactiplantibacillus plantarum* can enhance the silage quality of *Lotus corniculatus* and (2) *Lactiplantibacillus plantarum* improves silage quality by influencing the diversity and functionality of bacterial communities within the silage. The findings of this study provide a theoretical foundation for the promotion and utilization of *Lotus corniculatus*.

2. Materials and Methods

2.1. Silage Preparation

The "Qiangui No. 1" *Lotus corniculatus* L. was cultivated in Ronglei Village, Changshun County, Guizhou Province (Longitude 106°26′48.14″, Latitude 25°58′45.92″), an area characterized by a subtropical monsoon climate. On 25 September 2023, the grass was cut at a height of 10 cm above the ground. The harvested material was transported to the laboratory on the same day and chopped into 1–2 cm pieces. A portion of the material was used for chemical composition and microbial diversity analysis, while the remainder was ensiled. Table 1 presents the chemical composition of the fresh *Lotus corniculatus*.

The experimental treatments included (1) LP (addition of *Lactiplantibacillus plantarum* at a dosage of 10^5 cfu/g FW) (Zhongke Jiayi Biotechnology, Shandong, China) and (2) CK (distilled water). The additives were dissolved in deionized water, with 15 mL of the solution added per kilogram of *Lotus corniculatus* forage; the CK group received an equivalent volume of deionized water. Approximately 300 g of each mixture was sealed in a

polyethylene bag measuring 30 cm × 25 cm and vacuum-sealed, yielding a total of 24 bags. Samples from each treatment were opened at 3, 7, 15, and 45 days of ensiling. Three bags were randomly selected for each treatment at each time point for chemical composition and fermentation parameter analysis, and the bacterial community was analyzed for samples taken at 3 and 45 days.

Table 1. Chemical characteristics of fresh *Lotus corniculatus*.

Item	Fresh Material
DM	253.4 ± 1.17
WSC (g/kgDM)	75.3 ± 0.76
CP (g/kgDM)	183.8 ± 0.36
NDF (g/kgDM)	402.5 ± 1.28
ADF (g/kgDM)	346.3 ± 0.44

Note: DM denotes dry matter, WSC denotes water-soluble carbohydrate, CP denotes crude protein, NDF denotes neutral detergent fiber, and ADF denotes acid detergent fiber.

2.2. Fermentation Quality Analysis

To evaluate the fermentation characteristics of the silage, 10 g of fresh silage samples was taken from each bag, mixed with 90 mL of sterile water, and stored at 4 °C for 24 h. The mixture was then filtered through four layers of medical gauze. The pH of the supernatant was measured immediately using a pH meter (PHS-3E, Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China). Subsequently, the lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were measured using high-performance liquid chromatography (HPLC) with a KC-811 chromatographic column (Shodex; Shimadzu Co., Ltd., Tokyo, Japan); the SPD was 210 nm, the column temperature was 50 °C, and the flow rate was 1 mL/min [15]. The ammonia nitrogen (NH_3-N) was measured using the phenol colorimetric method [16].

2.3. Chemical Composition Analysis

The remaining silage samples from each bag were dried in an oven at 65 °C for 48 h to determine the dry matter (DM) weight. After weighing, the dried samples were ground and sieved through a 1 mm screen. The crude protein (CP) content was measured using a Kjeldahl nitrogen analyzer (Kjeltec 8400, FOSS, Hillerød, Denmark) [17]. The neutral detergent fiber (NDF, without α-amylase) and acid detergent fiber (ADF) were analyzed using the method reported by [18]. The water-soluble carbohydrate (WSC) content was quantified using the anthrone method [19].

2.4. Bacterial Community Analysis

Genomic DNA from the samples was extracted using the CTAB method. A 1000 μL CTAB lysis solution was added to a 2 mL EP tube, followed by the addition of lysozyme. Then, 2 g of fresh samples was added to the lysis solution, and the mixture was incubated in a 65 °C water bath. The supernatant was centrifuged at 12,000 rpm for 10 min with phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1). This extraction step was repeated using chloroform and isoamyl alcohol. Afterward, isopropanol was added to the supernatant, and the mixture was incubated at −20 °C to precipitate the DNA. Following centrifugation, the supernatant was discarded, and the DNA was dissolved in deionized water. Finally, 1 μL RNase A was added to digest RNA. The bacterial 16S rRNA was amplified using PCR with the primers AGAGTTTGATCCTGGCTCAG and GNTACCTTGTTACGACTT (R). The sequencing was conducted on the PacBio platform, with the original sequencing performed by inner Mongolia Baianle Biotechnology Co., Ltd. in Inner Mongolia, China. Finally, α-diversity and β-diversity analyses, as well as linear discriminant analysis effect

size (LEfSe) analysis (using LDA scores > 3 and $p < 0.05$ as thresholds), were performed on the Novogene platform (https://magic.novogene.com/, accessed on 2 May 2024), and the functional profiles of the bacterial communities were predicted.

2.5. Statistical Analysis

IBM SPSS 26.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis of the data. One-way and two-way analyses of variance were conducted for the effects of additives and time. Duncan's multiple comparison test was applied to the mean values for each additive and time point. The statistical significance was determined when $p < 0.05$. The Spearman correlation coefficient between bacterial populations and fermentation quality was calculated using SPSS. All the graphics were created using GraphPad Prism 9.5.

3. Results

3.1. Chemical Composition of Lotus corniculatus L. After Silage

The chemical composition of the silage was influenced by fermentation duration (D), additives (A), and their interaction (D*A) (Table 2). The fermentation duration (D) significantly affected the water-soluble carbohydrates (WSCs) ($p < 0.05$), and additives (A) also significantly affected the WSCs ($p < 0.05$). With the extension of the fermentation time, the dry matter (DM) content of the control (CK) silage gradually decreased, with a significant reduction at 45 days compared to other time points. The dry matter (DM) content of the LP-treated silage was consistently higher than that of the CK silage at all the sampling points.

Table 2. Chemical composition of *Lotus corniculatus* after silage.

