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

Yu Gao  
China Agricultural University

Hongzhang Zhou  
China Agricultural University

yuan Wan  
China Agricultural University

Fuyu Yang  
China Agricultural University

Kuikui Ni  
nikk@cau.edu.cn

China Agricultural University

Research Article

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

Posted Date: February 27th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3981175/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.
Abstract

Background

Oxidative stress is currently one of the main threats to animal health, and flavonoids in feed have good antioxidant activity. However, the impact of fermentation on flavonoids and their antioxidant activity in feed 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 feed ($p<0.05$). The addition of lactic acid bacteria significantly improved the antioxidant capacity of alfalfa silage feed ($p<0.05$). 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'-trihydroxyflavonoids, 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.

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-C skeleton composed of two benzene rings (A and B) connected through a heterocyclic pyran ring (C) [2]. Based on the structural variations, flavonoids can be 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].

Generally, 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].

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, and the duration of fermentation [12, 13]. 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 [14], apple juice [15], blueberry and pear [16]. Hole et al. [14] 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. [17] 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 play a crucial role in the fermentation of human food products and serve as essential starter strains in silage fermentation [18]. However, current research on the involvement of lactic acid bacteria in the fermentation of flavonoids-containing raw materials mainly focuses on assessing their impact on the total flavonoids content. A recent study conducted a quantitative analysis of quercetin, kaempferol, and isorhamnetin in silage with lactic acid bacteria [19]. 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 used in this study was sourced from the Ewenke Experimental Station, Chinese Academy of Sciences, located in Hulunbuir city, Inner Mongolia Autonomous Region (E: 116.33°, N: 39.98°). The alfalfa plants were selected at the first flowering stage and sampled 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) screened from alfalfa in our laboratory. Four treatment groups were set up in this experiment: control group without addition, *Lactiplantibacillus pentosus* group (LPe), *Pediococcus pentosaceus* (PP) and *Lactiplantibacillus plantarum* group (LPl). After the fresh alfalfa was cut, it was naturally dried in the field for 5 hours, 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 $1.0 \times 10^6$ 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°C to constant weight. After sieving through a 40-mesh sieve, a weight of 0.02 g was obtained. Subsequently, shock extraction was performed by adding 60% ethanol at a temperature of 60°C for a duration of 2 hours. The resulting mixture was then centrifuged at 25°C for 10 minutes, and the supernatant was collected for further analysis. The content of total flavonoids in the samples was quantified using the total flavonoids 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°C for 6 minutes. Subsequently, 6 µl of reagent 2 was added and allowed to react at 25°C for another 6 minutes. After that, 80 µl of reagent 3 was added and left standing at room temperature for 15 minutes before measuring the absorption value at 510 nm using an enzyme label.

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

The fresh alfalfa raw materials and silage samples at 60 days were stored at -80°C for subsequent analyses. A 0.1 g sample was homogenized in an ice bath by adding it to 1 ml of ice-cold extraction solution (Suzhou Keming Biology, Suzhou, China). After centrifugation at 4°C for 10 minutes, the resulting supernatant was collected and kept on ice for further measurements. The DPPH antioxidant capacity of the sample was assessed by combining 20 µl of the extract with 380 µl of the detection reagent, followed by incubation at room temperature in the dark for 20 minutes. The absorbance at 515 nm was measured using a 200 µl volume in a standard microplate. In the control group, a standard curve was established using Trolox antioxidant, with umol Trolox/g fresh weight representing the antioxidant capacity of the samples.

**FRAP (Ferric Reducing Antioxidant Power) assay**
A 0.1 g sample was homogenized in an ice bath with 1 ml of ice extract (Suzhou Keming Biology, Suzhou, China) and then centrifuged at 4 °C for 10 minutes. The resulting supernatant was collected and maintained on ice for subsequent analysis. 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.

**Flavonoids-targeted metabolomic analysis**

Fresh alfalfa raw materials and silage samples were freeze-dried under vacuum for 60 days. The dried samples were ground into powder form using a ball mill at a frequency of 30 Hz for 1.5 minutes. 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 minutes. Afterward, the mixture was centrifuged at 4°C and 12000 r/min for 5 minutes. 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 ultrapure 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°C, 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.

