Effect of Condensed Tannins on Nitrogen Distribution and Metabolome after Aerobic Exposure of Sainfoin Silage

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Abstract: (1) Background: Previous studies have indicated that proteolysis is inhibited by the condensed tannins (CTs) that are present during sainfoin ensiling. Whether inhibiting this effect of CTs on proteolysis is functional during aerobic exposure is still unclear. (2) Methods: the present study investigated the effect of CTs on metabolite composition during the aerobic exposure of sainfoin silage via the use of polyethylene glycol (PEG), leading to the inactivation of CTs. (3) Results: The neutral detergent-insoluble protein (NDIP) and acid detergent-insoluble protein concentrations were both more concentrated in the control group than in the PEG-treated group. There were 587 and 651 different metabolites present in the control and PEG-treated groups after 3 and 7 days, respectively, of aerobic exposure of silage. Flavonoids (72 metabolites) were the most abundant among these different metabolites. The addition of PEG upregulated histidine, threonine, asparagine, tryptophan, and glutamine, but downregulated phenylalanine. The relative abundances of Lactococcus, Fructobacillus, Enterobacter, Cutibacterium, Citrobacter, and Rosenbergiella differed significantly between the control and PEG-treated groups ($p < 0.05$); all of these bacteria showed significant correlation with some of the 50 most abundant metabolites. (4) Conclusions: the results suggest that the antioxidant status of the silage increased and inhibited the activity of a variety of bacteria that coexist with CTs, and decreased the production of certain amino acids after the aerobic exposure of silage.

Keywords: sainfoin; aerobic exposure; proteolysis; condensed tannins

1. Introduction

Proteolysis can occur in all forages during ensiling, and it is attributed to the enzyme activity of microbes or of the plant itself [1]. Among different forages, legumes cause extensive proteolysis during ensiling, especially for alfalfa, and 44–87% of the total nitrogen is non-protein nitrogen (NPN) [2]. NPN from silage is less used for rumen microbial protein synthesis compared with NPN from fresh forage [3]; thus, protecting protein during ensiling is essential for maintaining the nutritiousness of silage. Condensed tannins (CTs) are plant secondary metabolites with the ability to bind proteins; they are stabilized during ensiling, resulting in the inhibition of protein degradation [4]. The ability of tannins to bind with proteins is attributed to hydrogen bonds formed between protein residues and a hydroxyl group [5]. CTs, including sainfoin, are widely distributed in a variety of plants. CTs from sainfoin contain both procyanidin (catechin/epicatechin) and prodelphinidin (gallocatechin/epigallocatechin) units, all of which have two or three hydroxyl groups located at the B-ring, indicating the potential for more effective binding with protein [6]. CTs from sainfoin could serve as a more effective protein protector used for animals due to their high capacity to bind protein, and CTs have shown low inhibition of cellulose digestion in the rumen [7]. The strong tannin–protein binding effect has been observed as a result of the level of buffer-soluble N (BSN) being decreased due to the CTs present in sainfoin silage [8]. Wang et al. reported that the NPN content decreased when alfalfa...
and sainfoin were mixed during ensiling [9]. The mechanisms of this effect of sainfoin CTs on proteolysis inhibition during ensiling are partly related to the CT combination with protein, and could inhibit not only microbial and enzyme activity but also partly the formation of stabilized compounds between CTs and proteins. Peng et al. (2018) observed that CTs from purple prairie clover (PPC; Dalea purpurea Vent.) could decrease the NPN content during ensiling and upon the aerobic exposure of silage. Furthermore, CTs from PPC could prolong the aerobic stability of silage [10]. However, these effects of CTs on silage were not present when CTs from the quebracho plant were used [11]. These different results are probably due to the composition of the CTs of different plants, which exhibit different respective biological activities [5]. A previous study showed that CTs from sainfoin showed a strong ability to reduce protein degradation because of their great ability to inhibit protease activity and partly inhibit Pediococcus activity, resulting in an indirect decrease in protein degradation being induced by Enterobacter [12]. Many studies have focused on sainfoin proteolysis only during ensiling [4,9,13,14]. However, whether this effect of sainfoin CTs on protein is still functional upon aerobic exposure is unclear. Notably, profiling the silage metabolome could enhance our understanding of the biological processes underlying silage fermentation [15]. Therefore, investigating the metabolites could lead to a better understanding of the mechanism of proteolysis during the aerobic exposure of silage. This study investigated the effect of CTs on metabolite composition during the aerobic exposure of sainfoin silage, and aimed to explain how CTs influence nitrogen distribution during the aerobic exposure of silage.