Item	Dispose\Days	3	7	15	45	SEM	*p*-Value D	*p*-Value A	*p*-Value D*A
DM (g/kgFM)	CK	251.1 Aa	240.9 Aa	240.3 Aa	233.2 Ba	0.21	NS	NS	NS
	LP	253 Aa	244.1 Aa	243.6 Aa	238.1 Aa				
WSC (g/kgDM)	CK	74.5 Aa	67.4 Ba	57.4 Ca	47.9 Da	0.25	0.005 **	0.008 **	NS
	LP	71.6 Ab	61.5 Ab	50.1 Bb	41.8 Bb				
CP (g/kgDM)	CK	180.6 Aa	177.7 Aa	176.6 Aa	175.5 Ab	0.07	NS	NS	NS
	LP	181.5 Aa	178.4 Aa	177.5 Aa	178.5 Aa				
NDF (g/kgDM)	CK	404.9 Aa	410.5 Aa	403.1 Aa	395.8 Aa	0.2	NS	NS	NS
	LP	404 Aa	405 Aa	407.6 Aa	395.2 Aa				
ADF (g/kgDM)	CK	351.8 Aa	350.3 Aa	351.9 Aa	349.7 Aa	0.16	NS	NS	NS
	LP	347.6 Aa	350.7 Aa	352.1 Aa	336.7 Aa				

Note: Capital letters represent significant differences between different days for the same treatment, while lowercase letters indicate significant differences between different treatments on the same day. DM denotes dry matter, WSC denotes water-soluble carbohydrate, CP denotes crude protein, NDF denotes neutral detergent fiber, ADF denotes acid detergent fiber, SEM denotes standard error of the mean, D represents fermentation duration, A represents additives, and D*A indicates the interaction between fermentation duration and additives. ** indicates extremely significant differences ($p < 0.01$). NS indicates that it is not significant.

Throughout the fermentation period from 3 to 45 days, the water-soluble carbohydrate (WSC) content in all the silage samples significantly decreased ($p < 0.05$), with the CK group consistently exhibiting higher water-soluble carbohydrate (WSC) levels than the LP-treated group ($p < 0.05$). The lowest water-soluble carbohydrate (WSC) content for the LP treatment was recorded at 41.8 g/kgDM at 45 days, while the highest water-soluble carbohydrate (WSC) content for the CK treatment was observed at 74.5 g/kgDM after 3 days of fermentation (Table 2).

In terms of crude protein (CP) content, the LP silage consistently showed higher levels than the CK silage throughout the fermentation process (Table 2). Specifically, the crude

protein (CP) content in the LP-treated silage reached 178.5 g/kgDM at 45 days, significantly surpassing that of the CK treatment. However, there were no significant changes observed in the neutral detergent fiber (NDF) and acid detergent fiber (ADF) across all the treatment groups throughout the silage process, indicating that the addition of LP did not significantly alter the neutral detergent fiber (NDF) and acid detergent fiber (ADF) content.

3.2. Fermentation Characteristics of Lotus corniculatus L. After Silage

The fermentation characteristics of the *Lotus corniculatus* silage were influenced by fermentation duration (D), additives (A), and their interaction (D*A) (Table 3). Both D and A had extremely significant effects on the pH, ammonia nitrogen (NH_3-N), lactic acid (LA), and acetic acid (AA) ($p < 0.01$). During the fermentation process from 3 to 45 days, the pH of all the treatments showed a significant downward trend ($p < 0.05$). At fermentation days 3, 15, and 45, the pH of the LP treatment group was significantly lower than that of the CK group ($p < 0.05$), with the lowest pH of 4.18 recorded for the LP treatment at 45 days.

Table 3. Fermentation characteristics of *Lotus corniculatus* after silage.

Item	Dispose\Days	3	7	15	45	SEM	*p*-Value D	*p*-Value A	*p*-Value D*A
pH	CK	5.46 Aa	5.3 ABa	5.24 ABa	5.02 Ba	0.085	0.004 **	0.006 **	NS
	LP	4.9 Ab	4.77 ABa	4.55 Bb	4.18 Cb				
NH_3-N (g/kgTN)	CK	24.2 Ca	39.5 Ba	42 ABa	44.9 Aa	0.16	0.004 **	0.003 **	NS
	LP	24.2 Ca	31.3 Bb	34.5 ABb	39.6 Ab				
LA (g/kgDM)	CK	33.5 Bb	33.6 Bb	42.8 Ba	46.1 Ab	0.15	0.007 **	0.009 **	NS
	LP	39.1 Ba	42 ABa	46.1 ABa	52.4 Aa				
AA (g/kgDM)	CK	20.5 Aa	14.8 Ba	15 Ba	14.1 Ba	0.06	0.009 **	0.006 **	NS
	LP	16.1 Ab	12.4 Bb	11.7 Ba	9.1 Cb				
PA (g/kgDM)	CK	ND	ND	ND	ND				
	LP	ND	ND	ND	ND				
BA (g/kgDM)	CK	ND	ND	ND	ND				
	LP	ND	ND	ND	ND				

Note: Capital letters represent significant differences between different days for the same treatment, while lowercase letters indicate significant differences between different treatments on the same day. NH_3-N represents ammonia nitrogen, LA represents lactic acid, AA represents acetic acid, PA represents propionic acid, and BA represents butyric acid. ND means not detected, SEM denotes standard error of the mean, D represents fermentation duration, A represents additives, and D*A represents the interaction between fermentation duration and additives. ** indicates extremely significant differences ($p < 0.01$). NS indicates that it is not significant.

The NH_3-N levels exhibited significantly different trends among the treatment groups ($p < 0.05$). As the fermentation time increased, the NH_3-N levels in all the treatment groups showed a significant upward trend ($p < 0.05$). The NH_3-N level in the LP treatment group was significantly lower than that of the CK group ($p < 0.05$), reaching the lowest value of 39.6 g/kgTN at 45 days of fermentation (Table 3).

During the fermentation period from 3 to 45 days, both the CK and LP treatment groups showed a significant increase in LA levels ($p < 0.05$), with the LA content in the LP treatment group significantly higher than that in the CK group ($p < 0.05$). The highest LA content recorded in the LP treatment group was 52.4 g/kgDM at 45 days (Table 3).

