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DYNAMIC EFFECTS OF ANAEROBIC FERMENTATION ON PROTEIN, FLAVONOIDS, NITRATE, AND NITRITE CONTENT AND BIOACTIVITIES OF MATURE LEAVES OF *TOONA SINENSIS*

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ABSTRACT

Toona sinensis (TS) is a widely cultivated and economically valued tree in Asia. However, millions of tons of its nutritious mature leaves go unutilized due to excessive amount of nitrate and nitrite. In this study, the mature leaves of TS were chopped, vacuum-packed, and fermented for up to 18 weeks to evaluate their feeding potential. The dynamic effects of fermentation on quality indicators, including crude protein, flavonoids, nitrate and nitrite content, and antioxidant activity [radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, ferric-reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC)] of mature leaves of TS were quantified every two weeks. Results showed that after fermentation for 6 weeks, the total content of nitrate and nitrite of TS leaves was significantly decreased ($p \leq 0.05$) from 4523.36 to 1102.20 mg/kg. The mean content of nutritional components, including crude protein (17.88 ± 0.49%), flavonoids (792.24 ± 50.78 mg/100 g), and antioxidant activity (317.62 ± 44.70 mg/100 g for DPPH, 400.44 ± 25.87 mg/100 g for FRAP, 473.74 ± 37.17 μM/g for ORAC) of TS leaves were well preserved between 6 to 14 weeks of fermentation. These results indicated that fermentation may provide a new way to utilize TS resources, and the fermented mature leaves of TS could be a novel, valuable feed supplement, especially for ruminants. Screening and inoculating TS leaves with an appropriate starter culture, such as lactic acid bacteria inoculants, during fermentation is recommended to further improve TS feed quality.

Keywords: Feed, Quality, Nitrate and nitrite, Flavonoid, Antioxidant activity.

INTRODUCTION

With rising incomes, the fast-growing population has expanded the global demand for animal products, especially meat, eggs, and dairy. Livestock production has been projected to expand by 21% between 2010 and 2025 (Kim *et al*., 2019). This expansion may add pressure to the livestock feed supply system. Protein is the most expensive and limiting feed ingredient, and the global protein market is projected to grow at a rate of 9.1% from 2020 to 2027 (I,smail *et al*., 2020; Kim *et al*., 2019). To provide the demanded protein in an environmentally and socially sustainable way, a renewed interest in exploring feed protein sourced from woody plants has emerged (Alatürk *et al*., 2023; Azim *et al*., 2011; Sample *et al*., 2023). Trees and shrubs with wide adaptability and strong resistance have been explored as fodder supplements to bridge the gap between protein supply and demand. Among them, mulberry, with a crude protein content of leaves around 15%-35% (dry matter), is a traditional woody forage crop used worldwide and is suitable to feed silkworms, livestock, poultry, and aquatic animals (Huang *et al*., 2022; Jiang *et al*., 2022; Mwai *et al*., 2021). *Moringa oleifera* (Valdivié-Navarro *et al*., 2020) and *Broussonetia papyrifera* (Xiong *et al*., 2023) have recently been developed as perennial tree fodder crops are noted for rapid regrowth after heavy shearing. Incorporating woody fodder in feed can improve the growth performance and nutrient digestion of animals (Bhatt *et al*., 2023; Wang *et al*., 2022; Xiong *et al*., 2023). The widespread use of woody fodder is beneficial due to its abundant bioactive components. The secondary metabolites, especially flavonoids, in woody forage commonly improve the serum antioxidative capacity and muscle lipid metabolism of animals, which are directly related to animal product quality (Huang *et al*., 2022; Liu *et al*., 2022). However, when considering the industrialization of woody fodder, both the traditionally used and newly developed woody trees need new investments to start over, as their natural distributions are sporadic, and intensive replanting is demanded.

Toona sinensis (Juss. M.) Roem (TS), commonly known as Chinese toon or Chinese mahogany, belongs to the Meliaceae family and is a perennial tree widely distributed in eastern and southeastern Asia. The young buds of TS have been treasured as a forest vegetable for thousands of years, mainly for their distinctively tangy flavor (Wang *et al*., 2023). Recently, TS has aroused considerable public interest due to its high level of flavonoid content and strong health benefits, such as anti-tumor, anti-diabetic, and anti-inflammatory effects (Peng *et al*., 2019; Su *et al*., 2020b). The antioxidant activity of TS is reported to be the highest among 110 kinds of vegetables and fruits (Liu *et al*., 2014b). As a result, except in several northwest Chinese provinces, the cultivation of TS occurs throughout China. The area of TS under intensive cultivation in China is reported to be more than 133,000 hectares (Su *et al*., 2020a). However, the mature leaves of TS, with decreased aroma compared to young buds, have never been utilized. Under the current cultivation pattern, the biomass of mature leaves of TS is estimated to be over 100 tons/ha; thus, millions of tons of mature leaves are wasted every year.

Previous research has indicated that the mature leaves of TS are rich in crude protein, essential amino acids, minerals, and flavonoids, and possess outstanding biological activity (Geng *et al*., 2019; Liu *et al*., 2014a). All of these factors demonstrate the potential of mature TS leaves as a functional protein feed resource. However, the forage value is severely restricted by its excessive accumulation of nitrate and nitrite (Chen, 2010; Geng *et al*., 2019; Qiao *et al*., 2016). Nitrates themselves are not toxic but can be reduced to nitrite during digestion. Nitrite is regulated in feed due to its potential to cause methaemoglobin (MetHb) formation, leading to cyanosis and death at excessive levels (Schrenk *et al*., 2020). Nitrate in forage of less than 3000 mg/kg is thought to be safe for animal and human health (Obour *et al*., 2018), and the maximum content of nitrite in commercial feedstuff (with a moisture content of 12%) is restricted to 60 mg/kg (Cockburn *et al*., 2013). However, the content of nitrate and nitrite in leaves of TS can be 2300 to 7300 mg/kg and 60 to 1800 mg/kg, respectively (Chen, 2010; Geng *et al*., 2019; Qiao *et al*., 2016), far higher than the suggested content.

Processing technologies such as fermentation are conventionally used to retain or increase the concentration of essential nutrients and valuable bioactive compounds or to remove potentially toxic substances (Fan *et al*., 2020; Marco *et al*., 2017). It was previously reported that fermentation could decrease nitrate and nitrite content up to 99.60% (Liao *et al*., 2018; Yan *et al*., 2008). Fermentation can also increase protein quality and is known to have other positive biological functions, such as increasing antioxidant activity (He *et al*., 2020; Jiang *et al*., 2022; Zhang *et al*., 2012). Besides, silage fermentation is the dominant process allowing long-term storage and long-distance transportation of high-quality preserved feeds (Gentu *et al*., 2018). Thus, fermentation may provide a new means for utilizing TS resources. The present study was designed to explore the potential of using fermented mature leaves of TS for animal feed while clarifying the dynamic effects of fermentation on the key feed quality indicators: crude protein, flavonoid, nitrate and nitrite content, and antioxidant activity. This research will have the potential to significantly reduce the agricultural waste in TS production while providing a new source of animal feed to meet the growing needs.