2. Materials and Methods

2.1. Silage Preparation

Sainfoin (Onobrychis viciifolia soosp. cv. Qi-Tai, BY2020-003, extensive grassland farming, Xinjiang, China) plants were grown in the experimental field at the Shihezi University on 1 May 2022 (44.21 N; 85.57 E, Xinjiang, China). Whole-plant sainfoin was harvested in July 2022 at the early-flowering stage. Wilting was conducted for dry-matter content; it yielded approximately 250 g/kg of fresh weight, which was then chopped into 1–2 cm pieces. We used polyethylene glycol (PEG) (Sigma-Aldrich, Shanghai, China; molecular weight, 6000) to inactivate CT activity. The sample was then sprayed with a solution of 640 g/L PEG at the rate of 217 mL/kg DM to achieve a CTs:PEG ratio of 1:2 [10]. The PEG (molecular weight 6000) had the most efficient capacity to bind with CTs through hydrogen bonds [16]. After they were treated, 1000 g samples were packed into polyethylene plastic bags (30 cm × 50 cm), then compacted and sealed using a vacuum sealer. Five replicates were prepared for control and PEG treatment. All bags were stored indoors at 20 °C.

2.2. Nitrogen Content Analysis of Sainfoin Silage

A total of 18 samples were collected from each treatment for two treated (control and PEG-treated) and three ensiling times (60 d of ensiling, 3 and 7 days of aerobic exposure) × three replicates. Silage samples were dried at 65 °C for 48 h and ground using a 1.0 mm grinder in preparation for further analysis. The NPN, neutral detergent-insoluble protein (NDIP), and acid detergent-insoluble protein (ADIP) concentrations were analyzed according to the methods described above [17]. Soluble protein (SOLP) concentrations were analyzed according to the methods described above [18]. Briefly, 0.5 g samples from each treatment were added into a 250 mL conical flask, as well as 50 mL of still water. The samples were left to stand for 30 min at room temperature. Then, 10 mL of 10% trichloroacetic acid (TCA) was added and left to stand for 30 min. We filtered the solution with Whatman #54 paper and collected the residues after the samples were washed twice with a TCA solution. The NPN was calculated through determining the nitrogen content of the residues.
2.3. Metabolite Analysis

A total of 24 samples were collected from each treatment for two treated (control and PEG-treated) \( \times \) two ensiling times (3 and 7 days of aerobic exposure) \( \times \) six replicates using a methanol: water (4:1, \( v/v \)) solution to extract the metabolites from each sample. We followed the methods described in [12] to further analyze metabolites through ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). All the metabolite variables were scaled to unit variances before conducting the PCA. Orthogonal partial least squares discriminate analysis (OPLS-DA) was used for statistical analysis to determine global metabolic changes between comparable groups. All the metabolite variables were subjected to Pareto Scaling before the OPLS-DA. The model validity was evaluated based on model parameters R2 and Q2, which provide information on interpretability and predictability, respectively, of the model, and minimize the overfitting risk. Variable importance in projection (VIP) was calculated using the OPLS-DA model. The \( p \)-values were estimated using paired Student’s \( t \)-test on single-dimensional statistical analysis. Statistical significance among groups was identified with variable importance in projection (VIP) values > 1 and \( p < 0.05 \). The Majorbio Cloud Platform (Majorbio Bio-pharm Technology Corporation, Shanghai, China) was used for further analyses.

2.4. Statistical Analysis

The bacterial community analysis was conducted by the use of the 16S rRNA gene sequencing technology. Primers targeting were V3-V4 (338F: ACTCCTACGGGAGGCAGCAG; 806R: GGACTACHVGGGTWTCTAAT). Methods of DNA extraction and sequence analysis followed our previous study [19]. The sequences of bacteria upon aerobic exposure were deposited in the National Center for Biotechnology (NCBI), Sequence Read Archive (SRA) under accession number “PRJNA948115”. It should be noted that the data of cured protein and ammonia nitrogen were already used in our former study (in the process of publishing), which are same as those of this present study. The characteristics of sainfoin silage were subjected to a two-way analysis of variance, 2 (treatment) \( \times \) 3 (ensiling days) factorial Complete Randomized Design (Treatment: PEG and control groups, ensiling days: 60 fermentation days, 3 and 7 aerobic exposure days). Data were analyzed using IBM SPSS 22 Statistics (IBM Corp., Armonk, NY, USA). Significant differences between treatments were determined using Tukey’s test at \( p < 0.05 \).