Conversely, the AA content showed a significant decreasing trend across all the treatment groups throughout the fermentation period ($p < 0.05$). The AA content in the LP treatment group was significantly lower than that in the CK group ($p < 0.05$). The CK treatment group had the highest AA content of 20.5 g/kgDM at 3 days, while the LP treatment group recorded the lowest AA content of 9.1 g/kgDM at 45 days (Table 3).

3.3. Changes in Bacterial Communities During the Silage of Lotus corniculatus L.

3.3.1. Alpha Diversity

The goods coverage index for all the treatment groups (excluding FM) was above 0.97, indicating a high level of coverage in the sampling (Table 4). The microbial diversity indices observed in the fresh samples of *Lotus corniculatus* (FM) were significantly higher than those in the silage treatments, with the highest values for the observed species, Shannon, Simpson, Chao1, and Ace. In all the silage treatment groups, these indices showed significant changes ($p < 0.05$). Specifically, the indices for silage at 3 days were higher than those at 45 days. Additionally, the Shannon and Simpson indices for the LP treatment group were significantly lower than those for the CK group.

Table 4. Bacterial microbial community diversity and richness in fresh and silaged *Lotus corniculatus*.

Treatments	Observed_Species	Shannon	Simpson	Chao1	Ace	Goods_Coverage
FM	24 a	3.883 a	0.832 a	28.944 a	33.24 a	0.847 b
CK3	13 b	3.075 a	0.822 a	17 ab	8.95 b	0.929 a
LP3	4 bc	0.967 b	0.309 b	4.667 b	5.11 b	0.989 a
CK45	3 bc	0.378 b	0.114 c	3 b	3.8 b	0.984 a
LP45	2 c	0.161 b	0.043 c	2.667 b	1.67 b	0.987 a
SEM	2.511	0.428	0.09	3.69	3.36	0.019
D *p*-value	**	**	**	**	**	0.133
A *p*-value	**	**	**	**	**	0.23
D*A *p*-value	**	**	**	**	0.086	0.593

Note: Different lowercase letters indicate significant differences between treatments ($p < 0.05$). SEM represents the standard error of the mean, D represents fermentation duration, A represents additives, D*A represents the interaction between fermentation duration and additives, and ** indicates extremely significant differences ($p < 0.01$). FM represents the fresh *Lotus corniculatus*; CK3 represents the control silage on day 3; LP3 represents the silage on day 3 with added LP; CK45 represents the control silage on day 45; and LP45 signifies the silage on day 45 with added LP.

3.3.2. PCOA Analysis

The principal coordinate analysis (PCOA) revealed that the first principal coordinate (PCOA1) accounted for 56.76% and the second principal coordinate (PCOA2) accounted for 21.17% of the total variance, resulting in a cumulative variance of 77.93%. The fresh *Lotus corniculatus* samples (FM) were primarily distributed in the third quadrant, while the control samples at 3 days (CK3) were located in the second quadrant. Both LP samples at 3 days (LP3) and the control samples at 45 days (CK45) were mainly concentrated in the first quadrant, showing distinct separation. Notably, the LP samples at 45 days (LP45) were located in the fourth quadrant (Figure 1).

3.3.3. Bacterial Community Compositions

During the silage fermentation process, the predominant microorganisms play a crucial role in determining the fermentation quality of the silage feed. In this study, the main genera identified in the fresh *Lotus corniculatus* sample (FM) included *Chryseobacterium* (20.24%), *Methylobacterium Methylorubrum* (9.38%), and *Aureimonas* (6.42%), while the abundance of *Lactiplantibacillus plantarum* was relatively low at 0.99%. In the control treatment group after 3 days of fermentation (CK3), the predominant genera were *Enterococcus* (52.1%), *Lactococcus* (18.27%), *Companilactobacillus* (6.18%), *Methylobacterium Methylorubrum* (4.19%), and *Enterobacter* (3.95%). Notably, the addition of *Lactiplantibacillus plantarum* resulted in a significant increase in *Lactiplantibacillus plantarum* abundance. In the *Lactiplantibacillus plantarum* treatment group after 45 days (LP45), the abundances of *Chryseobacterium*, *Methylobacterium Methylorubrum*, and *Aureimonas* significantly decreased to 0%, while *Lactiplantibacillus plantarum* increased dramatically from 0.99% to 95.8%. Compared with the control treatment group after 3 days of fermentation (CK3), the relative abundances of *Enterococcus*, *Lactococcus*, *Methylobacterium Methylorubrum*, and *Enterobacter*

markedly declined, decreasing from 52.1% to 0.25%, from 18.27% to 0%, from 4.19% to 0%, and from 3.95% to 0.24%, respectively (Figure 2A).

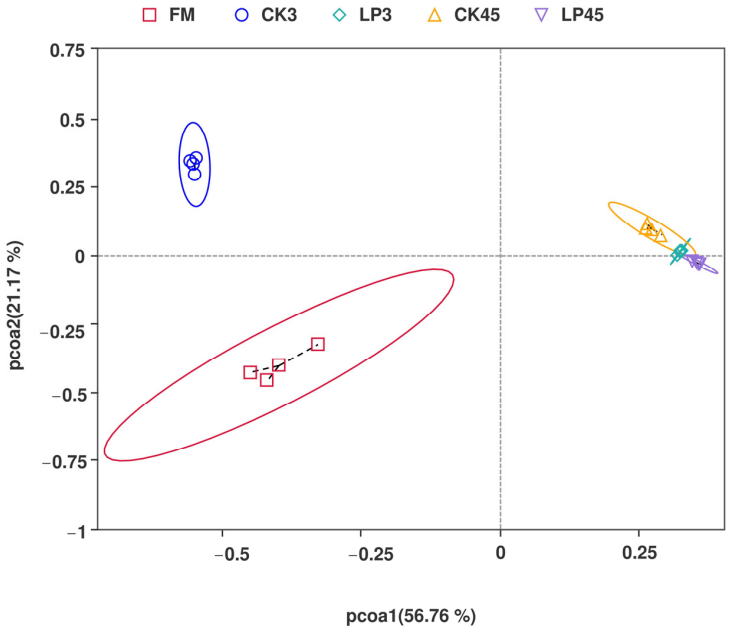

Figure 1. Principal coordinate analysis (PCOA) of bacterial communities in fresh and silaged *Lotus corniculatus*. FM represents the fresh *Lotus corniculatus*; CK3 represents the control silage on day 3; LP3 represents the silage on day 3 with added LP; CK45 represents the control silage on day 45; and LP45 signifies the silage on day 45 with added LP.