Chemicals: Quercetin 3-*O*-rutinoside (rutin) and gallic acid (GA) were purchased from Yuanye (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH), 2,4,6-tripyridyl-S-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchromate-2-carboxylic acid (Trolox), fluorescein, and 2,2'-azobis (2-methylpropionamide) dihydrochloride were purchased from Sigma-Aldrich (St. Louis, USA). Nitrate and nitrite standards were purchased from Institute for Environmental Reference Materials, Ministry of Environmental Protection (Beijin, China). Ultrapure water was obtained from a Milli-Q System (Millipore, Billerica, USA). Formic acid, methanol, sodium nitrite, aluminum nitrate nonahydrate, sodium hydroxide, and potassium hydroxide were of analytical grade and obtained from Beijing Chemical Works (Beijing, China).

Plant materials: *Toona sinensis* used in this study was grown in the Golden Sun Farm (Beijing, China) for five years. The fermentation process was conducted as shown in Figure 1. Briefly, healthy mature leaves were hand-harvested on a sunny morning (20th July 2018). All leaves were cut into small pieces using a shredder such that the pieces had a final length of approximately 1 cm. After evenly mixing with a blender, small pieces were randomly subsampled and placed into clean plastic bags with a one-way air valve (200 g of leaves per bag). Then, each bag was vacuum-packed and heat-sealed using a vacuum sealer. All bags were moved into a room without direct sunlight for anaerobic fermentation. Three replicate bags were then collected as the nonfermented control samples (CK) and frozen at -20 °C until future analysis. Additional samples were collected in triplicates every 2 weeks and frozen at -20 °C as well. The final samples were collected 18 weeks after sealing. During fermentation (0-18 weeks), bags were checked to ensure the vacuum seals were intact and no fungal masses were visible. After the final sample collection, fermented TS leaves in each bag were ground in liquid nitrogen for further analysis.

Extraction and quantitative analysis of crude protein content: For each sample, 5.0 g of ground tissue was dried in a 60°C drying oven until a constant weight was reached. Then, the crude protein (CP) content was analyzed with the Kjeldahl method (AOAC, 2000).

Extraction and quantitative analysis of flavonoid content: For each sample, 0.5 g of the ground tissue was extracted with methanol containing 0.2% formic acid following methods from Su *et al*. (2020b). The total flavonoid content (TFC) of each sample was analyzed using the $NaNO_2$-$Al(NO_3)_3$ spectrophotometric method following Chen *et al*. (2007) with methanol containing 0.2% formic acid was used to prepare the reaction solutions. Rutin standards (12.5 to 300 mg/L) were used for the quantitation of flavonoid content. The TFC of each sample was determined by comparing absorption value to the calibration curve of rutin: Y = 0.0009 X - 0.0020 (r^2 = 0.9998). The results were expressed as mg of rutin equivalent per 100 g of sample (n=3).

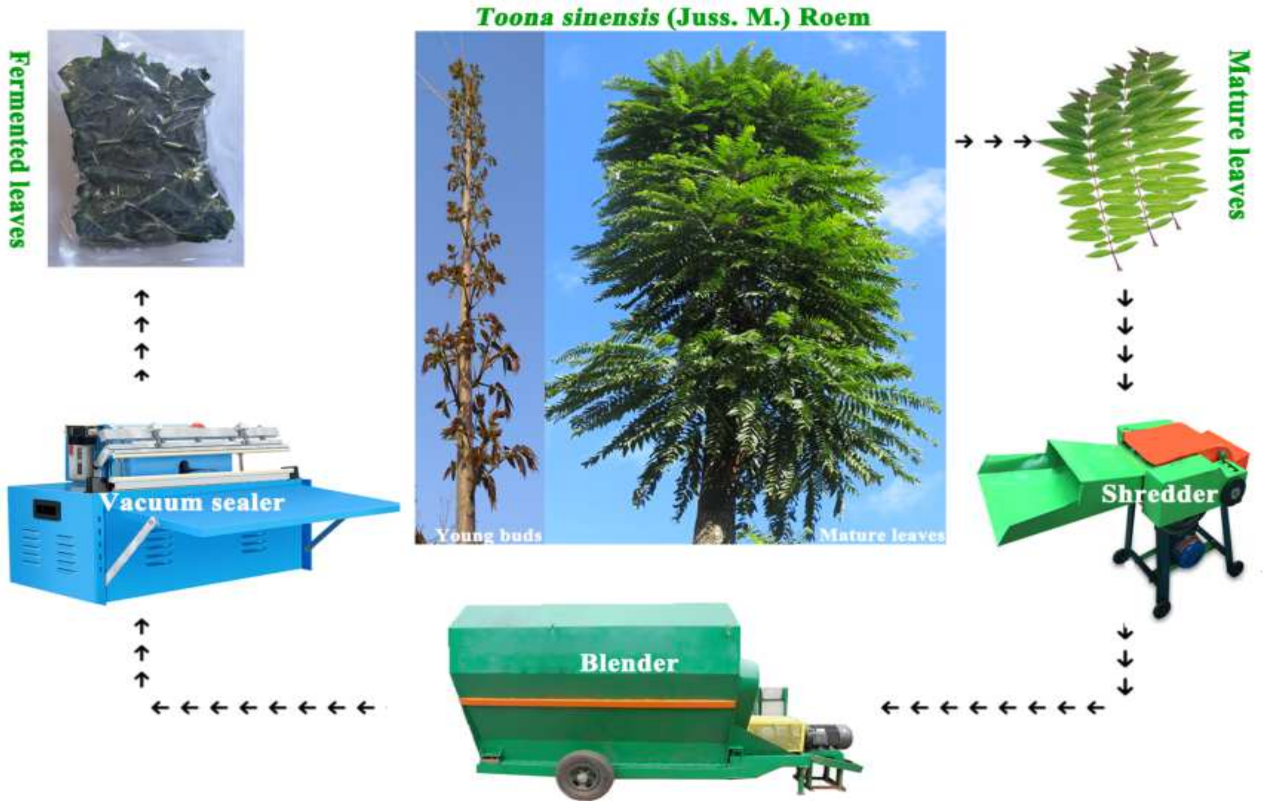

Fig. 1. Schematic diagram of the making process of fermented mature leaves of *T. sinensis*.

Extraction and quantitative analysis of total nitrate and nitrite content: Before the start of this experiment, the ultrapure water was boiled and allowed to cool to room temperature. For each sample, 1.0 g of the ground tissue was placed into a 50 mL centrifuge tube. Then, 20 mL of treated ultrapure water was aliquoted into each centrifuge tube. Finally, 0.25 mL of 1 M NaOH was added to each tube. Centrifuge tubes were mixed and sonicated in a KQ-500DE ultrasonic cleaner (Shangyi, China) at 25°C for 30 min, then placed in a water bath at 75 °C for 5 min. After the tubes were cooled to room temperature, the contents were diluted with treated ultrapure water to a final volume of 25 mL. Tubes were then centrifuged at 10,000 rpm for 20 min. The supernatant was collected and filtered through 0.22 µm reinforced nylon membrane filters (Anpel, Shanghai, China) prior to the ion chromatographic analyses.