3. Results

3.1. Nitrogen Distribution in Sainfoin Silage upon Aerobic Exposure

As shown in Table 1, the SOLP was higher in PEG-treated samples compared with control during the ensiling and aerobic exposure of silage (\( p < 0.05 \)). The content of SOLP was not different in different times in the control group (\( p > 0.05 \)), same as in the case of the PEG-treated group. The content of NDIP was higher in the control than in the PEG-treated group (\( p < 0.05 \)). The content of NDIP experienced a significant increase in the control group with aerobic exposure time prolonged (\( p < 0.05 \)), but stabilized in the PEG-treated group (\( p > 0.05 \)). The content of ADIP increased in both control and PEG-treated groups after 3 d of aerobic exposure (\( p < 0.05 \)); the control value was higher than the PEG-treated one (28.59 g/kg DM vs. 15.76 g/kg DM, \( p < 0.05 \)). The content of NPN decreased in the control group with aerobic exposure time prolonged (\( p < 0.05 \)), but increased in the PEG-treated group after 7 d of aerobic exposure (\( p < 0.05 \)). The content of NPN was lower in the control compared with the PEG-treated group during ensiling and 7 d of aerobic exposure (\( p < 0.05 \)). The content of AA decreased in the control group control after 7 d of aerobic exposure (\( p < 0.05 \), and decreased in the PEG-treated group with aerobic exposure time prolonged (\( p < 0.05 \). The content of AA was higher in PEG-treated samples than in control during ensiling and 7 d of aerobic exposure (\( p < 0.05 \)).
Table 1. Nitrogen distribution in sainfoin silage upon aerobic exposure.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>Day of Ensiling</th>
<th>Day of Aerobic Exposure</th>
<th>SEM</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>CK</td>
<td>202.93 Aa</td>
<td>193.77 Ab</td>
<td>196.02 Ab</td>
<td>1.407</td>
</tr>
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<td></td>
<td>PEG</td>
<td>202.11 Aa</td>
<td>182.92 Bb</td>
<td>183.72 Bb</td>
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</tr>
<tr>
<td>SOLP (g/kg DM)</td>
<td>CK</td>
<td>5.00 Ba</td>
<td>5.42 Ba</td>
<td>5.57 Ba</td>
<td>3.944</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>7.77 Aa</td>
<td>8.04 Aa</td>
<td>8.31 Aa</td>
<td>2.030</td>
</tr>
<tr>
<td>NDIP (g/kg DM)</td>
<td>CK</td>
<td>56.54 Ac</td>
<td>64.04 Ab</td>
<td>83.19 Aa</td>
<td>1.049</td>
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<tr>
<td></td>
<td>PEG</td>
<td>25.36 Ba</td>
<td>23.38 Ba</td>
<td>23.68 Ba</td>
<td>1.348</td>
</tr>
<tr>
<td>ADIP (g/kg DM)</td>
<td>CK</td>
<td>22.57 Ab</td>
<td>28.59 Aa</td>
<td>27.99 Aa</td>
<td>1.108</td>
</tr>
<tr>
<td></td>
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<td>12.75 Bb</td>
<td>15.76 Ba</td>
<td>16.55 Ba</td>
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<td>CK</td>
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<td>39.67 Bb</td>
<td>31.67 Bc</td>
<td>1.108</td>
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<tr>
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<td>65.50 Aa</td>
<td>62.57 Aa</td>
<td>60.45 Ab</td>
<td>1.348</td>
</tr>
<tr>
<td>AA-N (%TN)</td>
<td>CK</td>
<td>13.39 Ba</td>
<td>16.11 Aa</td>
<td>8.19 Bb</td>
<td>2.030</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>27.58 Aa</td>
<td>23.07 Ab</td>
<td>18.75 Ac</td>
<td>1.348</td>
</tr>
<tr>
<td>AN (%TN)</td>
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<td>2.06 Bb</td>
<td>2.30 Ba</td>
<td>1.02 Ac</td>
<td>0.134</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>2.38 Ab</td>
<td>3.22 Aa</td>
<td>1.00 Ac</td>
<td>0.134</td>
</tr>
</tbody>
</table>

1 n = 3. CK: control. PEG: polyethylene glycol; CP: crude protein (already published); SOLP: soluble protein; NDIP: neutral detergent-insoluble protein; ADIP: acid detergent-insoluble protein; NPN: non-protein nitrogen; AN: ammonia nitrogen (already published); AA-N: amino acid nitrogen. A, B: Means in the same column followed by different uppercase letters differ (p < 0.05); a–c: Means in the same row followed by different lowercase letters differ (p < 0.05).