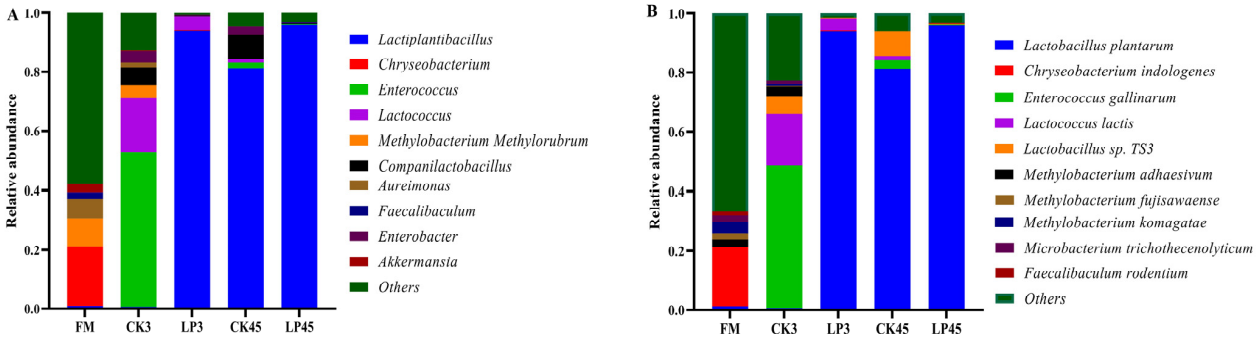

Figure 2. Relative abundance of bacterial genera (**A**) and species (**B**) in fresh and silaged *Lotus corniculatus* after 3 and 45 days of fermentation. FM represents the fresh *Lotus corniculatus*; CK3 represents the control silage on day 3; LP3 represents the silage on day 3 with added LP; CK45 represents the control silage on day 45; and LP45 signifies the silage on day 45 with added LP.

At the species level, the major species in the fresh *Lotus corniculatus* sample (FM) included *Chryseobacterium indologenes* (23.3%), *Methylobacterium komagatae* (3.7%), and *Methylobacterium adhaesivum* (2.46%). In the control treatment group after 3 days of fermentation (CK3), the predominant species were *Enterococcus gallinarum* (47.9%), *Lactococcus lactis* (17.3%), and *Lactobacillus* sp. *TS3* (6.17%). Compared to the fresh samples, the abundance of *Lactiplantibacillus plantarum* significantly increased in the LP45 silage, rising from 1.23% to 81.23% after 45 days without *Lactiplantibacillus plantarum* addition. Conversely, the relative abundances of *Chryseobacterium_indologenes*, *Methylobacterium adhaesivum*, and *Methylobacterium komagatae* significantly decreased, dropping to 0% for all three.

In comparison to the control treatment group after 3 days of fermentation (CK3), the abundance of *Lactiplantibacillus plantarum* significantly increased in the *Lactiplantibacillus*

plantarum-treated samples after 45 days, rising from 0.7% to 95.8%. Meanwhile, the relative abundances of *Enterococcus gallinarum*, *Lactococcus lactis*, *Lactobacillus* sp. *TS3*, and *Methylobacterium adhaesivum* significantly declined, decreasing from 47.9% to 0.24%, from 17.3% to 0%, from 6.17% to 0.24%, and from 2.96% to 0%, respectively.

3.3.4. LEfSe Analysis

LEfSe analysis was employed to evaluate the differences in bacterial communities among fresh samples of *Lotus corniculatus*, and silages after 3 and 45 days of fermentation, identifying specific bacterial taxa for each treatment group (LDA score > 4). The presence of *Lactiplantibacillus plantarum* significantly influenced the changes in the bacterial community of the silage (Figure 3). In the fresh material (FM), 39 bacterial taxa were significantly enriched, with the highest LDA score attributed to *p Proteobacteria*. In the CK3 treatment, 12 bacterial taxa were significantly enriched, led by *g Enterococcus* with the highest LDA score. The LP3 treatment showed the enrichment of three taxa, with *o Lactobacillales* having the highest score. For CK45, four bacterial taxa were significantly enriched, with *s Lactobacillus* sp. *TS3* recording the highest score. The LP45 treatment exhibited enrichment of three taxa, prominently featuring *s Lactiplantibacillus plantarum* with the highest LDA score. These findings highlight the impact of different fermentation times and the addition of specific additives on the bacterial community structure in the silage, particularly the role of *Lactiplantibacillus plantarum* in enhancing beneficial bacterial populations during fermentation.