The triple quadrupole mass spectrometry was employed in multi-reaction monitoring mode for quantitative analysis. A calibration curve was established using a standard solution 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.

\[
\text{Flavonoids content (nmol/g) = } c \times \frac{V}{1,000,000} / m
\]

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

The identification of flavonoids compounds was accomplished by comparing the retention time and mass spectra of the detected peaks to the standard Metware Database. To confirm the identity of the
compounds, we relied on the retention time and matching of at least two characteristic mass spectrum fragments.

**Statistical analyses**

The mass spectrum data were processed using Analyst 1.6 and Multi Quant 3.0.3 software, and the metabolite content was normalized. Hierarchical clustering analysis of the accumulation patterns of metabolites among different samples was performed using the Complex Heatmap package in R, and heat maps were generated. Principal Component Analysis (PCA) was conducted using the built-in statistical prcomp function in R, with the prcomp function parameter scale = True. After centralizing the original data, OPLS-DA was performed using the Metabo AnalystR package and the OPLSR.Anal function in R. All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out 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).

**Results**

**Effects of lactic acid bacteria on total flavonoids content**

As presented in Table 1, the impact of lactic acid bacteria on total flavonoids content in alfalfa ensiling fermentation was examined across distinct treatment groups, including control and those treated with *Lactiplantibacillus pentosus* (LPe), *Pediococcus pentosaceus* (PP), and *Lactiplantibacillus 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 *Pediococcus pentosaceus* (PP) and *Lactiplantibacillus plantarum* (LPl) treatments exhibited significant increases in total flavonoid content, measuring 8.30 and 8.42 mg/g, respectively. Conversely, the *Pediococcus pentosus* (LPe) group showed marginal changes, with a content of 7.07 mg/g. The findings emphasize the potential of specific lactic acid bacteria, especially PP and LPl, in enhancing the total flavonoids content during alfalfa ensiling fermentation.

**Effect of lactic acid bacteria on antioxidant activity**

The antioxidant capacity was assessed using both the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) methods. The DPPH method 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 [20]. Meanwhile, the FRAP method 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 [21]. Both the DPPH and FRAP methods are widely used and considered to be fast and effective for evaluating the antioxidant capacity of substances [22].
In this study, the antioxidant capacities of raw materials and silage were assessed using the DPPH assay in an ethanol system and the FRAP assay in a water system, respectively (Table 2). The results from both methods were nearly consistent. This study revealed that the antioxidant capacity was the lowest in the alfalfa raw material, followed by the silage without the addition of lactic acid bacteria. In the assessment of antioxidant capacity using both methods, the alfalfa ensiling treated with Lactiplantibacillus plantarum exhibited significantly higher antioxidant activity compared to all other groups. These results collectively indicate that the incorporation of lactic acid bacteria, particularly Lactiplantibacillus plantarum (LPl), significantly augmented the antioxidant capacity during ensiling fermentation.

**Correlation between total flavonoids content and antioxidant activity**

To assess the relationship between flavonoids and antioxidant activity, we calculated the Pearson correlation coefficient between total flavonoids content and its antioxidant activity in all treatment groups (Table 3). The results revealed a significant positive linear correlation between the total flavonoids content and antioxidant activity, as indicated by the DPPH ($R = 0.62, P < 0.05$) and FRAP ($R = 0.70, P < 0.01$) values.

**Overview of flavonoids metabolite**

Figure S1 presented the total ion chromatogram (TIC) and extracted ion chromatogram (XIC). The horizontal axis represents the retention time (min) of the detection, and the vertical axis represents the ion flow intensity (cps) of the detection. Using a local metabolite database, we conducted quantitative and qualitative analysis of flavonoids metabolites using mass spectrometry. Fermentation resulted in an increase in the total flavonoids content in alfalfa and altered the composition of flavonoids. We identified a total of 90 flavonoids from the raw materials, while the control and PP group identified 89 flavonoids. The LPe group identified 88 flavonoids, and the LPI group identified 85 flavonoids. The compound with the highest content in the raw material is scutellarin, and post-fermentation, apigenin becomes the most abundant compound. The specific substances contents are shown in Table S1. 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.