Chromatographic analysis of total nitrate and nitrite content (TNC) was performed with an ion chromatograph Dionex ICS-3000 (Dionex, USA) with the associated conductivity detector, ASRS 300 suppressor, and SP6949 analytical column. The mobile phase included two solvents; A, ultrapure water, and B, 200 mM NaOH. Isocratic elution with 85% A and 15% B was kept for 10 min for each sample. A total of 20 uL of analyte solution was injected with a flow rate of 1 mL/min at a column temperature of 30°C. The total nitrate and nitrite content in sample was quantified by comparing chromatographic peak areas to the calibration curves of commercial standards of nitrate and nitrite (6.25 to 400 mg/L). The resultant calibration curves for nitrate and nitrite were Y = 0.0544 X - 0.249 (r^2 = 0.9991) and Y = 0.0476 X - 0.0023 (r^2 = 0.9994), respectively. Results were expressed as mg of standard equivalents per kg samples (n=3).

Analysis of antioxidant activity: Radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, ferric-reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) were evaluated following the methods described by Su *et al.* (2016). For DPPH analysis, GA was used to create the calibration curve, and the amount of GA equivalents in each sample was calculated using a linear regression: Y (scavenging ratio) = 1.6895 X (GA content) + 0.0388 (r^2 = 0.9990). Results were expressed as mg of GA equivalents per 100 g sample. For FRAP analysis, GA was used to create the calibration curve, and the amount of GA equivalents in each sample was calculated using a linear regression: Y (scavenging ratio) = 3.0343X (GA content) - 0.0660 (r^2 = 0.9994). Results were expressed as mg of GA equivalents per 100 g sample. For ORAC analysis, Trolox was used to create the calibration curve, and ORAC values were calculated using a quadratic regression: Y (mg/mL) = -3.389X^2 (net AUC) + 20.341X - 0.0985 (r^2 = 0.9998) (net AUC, area between the decay curve of fluorescein of blank and sample). The final ORAC values were expressed as µM of Trolox equivalents (TE) per gram sample.

Statistical analysis: Data was analyzed using SPSS 20.0 (IBM, USA). The final results were presented as means ± standard errors of the triplicates. Least significant difference (LSD) test was used to determine significant differences between treatments, following the one-way analysis of variance (ANOVA). Hierarchical cluster analysis was performed with Ward's method to describe the characters of samples from different fermentation stages.

RESULTS AND DISCUSSION

Effect of anaerobic fermentation on the crude protein content: There were no statistically significant differences (p = 0.63) in CP content among mature leaves fermented for different durations (Figure 2). As the fermentation progressed, the mean values of CP content numerically decreased in the early stages of fermentation (0 to 4 weeks) from 16.76% to 16.52% and then slightly increased in the later stages (6 to 18 weeks) from 18.23% to 18.48%. This pattern was consistent with the report of Tabaszewska *et al.* (2018), which showed that fermentation and storage had no significant effect on the total protein content of white asparagus (*Asparagus officinalis* L.), although a slight decrease of CP from 2.12% to 2.02% was detected in samples stored for 1 month. The study supposed that both protein-degrading enzymes and the activity of bacteria influenced the protein content. During the fermentation, with the rapid growth of lactic acid bacteria (LAB), the pH dropped rapidly, and protease activity decreased. Meanwhile, the proteins synthesized by the LAB might have contributed to the observed later increase in the protein content (Li, 2018). As reported by Sikora *et al.* (2021), silage of alfalfa (*Medicago sativa*) generally exhibited extensive protein degradation during the initial stage (21 days) of ensiling, after which the reduced pH limited the further degradation of CP. He *et al.* (2020) reported that the addition of 1% gallic acid, which possesses great protein binding capacity, prior to the ensiling of *M. oleifera* leaves, could help protect proteins from degradation by protease and microorganisms. Gallic acid is one of the major bioactive compounds in mature TS leaves, whose mean content is up to 0.87% by dry weight (Liu *et al.*, 2014a). The abundant gallic acid in TS leaves may explain the low protein degradation rate observed during fermentation in this study.

Compared with natural fermentation, the addition of microbial inoculants can greatly alter the effects of fermentation, especially on pH alteration and protein preservation. Jiang *et al.* (2022) reported that the mulberry leaves fermented for 61 hours following inoculation with *Bacillus zhangzhuensis* had a significant increase of 24.45% in CP content (from 14.03% to 17.46%) as compared to the non-fermented leaves. However, Gouvêa *et al.* (2020) examined the effects of 7 commercial microbial inoculants on fermentation characteristics and nutritive value of bermudagrass silage, and no significant differences were observed after 100 days of fermentation. These results indicate that the careful selection of suitable microbial inoculants might further improve the fermentation quality of mature TS leaves.

The mean CP content of fermented TS leaves was 17.49 ± 0.34%, which was much higher than traditionally used grain feeds (commonly less than 15%), such as *Sorghum bicolor* (8.7%), *Zea mays* (9.4%), and *Triticum aestivum* (13.4%) (Geng *et al.*, 2019). The CP value of TS was also higher than commonly used grass silage (averagely 14%), such as *Cleistogenes squarrosa* (7.94%), *Pulsatilla turczaninovii* (8.45%), and *Melissilus ruthenicus* (13.51%) (Gentu *et al.*, 2018; Weiby *et al.*, 2022). In addition, the CP value of fermented TS was also higher than tree fodders reported from Pakistan, such as *Melia azedarach* (14.09%), *Morus alba* (15.43%), and *Acacia modesta* (16.26%) (Azim *et al.*, 2011), and was comparable to the newly explored high protein woody forage *Moringa oleifera* (17.18%) (Valdivié-Navarro *et al.*, 2020). Therefore, the long-term preservation of CP by fermentation demonstrated in this study indicates that fermented mature leaves of TS have great potential as a novel high-protein feed ingredient.

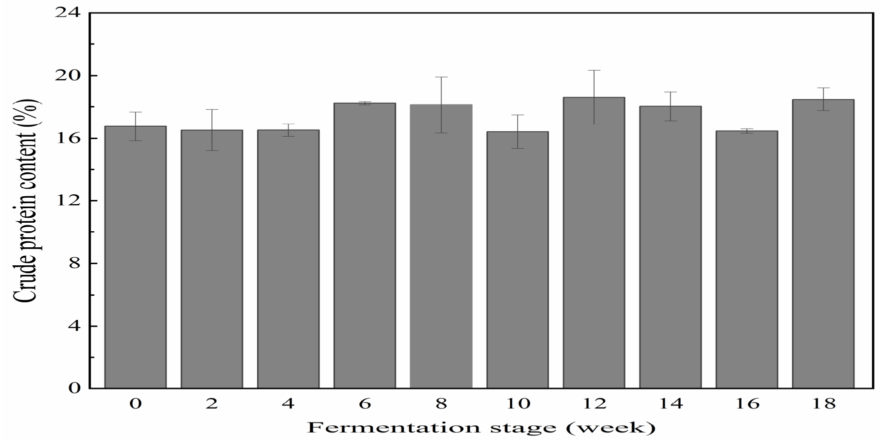

Fig. 2. Dynamic effect of anaerobic fermentation on crude protein content of mature leaves of *T. sinensis* (Mean ± SE, n = 3). Means were not significantly different according to the one-way analysis of variance ($p = 0.63$).