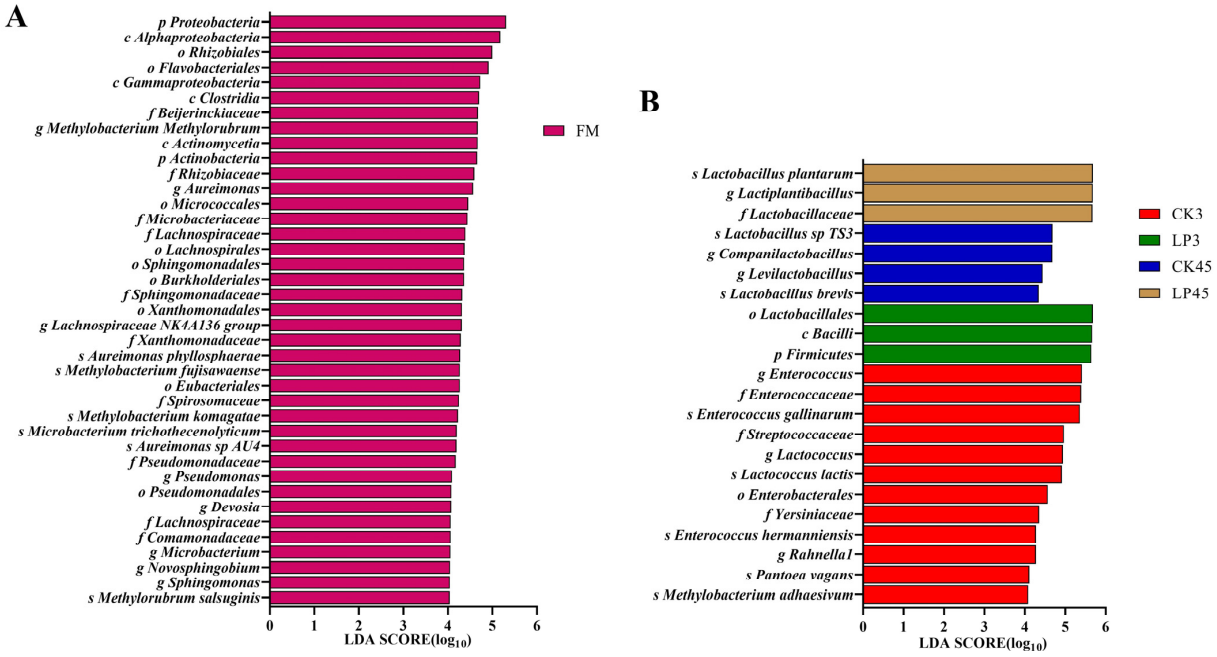

Figure 3. Comparison of microbial variation in fresh *Lotus corniculatus* samples (**A**) and silage after 3 days and 45 days (**B**) using the LEfSe online tool. This analysis identifies indicator microorganisms in the silage community with linear discriminant analysis (LDA) scores of 4 or higher under different treatments. FM represents the fresh *Lotus corniculatus*; CK3 represents the control silage on day 3; LP3 represents the silage on day 3 with added LP; CK45 represents the control silage on day 45; and LP45 signifies the silage on day 45 with added LP.

3.4. Relationship Between Silage Quality and Bacterial Communities

Fermentation products are typically the result of interactions among resident microorganisms. A correlation analysis between bacterial communities and fermentation products helps to enhance the evaluation of the effectiveness of *Lotus corniculatus* silage.

The relationship between the fermentation quality of *Lotus corniculatus* silage at 3 and 45 days and the relative abundance of bacterial species was analyzed using the Spearman correlation (Figure 4). The results showed that the pH, acetic acid (AA), and water-soluble carbohydrates (WSCs) exhibited highly significant or significant negative correlations with *Lactiplantibacillus plantarum* ($p < 0.01$). In contrast, these parameters showed significant positive correlations with other bacterial species ($p < 0.05$), except for *Chryseobacterium indologenes*. Conversely, the correlation between lactic acid (LA) and bacterial abundance demonstrated an opposite trend. LA was significantly positively correlated with *Lactiplantibacillus plantarum* ($p < 0.01$), while it exhibited significant negative correlations with most other bacteria, excluding *Chryseobacterium indologenes* and *Lactobacillus* sp. *TS3* ($p < 0.01$).

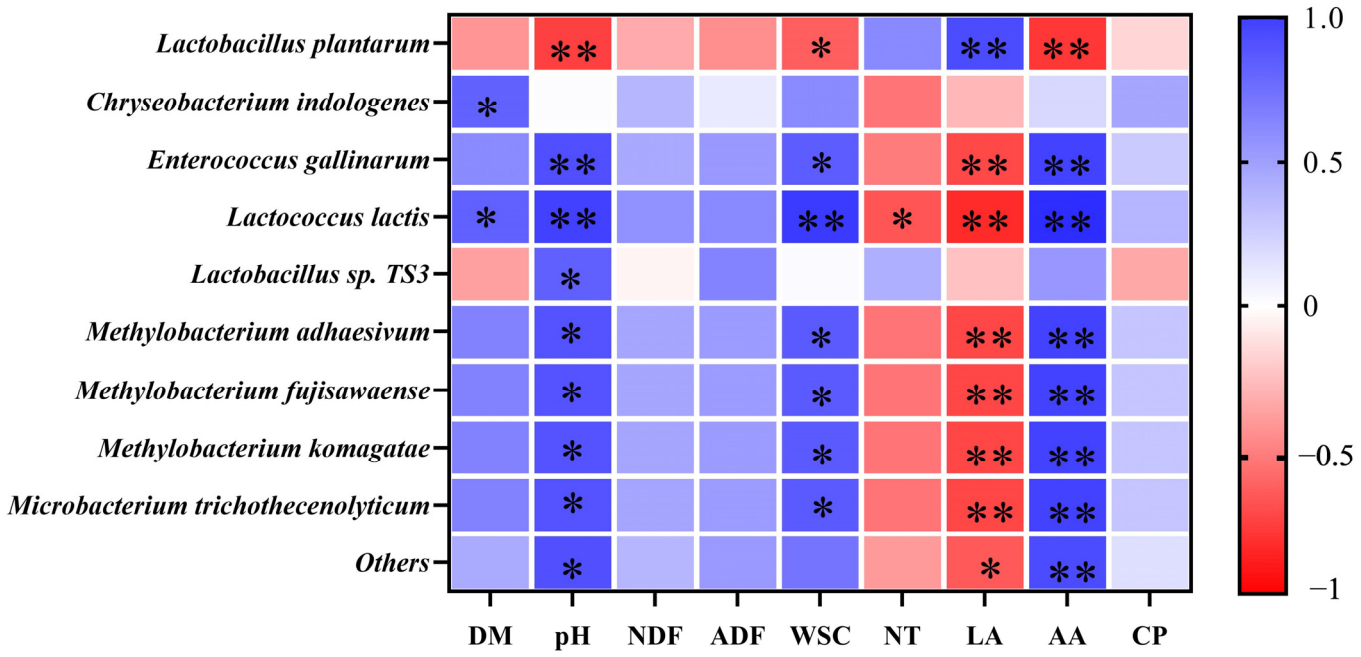

Figure 4. Heatmap showing the correlation between silage fermentation quality and the relative abundance of bacterial species. DM represents dry matter, NDF represents neutral detergent fiber, ADF represents acid detergent fiber, WSC represents water-soluble carbohydrate, NT represents ammonia nitrogen, LA represents lactic acid, AA represents acetic acid, and CP represents crude protein. * $p < 0.05$; ** $p < 0.01$.