Generally, 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 in other types of flavonoids during fermentation were more intricate, with some substances exhibiting higher content compared to the raw materials and vice versa. To investigate the impact of various lactic acid bacteria on flavonoids metabolism during fermentation, we employed the OPLS-DA model. The criteria used for screening and comparing different metabolites between the two groups were VIP > 1 and $|\log_2 FC| \geq 1$, as depicted in Fig. 1d. As presented in Fig. 2, we observed 7 significantly different metabolites
between the LPe group and the control group, 7 significantly different metabolites between the PP group and the control group, and 8 significantly different metabolites between the LPI group and the control group. 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.

### Analyzing the difference in flavonoids and its relationship with antioxidant ability

Metabolites derived from various comparison combinations were aggregated to yield a total of 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 metabolic mechanism of flavonoids. The result showed that differential metabolites were mainly enriched in the secondary metabolites pathway including flavone and flavonol biosynthesis, isoflavonoid biosynthesis, flavonoid biosynthesis and biosynthesis. 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 (Fig. S4). Further investigation into flavonoids-related pathways revealed the detection of a total 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.

### 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[23–25]. 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[26]. On the contrary, after fermenting moringa, polyphenols were significantly reduced while accumulation of free amino acids
and small peptides led to an increase in its antioxidant activity\[27\]. In this study, the additions of PP and LPI resulted in a significantly higher total flavonoids content compared to other groups, suggesting that PP and LPI demonstrated superior conversion/release efficiency for flavonoids during fermentation. The similar results were also found during the mulberry leaf fermentation\[28\]. Overall, the antioxidant activity increased after fermentation, in accordance with previous research on the fermentation of fruit and vegetable juices\[29, 30\] and tea\[31, 32\]. This improvement in antioxidant activity is believed to be attributed to the various enzymes produced by lactic acid bacteria during the fermentation process.

Furthermore, the antioxidant capacity of the LPI group was significantly higher than that of other groups in this study. 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\[33\]. 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. Specific strains of lactic acid bacteria demonstrate heightened enzymatic production, releasing a greater quantity of flavonoids compounds and exhibiting a more efficient biotransformation capability.

Pearson correlation coefficient suggested that flavonoids content has close relationship with the antioxidant activity. Similar results were also observed in other studies, such as the yellowing process of rice\[34\] and the fermentation of seaweed by lactic acid bacteria\[35\]. For instance, the fermentation of Sargassum by lactic acid bacteria led to an increase in total flavonoids content and DPPH free radical scavenging activity\[35\]. To identify the specific compounds that play a key role in this process, further investigation using targeted metabolome analysis was performed to determine the type and content changes of flavonoids during fermentation.

Compared to natural fermentation, the addition of lactic acid bacteria resulted in distinctive biotransformation of flavonoids. The common substance in the lactic acid bacteria treatment group is 3,7,4'- trihydroxyflavonoid, also known as resokaempferol, is a flavonoid compound with hydroxyl substitutions at positions 3, 7, and 4'. This compound has been demonstrated to possess the ability to scavenge DPPH free radicals and exhibit antibacterial activity\[36, 37\]. Additionally, it manifests excellent anti-inflammatory effects in vivo, contributing significantly to animal health\[38\]. Notably, the LPI group with \textit{Lactiplantibacillus plantarum} exhibited distinct differences from control in terms of substance composition. This indicates that \textit{Lactiplantibacillus plantarum} possessed a distinctive capability for flavonoids biotransformation during fermentation. For instance, adding LPI resulted in complete reduction of narcissin content. It is speculated that \textit{Lactiplantibacillus plantarum} treatment induced high production of α-L-rhamnosidase, leading to glucoside bond cleavage within narcissin. Mueller et al. \[39\] assessed the hydrolytic capacity of 14 \textit{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 \textit{Lactiplantibacillus plantarum} exhibited the most complete hydrolysis of narcissin. The characteristic flavonoid in the LPe group was identified as morusin, a kind of pyran-containing isopentenylation flavonoids. According to Pearson correlation coefficient, it was found that pedalitin,
engeletin, 7-Methoxyisoflavone, 3,4'-Dihydroxyflavone, puerarin, 2'-Hydroxydaidzein were significantly negatively correlated with morusin (Fig. S2). 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. [40] highlighted that isopentenylation enhanced the antibacterial efficacy of flavonoids. Hoi et al. [41] also confirmed the inhibitory effects of morusin extracted from Artocarpus nigrifolius on Bacillus subtilis and Staphylococcus aureus. The presence of this unique flavonoids may explain the near absence of Bacillus in the LPe group (Fig. S3). Acacetin, extracted from Glycyrrhiza glabra, has demonstrated potent DPPH radical scavenging activity [42]. In vivo experiments on mice [43], zebrafish [44] and other animals [45], acacetin has been confirmed to exert antioxidant capabilities. Various lactic acid bacteria strains demonstrated different abilities in biotransforming flavonoids [46].