Effects of anaerobic fermentation on the flavonoid content: Overall, fermentation decreased the flavonoid content of mature TS leaves (Figure 3). The TFC rapidly dropped from 1168.50 ± 45.81 to 625.93 ± 192.51 mg/100 g in the first 6 weeks of fermentation ($p \leq 0.05$). Then, the TFC remained relatively stable from 8 to 18 weeks of fermentation, with a mean value of 762.67 ± 44.03 mg/100 g. The overall decrease of TFC observed in fermented TS leaves was consistent with previous studies, such as Jiang *et al*. (2022) and Zhang *et al*. (2012). These studies detected decreases in TFC in fermented mulberry leaves (from 11.96% to 10.30%) and *Ginkgo biloba* leaves (from 9.7 mg/g to 9.4 mg/g). According to Tabaszewska *et al*. (2018), one month of fermentation could cause a slight decrease in TFC (from 4.92 ± 0.4 to 3.71± 0.5 mg/g), while 3 months of fermentation could cause a mild increase in TFC (from 3.71 ± 0.5 to 4.53 ± 0.5 mg/g) in white asparagus. Observed decreases in TFC are thought to be related to the oxidative degradation of phenolic compounds, which may be caused by the undesirable bacteria on the surface of leaves (Lin *et al*., 2021). Polyphenol oxidases are responsible for the degradation of phenolic compounds during ensilage. This is especially true in the early stage of fermentation, as the enzyme activity will be inhibited during later stages of fermentation as the pH drops and conditions become anaerobic (He *et al*., 2019).

Adding LAB inoculants is often an effective method to rapidly increase the accumulation of lactic acid and lower pH, which could inhibit the growth of undesirable bacteria and the loss of nutritional content during early stage of ensiling (Jung *et al*., 2022). In the later stages of fermentation, various microbial enzymes, such as ligninase, cellulases, and esterases produced during the fermentation process break down the cell walls, hydrolyze some chemical bonds between phenolics, and liberate more soluble phenolic compounds, potentially contributing to the later increase of TFC (Wang *et al*., 2018). He *et al*. (2019) showed that mulberry leaves subjected to ensiling (60 days) with the addition of cellulase and *Lactobacillus casei* resulted in a higher TFC. Thus, screening for suitable additives, such as microbial inoculants, has the potential to further improve the preservation of bioactive content in TS leaves during fermentation.

The mean value of TFC of fermented TS leaves was 761.12 ± 36.78 mg/100 g. The TFC of all samples (excluding the final sample) of fermented TS leaves was higher than 600 mg/100 g, far higher than many commonly consumed vegetables and fruits (Cao *et al*., 2010). The importance of flavonoids to health is not only a hotspot in human health research but also a key area of study in livestock in recent years. Liu *et al*. (2022) demonstrated that dietary supplementation with flavonoid (200 to 1600 mg/kg) could increase the mean daily gain, lean meat percentage, loin-eye area, and n-3 polyunsaturated fatty acid content in the *longissimus lumborum* muscle of pig. Huang *et al*. (2022) indicated that a diet supplemented with mulberry-leaf flavonoid (30 to 60 mg/kg body weight) in aged breeder hens improved their reproduction performance by improving ovary function and hepatic lipid metabolism. De Feo *et al*. (2006) found that goats ingested about 435 mg/week of flavonoid compounds excreted about 105 mg/week of these compounds in their milk. Ma *et al*. (2017) evaluated the effects of flavonoids on methanogenesis and microbial flora in Dorper × thin-tailed Han crossbred ewes. They found that a diet supplemented with flavonoids (2 g/head/day) improved the digestibility of organic matter and decreased CH_4 output by inhibiting the populations of microbes involved in methanogenesis. Hence, fermented mature leaves of TS may represent an excellent source of TFC for use in functional feed supplements.

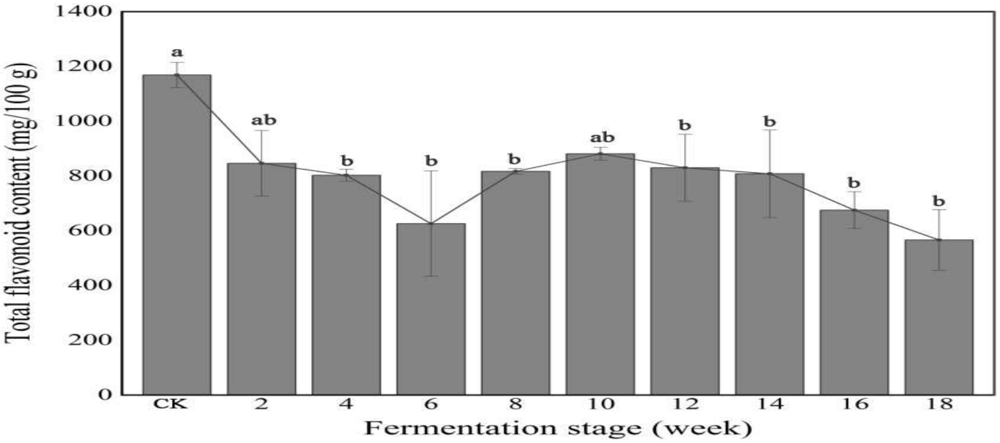

Fig. 3. Dynamic effects of anaerobic fermentation on flavonoid content of mature leaves of *T. sinensis* (Mean ± SE, n = 3). Means with different superscript letters were significantly different according to the least significant difference analysis ($p \leq 0.05$).

Effect of anaerobic fermentation on the antioxidant activity: High flavonoid content contributes to antioxidant activity in TS leaves. The antioxidant activity of fermented TS leaves, as indicated by DPPH, FRAP, and ORAC assays, changed over time during fermentation (Figure 4). All these values decreased during the initial and the final phases of fermentation but had slight upward trends during the middle of the fermentation period. However, most changes were not statistically significant (Figure 4). The antioxidant activity is reported to be positively correlated with TFC content in woody forage (He *et al*., 2019). Besides the total content, the antioxidant activity of phenolic compounds also depends on the number of hydroxyl groups, physical location, and interactions with other compounds. According to Fan *et al*. (2020), fermentation alters the composition of secondary metabolites in plant tissue. For example, the chlorogenic acid content of *Cynara scolymus* decreased from 6302.87 to 64.11 mg/kg after 60 days of fermentation, while the content of luteolin increased from 67.67 mg/kg to 3627.24 mg/kg. Thus, in addition to the decline of TFC, the changes of other antioxidants, such as phenolic acids, may also contribute to the reduction in antioxidant capacity of fermented TS leaves. The mild increase in antioxidant activity observed in the middle of the fermentation period might be due to the increase in the proportion of low molecular weight peptides and free amino acids originated from zymohydrolysis, which were reported to exhibit strong radical scavenging activity (He *et al*., 2020; Jiang *et al*., 2022; Zhang *et al*., 2012).