3.5. PICRUSt Prediction of Potential Bacterial Community Functions

This study utilized PICRUSt to predict the potential functions of the bacterial community, classifying them into three levels of pathways. At the first pathway level, the metabolism category in the LP45 treatment group was significantly lower than that in the CK45 group ($p < 0.05$). Both the 3-day and 45-day LP treatment groups exhibited significantly higher levels of genetic information processing and environmental information processing compared to the CK groups (Figure 5A).

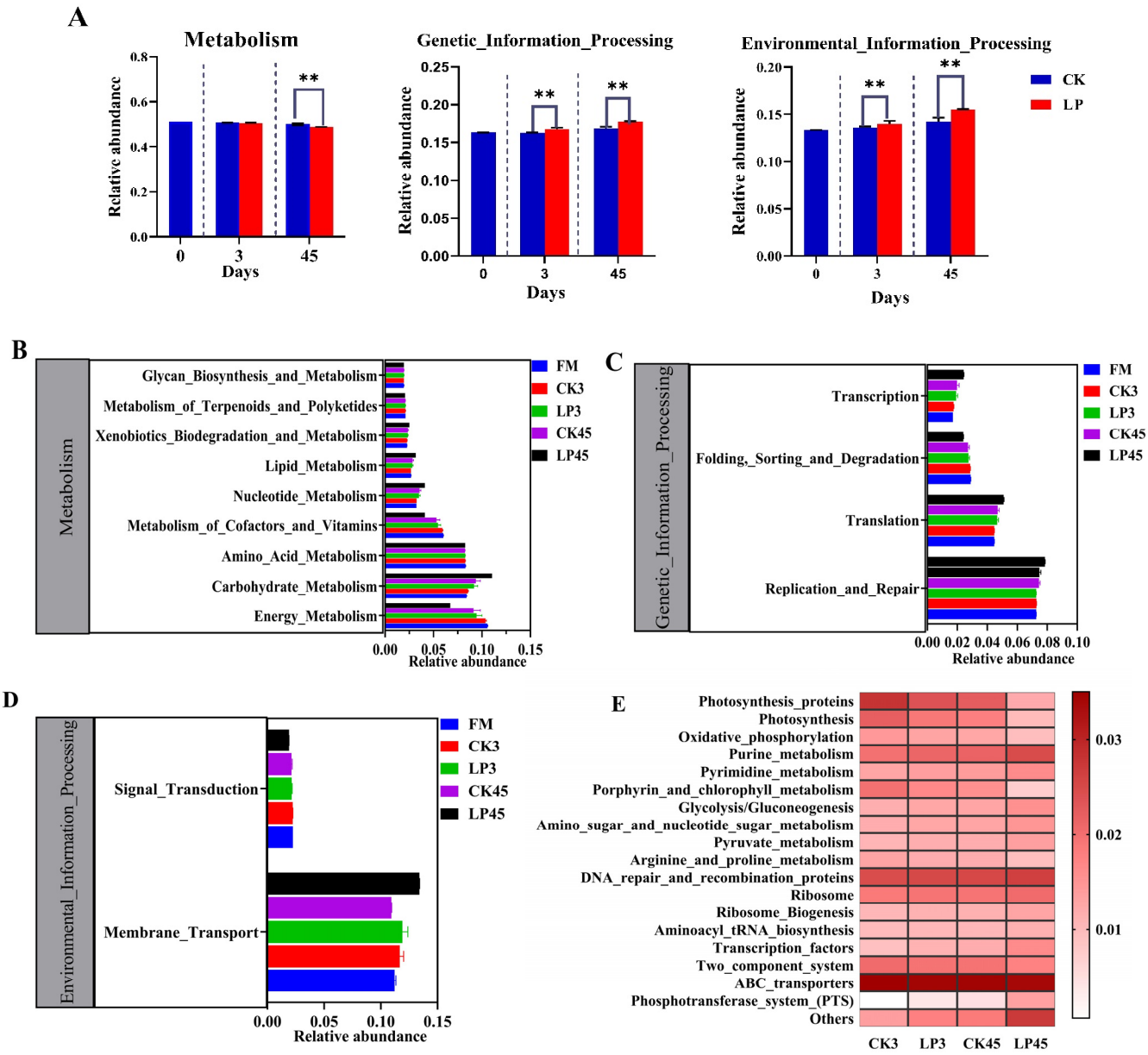

Figure 5. Prediction of potential functions of bacterial communities in fresh *Lotus corniculatus* samples and silage after 3 and 45 days fermentation using PICRUSt. (**A**) The predicted first-level KEGG pathways; (**B**–**D**) the predicted second-level KEGG pathways. (**E**) Tshe heatmap of relative abundances for the predicted third-level KEGG pathways. ** Indicates a significant difference. ($p < 0.05$).

At the second pathway level, the metabolism of the silage predominantly focused on Energy Metabolism, carbohydrate metabolism, and Amino Acid Metabolism. The FM group had the highest abundance of Energy Metabolism, while the LP45 group had the lowest. Conversely, the LP45 group exhibited the highest abundance of carbohydrate metabolism, with the FM group showing the lowest levels (Figure 5B). Within genetic information processing, the LP45 treatment demonstrated higher abundances of replication and repair and translation (Figure 5C). For environmental information processing, the LP45 group also showed elevated levels of membrane transport (Figure 5D).

At the third pathway level, the silage samples from the CK3, LP3, CK45, and LP45 treatments were relatively enriched in ABC Transporters. Notably, the LP45 treatment group displayed higher relative abundances of Purine Metabolism, Pyrimidine Metabolism,

Glycolysis/Gluconeogenesis, and DNA Repair and Recombination Proteins compared to the other treatments (Figure 5E).