3.2. Bacterial Community Structure of Sainfoin Silage upon Aerobic Exposure

Based on the comparison of microbial variations by the use of the LefSe analysis in Figure 1, on a genus level, *Enterobacter*, *Aerococcus*, *Massilia* and *Citrobacter* were the bacteria with the most significant difference in the control and *Lactococcus* and *Fructobacillus* were the bacteria with the most significant difference in the PEG-treated group after 3 d of aerobic exposure (p < 0.05). *Rhodococcus*, *Phyllobacterium*, *Cutibacterium* and *Rosenbergiella* were the most significant difference bacteria in the control group after 7 d of aerobic exposure (p < 0.05).

3.3. Metabolomic Profiles

In total, there were 587 and 651 different metabolites between control and PEG-treated groups after 3 d and 7 d of aerobic exposure of silage, respectively. As shown in Figure 2, according to the KEGG data, 105 metabolites were compounds with biological roles, 345 metabolites were lipids and 269 metabolites were phytochemical compounds. Among these compounds with biological roles, amino acids were the most abundant (19 metabolites), followed by carboxylic acids (11 metabolites), fatty acids (11 metabolites) and eicosanoids (9 metabolites). Among these phytochemical compounds, flavonoids were dominant (59 metabolites), followed by monolignols (29 metabolites), monoterpenoids (21 metabolites) and diterpenoids (15 metabolites). As shown in Figure 3a, there was a clear difference between control and PEG-treated groups in 3 d and 7 d of aerobic exposure, respectively. As shown in Figure 3b, the R² and Q² were both close to one, indicating that the model could represent the real situation of sample data, and the addition of a new sample to the model should provide the same results.

The heatmap of variable importance in the project (VIP) describing the differences between the treated groups of these metabolites is shown in Figure 4a,b. Among these 30 most VIP metabolites, 11 metabolites were down-regulated, but 19 metabolites were up-regulated when PEG was added after 3 d of aerobic exposure. A total of 14 metabolites were down-regulated, but 16 metabolites were up-regulated when PEG was added after 7 d of aerobic exposure. In total, among these 60 most VIP metabolites, there were 44 different VIP metabolites during 7 d of aerobic exposure.
3.3. Metabolomic Profiles

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Figure 1. Comparison of microbial variations using LefSe analysis of sainfoin silage upon aerobic exposure. CK3: control after 3 days of aerobic exposure, PEG3: polyethylene-2glycol (PEG)-treated group after 3 d of aerobic exposure, same as others.

Figure 2. Different metabolites annotated by Kyoto Encyclopedia of Genes and Genomes after aerobic exposure of sainfoin silage; (a) compounds with biological roles, (b) lipid, (c) phytochemical compounds.
Figure 2. Different metabolites annotated by Kyoto Encyclopedia of Genes and Genomes after aerobic exposure of sainfoin silage; (a) compounds with biological roles, (b) lipid, (c) phytochemical compounds.

Figure 3. Principal component analysis (PCA) and orthogonal projections to latent structure-discriminant analysis (OPLS-DA) of sainfoin silage. (a) Score scatter plot of the PCA model for the control versus PEG-treated groups. CK3: control group after 3 d of aerobic exposure, PEG 3: polyethylene-glycol (PEG)-treated group after 3 d of aerobic exposure, same as others. (b) Permutation test of the OPLS-DA model for the control versus PEG-treated groups.

Figure 4. Variable importance in project (VIP) heatmap for the differentially accumulated metabolites in aerobic exposure of sainfoin silage. (a) Different metabolites between two groups after 3 d of aerobic exposure, (b) different metabolites between two groups after 7 d of aerobic exposure. ***: \( p < 0.001 \).
As shown in Figure 5a, the most activated pathway of metabolites was biosynthesis of phenylpropanoids (14 metabolites), followed by biosynthesis of plant secondary metabolites (11 metabolites), flavone and flavonol biosynthesis (9 metabolites) and flavonoid biosynthesis (9 metabolites) after 3 d of aerobic exposure. The same results were observed, except in the case of the alpha-libolenic acid metabolism, which became the third activated pathway among these pathways after 7 d of aerobic exposure.

Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially accumulated metabolites in aerobic exposure of sainfoin silage. (a) Different metabolites between two groups after 3 d of aerobic exposure, (b) different metabolites between two groups after 7 d of aerobic exposure.

3.4. Correlation between Bacteria Relative Abundance and Metabolites

As shown in Figure 6, Lactococcus showed the highest correlation with these 50 most abundant metabolites during aerobic exposure of the silage. Among these 50 metabolites, 28 metabolites such as asitrilobin D, lavoltidine, asparagine, leucylproline, etc., were significant positively associated with Lactococcus, and 21 metabolites such as 2′-hydroxygenistein, stearidonic acid, neocnidolide, betaxolol, tricetin, etc., were significant negatively associated with it (p < 0.05). The same results followed with Fructobacillus, except N-palmitoyl valine and 2′-hydroxygenistein displayed no correlation with these bacteria (p > 0.05). The correlation of Rosenbergiella and metabolites showed results contrasting with those of Lactococcus. A total of 28 metabolites (the same number as that of metabolites positively correlated with Lactococcus) were significantly negatively correlated with Rosenbergiella and 21 metabolites (the same number as that of metabolites negatively correlated with Lactococcus) were significantly positively correlated with Rosenbergiella (p < 0.05). The same results followed with Citrobacter, except 2′-hydroxygenistein, N-palmitoyl valine, 13(S)-HpOTrE and 9(S)-HpOTrE displayed no correlation with these bacteria (p > 0.05).
Figure 6. Heatmap of correlation between bacteria (top 20 of relative abundant) and metabolites (top 50 of relative abundant) after 7 d of aerobic exposure of sainfoin silage. "*": 0.01 < \( p \) < 0.05, "**": 0.001 < \( p \) < 0.1, ***": \( p \) < 0.001.

4. Discussion

4.1. Nitrogen Distribution after Aerobic Exposure of Sainfoin Silage

The content of NDIP increased in the control group with aerobic exposure time prolonged (increased by 47.13% and after 7 d of aerobic exposure), but it stabilized in the PEG-treated group of silage. Consequently, the content of NPN decreased in the control group with aerobic exposure time prolonged (decreased by 25.90% after 7 d of aerobic exposure), but it stabilized in the PEG-treated group after 3 d of aerobic exposure. The content of SOLP was stabilized in both control and PEG-treated groups. Overall, the contents of NDIP and ADIP in the control group were 251.30% and 69.12% higher than those in the PEG-treated groups, respectively, but NPN in the control group was 47.60% lower than in the PEG-treated group. CT from PPC showed that NDIP was not affected by CT, but ADIP levels were increased due to the presence of CT after 7 d of aerobic exposure [10]. Considering of the different biological activity of CT in a variety of plants [5], the results suggest that CT from sainfoin could still inhibit protein degradation via an increase in the content of NDIP but a decrease in NPN upon aerobic exposure of silage.