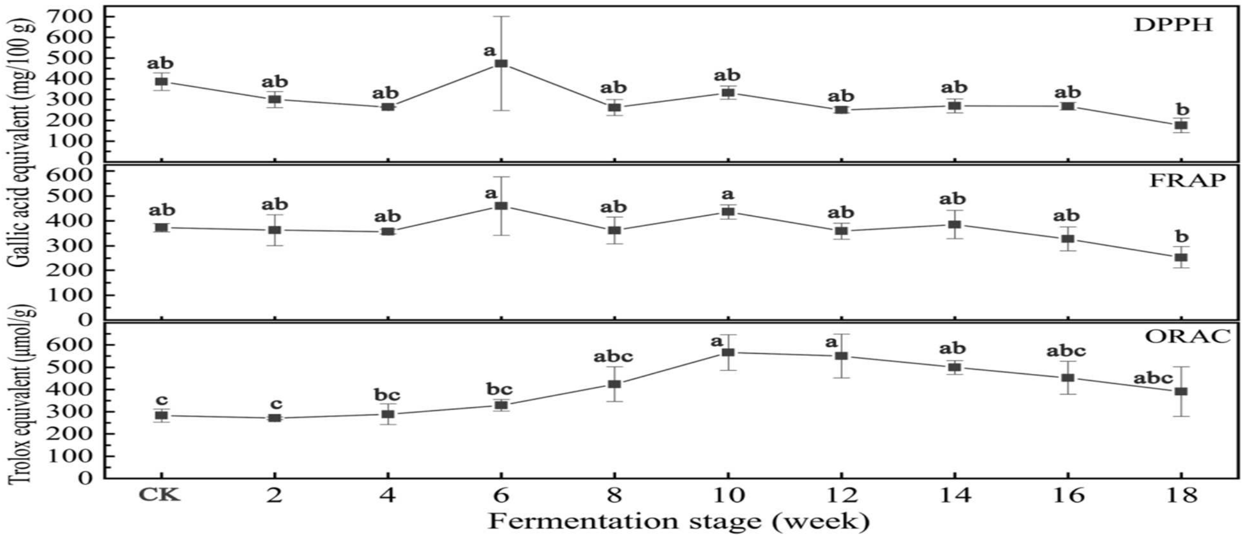

Fig. 4. Effect of anaerobic fermentation on the antioxidant activity of mature leaves of *T. sinensis* detected by DPPH, FRAP, and ORAC assay (Mean ± SE, n = 3). Means in the same assay with different superscript letters were significantly different according to the least significant difference analysis ($p \leq 0.05$).

There is a trend towards utilizing natural antioxidants, instead of synthetic ones, as additives to prevent lipid and protein oxidation in meat and poultry production (Oswell *et al.*, 2018). Recently, numerous studies have shown that dietary supplementation with natural antioxidants, such as plant flavonoids, can improve both growth performance and meat quality of farmed animals (Huang *et al.*, 2022; Liu *et al.*, 2022). The antioxidant activity of fermented TS leaves remained at biologically significant levels throughout fermentation, with the mean values of DPPH, FRAP, and ORAC being 288.47 ± 26.95 mg/100 g, 367.02 ± 19.54 mg/100 g, and 419.09 ± 30.05 μM/g, respectively. These values are much higher than in commonly consumed vegetables and fruits (Liu *et al.*, 2014b). Thus, fermented TS could be considered as an attractive alternative for feed supplementation or as an additive to improve meat quality and extend the shelf life of animal products. According to He *et al.* (2019) and Wang *et al.* (2021), the addition of cellulase (50-100 U/g), sucrose (20 g/kg), or LAB (10^6 colony forming unit/g) can accelerate pH decline, promote the breeding of beneficial bacteria, and increase the antioxidant activity of woody forage. Thus, the fermentation process for TS feed could be further optimized with suitable silage additives.

Effect of anaerobic fermentation on the nitrate and nitrite content: Fermentation effectively decreased the content of nitrate and nitrite of TS ($p \leq 0.05$, Figure 5). A dramatic decrease (75.63%) was detected in the first 6 weeks, with the TNC value dropping from 4523.36 mg/kg (CK) to 1102.20 mg/kg (week 6). Following this decrease, the TNC value remained relatively stable until the end of sampling. There were no statistically significant differences in TNC among TS leaves fermented for 4 to 18 weeks ($p > 0.05$). These results agree with previous experiments that report the content of nitrate and nitrite can rapidly decrease during fermentation, especially in the early stages of fermentation (Jagannath *et al.*, 2015; Liao *et al.*, 2018). During fermentation, the nitrate present in plant tissue is reduced to nitrite by nitrate reductase-producing bacteria, especially the Gram-negative bacteria, which dominate the flora during the initial stage of the fermentation process (Wang *et al.*, 2010). As the fermentation continues, LAB strains generally proliferate, and the pH drops rapidly. Almost all LAB possess nitrite reductase, which reduces nitrite to NH_4^+ or N_2 under anaerobic conditions (Liao *et al.*, 2018). Meanwhile, it has been demonstrated that nitrite is unstable in acidic environments, with 53.5% of nitrite being depleted at pH 6 while 98.6% of nitrite is lost at pH 3 (Wang *et al.*, 2010). Recently, Li *et al.* (2022) investigated the mechanisms underlying nitrite degradation by *Lactiplantibacillus plantarum*. They demonstrated that it was the upregulation of genes in *L. plantarum* related to pyruvate metabolism and energy and nucleotide synthesis that resulted in enhanced cell growth and acid production, leading to the degradation of nitrite.

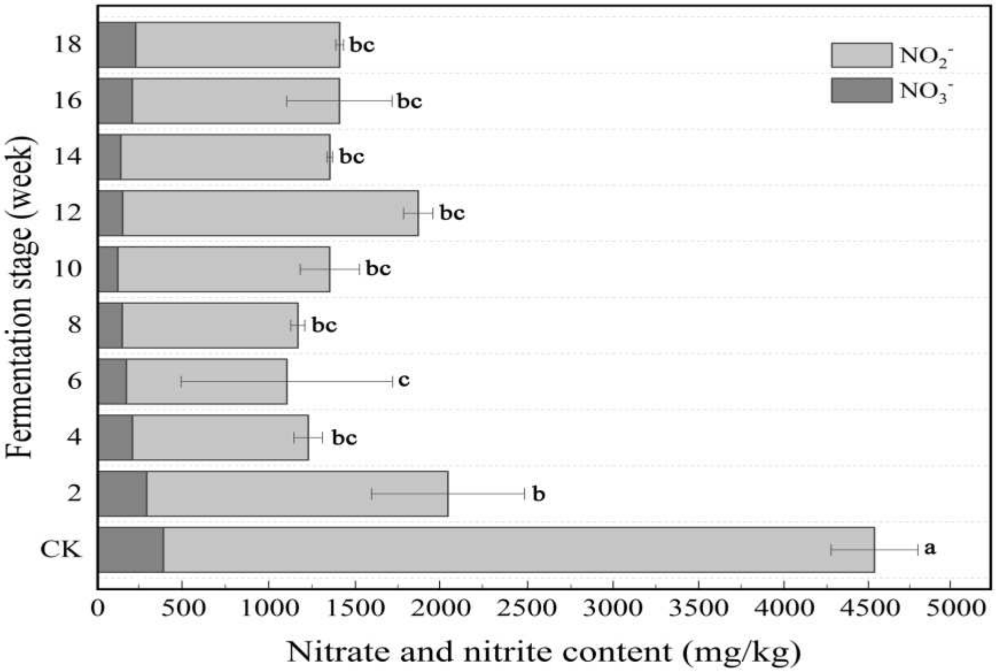

Fig. 5. Dynamic effects of anaerobic fermentation on nitrate and nitrite content of mature leaves of *T. sinensis*. Means with different letters are significantly different according to least significant difference analysis ($p \leq 0.05$).

Because of the high potential for MetHb formation and animal death at excessive levels of intake, nitrate and nitrite are regulated in feed. According to the review of Cockburn *et al*. (2013), the physiological and anatomical differences between animal species result in their differential sensitivity to nitrate and nitrite. Ruminants commonly possess higher nitrate and nitrite tolerance than non-ruminants. In ruminants, these compounds can be metabolized by rumen microorganisms to amino acids or eliminated with other gases during eructation, and only about 10-20 % of nitrite and nitrate present in the rumen pass into the bloodstream as nitrite (Schrenk *et al*., 2020). It has been reported that the lethal dose LD_{50} for ruminants is between 160 and 224 mg/kg body weight (BW) (Wright and Davison, 1964). Calves weighing 75 kg receiving artificial milk supplemented with varying concentrations of nitrate (about 18-10,000 mg NO_3/calves per day) did not show adverse effects (Berende, 1977). In addition, cattle treated with 6.48 g NO_3/kg dry matter in their feed intakes did not develop clinical signs of nitrite toxicity (MetHb in blood > 20%) throughout a 70 day study (Benu *et al*., 2021). A diet supplemented with 18 g NO_3/kg dry matter was reported to improve the ruminal fermentation, milk quality, and methane inhibition of lactating cows (Sharifi *et al*., 2022). In sheep, a dose of 260 mg/kg BW nitrate per day had no immediate effects (Setchell and Williams, 1962). In goat, feed intake, nutrient utilization, and weight gain were improved when *Asystasia gangetica*, with a nitrate concentration as high as 11 g/kg dry matter, was offered for feeding (Ali *et al*., 2021).

Nitrite in feeds, including inter-conversion from nitrate, could cause acute or chronic adverse effects in animals. For pigs, a single oral dose of nitrite above 20 mg/kg BW was lethal (Muirhead and Alexander, 1997). MetHb formation and cyanosis were observed in calves after an oral administration of 2600 mg of nitrite, corresponding to 34 mg/kg BW (Bartik and Piskac, 1981). A single 50 mg/kg BW dose of nitrite resulted in a rise of MetHb up to 10%, and the oral lethal dose of nitrite was 67 to 110 mg/kg BW for sheep (Trif *et al*., 1993). Breed sensitivity, age, the timing and length of exposure, and the progressive adaptation of the gastrointestinal flora to high levels of nitrate and nitrite in feed are important factors determining animal tolerance (Schrenk *et al*., 2020). Thus, given the proper precautions, the fermented mature leaves of TS, with nitrate and nitrite content around 1400 mg/kg, could be used as feed raw materials or additives for ruminants, such as sheep.

It should be noted that the content of nitrite in both the CK and all fermented TS treatments (Figure 5) was higher than the feed limit of European Union (60 mg/kg) (Cockburn *et al*., 2013). The nitrite content of fresh young buds of TS can be as high as 1800 mg/kg, which can increase with maturity of leaves (Chen, 2010; Qiao *et al*., 2016). Researchers have demonstrated that fermentation using inoculation of specific microbial starter cultures is more effective in lowering nitrite concentration compared to spontaneous fermentation. Liao *et al*. (2018) evaluated the nitrite degradation ability of 3 different microbial strains isolated from fermented fish products and found that the highest nitrite degradation rate was from *Lactobacillus plantarum* 120 (90.70%), followed by *Saccharomyces cerevisiae* 2018 (78.75%), and *Staphylococcus xylosus* 135 (13.09%). Yan *et al*. (2008) isolated 6 kinds of LAB from Chinese paocai, and found that LAB depleted 97.0% to 99.6% of nitrite during fermentation. Recently, Zhang *et al*. (2023) summarized 28 kinds of microbes capable of nitrite reduction during the preparation of cured or salted meat products. These types of microbial inoculants should be explored for their potential to reduce TNC in TS leaves to improve the safety and expand the application of fermented TS feed.

Cluster analysis: Measurements of protein, flavonoids, nitrate, nitrite, and antioxidant activity were standardized and used as variables in Ward's method cluster analysis. Fermented (cluster I and II) and non-fermented leaves (CK, cluster III) were visibly distinct (Figure 6 A). TS leaves fermented for 2, 4, 16, and 18 weeks were clustered together (cluster II) by their relatively large quality fluctuations, mainly in TFC and antioxidant activity (Figure 6 B). The other TS leaves (6 to 14 weeks of fermentation) were gathered in cluster I by their relatively stable values for CP (average value as 17.88 ± 0.49%), TFC (792.24 ± 50.78 mg/100 g), TNC (1367.60 ± 131.52 mg/kg), and antioxidant activity (317.62 ± 44.70 mg/100 g for DPPH, 400.44 ± 25.87 mg/100 g for FRAP, 473.74 ± 37.17 μM/g for ORAC). These results suggest that fermentation effectively reduced the nitrate and nitrite content of mature leaves of TS, but the fermentation duration also affected the nutritional quality of feed. This study shows that the feed quality of fermented TS leaves could remain relatively stable from 6 to 14 weeks of fermentation.

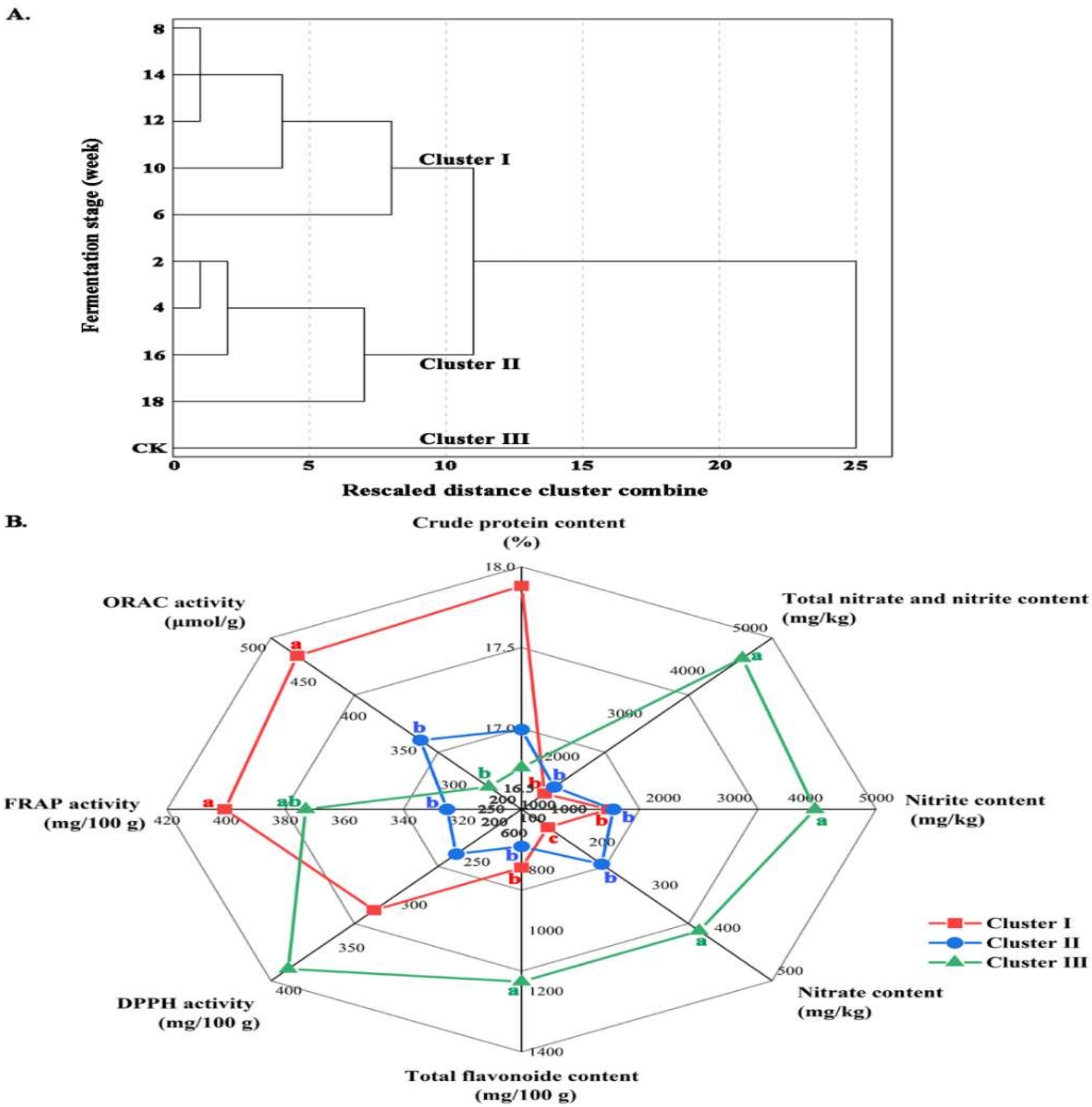

Fig. 6. Hierarchical cluster dendrogram (A) of mature leaves of *T. sinensis* from different fermentation stages and their quality characteristics (B). Means in the same axis with different letters were significantly different according to the least significant difference analysis ($p \leq 0.05$).

Conclusion: This study demonstrated that anaerobic fermentation significantly decreased the content of nitrate and nitrite in mature leaves of TS, and effectively preserved their protein and flavonoid content along with their antioxidant activity. Within the fermentation period of 6 to 14 weeks, mature leaves of TS maintained outstanding content of CP (17.88 ± 0.49%), TFC (792.24 ± 50.78 mg/100 g), and antioxidant activity (317.62 ± 44.70 mg/100 g for DPPH, 400.44 ± 25.87 mg/100 g for FRAP, 473.74 ± 37.17 μM/g for ORAC). These results indicate the great potential of fermented TS leaves to serve as a high-quality animal feed, especially for ruminants. Screening for suitable fermentation additives (e.g. lactic acid bacteria) for further lowering the nitrate and nitrite content, and clarifying the optimal type and dosage of these additives are recommended to improve the feed quality of fermented TS leaves.

Conflict of interest: There is no conflict of interest.

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Authors' contribution: S. Su and X. Q. Xu designed the study. Y. H. Geng, J. W. Ni and W Wang executed the experiment. S. Su and Y. H. Geng analyzed the data and wrote the first draft. All authors interpreted the data, critically revised the manuscript and approved the final version.

REFERENCES

Alatürk, F., H. Hanoglu Oral, A. Gokkus and B. Ali (2023). Annual changes in biomass amount and feeding potential of shrubby rangelands in maquis formation. Peer J. 11: e15204. https://doi.org/10.7717/peerj.15204

Ali, A. I. M., S. Sandi, Riswandi, M. N. Rofiq and Suhubdy (2021). Effect of feeding *Asystasia gangetica* weed on intake, nutrient utilization, and gain in Kacang goat. Ann. Agr. Sci. 66(2): 137-141. https://doi.org/10.1016/j.aoas.2021.10.002

AOAC (2000). Official methods of analysis of AOAC international. 17th Ed. Association of Official Analytical Chemists, Arlington.

Azim, A., S. Ghazanfar, A. Latif and M. A. Nadeem (2011). Nutritional evaluation of some top fodder tree leaves and shrubs of district Chakwal Pakistan in relation to ruminants requirements. Pakistan J. Nut. 10(1): 54-59. https://doi.org/10.3923/pjn.2011.54.59

Bartik, M. and A. Piskac (1981). Veterinary toxicology. Elsevier Scientific publishing Company, Amsterdam. 346 p.

Berende, P. L. (1977). Dynamic and kinetic aspects of nitrate in rations for milk-fed calves. ILOB Rep. 430-430a.

Benu, I., M. J. Callaghan, N. Tomkins, G. Hepworth, L. A. Fitzpatrick and A. J. Parker (2021). The effects of feeding nitrate on the development of methaemoglobinaemia in sedentary *Bos indicus* cattle. Anim. Prod. Sci. 61(16): 1680-1685. https://doi.org/10.1071/AN20148

Bhatt, R. S., S. Sarkar, S. R. Sharma and A. Soni (2023). Use of *Moringa oleifera* leaves (sole or combined with concentrate) in rabbit feeding: effects on performance, carcass characteristics and meat quality attributes. Meat Sci. 198: 109108. https://doi.org/10.1016/j.meatsci.2023.109108

Cao, J., W. Chen, Y. Zhang, Y. Q. Zhang and X. J. Zhao (2010). Content of selected flavonoids in 100 edible vegetables and fruits. Food Sci. Technol. Res. 16(5): 395-402. https://doi.org/10.3136/fstr.16.395

Chen, C. J., K. Y. Huang, D. L. Li and G. W. Wang (2007). $NaNO_2$-$Al(NO_3)_3$ spectrophotometric determination of total flavonoids in *Toona sinensis* (A. Juss) leaves. Food Res. Dev. 28: 104-107. https://doi.org/10.3969/j.issn.1005-6521.2007.05.035

Chen, X. (2010). Research on the variation of nitrite content of *Toona sinensis* and the technology to reduce nitrite content. M.Sc. thesis. Shandong Univ., Jinan (China).

Cockburn, A., G. Brambilla, M. L. Fernandez, D. Arcella, L. R. Bordajandi, B. Cottrill, C. van Peteghem and J. L. Dorne (2013). Nitrite in feed: from animal health to human health. Toxicol. Appl. Pharm. 270(3): 209-217. https://doi.org/10.1016/j.taap.2010.11.008

De Feo, V., E. Quaranta, V. Fedele, S. Claps, R. Rubino and C. Pizza (2006). Flavonoids and terpenoids in goat milk in relation to forage intake. Ital. J. food Sci. 18(1): 85-92.

Fan, Z. Y., K. Chen, L. Y. Ban, Y. Mao, C. Y. Hou and J. M. Li (2020). Silage fermentation: a potential biological approach for the long-term preservation and recycling of polyphenols and terpenes in globe artichoke (*Cynara scolymus* L.) by-products. Molecules. 25(14): 3302. https://doi.org/10.3390/molecules25143302

Geng, Y. H., X. Q. Xu, J. W. Ni and S. Su (2019). Effect of harvest time on forage quality of *Toona sinensis*. Forest Res. 32(2): 145-151. https://doi.org/10.13275/j.cnki.lykeyj.2019.02.021

Gentu, G., M. L. Hou, T. Y. Liu, Y. S. Jia and Y. M. Cai (2018). Microbial population, chemical composition and silage fermentation of native grasses growing on the Inner Mongolian Plateau. Grassl. Sci. 64(4): 226-233. https://doi.org/10.1111/grs.12207

Gouvêa, V. N., J. M. B. Vendramini, L. E. Sollenberger, F. C. L. Oliveira, J. C. B. Dubeux Jr, P. Moriel, U. Cecato, C. V. Soares Filho, J. M. D. Sanchez, J. K. Yarborough and F. Kuhawara (2020). Inoculant effects on fermentation characteristics, nutritive value, and mycotoxin concentrations of bermudagrass silage. Crop Forage Turfgrass Mgmt. 6(1): e20054. https://doi.org/10.1002/cft2.20054

He, L. W., H. J. Lv, N. Chen, C. Wang, W. Zhou, X. Y. Chen and Q. Zhang (2020). Improving fermentation, protein preservation and antioxidant activity of *Moringa oleifera* leaves silage with gallic acid and tannin acid.

Bioresour. Technol. 297: 122390. https://doi.org/10.1016/j.biortech.2019.122390

He, L. W., W. Zhou, C. Wang, F. Y. Yang, X. Y. Chen and Q. Zhang (2019). Effect of cellulase and *Lactobacillus casei* on ensiling characteristics, chemical composition, antioxidant activity, and digestibility of mulberry leaf silage. J. Dairy Sci. 102(11): 9919-9931. https://doi.org/10.3168/jds.2019-16468

Huang, Z. W., Z. P. Lv, H. J. Dai, S. M. Li, J. L. Jiang, N. W. Ye, S. L. Zhu, Q. W. Wei and F. X. Shi (2022). Dietary mulberry-leaf flavonoids supplementation improves liver lipid metabolism and ovarian function of aged breeder hens. J. Anim. Physiol. Anim. Nutr (Berl). 106(6): 1321-1332. https://doi.org/10.1111/jpn.13658

Ismail, B. P., L. Senaratne-Lenagala, A. Stube and A. Brackenridge (2020) Protein demand: review of plant and animal proteins used in alternative protein product development and production. Anim. Front. 10(4): 53-63. https://doi.org/10.1093/af/vfaa040

Jagannath, A., M. Kumar and P. S. Raju (2015). The recalcitrance of oxalate, nitrate and nitrites during the controlled lactic fermentation of commonly consumed green leafy vegetables. Nutr. Food Sci. 45: 336-346. https://doi.org/10.1108/NFS-08-2014-0078

Jiang, W. Q., Y. Lin, L. J. Qian, L. H. Miao, B. Liu, X. P. Ge and H. S. Shen (2022). Mulberry leaf meal: a potential feed supplement for juvenile *Megalobrama amblycephala* "Huahai No. 1". Fish Shellfish Immunol. 128: 279-287. https://doi.org/10.1016/j.fsi.2022.07.022

Jung, J. S., B. Ravindran, I. Soundharrajan, M. K. Awasthi and K. C. Choi (2022). Improved performance and microbial community dynamics in anaerobic fermentation of triticale silages at different stages. Bioresour. Technol. 345: 126485. https://doi.org/10.1016/j.biortech.2021.126485

Kim, S. W., J. F. Less, L. Wang, T. H. Yan, V. Kiron, S. J. Kaushik and X. G. Lei (2019). Meeting global feed protein demand: challenge, opportunity, and strategy. Annu. Rev. Anim. Biosci. 7: 221-243. https://doi.org/10.1146/annurev-animal-030117-014838

Liao, E., Y. S. Xu, Q. X. Jiang and W. S. Xia (2018). Characterisation of dominant autochthonous strains for nitrite degradation of Chinese traditional fermented fish. Int. J. Food Sci. Tech. 53(12): 2633-2641. https://doi.org/10.1111/ijfs.13914

Lin, H. Y., S. Q. Lin, M. K. Awasthi, Y. F. Wang and P. Xu (2021). Exploring the bacterial community and fermentation characteristics during silage fermentation of abandoned fresh tea leaves. Chemosphere. 283: 131234. https://doi.org/10.1016/j.chemosphere.2021.131234

Liu, C. J., H. Y. Wan, J. Zhang and Z. T. Hua (2014a). Quantitative identification and antioxidant activity in vitro of phenolic compounds from the old leaves of *Toona sinensis*. Proc. 2012th Int. Co. Appl. Biotech.Tianjin (China), 249: 523-533. https://doi.org/10.1007/978-3-642-37916-1_54

Liu, C. J., Y. L. Zhao, X. J. Li, J. Y. Jia, Y. Y. Chen and Z. T. Hua (2014b). Antioxidant capacities and main reducing substance contents in 110 fruits and vegetables eaten in China. Food Nutr. Sci. 5(4): 293-307. https://doi.org/10.4236/fns.2014.54036

Liu, Y., Y. Xiao, J. Xie, Y. Peng, F. Li, C. Chen, Y. Li, X. Zhang, J. He, D. Xiao and Y. Yin (2022). Dietary supplementation with flavonoids from mulberry leaves improves growth performance and meat quality, and alters lipid metabolism of skeletal muscle in a Chinese hybrid pig. Anim. Feed Sci. Tech. 285: 115211. https://doi.org/10.1016/j.anifeedsci.2022.115211

Li, X. J. (2018). Research on mechanism and modification of protein degradation in alfalfa silage. D.Sc. thesis. China Agri. Univ., Beijing.

Li, Y. Y., D. Xiong, L. Y. Yuan, P. F. Fan, Y. Xiao, J. P. Chen and W. