

# Effects of intrinsic tannins on metabolome during sainfoin ensiling

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**Abstract:** Condensed tannins (CT) from sainfoin have a high capacity to inhibit proteolysis. The objective of the present study was to investigate the effects of CT (following supplementation of deactivated CT with polyethylene glycol [PEG]) on metabolome during sainfoin ensiling. As results, in total, 510 metabolites were identified after 60 d of sainfoin ensiling, with 33 metabolites were annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Among those metabolites, phospholipids were the most abundant (72.7% of total 33 metabolites). In addition, 10 up-regulated and 23 down-regulated metabolites, respectively, were identified in the PEG treated group when compared with the control group, after 60 d of ensiling ( $p < 0.05$ ). *Pediococcus* (correlated with 20 metabolites,  $R^2 > 0.88$ ,  $p < 0.05$ ) and *Lactobacillus* (correlated with 16 metabolites,  $R^2 > 0.88$ ,  $p < 0.05$ ) were the bacteria most correlated with metabolites. The results suggested antagonistic effects between *Lactobacillus* and *Pediococcus* during ensiling. The proteolysis decreased partly due to CT inhibiting *Pediococcus* activity during ensiling, with *Pediococcus* being significantly and positively correlated with dopamine after 60 d of ensiling ( $R^2 = 0.8857$ ,  $p < 0.05$ ).

**Key words:** sainfoin silage; intrinsic tannins ;metabolome; *Pediococcus*; proteolysis

## Introduction

Sainfoin is widespread in North America, Europe, and the middle East, as a perennial forage and fodder legume (Carbonero *et al.*, 2011). CT from sainfoin reportedly reduces protein degradation (Aufrere *et al.*, 2014), reduces methane production (Wang *et al.*, 2021), and has antihelmintic potential against gastrointestinal nematodes (Novobilsky *et al.*, 2013), which improve animal performance and better for environment. However, as far as we know, few researchers focus on effects of CT from sainfoin on metabolome during sainfoin ensiling (Theodoridou *et al.*, 2012). Previous study found that CT inhibit *Pediococcus* activity without effects on *Enterobacter* activity, *Enterobacter* is a one bacterial that can produce ammino nitrogen (AN) during ensiling, however, CT decreased AN content during entire ensiling process (Huang *et al.*, 2022). *Enterobacter* relative abundance still was 1.04%–17.96% during sainfoin ensiling (Huang *et al.*, 2022), it is not clear whether microbe activity association with protein degradation. Profiling the silage metabolome can improve the available understanding of the biological processes underlying silage fermentation (Guan *et al.*, 2020). Therefore, the present study was conducted to investigate the effects of CT on metabolome during sainfoin ensiling.

## Methods

Sainfoin (*Onobrychis viciifolia*) was planted in Shihezi City, Xinjiang Province, China. Whole-plant sainfoin was harvested in July 2021 at the early flower stage. After wilting for 12 h to a dry matter (DM) content of approximately 240 g/kg fresh weight, the sample was chopped into 1–2 cm pieces. The sample was then sprayed with a solution of 640 g/liter PEG (Sigma, molecular weight, 6000) at the rate of 217 mL/kg DM to achieve a CT:PEG ratio of 1:2 in the samples (Peng *et al.*, 2018). According to Jones and Mangan, (1977) the polyethylene glycol (PEG) to inactivate condensed tannins biological activity due to its specifically form stable PEG-CT complexes attributed to binds with CT by H-bonds. The control samples were sprayed with an equivalent amount of distilled water. After spraying, 1000 g samples of both the treated and control chopped sainfoin were packed into a polyethylene plastic bags (30× 50 cm), then compacted and sealed using a vacuum sealer. Three replicates were prepared of the control and treated samples. The bags were stored indoors at 23°C. After 60 d of ensiling, the ensiled sainfoin samples were taken 50 mg, the metabolites extracted using a 400  $\mu$ L methanol: water (4:1, v/v) solution. The mixture was allowed to settle at -20°C and treated by High throughput tissue crusher Wonbio-96c (Shanghai wanbo biotechnology co., LTD) at 50 Hz for 6 min, then followed by vortex for 30 s and ultrasound at 40 kHz for 30 min at 5°C. The samples were placed at -20°C for 30 min to precipitate proteins. After centrifugation at 13000g at 4°C for 15min, the supernatant was collected for LC-MS/MS analysis. The metabolites analysis

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were accomplished through Majorbio Cloud Platform (Majorbio Bio-pharm Technology Corporation, Shanghai, China, <https://www.majorbio.com>).