4.2. Metabolite Analysis after Aerobic Exposure of Sainfoin Silage

In total, there were 587 and 651 different metabolites between control and PEG-treated groups after 3 d and 7 d of aerobic exposure of silage, respectively. According to the KEGG database, 105 metabolites were attributed to compounds with biological roles category, 269 metabolites were attributed to phytochemical compounds category, 345 metabolites were attributed to the lipids category. Flavonoids (72 metabolites) were most abundant metabolites among these categories. The present study showed that the metabolite composition between control and PEG-treated groups is clearly different. Among these top 30 VIP metabolites, the 11 metabolites of apiin, kaempferol-3-O-rutinoside, 4-p-coumaroylquinic acid, 4-hydroxycinnamoylagmatine, omadacycline, leucoside, isoscoparin 2’-(6-(E)-ferulylglucoside), 2-Isopropyl-5-methylphenol acetate, 3-tert-butylphenol, 8-Methoxykynurenate and birabresib were down-regulated in the PEG-treated group, contrary to those in the control group, after 3 d of aerobic exposure. Apiin, isoscoparin
2”-(6-(E)-ferulylglucosidde) and birabresib were still down-regulated in PEG with aerobic exposure time prolonged to 7 days. Apiin is a kind of a major flavonoid glycoside isolated from parsley, which showed a high capacity for antioxidant activity [20]. The results probably suggest that the antioxidant activity of silage upon aerobic exposure enhance when existing with CT. Kaempferol-3-O-rutinoside, also called nicotiflorin, showed good capacity for anti-inflammatory and myocardial protection [21]. Nicotiflorin was scarce to inhibit bacteria, but it showed a strong ability to inhibit α-glucosidase activity [22]. Some strains of Lactobacillus had α-glucosidase activity, which is involved in the glucoside metabolic process [23]. The present results probably suggest that CT inhibited glucose metabolism on some level after aerobic exposure. Omadacycline is a potent aminomethylcycline; it can decrease the populations of Lactobacillus and Enterococcus in broth models [24]. Thus, some LAB could be inhibited when existing with CT during aerobic exposure of sainfoin silage. Among these 30 top VIP metabolites, 19 metabolites such as digitoxigenin bisdigitoxide, triterpenoids, dihydrodigitoxin, phosphatidylethanolamine (PE, 22:6), 4-hydroxy-3-nitrophenylacetate, sphingomyelin (SM, d18:2), ophiopogonin C’, temporins, 7-acetamidonitrazepam, 4-(8-Methyl-9H-1,3-dioxolo(4,5-h)(2,3)benzodiazepin-5-yl)benzenam, didemmin A, phosphatidic acid(IPA,22:6), cardiolipin(CL,18:1), butyl 2-decenoate, 6-[4-(4-carboxy-2-hydroxybutyl)-2-hydroxyphenoxyl]-3,4,5-trihydroxyoxane-2-carboxylic acid, Leucyl-Threonine, etc., were up-regulated in the PEG-treated group, contrary to those in the control group, after 3 d of aerobic exposure. Digitoxigenin bisdigitoxide, dihydrodigitoxin, ophiopogonin C’, 7-acetamidonitrazepam, PE, PA, CL, 4-(8-Methyl-9H-1,3-dioxolo(4,5-h)(2,3)benzodiazepin-5-yl)benzenamine and didemmin A were still up-regulated in PEG groups after 7 d of aerobic exposure. The PE and CL were the most important elements for amphiphilic lipids of bacteria cell membranes, such as CL for Lactobacillus spp., PE and CL for Bacillus subtilis and Escherichia coli, PE for Clostridium perfringens, etc. [25]. A previous study observed that CT could inhibit phospholipid activity during ensiling [12]. The present results probably suggest that the effects of CT from sainfoin on the inhibited phospholipid synthesis of the cell membrane were still functional during aerobic exposure of silage. Temporins are a kind of peptides with antibacterial and antifungal properties which were initially isolated from frogs; they usually show the most capacity against Gram-positive bacteria such as Enterococcus faecium than Gram-negative bacteria and fungi [26]. Didemmins A was initially discovered from marine depsipeptides; it shows antiviral and immunosuppressive activity on some level [27]. The results of the present study probably suggest that there were still alternative compounds of anti-microbial activity when CT was inactivated during aerobic exposure of sainfoin silage. The mechanism of temporins and didemmin A production in the silage system needs further study.

A total of 23 metabolites belong to peptides category, as shown in Supplementary Table S1. Six amino acids among these peptides displayed a significant difference between the control and PEG-treated groups (VIP > 1, p < 0.05), namely phenyl-alanine, histidine, threonine, asparagine, tryptophan and glutamine. The addition of PEG down-regulated phenyl-alanine but up-regulated the other five amino acids among these six metabolites during 7 d of aerobic exposure. This was in accordance with the fact that the content of AA was lower in the control group after 7 d of aerobic exposure of silage. Usually, N-carbobenzoxy-L-phenyl-L-alanine is used as a substrate to determine the activity of carboxypeptidase (CPs) during ensiling [28]. A previous study found that CT showed strong ability to inhibit CP activity [12]. Therefore, as per present results, the inhibition of CPs from CT is probably still functional during aerobic exposure.

As shown in the results of the present study in Figure 6, the most activated pathway of metabolites was biosynthesis of phenylpropanoids (14 metabolites), biosynthesis of plant secondary metabolites (12 metabolites), flavone and flavonol biosynthesis (9–12 metabolites), and flavonoid biosynthesis (9 metabolites) during 7 d of aerobic exposure. A previous study showed that PEG addition had no effect on the flavonoid biosynthesis pathway during sainfoin ensiling [12]. This effect of PEG on flavonoid biosynthesis probably showed
up when silage was subjected to aerobic exposure. The mechanism of this effect needs further study.