4. Discussion

To improve the silage quality of *Lotus corniculatus* L. and accelerate the promotion of cultivated varieties, this study examines the effects of *Lactiplantibacillus plantarum* on the fermentation characteristics, bacterial community composition, and functional dynamics of silage.

4.1. Changes in Chemical Composition and Fermentation Characteristics of Lotus corniculatus L. During Silage

The loss of dry matter (DM) in silage feed is attributed to the decomposition of nutrients, wherein aerobic microorganisms convert carbohydrates into water, carbon dioxide, and heat. In this study, the DM consistently decreased with extended silage time. Notably, the DM content in the *Lactiplantibacillus plantarum* (LP) treatment group was higher than that in the control group (CK), indicating that the addition of LP effectively limits the growth of undesirable microorganisms in *Lotus corniculatus* silage and reduces nutrient consumption [20]. Previous research has demonstrated that successful silage fermentation requires 5% water-soluble carbohydrates (WSCs) in DM [21]. In our study, the WSC content of *Lotus corniculatus* was 75.3% DM, which provides the necessary conditions for producing high-quality silage [22].

Silage fermentation relies on lactic acid bacteria rapidly degrading WSCs into organic acids, thereby lowering the pH and inhibiting the activity of aerobic microorganisms [23]. Among these organic acids, lactic acid (LA) is one of the primary fermentation products and the most crucial organic acid for pH reduction [24]. In our research, as the fermentation time increased, the WSC content in the *Lotus corniculatus* silage showed a significant decline, while the pH decreased significantly, and the LA levels increased markedly. Furthermore, the LP treatment group exhibited significantly lower WSC and pH values than the CK group, and the LA levels in the LP group were significantly higher ($p < 0.05$), consistent with the findings of [25]. This phenomenon suggests that during the silage process, WSCs in *Lotus corniculatus* are rapidly degraded, resulting in substantial lactic acid production. The addition of LP typically leads to higher WSC consumption and more lactic acid production, swiftly lowering the pH to maintain the acidic anaerobic environment required for fermentation [20,26]. This finding aligns with the higher crude protein (CP) content in the LP group compared to the CK group, further confirming that the addition of LP facilitates quicker maintenance of the acidic environment necessary for fermentation, thereby inhibiting protease hydrolysis and reducing crude protein loss [27]. Moreover, the pH of the *Lotus corniculatus* silage with LP added for 45 days reached 4.18, which meets the pH standard for quality silage [28,29].

In our study, no significant changes were observed in the neutral detergent fiber (NDF) and acid detergent fiber (ADF) levels among the various treatment groups, indicating that the addition of LP does not significantly alter the NDF and ADF contents in *Lotus corniculatus*. This finding is consistent with results obtained from studies on paper mulberry silage [25] and mixed silage of alfalfa and *Leymus chinensis* [30] but contrasts with findings from perennial ryegrass silage [30], potentially due to differences in the silage materials used.

Acetic acid (AA) serves as a promoter of aerobic stability during silage and an effective inhibitor of fungi. In this study, AA was present at various stages of silage, consistent with findings from [31]. The initial stages of silage exhibited higher AA levels, which may contribute to a reduction in the abundance of undesirable microorganisms during the

early fermentation phase [32]. In our research, the AA content in the CK3 group was the highest, possibly due to the elevated levels of *Enterococcus gallinarum* and *Lactococcus lactis*, as *Enterococcus gallinarum* can produce acetic acid during the early stages of silage, thereby reducing the growth of undesirable microorganisms.

The ratio of ammonia nitrogen (NH_3-N) to total nitrogen (TN) serves as an indicator of protein degradation. In silage feed, NH_3-N is typically generated from amino acid decarboxylation and deamination activities caused by microbial and plant proteinase activities. The accumulation of NH_3-N diminishes the nutritional value of silage feed [33]. In our study, the NH_3-N levels in *Lotus corniculatus* silage were determined to be below the threshold level of 80 g/kgTN [19]. Notably, after the 7 days of silage, the NH_3-N content in the LP treatment group was significantly lower than that in the control group. This finding indicates that the addition of LP can facilitate the early production of substantial amounts of lactic acid in *Lotus corniculatus* silage, thereby inhibiting protease hydrolysis as well as the activities of microbial enzymes and *Enterobacter*, leading to a reduction in NH_3-N concentration [34]. This observation was validated through the bacterial community composition analysis.

4.2. Diversity Levels of Bacterial Communities in Lotus corniculatus L. During Silage

The 16S rRNA gene is a highly conserved fragment found in both bacteria and archaea, with a certain degree of variability. Sequencing the 16S rRNA gene allows for the identification of different bacterial genera and species present in the microbial community, as well as the evaluation of their relative abundance [24]. Alpha diversity reflects the bacterial abundance and species diversity within a single sample. Observed_species represents the number of species in the sample. To assess bacterial abundance, the Chao1 and Ace indices were used, while the Shannon and Simpson indices were employed to evaluate species diversity. The Shannon index is based on information entropy; a larger Shannon index indicates greater uncertainty, suggesting higher diversity in the community due to more unknown factors. Goods_coverage, which refers to microbial coverage, is also used to assess sampling completeness. A higher value indicates a lower probability that new, unmeasured species exist in the sample [35]. The goods coverage index for the various silage treatments of *Lotus corniculatus* was consistently above 0.97, indicating that the sequencing process sufficiently characterized the dynamic changes in the bacterial community [36]. The fresh samples (FMs) were found to favor the proliferation of aerobic microorganisms due to their aerobic and neutral environment, resulting in higher observed species, Simpson, Chao1, and Shannon diversity indices compared to the silage feed [29]. Over the course of silage, from day 3 to day 45, the alpha diversity indices continuously decreased. The addition of *Lactiplantibacillus plantarum* (LP) promoted significant development of the acidic and anaerobic environment in the silage, further leading to a decline in bacterial diversity. By day 45 of silage, the LP treatment group exhibited the lowest diversity indices, indicating stabilization of the silage fermentation process.

The principal coordinate analysis (PCOA) revealed that the addition of LP and the duration of silage significantly altered the microbial community diversity. Notably, the degree of separation due to the silage time was significantly greater than that caused by the addition of the LP, suggesting that silage duration had a more pronounced effect on the bacterial community of Pueraria lobata than the additives [37].

The predominant bacterial genus in the fresh *Lotus corniculatus* samples was *Chryseobacterium*, an aerobic bacterium commonly found attached to plant surfaces and known for its ability to inhibit certain plant pathogenic bacteria [38]. The principal species identified was *Chryseobacterium indologenes*, capable of degrading complex organic compounds, including proteins [39]. Another significant genus identified was *Methylobacterium Methy-*

lorubrum, a pink facultative methylotroph reported in various silage feeds [40]. Species such as *Methylobacterium komagatae* and *Methylobacterium adhaesivum* within this genus can influence methanol utilization and metabolism, classifying them as undesirable bacteria in silage [41].

After 3 days of fermentation, anaerobic conditions inhibited the growth of the strictly aerobic *Chryseobacterium*, allowing *Enterococcus* and *Lactococcus* to become the dominant genera. Both *Enterococcus* and *Lactococcus* produce lactic acid, lowering the pH and driving the fermentation of silage feed. However, under suitable conditions, *Enterococcus* can transform into pathogenic symbionts, posing a potential health risk. Despite this shift, certain undesirable bacteria, such as *Methylobacterium Methylorubrum*, remained present in the silage. Lactic acid bacteria can convert one molecule of glucose into two molecules of lactic acid [42]. In *Lotus corniculatus* silage treated with LP, *Lactiplantibacillus plantarum* and *Lactiplantibacillus plantarum* emerged as the dominant genera and species after 3 days of fermentation. The pH in this treatment group was significantly lower than that in the untreated group, while the lactic acid (LA) levels were significantly higher. This combination of lower pH and higher lactic acid effectively reduced the abundance of *Enterococcus*, thereby mitigating the potential negative impacts of *Enterococcus-derived* toxins on livestock health. Ref. [43] demonstrated that the addition of *Lactiplantibacillus plantarum* decreased the growth of undesirable microorganisms and improved the quality of wheat silage. Similarly, Ref. [44] reported that the addition of *lactobacilli* increased lactic acid and crude protein content, enhancing the quality of alfalfa silage. These findings align with the results of the present study.

As the silage time extended to 45 days, *Lactiplantibacillus plantarum* remained the dominant genera and species in the silage. *Lactiplantibacillus plantarum* exhibited a highly significant or significant negative correlation with pH, acetic acid (AA), and WSCs while showing a highly significant positive correlation with LA. This further indicates that the homolactic fermentation bacterium *Lactiplantibacillus plantarum* utilizes WSCs to produce lactic acid, lowering pH and creating an acidic anaerobic environment that suppresses the growth of undesirable microorganisms, ultimately enhancing the silage quality of *Lotus corniculatus* [45]. In the untreated silage at 45 days, *Methylobacterium Methylorubrum* was no longer detected, suggesting that it may be an acid-sensitive bacterium or that its growth was inhibited by competition with the rapidly proliferating lactic acid bacteria.

4.3. Predicted Metabolic Functions of Bacterial Communities

The PICRUSt principle is to infer the functional spectrum of their common ancestor genes (homologous genes) based on the 16 S r RNA full-length sequence of the measured bacterial genome, infer the gene function spectrum of other unmeasured species in the Greengenes database, and construct the gene function prediction spectrum of the bacterial domain. Finally, the composition of the sequenced flora was "mapped" to the database to predict the metabolic function of the flora. The prediction of potential functions of bacterial communities using PICRUSt provides a detailed insight into the changes in silage feed quality [46]. In this study, the relative abundance of bacterial communities in the *Lotus corniculatus* silage was predominantly associated with metabolism, genetic information processing, and environmental information processing. This is similar to the metabolic abundances identified in Italian ryegrass silage by [47], which observed higher bacterial community activity at 60 days of flowering ryegrass silage due to lower NH_3-N levels, leading to increased abundances in environmental information processing, cellular processes, metabolism, biosystems, and genetic information processing. In our study, the relative abundances of genetic information processing and environmental information processing in the LP treatment groups of *Lotus corniculatus* silage on days 3 and 45 were

significantly higher than those in the control group, potentially related to the lower NH_3-N content observed in the LP treatment. The bacterial community in the LP-treated *Lotus corniculatus* silage exhibited enhanced carbohydrate metabolism, which accelerates the conversion of water-soluble carbohydrates (WSCs) to facilitate sufficient lactic acid accumulation. Conversely, the Energy Metabolism was lower, likely due to reduced microbial diversity in the silage associated with the addition of LP.

The elevated levels of replication and repair, translation, and membrane transport indicate an improved proliferative capacity of beneficial bacteria, contributing to enhanced silage quality [39]. Notably, the replication and repair, translation, transcription, and membrane transport functions of the bacterial communities in the LP treatment group on day 45 were significantly upregulated, indicating substantial proliferation of beneficial LP bacteria. Additionally, the bacterial communities showed notable upregulation in Purine Metabolism, Pyrimidine Metabolism, Glycolysis/Gluconeogenesis, and DNA Repair and Recombination Proteins, which further supports the proliferation of beneficial LP bacteria.

The relative enrichment of ABC Transporters in the *Lotus corniculatus* silage aligns with findings from [48], indicating that the membrane transport of the bacterial community in *Lotus corniculatus* silage may rely on ABC Transporters. It is important to note that these results are based on the functional predictions derived from 16S rRNA sequencing and do not represent direct measurements of metabolic pathways.

5. Conclusions

The *Lotus corniculatus* L. silage produced with the addition of *Lactiplantibacillus plantarum* exhibited superior fermentation quality, characterized by lower pH and NH_3-N levels, along with higher lactic acid content and the absence of butyric acid. The integration of high-throughput sequencing technology with the PICRUSt methodology revealed that *Lactiplantibacillus plantarum* influences the fermentation quality and microbial community of *Lotus corniculatus* silage through various metabolic pathways. Consequently, the addition of *Lactiplantibacillus plantarum* can facilitate the production of high-quality silage feed in karst regions.

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