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Insights into fermentation with lactic acid bacteria on the flavonoids biotransformation of alfalfa silage

Yu Gao[1], Hongzhang Zhou[1], Yuan Wang[1], Luiz Gustavo Nussio[2], Fuyu Yang[1,3] and Kuikui Ni[1*]

Abstract

Background Oxidative stress is currently one of the main threats to animal health, and flavonoids in forage have good antioxidant activity. However, the impact of fermentation on flavonoids and their antioxidant activity in forage is still unclear. This study aims to investigate the effect of lactic acid bacteria inoculation on the biological transformation of flavonoids in alfalfa silage fermentation and its relationship with antioxidant activity.

Results Compared with the raw materials, silage fermentation can increase the total flavonoid content of alfalfa. The addition of *Pediococcus pentosaceus* (CP115739.1) and *Lactiplantibacillus plantarum* (CP115741.1) can significantly increase the total flavonoid content in alfalfa silage ($P < 0.05$). The addition of lactic acid bacteria significantly improved the antioxidant capacity of alfalfa silage ($P < 0.05$). Pearson correlation analysis showed a significant correlation between total flavonoids and DPPH ($R = 0.62$, $P < 0.05$), and a highly significant correlation between total flavonoids and FRAP ($R = 0.70$, $P < 0.01$). Compared with natural silage fermentation, the addition of lactic acid bacteria leads to changes in the biological transformation process of flavonoids in alfalfa. Its unique products, 3,7,4′-trioxyflavonoids, as well as acacetin and taxifolin 7-O-rhamnoside, are significantly positively correlated with antioxidant activity.

Conclusions Silage fermentation contributes to the transformation of flavonoids, and inoculation with certain lactic acid bacteria can increase the content of flavonoids (including apigenin, luteolin, and other free flavonoids). It is worth noting that after fermentation, the antioxidant capacity of alfalfa is significantly improved, which may be attributed to the biotransformation of flavonoids related to acacetin, 3,7,4′-trihydroxyflavonoids, and taxifolin 7-O-rhamnoside. This study provides a potential pathway for obtaining value-added silage fermentation products by selecting specific lactic acid bacteria inoculants.

Keywords Silage, Targeted metabolomics, Lactic acid bacteria, Flavonoids, Antioxidant capacity

*Correspondence:
Kuikui Ni
nikk@cau.edu.cn
Full list of author information is available at the end of the article

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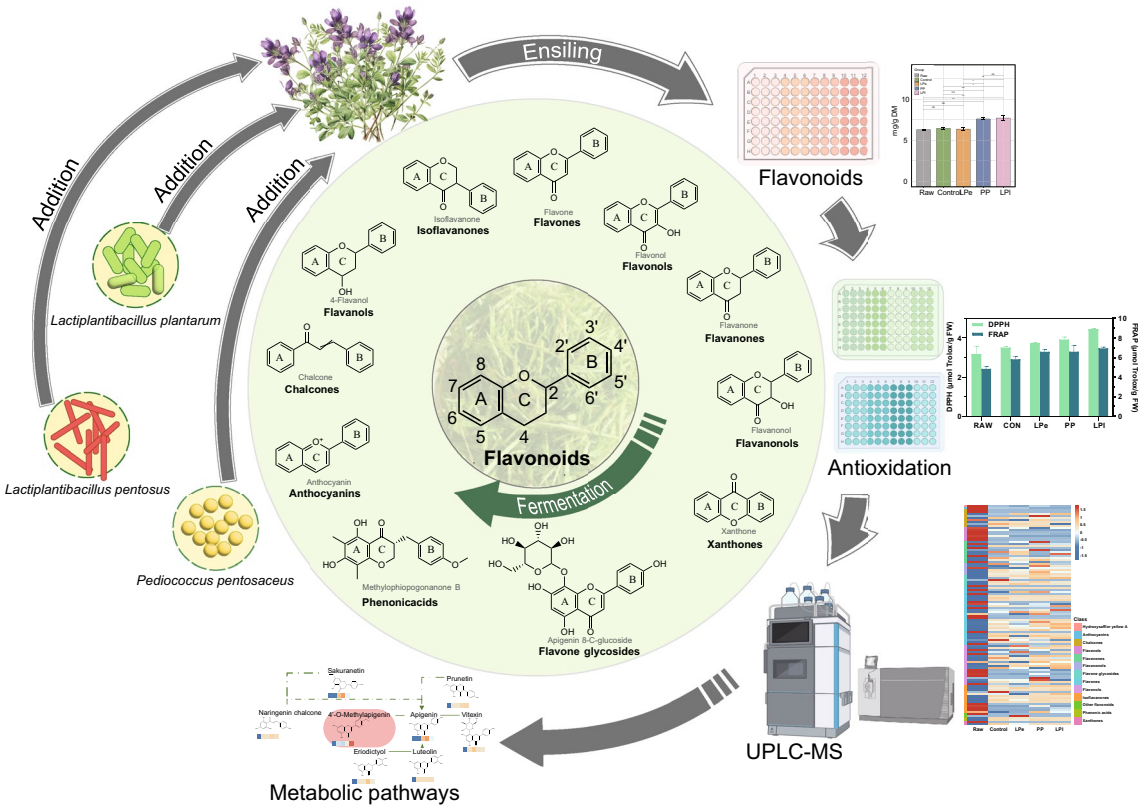
Graphical Abstract



Background

Flavonoids constitute a diverse group of polyphenolic compounds that are ubiquitously distributed in plants, exerting significant implications on human and animal health [1]. Structurally, flavonoids consist of a 15-carbon skeleton composed of two benzene rings (A and B) connected through a heterocyclic pyrane ring (C) [2]. Based on the structural variations, flavonoids are classified into various subclasses including flavonols, flavanones, isoflavones, dihydroflavones, dihydroflavonols, dihydroflavonoid glycosides, flavonoid carbosides, chalcones, xanthones, proanthocyanidins, phenolic acids and others [3]. The structural variations of flavonoids also result in significant disparities in their bioactivity [4]. In general, flavonoids are bound to cellulose, hemicelluloses, lignin, pectin or proteins within plant cell walls via -OH groups (O-glycosides) or carbon–carbon bonds (C-glycosides) [5, 6]. Microbial fermentation can enhance the release of conjugated flavonoids from plants to promote the development of their biological activities. Currently, it has been confirmed that microbial fermentation processes can convert flavonoids into glucosides [7, 8] and sulfonyl conjugates [9], as well as glucuronides [10]. Moreover, the glucosides of flavonoids exhibit higher antioxidant activity compared to glycosides [11, 12].

During the fermentation process, microorganisms possess the capacity to metabolize flavonoids into more bioavailable forms. This metabolic transformation is influenced by various factors, including the type of microorganisms, fermentation conditions, fermentation duration, and substrate nature [13–15]. In traditional Chinese medicine, microbial fermentation is a critical processing technology that releases a large amount of original active ingredients and synthesizes new substances [16]. This technology has been demonstrated to enhance the clinical efficacy of herbs [17]. Meanwhile, in the food field, how to further enhance the nutritional value and functionality of vegetables, fruits and edible fungi through microbial fermentation has also attracted extensive research interest from scholars [18]. Lactic acid

bacteria, in particular, have become the most commonly used strains because of their excellent substance transformation ability and high biological safety [19]. Over the past two decades, there has been a gradual elucidation of the mechanism by which lactic acid bacteria release conjugated flavonoids. For instance, several studies have assessed the capacity of different lactic acid bacteria to release conjugated flavonoids during the fermentation of barley and oats [20], apple juice [21], blueberry and pear [22]. Hole et al. [20] discovered that the inoculation with *Lactobacillus johnsonii*, *Lactobacillus reuteri*, and *Lactobacillus acidophilus* increased the total free phenolic acid content in fermented barley and oat by 20 times compared to the unfermented group. Various enzymes are produced during microbial growth and reproduction in fermentation processes, and one extensively studied enzyme is β-glucosidase. β-Glucosidase catalyzes the hydrolysis of glucoside bonds in alkanes and aromatic-β-D-glucosides, releasing their aglycone components. For example, Brochet et al. [23] found that *Lactobacillus melliventris* present in bee gut can produce glycosyl hydrolase to metabolize rutin (a flavonoid glycoside compound found in pollen) into quercetin (the aglycone part after deglycosylation).

As widely acknowledged, lactic acid bacteria not only play a crucial role in the fermentation of human food products but also serve as essential starter strains in silage fermentation [24–26]. It is of great significance to study the transformation of active substances by lactic acid bacteria in silage for animal health. Medicago sativa L. is the most ancient species cultivated as a fodder plant, and it is commonly known as the "father of all foods" (al-fal-fa) due to its high protein content and abundance of various bioactive substances [27]. It has been cultivated not only for animal feed but also as an ethnopharmaceutical remedy since ancient times [28]. Various types of flavonoids have been found in the extract of alfalfa leaves, which have been proven to possess antioxidant activity and anticancer properties [29, 30]. However, the effects of fermentation by lactic acid bacteria on flavonoids and the antioxidant activity of alfalfa are still unclear. Current research mainly focuses on assessing its impact on the total flavonoid content, while ignoring the impact of fermentation on various members of flavonoids. A recent study conducted a quantitative analysis of quercetin, kaempferol, and isorhamnetin in silage with lactic acid bacteria [31]. The study observed an increase in the content of these three compounds in silage and attributed it to the conversion of flavonoids glycosides from a bound state to a free state during ensiling. However, this study focused solely on the quantification of these three compounds, providing limited insight into the diverse changes in the abundant and varied flavonoids present in alfalfa during the ensiling process.

Therefore, this study employed three typical lactic acid bacteria strains and utilized targeted metabolomics technology to investigate the impact of lactic acid bacteria supplementation on the composition and content of flavonoids in silage, and to explore their relationship with antioxidant activity. These findings establish a theoretical foundation for screening novel inoculants, while also providing practical insights into the development of functional feeds enriched with enhanced biological activity to enhance animal health.

Methods

Materials collection and silage making

Alfalfa (*Medicago sativa* L. Zhongmu No. 1) was cultivated in the Ewenke Experimental Station, Chinese Academy of Sciences, located in Hulunbuir City, Inner Mongolia Autonomous Region (E: 116.33°, N: 39.98°), and harvested in early bloom stage on July 9, 2022, with a stubble height of 10 cm. Lactic acid bacteria inoculants included *Lactiplantibacillus pentosus* (CP115741.1), *Pediococcus pentosaceus* (CP115739.1) and *Lactiplantibacillus plantarum* (CP115480.1), which are screened from alfalfa and preserved in the laboratory. Four treatments were set up in this experiment: without addition (control), *L. pentosus* addition (LPe), *P. pentosaceus* addition (PP) and *L. plantarum* addition (LPl). After the fresh alfalfa was cut, it was naturally dried in the field for 5 h, and mechanically cut to 2 cm. Then, 1 kg wilted alfalfa were put into a vacuum bag, then the air was removed using a vacuum sealer (SQ-303; Asahi Kasei Pax, Tokyo, Japan). Lactic acid bacteria inoculants were sprayed at a rate of 1.0×10^6 colony forming units (cfu)/g of fresh weight. The bags per treatment were performed in triplicate and stored at room temperature for 60 days.

Total flavonoid assay

Samples were dried at 65 ℃ to constant weight and then sieved through a 40-mesh sieve. Each sample (0.02 g) was mixed with 10 mL 60% ethanol and then shaken at 160r min^{-1} for 2 h at 60 ℃. The mixture was centrifuged at 25 ℃ for 10 min, and the supernatant was collected for further analysis. The content of total flavonoid in the samples was quantified using the total flavonoid kit (Suzhou Keming Biology, Suzhou, China). This kit employs a colorimetric method to detect the reaction between flavonoids and aluminum ions. Briefly, 108 μL of sample extraction solution was added to each well of a 96-well plate along with 6 μL of the provided detection reagent, followed by incubation at 25 ℃ for 6 min. Subsequently, 6 μL of reagent 2 was added and allowed to react

at 25 ℃ for another 6 min. After that, 80 μL of reagent 3 was added and left standing at room temperature for 15 min before measuring the absorption value at 510 nm using an 96-well plate.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity assay

The fresh alfalfa and silage samples were stored at − 80 °C for subsequent assays. For each 0.1 g of sample, 1 mL of ice-cold extraction solution (Suzhou Keming Biology, Suzhou, China) was added, and the mixture was homogenized in an ice bath. After centrifugation at 4 °C for 10 min, the supernatant was collected and kept on ice for further measurements. The DPPH antioxidant capacity was assessed by mixing 20 μL of the extract with 380 μL of the detection reagent, followed by incubation at room temperature in the dark for 20 min. The absorbance was then measured at 515 nm using a 200 μL sample volume in a standard microplate. In the control group, a standard curve was established using Trolox antioxidant, with μmol Trolox/g fresh weight representing the antioxidant capacity of the samples.

FRAP (ferric reducing antioxidant power) assay

Each 0.1 g sample was homogenized with 1 mL of ice-cold extract (Suzhou Keming Biology, Suzhou, China) in an ice bath and then centrifuged at 4 ℃ for 10 min. The supernatant was collected and kept on ice for subsequent assays. The FRAP working solution, following the test kit instructions, was mixed thoroughly with the sample solution and incubated on a 96-well plate. The absorbance at 593 nm was measured, and the antioxidant capacity was quantified in μmol Trolox/g fresh weight using a standard curve generated with Trolox as an antioxidant reference.

ABTS [2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)] radical-scavenging activity assay

Each sample (0.1 g) were mixed with 1 mL extract (Suzhou Keming Biology, Suzhou, China) and then vigorously shook at 160 r min^{-1} for two hours at 4 °C. The mixture was centrifuged at 10,000 r min^{-1} for 10 min at 4 ℃, and the supernatant was collected for further analysis. Reagent 1 mixed with reagent 2 and shaked for 20 min as a working solution. The 10 μL extracts prepared samples mixed with 190 μL working solution. The absorbance at 734 nm was measured for each sample after incubation at 25 °C for 10 min.

Flavonoids-targeted metabolomic analysis

Fresh alfalfa raw materials and silage samples were freeze-dried under vacuum at − 50 °C for 5 days to a constant weight. The dried samples were ground into powder form using a ball mill at a frequency of 30 Hz for 1.5 min. A weight of 20 mg of the powdered sample was taken and mixed with 10 μL of internal standard working liquid, which had a concentration of 4000 nmol/L, and 500 μL of 70% methanol. The mixture was then subjected to ultrasonic treatment for 30 min. Afterward, the mixture was centrifuged at 4 ℃ and 1,2000 r/min for 5 min. The resulting supernatant was collected and filtered through a 0.22 μm filter into a sample bottle for LC–MS/MS analysis.

Ultra-high-performance liquid chromatography (ExionLC™ AD) and tandem mass spectrometry (QTRAP® 6500 +) were employed for the tests. The chromatographic conditions were as follows: a Waters ACQUITY UPLC HSS T3 C18 column (1.8 μm, 100 mm × 2.1 mm) was used. The mobile phase A consisted of ultra-pure water with 0.05% formic acid, and the B phase consisted of acetonitrile with 0.05% formic acid. The flow rate was 0.35 mL/min, the column temperature was maintained at 40℃, and the sample size was 2 μL. The elution gradient was as follows: 0 min A/B 90:10 (V/V), 1 min A/B 80:20 (V/V), 9 min 30:70 (V/V), 12.5 min A/B 5:95 (V/V), 13.5 min 5:95 (V/V), 13.6 min 90:10 (V/V), 15 min 90:10 (V/V). The mass spectrometer was operated in positive/negative ionization mode, and the parameters for flavonoids analysis were optimized. Multiple Reaction Monitoring (MRM) mode was employed for MS/MS detection, with each target flavonoids compound having a specific preproduct-ion transition. Data acquisitions were performed using Analyst 1.6.3 software (Sciex). Multiquant 3.0.3 software (Sciex) was used to quantify all metabolites.

The triple quadrupole mass spectrometry was employed in multi-reaction monitoring mode for quantitative analysis. Flavonoids contents were detected by MetWare (http://www.metware.cn/) based on the AB Sciex QTRAP 6500 LC–MS/MS platform. A calibration curve was established using a standard solution (Table S1) of the relevant flavonoids with known concentrations. The concentration of individual flavonoids in alfalfa extract was determined by comparing their peak areas to the standard curve.

$$\text{Flavonoids content}\left(\text{nmol/g}\right) = c * V/1,000,000/m$$

c is the concentration of the sample (nmol/L).
V is the volume of solution used for extraction (μL).
m is the mass of the sample (g).

Statistical analyses

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using ANOVA followed by Duncan's new multiple range test (MRT) with

$P < 0.05$ considered statistically significant. The statistical analysis was performed using SPSS software (Version 25, IBM Corp., Armonk, NY). The relationship between total flavonoid content and antioxidant activities was calculated through Pearson correlation analysis using SPSS software. The mass spectrum data were processed with Analyst 1.6 and Multi Quant 3.0.3 software. Metabolite contents were normalized, and hierarchical clustering analysis (HCA) was performed to examine metabolite accumulation patterns among different samples using the Complex Heatmap package in R. For HCA, normalized signal intensities of metabolites were visualized as a color spectrum by unit variance scaling. The Pearson correlation analysis of different flavonoids was calculated using SPSS software. Principal Component Analysis (PCA) was conducted using the prcomp function in R, with the parameter scale set to True. Orthogonal partial least square discriminate analysis (OPLS-DA) was performed using the Metabo AnalystR package and the OPLSR. Anal function in R. The data was log-transform (log_2) and mean-centred before analysis. The flavonoids were annotated by the KEGG database (https://www.genome.jp/kegg/compound/) and then mapped to the KEGG Pathway database (https://www.genome.jp/kegg/pathway.html). Pathways with significantly regulated metabolites were identified through metabolite set enrichment analysis (MSEA), with statistical significance determined by the hypergeometric test's *P* values.

Results

Effects of lactic acid bacteria on total flavonoid content

As presented in Table 1, the impact of lactic acid bacteria on total flavonoid content in alfalfa ensiling fermentation was examined across distinct treatment groups, including control and those treated with *L. pentosus* (LPe), *P. pentosaceus* (PP), and *L. plantarum* (LPl). The effects of different lactic acid bacteria on the overall flavonoid levels in alfalfa were diverse. Notably, compared to the control group, the *P. pentosaceus* (PP) and *L. plantarum* (LPl) treatments exhibited significant increases ($P < 0.05$) in total flavonoid content, measuring 8.30 and 8.42 mg/g, respectively, representing increases of 16.25% and17.93%. Conversely, the *L. pentosus* (LPe) group showed marginal changes ($P > 0.05$), with 7.07 mg/g content. The findings emphasize the potential of specific lactic acid bacteria, especially PP and LPl, to enhance the total flavonoid content during alfalfa ensiling fermentation.

Effect of lactic acid bacteria on antioxidant activity

The antioxidant capacity of fresh alfalfa and silage samples was assessed using DPPH, ABTS and FRAP assays. The DPPH assay was used to measure the antioxidant capacity by reacting DPPH free radicals with antioxidant substances. The antioxidant substances capture the electrons in DPPH free radicals, causing them to change from purple to colorless DPPH-H. The degree of color weakening is directly proportional to the antioxidant capacity [32]. Meanwhile, the FRAP assay evaluates antioxidant activity by measuring the capability to reduce iron ions Fe^{3+} to form Fe^{2+}, with the change in the concentration of the reduced product indicating antioxidant activity [33]. These methods are both widely recognized for their efficiency and effectiveness in determining antioxidant capacities [34]. The free radical scavenging capacity was also measured by using the ABTS radical cation decolorization assay, which is based on the reduction of $ABTS^{\bullet+}$ radicals by antioxidants [35].

The results from different methods were nearly consistent (Table 2), which revealed that the antioxidant capacity was the lowest in the alfalfa raw material, followed by the silage without adding lactic acid bacteria. Fermentation significantly improved the ferric-reducing antioxidant power and $ABTS^{\bullet+}$ radical cation scavenge capacity of alfalfa ($P < 0.05$). The *L.* plantarum inoculant increased

Table 1 Total flavonoid content of alfalfa samples

Treatment	Total flavonoids (mg/g DM)
Raw	6.99 ± 0.70^{b}
Control	7.14 ± 0.15^{b}
LPe	7.07 ± 0.22^{b}
PP	8.30 ± 0.12^{a}
LPl	8.42 ± 0.30^{a}

Raw, fresh alfalfa; Control, alfalfa silage without addition; LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addtiton; LPl, *Lactiplantibacillus plantarum* addtiton; DM, dry matter. a, b Significant difference in different treatment($P < 0.05$) according to one-way ANOVA and Duncan's new multiple range test (MRT) (means ± standard deviation, n = 3, biologically independent samples)

Table 2 Antioxidant activity of alfalfa samples

Treatment	DPPH μmol Trolox/g FW	FRAP μmol Trolox/g FW	ABTS μmol Trolox/g FW
Raw	3.18 ± 0.70^{c}	4.84 ± 0.43^{c}	8.56 ± 0.49^{b}
Control	3.51 ± 0.09^{bc}	5.82 ± 0.52^{b}	13.52 ± 1.49^{a}
LPe	3.75 ± 0.05^{bc}	6.59 ± 0.44^{a}	13.93 ± 0.90^{a}
PP	3.92 ± 0.24^{b}	6.60 ± 1.13^{a}	14.28 ± 0.61^{a}
LPl	4.45 ± 0.05^{a}	6.94 ± 0.23^{a}	14.35 ± 1.41^{a}

Raw, fresh alfalfa; Control, alfalfa silage without addition; LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPl, *Lactiplantibacillus plantarum* addition; FW, Fresh weight. The letters "a–c" indicates significant differences in different treatments ($P < 0.05$) according to one-way ANOVA and Duncan's new multiple range test (MRT) (means ± standard deviation, n = 3, biologically independent samples)

($P < 0.05$) DPPH radical scavenging activity compared with control. These results collectively indicate that the incorporation of lactic acid bacteria, particularly *L. plantarum* (LPl), significantly augmented the antioxidant capacity during ensiling fermentation.

Correlation between total flavonoid content and antioxidant activity

To assess the relationship between total flavonoid content and antioxidant activity, the Pearson correlation coefficient was calculated between the total flavonoid content and various measures of antioxidant activities (DPPH, FRAP, ABTS), as detailed in Table 3. The results revealed a significant positive linear correlation between the total flavonoid content and antioxidant activity, as indicated by the DPPH (R = 0.62, $P < 0.05$) and FRAP (R = 0.70, $P < 0.01$) values. Although there was also a positive relationship between total flavonoid content and ABTS antioxidant activity (R = 0.40), this correlation was not statistically significant. This suggests that factors other than total flavonoids may influence the antioxidant outcomes in the ABTS assay. In addition, the internal consistency among the antioxidant assays was highlighted by strong positive correlations, particularly between FRAP and ABTS, which had a correlation coefficient of 0.70 ($P < 0.01$). This indicates a generally consistent response to antioxidant components within the alfalfa samples across different testing methods.

Overview of flavonoids metabolite

Figure S1 displays both the total ion chromatogram (TIC) and extracted ion chromatogram (XIC), where the horizontal and vertical axes represent the retention time (min) and the ion flow intensity (cps), respectively. Using mass spectrometry and local metabolite database to conduct a quantitative and qualitative analysis of flavonoids metabolites. The results showed that fermentation increased the content of total flavonoids in alfalfa and changed the composition of flavonoids. There were 90 flavonoids identified in fresh alfalfa, while 89, 88, and 85 flavonoids were detected in the control, LPe, and LPl treatment groups, respectively. The primary compound in the raw material is scutellarin, while apigenin becomes the most abundant compound after fermentation. The specific substance contents are shown in Table S1.

Table 3 Pearson correlation between total flavonoid content and antioxidant activities of alfalfa samples

Item	Total flavonoid	DPPH	FRAP	ABTS
Total flavonoid	1	0.62*	0.70**	0.40
DPPH	0.62*	1	0.65**	0.45
FRAP	0.70**	0.65**	1	0.70**
ABTS	0.40	0.45	0.70**	1

DPPH, 2,2-diphenyl-1-picrylhydrazyl. FRAP, ferric reducing antioxidant power

*, significance at the 0.05 probability level. **, significance at the 0.01 probability level

According to Fig. 1a, PCA was utilized to analyze the overall differences in flavonoids metabolites between the treatment groups before and after fermentation. The results revealed significant variations between the raw materials and the ensiling treatment groups, indicating that fermentation significantly altered the characteristics of flavonoids in alfalfa. In general, both silage fermentation and the introduction of strains affected the component and content of flavonoids (Fig. 1b). The contents of all flavanols, anthocyanins, and phenolic acids decreased after fermentation, suggesting degradation during the fermentation process. However, the changes of other types of flavonoids in the fermentation process are more complicated, which means the contents of different members in the same subclass did not show the same trend; some substances increased, and some decreased after fermentation.

To further explore the impact of different LAB strains on flavonoid metabolism, orthogonal partial least squares discriminant analysis (OPLS-DA) was utilized. The criteria used for screening and comparing different metabolites between the two groups were VIP > 1 and $|\mathrm{Log_2\ FC}| \geq 1$, as depicted in Fig. 1d. Figure 2 shows that the LPe group differed significantly from the control, with notable increases in morusin, hesperetin, quercitrin, and 3,7,4′-trihydroxyflavone levels, and decreases in pedalitin, narcissin, and 2'-hydroxydaidzein. *P. pentosaceus* enhanced levels of isorhamnetin 3-O-glucoside, licochalcone E, taxifolin 7-O-rhamnoside, calycosin-7-O-β-D-glucoside, hesperetin, 3,7,4′-trihydroxyflavone, and isosakuranetin. *L. plantarum* significantly raised the levels of 3,7,4′-trihydroxyflavone, quercitrin, 4,4'-dimethoxychalcone, acacetin, and farrerol, while reducing narcissin, daidzin, and benzylideneacetophenone. The unique and common differential metabolites between the comparison groups are illustrated in Fig. 1c. The common differential metabolite among treatments and control group is a kind of flavonols called 3,7,4′-trihydroxyflavone, which is upregulated compared to the control group. This substance is absent in unfermented alfalfa raw material and the control group, indicating that lactic acid bacteria fermentation can metabolize certain compounds in alfalfa into 3,7,4′-trihydroxyflavone. In addition, there were seven significantly different metabolites between the PP and LPe groups, the LPl and LPe groups, and the LPl and PP groups,

emphasizing the unique metabolic signatures induced by each LAB strain.

Analyzing the difference in flavonoids and its relationship with antioxidant ability

Metabolites derived from various comparison combinations were aggregated to yield 22 distinct flavonoids. To investigate the correlation between distinct flavonoids and their varying antioxidant activities, Pearson correlation analysis was conducted on the 22 distinct flavonoids and the antioxidant activities (Fig. 3). The analysis revealed a significant negative correlation between narcissin and DPPH, while acacetin and 3,7,4′-trihydroxyflavone demonstrated a significant positive correlation with DPPH. Further, both 3,7,4′-trihydroxyflavone and taxifolin 7-O-rhamnoside exhibited a significant positive correlation with FRAP.

The flavonoids metabolic processes

KEGG analysis was conducted to understand the metabolic mechanism of flavonoids. The result showed that differential metabolites were mainly enriched in the secondary metabolites pathway (Fig. S2), including flavone and flavonol biosynthesis, isoflavonoid biosynthesis, flavonoid biosynthesis and biosynthesis. Specifically, the *L. pentosus* treatment group exhibited significant enrichment in the flavonoid biosynthesis, flavone and flavonol biosynthesis, isoflavonoid biosynthesis, and biosynthesis of secondary metabolites pathways compared to the control. Similarly, *P. pentosaceus* treatment influenced mainly the flavonoid biosynthesis and secondary metabolites pathways, while *L. plantarum* treatment were predominantly associated with the flavone and flavonol biosynthesis, and isoflavonoid biosynthesis pathways. The differences were observed between PP and LPe groups, as well as between LPl and LPe groups, with each set showing enrichment in isoflavonoid biosynthesis, flavone and flavonol biosynthesis pathways, and biosynthesis of secondary metabolites pathways. In addition, the differential flavonoids between LPl and LPe groups are also enriched in flavonoid biosynthesis pathway. The results indicated significant variations in the biotransformation capacity inoculated by different lactic acid bacteria, particularly towards secondary metabolites such as flavonoids, flavonols, and isoflavones.

Further investigation into flavonoids-related pathways revealed the detection of 42 substances in the metabolic pathways. By combining the identified substances with their structures and contents, the metabolic process of flavonoids was depicted as shown in Fig. 4. It can be observed that the degradation of flavonoids compounds in fermentation varied depending on different lactic acid bacteria treatments. Overall, high-molecular-weight flavonoids exhibited higher content in raw materials but lower content in each fermentation group. The macromolecular substances daidzin, dihydromyricetin, phlorizin, cynaroside, formononetin 7-O-glucoside, glycitein 7-O-glucoside, afzelechin, astragalin, baimaside, rutin, (-)-epicatechin, and epigallocatechin, at the end of the synthesis pathway, have higher content in the fresh alfalfa while lower content in fermentation treatments. However, the upstream components of the synthesis pathway, for example, naringenin chalcone and eriodictyol, have very low content in the raw materials but are higher in fermentation treatments.

Discussion

Within the realms of food and traditional Chinese medicine, researchers have directed their efforts toward investigating the transformations in substances and antioxidant activity induced by fermentation [16, 36, 37]. Fermentation concurrently facilitates the release and degradation of various substances; thus, the effects often vary across different plant materials. For instance, the fermentation enhanced the release of phenolic compounds in pumpkin (*Cucurbita maxima* D.) silage, thereby mitigating the decline in antioxidant capacity resulting from a reduction in substances such as carotenoids [38]. On the contrary, after fermenting moringa, polyphenols were significantly reduced, while the accumulation of free amino acids and small peptides led to an increase in its antioxidant activity [39]. In this study, the additions of *P. pentosaceus* and *L. plantarum* resulted in a significantly higher total flavonoid content than other groups, suggesting that PP and LPl demonstrated superior conversion/release efficiency for flavonoids during fermentation. Similar results were also found during the

(See figure on next page.)

Fig. 1 Overview of flavonoids metabolomics analysis during ensiling process. **a** PCA analysis of flavonoids metabolic profiles. **b** Heatmap analysis of the composition and content of flavonoids. In the heat map, different colors represent the degree of accumulation of each metabolite, with red to blue indicating high to low. **c** Venn diagram of differential flavonoids metabolites. **d** Differential metabolite analysis for LPe vs Control, PP vs Control, LPl vs Control, PP vs LPe, LPl vs LPe, and LPl vs PP. The green dots represent downregulated metabolites, red dots represent upregulated metabolites, and gray dots represent metabolites with no significant differences. Points with $\log_2$FC values equal to positive or negative infinity are not displayed in the figure. LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPl, *Lactiplantibacillus plantarum* addition

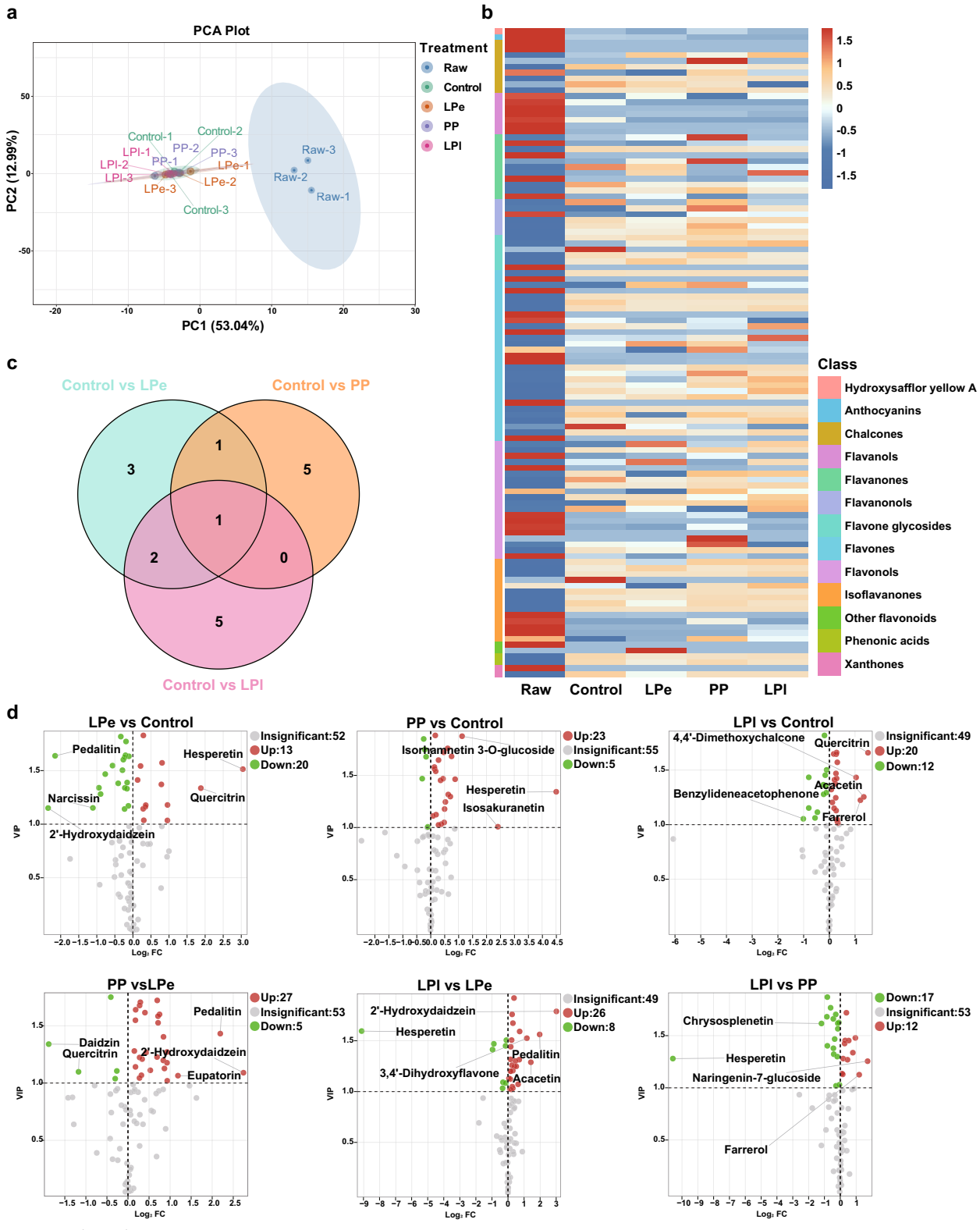


Fig. 1 (See legend on previous page.)

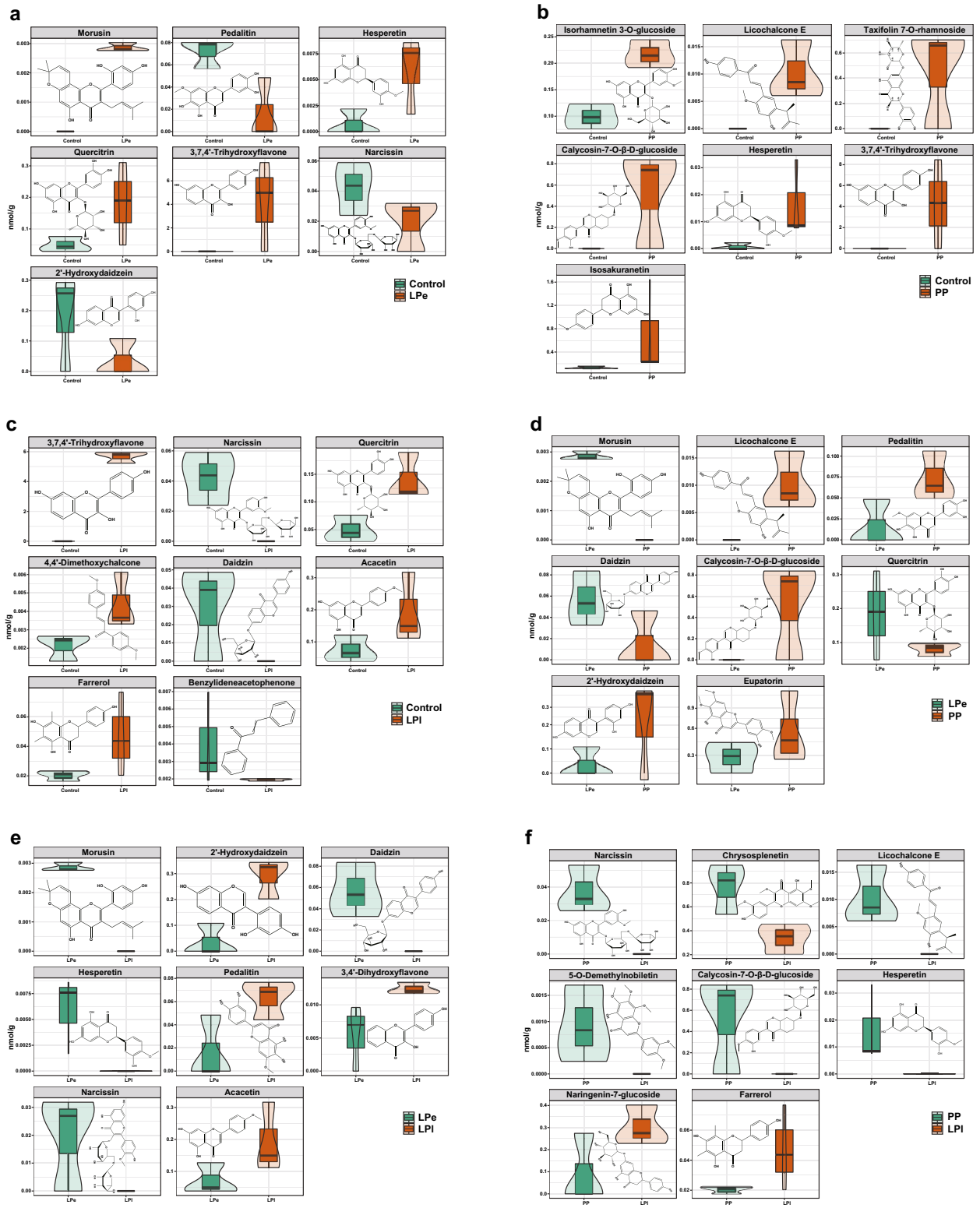


Fig. 2 Violin plot of differential metabolites and their chemical structures. A combination of a box line plot and a density plot is used to show the data distribution and its probability density. The box in the middle indicates the interquartile range, the thin black line represents the 95% confidence interval, the black horizontal line in the middle is the median, and the outer shape indicates the density of the data distribution. **a** LPe vs Control, **b** PP vs Control, **c** LPI vs Control, **d** PP vs LPe, **e** LPI vs LPe, **f** LPI vs PP. LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPI, *Lactiplantibacillus plantarum* addition

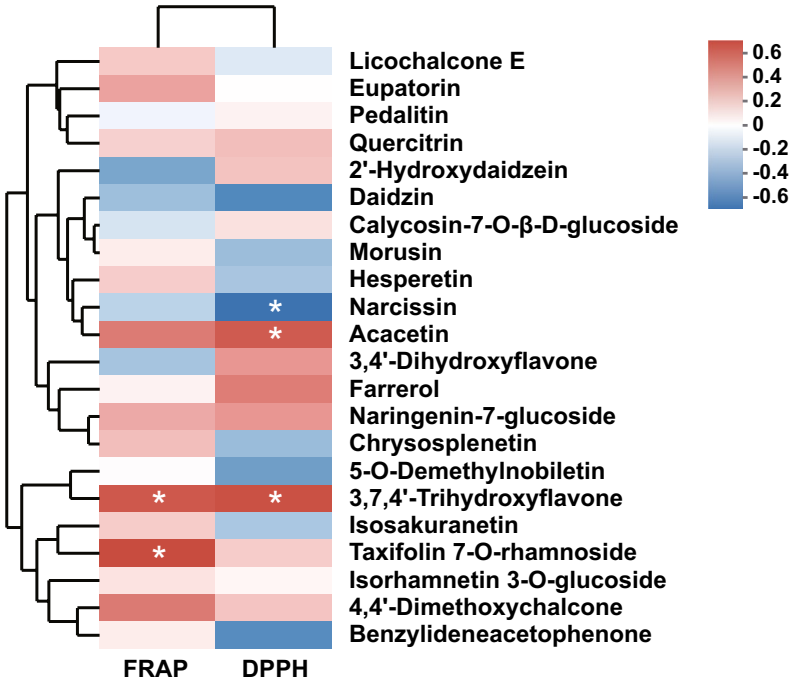


Fig. 3 Pearson correlation between differential metabolites and antioxidant activity. *, $P < 0.05$. Due to the lack of statistical significance in the correlation between ABTS assay results and total flavonoid content, this analysis was not included in the graphical representation

mulberry leaf fermentation [40]. Overall, the antioxidant activity increased after fermentation, in accordance with previous research on the fermentation of fruit and vegetable juices [41, 42] and tea [43, 44]. This improvement in antioxidant activity is believed to be attributed to the various enzymes produced by lactic acid bacteria during the fermentation process. In this study, the antioxidant capacity of the LPl group was significantly higher than that of other groups. The enhancement of antioxidant capacity varied among different strains of lactic acid bacteria, indicating that this process is highly strain-specific, likely associated with the inherent metabolic machinery of each strain [45]. Throughout the ensiling process, the intricate microbial community structure experiences intense competition, leading to diverse alterations in substances that collectively influence the antioxidant activity of alfalfa post-fermentation.

The Pearson correlation coefficient suggested that the total flavonoid content has close relationship with the antioxidant activity. Similar results were also observed in other studies, such as the yellowing process of rice [46] and the fermentation of seaweed by lactic acid bacteria [47]. For instance, the fermentation of Sargassum by lactic acid bacteria led to an increase in total flavonoid content and DPPH free radical scavenging activity [47]. To identify specific compounds that play a pivotal role in this process, targeted metabolomics analysis was conducted to ascertain the types and changes in flavonoid content during fermentation, analyzing flavonoid members directly linked to antioxidant activity. The results revealed diverse capabilities among lactic acid bacteria strains in biotransforming flavonoids, further illustrating the complexity of microbial interactions during fermentation. Among the three compounds identified in this study with a significant positive correlation to antioxidant activity—acacetin, 3,7,4′-trihydroxyflavone, and taxifolin 7-O-rhamnoside—acacetin is particularly notable for its strong DPPH radical scavenging activity and antioxidant capability in vivo [48–51]. Levels of acacetin were found to increase post-fermentation, especially in groups treated with LAB. This underscores the potential of LAB fermentation to enhance the antioxidant properties of flavonoids through the selective activation of specific enzymatic pathways by chosen bacterial strains.

Various lactic acid bacteria strains demonstrated different abilities in biotransforming flavonoids [52]. The flavonoid metabolomics have shown that each group of samples exhibits distinct flavonoid metabolism and accumulation patterns, manifested in various pathways of differential metabolite enrichment, illustrating the significant impact of lactic acid bacteria on flavonoid biotransformation. The addition of LAB, compared to natural fermentation, induced a broader range of enzymatic reactions such as glycosidic bond cleavage, sugar

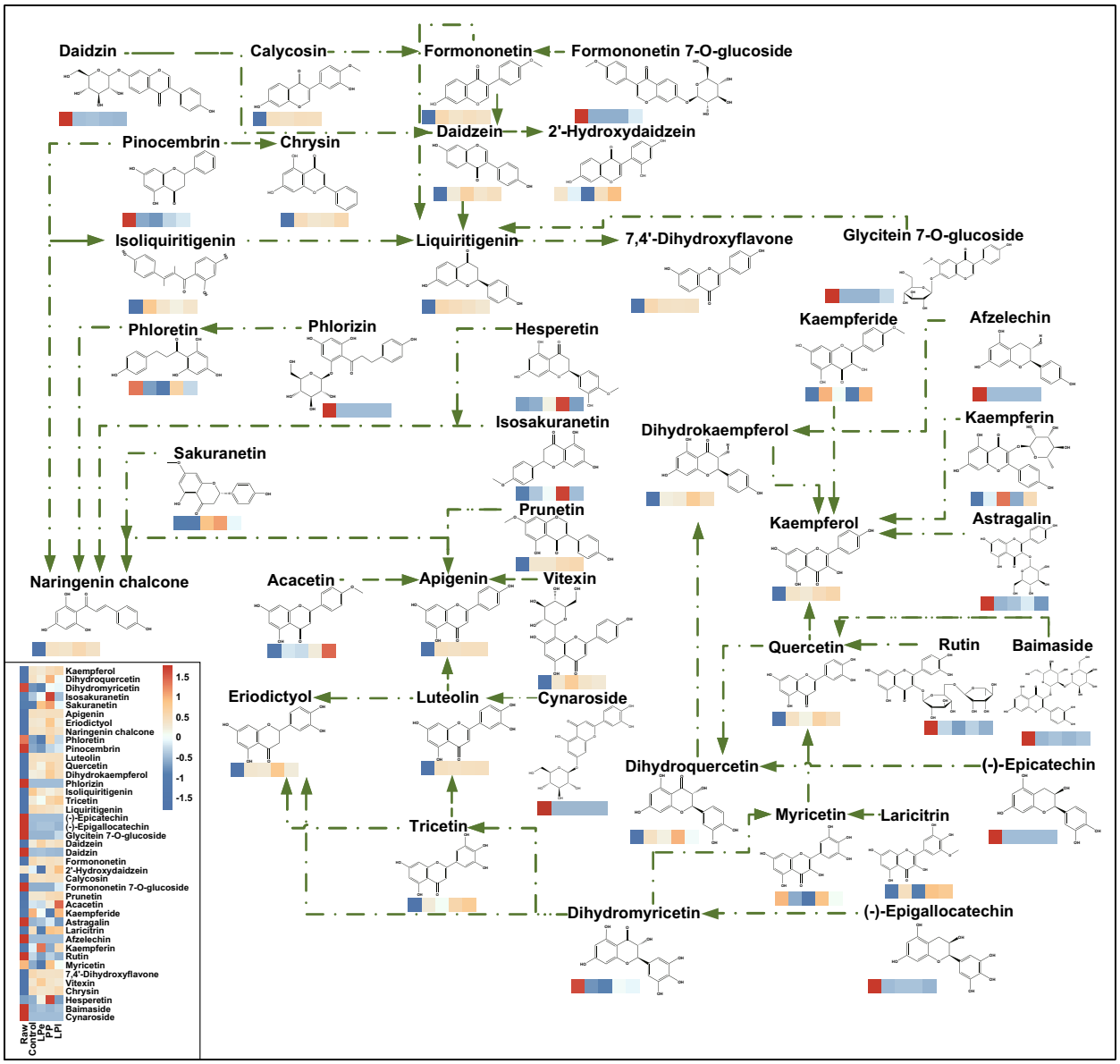


Fig. 4 Degradation process of flavonoids compounds in fermentation. The model diagram is based on the flavonoids-related pathway in the KEGG database. Dashed lines and arrows indicate the predicted flavonoids degradation pathways. Each solid square in the corresponding metabolites represented a sample, the color bar in the diagram indicated the range of log2, and the order from left to right represents fresh alfalfa, Control group, LP group, PP group and LPl group, respectively. LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPl, *Lactiplantibacillus plantarum* addition

transfer, hydroxyl substitution, methoxy substitution, and ring opening. These transformations are facilitated by enzymes like glycosidases, dehydrogenases, and methyltransferases that are produced during the fermentation process [4, 18]. One notable biotransformation observed was the production of 3,7,4′-trihydroxyflavonoid, also known as resokaempferol. This flavonoid, predominantly found in the LAB-treated groups, is known for its potent antioxidant and antibacterial properties, as well as its significant anti-inflammatory effects in vivo [53–55], which contribute to its health benefits. In the alfalfa silage treated with LAB, the unique detection of 3,7,4′-trihydroxyflavone may result from the biotransformation of kaempferol 3-O-rhamnoside, facilitated by enzymatic activities specific to the microbial flora introduced with LAB. This transformation likely involves the cleavage of the rhamnose group by a rhamnosidase, reverting kaempferol 3-O-rhamnoside to its aglycone form, kaempferol.

Subsequent hydroxylation at the 3′ and 5′ positions by enzymes such as flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) would yield 3,7,4′-trihydroxyflavone. While, the absence of these specific enzymes in the control group, particularly the rhamnosidase and hydroxylases, may lead to the retention of the methoxy group at the 4′ position of kaempferol, resulting in the formation of 4′-O-methylkaempferol instead. This absence highlights a differential metabolic pathway wherein the lack of microbial intervention limits the structural modification of flavonoids. Particularly, *L. plantarum* demonstrated a unique ability to alter the flavonoid profile significantly, as evidenced by the complete degradation of narcissin. This was likely facilitated by the strain-specific enzyme α-L-rhamnosidase, which is effective in cleaving glucoside bonds within flavonoids, highlighting the strain-specific enzymatic capabilities of LAB. Mueller et al. [56] assessed the hydrolytic capacity of 14 *Lactobacillus* strains on narcissin and other flavonoids and noted that rhamnosidase's capacity for flavonoids hydrolysis was highly specific to the strain. This specificity might explain why *L. plantarum* exhibited the most complete hydrolysis of narcissin. Moreover, the *L. pentosus* showed a distinct biotransformation pathway, leading to the production of morusin, a pyran-containing isopentenylated flavonoid. According to the Pearson correlation coefficient, it was found that pedalitin, engeletin, 7-methoxyisoflavone, 3,4′-dihydroxyflavone, puerarin, 2′-hydroxydaidzein were significantly negatively correlated with morusin (Fig. S3). Among them, pedalitin had the strongest negative correlation. Given that the pedalitin levels in the LPe group were markedly lower compared to other groups, its structure was subjected to analysis. It was speculated that under the influence of LPe, pedalitin undergoes a substitution reaction to yield morusin. In the study on the structure–activity relationship of flavonoids' antibacterial properties, Xie et al. [57] highlighted that isopentenylation enhanced the antibacterial efficacy of flavonoids. Hoi et al. [58] also confirmed the inhibitory effects of morusin extracted from *Artocarpus nigrifolius* on *Bacillus subtilis* and *Staphylococcus aureus*. The presence of morusin may explain the near absence of *Bacillus* in the LPe group (Fig. S4).

Overall, high-molecular-weight flavonoids exhibited higher content in raw materials but lower content in each fermentation group. This suggests that the increase in the total content of alfalfa flavonoids after fermentation is not attributed to continued plant biosynthesis but is primarily a result of the release of bound flavonoids through microbial and enzymatic actions during fermentation. Simultaneously, high-molecular-weight flavonoids undergo degradation into smaller molecules and further engage in various biochemical reactions. For instance, a large amount of astragalin in the raw materials is deglucosided during fermentation and degraded into kaempferol. The degradation of luteolin to eriodictyol during microbial fermentation, Braune et al. [59] was also observed during the ensiling process in this study. Similarly, comparing samples before and after silage, a decrease in rutin levels and an increase in quercetin concentration were observed. This may be because certain microorganisms produce enzymes responsible for the degradation of rutin into quercetin under anaerobic conditions [60]. Given the variable effects of LAB on different flavonoids, future research should aim to dissect the specific enzymatic pathways engaged by these bacteria, enhancing our understanding of how fermentation can be tailored to optimize the health benefits of plant-based foods. Further studies exploring the structural–activity relationships of flavonoids could provide deeper insights into the mechanisms by which fermentation affects their bioactive properties.

Conclusions

This study comprehensively examined the impact of lactic acid bacteria (LAB) inoculation on the flavonoid profile and antioxidant activities in alfalfa silage, demonstrating that inoculation with specific strains of LAB, particularly *L. plantarum* and *P. pentosaceus*, significantly increased the total flavonoid content by 17.93% and 16.25% compared to the control group, respectively. In addition, *L. plantarum* significantly enhanced the antioxidant capacity during the ensiling fermentation process. Furthermore, reveal a robust positive correlation between the total flavonoid content and antioxidant activity, underscoring the dual benefits of LAB fermentation in boosting the nutritional and functional properties of silage. Significant variations were observed in the flavonoid composition due to anaerobic fermentation. While some flavonoids such as flavanols, anthocyanins, and phenolic acids uniformly decreased, others like apigenin significantly increased, becoming the most abundant post-fermentation compound. This differential response underscores the selective activation of metabolic pathways by specific LAB strains. Notably, 3,7,4′-trihydroxyflavone was significantly synthesized during LAB fermentation, indicating the activation of a unique metabolic pathway. The correlation between enhanced levels of specific flavonoids (acacetin, 3,7,4′-trihydroxyflavone, and taxifolin 7-O-rhamnoside) and improved antioxidant activities further supports the potential of LAB fermentation in fortifying silage with bioactive compounds tailored. In conclusion, the strategic use of LAB in alfalfa silage fermentation can significantly alter the flavonoid composition and improve the antioxidant properties of the feed.

These findings provide a solid foundation for future applications to enhance the nutritional and therapeutic values of animal feed through biotechnological innovations. Future research should continue to explore the specific metabolic pathways LAB activates and assess the long-term impacts of these interventions on animal health and productivity.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00594-w.

Supplementary Material 1: Fig. S1 (a) Total ion chromatogram (TIC); (b) Negative ion extracted ion chromatogram (XIC-n); (c) Positive ion extracted ion chromatogram (XIC-p). Fig. S2 Enrichment map of KEGG pathway database of differential flavonoids. The horizontal axis represents the Rich factor for each pathway, the vertical axis represents the pathway name, and the color of the point is *P* Value. The redder the point, the more significant the enrichment. The size of the point represents the number of differential metabolites enriched. (a) LPe vs Control, (b) PP vs Control, (c) LPl vs Control, (d) PP vs LPe, (e) LPl vs LPe. LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPl, *Lactiplantibacillus plantarum* addition. Fig. S3 Pearson correlation analysis of flavonoids. The redder the color is, the stronger the negative correlation is, and size of the circle is proportional to the degree of correlation. Fig. S4 Relative abundance of *Bacillus* in alfalfa samples. LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPl, *Lactiplantibacillus plantarum* addition. Table S1 Flavonoid standards used for LC–MS/MS analysis. Table S2 Content and types of flavonoids in alfalfa samples. LPe, *Lactiplantibacillus pentosus* addition; PP, *Pediococcus pentosaceus* addition; LPl, *Lactiplantibacillus plantarum* addition.

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Author contributions
Yu Gao: conceptualization, methodology, writing original draft. Hongzhang Zhou: methodology, writing, review and editing. Yuan Wang: investigation. Luiz Gustavo Nussio: revised the manuscript. Fuyu Yang: investigation. Kuikui Ni: supervision.

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Availability of data and materials
The datasets used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This research has been confirmed for publication in the journal.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]College of Grassland Science and Technology, China Agricultural University, Beijing 100193, China. [2]Department of Animal Science, Luiz de Queiroz College of Agriculture, University of São Paulo, São Paulo, Brazil. [3]College of Animal Science, Guizhou University, Guiyang 550025, China.

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