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. [47] 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 [48].

Conclusions

In conclusion, our results indicated that inoculation with lactic acid bacteria has the ability to transform flavonoids during anaerobic fermentation of alfalfa. Different types of lactic acid bacteria have different abilities to transform flavonoids, and inoculation with Lactiplantibacillus plantarum has a particularly significant impact on the composition and content of flavonoids in alfalfa silage. There is a significant positive correlation between the content of acacetin, 3,7,4'-trioxyflavone, taxifolin 7-O-rhamnoside and antioxidant capacity during the fermentation process of alfalfa. In summary, adding lactic acid bacteria during feed fermentation can alter the composition and content of flavonoids, affect their antioxidant capacity, and provide a promising way to improve feed function.

Declarations

Acknowledgements
Author contributions

KN and YG designed the experiment and wrote the manuscript. YG and HZ performed the experiment. YW and FY helped in data collection. KN supervised the study and provide funding. All authors contributed to the article and approved the submitted version.

Funding

We would like to thank the National Key Research and Development Program of China (Grant No.2021YFD1300300) for financial support.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

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 College of Animal Science, Guizhou University, Guiyang 550025, China

References


15. Ankolekar C, Johnson K, Pinto M, Johnson D, Labbe R, Greene D, Shetty K. Fermentation of whole apple juice using lactobacillus acidophilus for potential dietary management of hyperglycemia,


Tables
### Table 1. Total Flavonoids Content

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total flavonoids (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>6.99 ± 0.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>7.14 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPe</td>
<td>7.07 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PP</td>
<td>8.30 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPl</td>
<td>8.42 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

LPe: *Lactiplantibacillus pentosus*; PP: *Pediococcus pentosaceus*; LPl: *Lactiplantibacillus plantarum*. Average of three replicates (average ± standard deviation). Values within a column followed by different lowercase letters are significantly different (*p* < 0.05).

### Table 2. Antioxidant Activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DPPH (μmol Trolox/g FW)</th>
<th>FRAP (μmol Trolox/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>3.18 ± 0.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.84 ± 0.43&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>3.51 ± 0.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.82 ± 0.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPe</td>
<td>3.75 ± 0.05&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.59 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PP</td>
<td>3.92 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.60 ± 1.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPl</td>
<td>4.45 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.94 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>LPe: *Lactiplantibacillus pentosus*; PP: *Pediococcus pentosaceus*; LPl: *Lactiplantibacillus plantarum*; FW: Fresh weight. Average of three replicates (average ± standard deviation). Values within a column followed by different lowercase letters are significantly different (*p* < 0.05).

### Table 3. Pearson Correlation Coefficients Between Total Flavonoids Content and Antioxidant Activity

<table>
<thead>
<tr>
<th></th>
<th>Total flavonoids</th>
<th>DPPH</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total flavonoids</td>
<td>1</td>
<td>0.62*</td>
<td>0.70**</td>
</tr>
<tr>
<td>DPPH</td>
<td>0.62*</td>
<td>1</td>
<td>0.65**</td>
</tr>
<tr>
<td>FRAP</td>
<td>0.70**</td>
<td>0.65**</td>
<td>1</td>
</tr>
</tbody>
</table>
At the 0.01 level (double tailed), the correlation is significant.

Figures

Figure 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; (c) Venn diagram of differential flavonoids metabolites; (d) Differential metabolite analysis for LPe vs Control, PP vs Control, LPI vs Control, PP vs LPe, LPI vs LPe, and LPI 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 Figureure.
Figure 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) LPl vs Control, (d) PP vs LPe, (e) LPl vs LPe, (f) LPI vs PP.

Figure 3

The correlation between differential metabolites and antioxidant activity.
Figure 4

The metabolic potentials of flavonoids.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary1.docx