4.3. Correlation Analysis between Bacteria and Metabolites after Aerobic Exposure of Sainfoin Silage

In order to further understand the relationship between microbes and metabolites, the heatmap of correlation between these two factors was established via Spearman’s correlation analysis in Figure 6. *Lactococcus, Fructobacillus, Enterobacter, Cutibacterium, Citrobacter* and *Rosenbergiella* all showed significant correlation with metabolites ($p < 0.05$). All of these bacteria displayed significant difference between control and PEG-treated groups (Figure 2). The results probably suggest that these bacteria play a certain role during aerobic exposure of sainfoin silage. *Lactococcus* had a significant correlation with these top 49 abundant metabolites such as asitrilobin D, asparagine, leucylproline, phenyl-alanine, N-lactoylleucine, etc. *Lactococcus* had a specific, significantly positive for an amino acid correlation with asparagine, leucylproline, threonine, tryptophan, histidine ($R^2 = 0.7293, 0.7329, 0.7563, 0.7681$ and $0.7529$, respectively, $p < 0.05$). Asparagine is an essential substrate for *Lactococcus* to produce nisin [29]. Dipeptides such as leucylproline are important for the regulation of the proteinase production system for *Lactococcus* [30]. 2-ketoisovalerate decarboxylase from *Lactococcus lactis* could use threonine as substrate to produce propionate [31]. In addition, *Lactococcus lactis* has the ability to uptake amino acids such as histidine, lysine, serine, phenyl-alanine, tyrosine, arginine, etc., via amino acid transporters [32]. Theoretically, the activity of *Lactococcus* in the PEG-treated group must be lower than that in the control group due to the fact that it can utilize them. However, procyanidins and catechin derivatives from fruit shows strong ability to inhibit *Lactococcus* activity [33]. Considering CT from sainfoin is mainly composed of catechin and procyanidins [5], CT from sainfoin could probably inhibit *Lactococcus* activity. In the silage system, theoretically, the results in these amino acids should be up-regulated in the control group (present study). As suggested by the present study results, asparagine and threonine were up-regulated, but phenyl-alanine was down-regulated in the PEG-treated group. Therefore, the mechanism of *Lactococcus* activity for these amino acids must be complicated during aerobic exposure of sainfoin silage. Some strains of *Enterobacter* had certain genes to encode the enzymes for the metabolism of amino acids such as glutamine, ornithine, arginine, tyrosine, histidine, etc., to produce amine [34]. In fact, in the present study, *Enterobacter* showed a negative correlation with these amino acids. *Citrobacter* led to increases in lipoxygenase activity during alfalfa ensiling, which had a strong correlation with lipid-related enzymes [35]. The present study also observed that some lipids such as corchorifatty acid A, corchorifatty acid F, 13-hydroperoxyoctadecatrienoic acid (13-HpOTrE), etc., showed a significant correlation with *Citrobacter* ($R^2 = 0.5052, 0.5338$ and $0.3925$, respectively, $p < 0.05$) during aerobic exposure of sainfoin silage. Notably, among these 50 top abundant metabolites, *Rosenbergiella* showed absolutely opposite results (correlation with metabolites) with *Lactococcus*, indicating that these two bacteria probably have some antagonistic effects between each other during aerobic exposure of silage.

5. Conclusions

During aerobic exposure of sainfoin silage, the degraded proteins were inhibited mainly through the formation of NDIP by a combination between CT and protein. The inhibition of a degraded protein partly related with CT decreased the content of some amino acids. CT could inhibit a variety of bacteria via decreased cell membrane phospholipid synthesis. The most activated pathways of metabolites were biosynthesis of phenylpropanoids, plant secondary metabolites, flavone, flavonol and flavonoid after aerobic exposure of sainfoin silage. Among these pathways of metabolites, flavonoids such as apiin and nictiflorin showed anti-oxidant and anti-inflammatory activities, respectively, indicating that CT could enhance the biological nutrition of silage.
Supplementary Materials: The following supporting information can be downloaded at: https://data.mendeley.com/datasets/fvx74zjy98/1, accessed on 19 June 2023. Supplementary Table S1 can be found in Mendele Data: Metabolites of sainfoin silage upon aerobic exposure.

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References


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