## Results and Discussion

The total 510 metabolites were identified after 60 d of sainfoin ensiling, which 300 metabolites were difference between CK and PEG treated group. The 97 metabolites were up-regulated and 203 were down-regulated among those 300 difference metabolites through addition with PEG. Among those difference metabolites, 33 metabolites annotated to KEGG database, which 10 were up-regulated and 23 were down-regulated through addition with PEG (Figure 1). The most abundant of those difference metabolites was phospholipid (17 metabolites annotated to KEGG database), followed by eicosanoids (5 metabolites annotated to KEGG database), nucleosides and 24-carbon atoms (5 metabolites annotated to KEGG database), and others (1 metabolites annotated to KEGG database among each of them). Catechin inhibit bacteria through damage the lipid bilayer membrane (Tsuchiya, 2001). The mechanism of this inhibit attributed to hydrogen bonding of catechin between its polyhydroxyl groups and phosphocholine moiety, interaction with phospholipids (Li et al., 2018). Consider of CT contain four type of catechin (Zeller, 2019), the results suggest that CT from sainfoin have strong effects on bacteria phospholipids synthesis. The PCA analysis indicate that there were clearly distinct of metabolites between control and PEG treated groups (Figure 2). Previous study showed that control and PEG treated groups had the same microbial community at the genus level (Huang et al., 2022). Thus, the mainly reason for those variation could account for CT, which had strong effect on microorganism metabolize during ensiling.

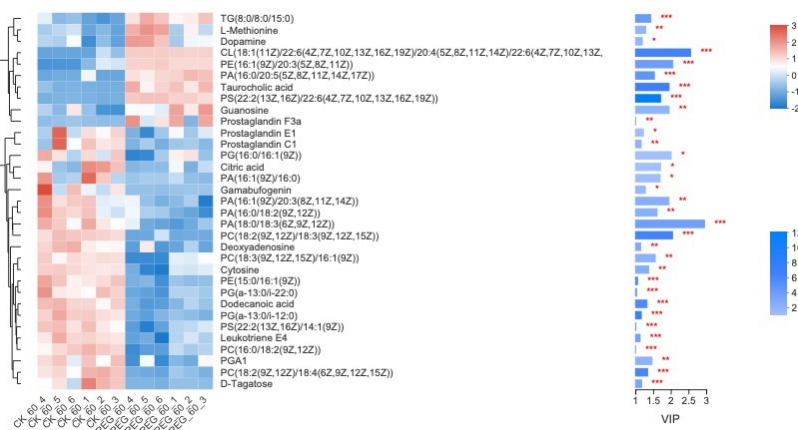


Figure 1. Heatmap of the differentially accumulated metabolites in sainfoin silage (CK 60, control group after 60 days of ensiling; PEG 60, PEG treated group after 60 days of ensiling). \* $p < 0.05$ , \*\* $0.01 < p < 0.05$ , \*\*\* $p < 0.01$ .

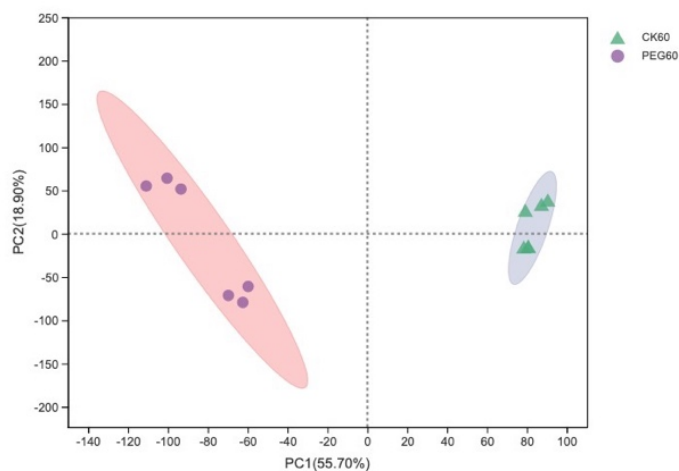


Figure 2. The principal component analysis (PCA) score plot of the metabolites in sainfoin silage (CK 60, control group after 60 days of ensiling; PEG 60, PEG-treated group after 60 days of ensiling).

One amino acid (L-methionine) was up-regulated through addition with PEG. The citric acid was down-regulated through addition with PEG. Two nucleosides include cytosine and deoxyadenosine were down-

regulated and one guanosine of nucleosides was up-regulated through addition with PEG. The mechanism of catechin inhibit bacteria is cause DNA degradation, and catechin was more toxic towards gram positive than gram negative (Fathima and Rao, 2016). Enterobacter as gram negative bacteria was not affected by CT during sainfoin ensiling, but *Pediococcus* as gram positive bacteria was highly inhibit by CT during sainfoin ensiling (Huang et al., 2022). The results suggested that CT affected gram positive bacteria through inhibit phospholipids and genes synthesis during ensiling.

In present study (Figure 3), addition with PEG up-regulated 10 metabolites and down-regulated 23 metabolites. The *Lactobacillus* had significant highly negative correlated with six metabolites among those up-regulated metabolites, and had significant positive correlated with ten metabolites among those down-regulated metabolites. On the contrary, The *Pediococcus* had significant highly positive correlated with five metabolites among those up-regulated metabolites, and had significant highly negative correlated with fifteen metabolites among those down-regulated metabolites. Additionally, the phospholipid were most affected metabolites both correlated with *Lactobacillus* and *Pediococcus*. The results suggested that there might had antagonistic effects between *Lactobacillus* and *Pediococcus* during ensiling. Among those up-regulated through addition with PEG, dopamine converted by tyrosine. The *Pediococcus* had significant highly positive correlated with dopamine but *Lactobacillus* had significant highly negative correlated with dopamine. The cell-free supernatants (CFSs) of some *Pediococcus* (*P. acidophilus*) have strong ability to stimulation effect on dopamine produced through *Enterobacter* (*E. coli*) than *Lactobacillus* (*Lac. lactis* subs. *lactis*) (Ozogul et al., 2017). Present results showed that addition with PEG up-regulated dopamine after 60 d of sainfoin ensiling, which correlated with CT strongly inhibit *Pediococcus* activity during sainfoin ensiling.

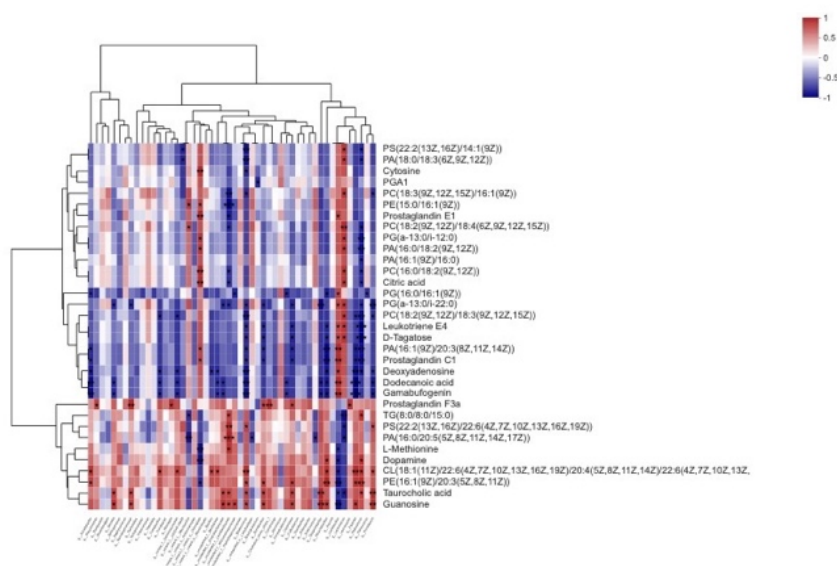


Figure 3. Heatmap of the correlation analysis for bacteria and metabolites in sainfoin silage.  $*p < 0.05$ ,  $**0.01 < p < 0.05$ ,  $***p < 0.01$ .

## Conclusions

The proteolysis decreased partly attributed to CT inhibit *Pediococcus* activity results from highly positive correlated between *Pediococcus* and dopamine.

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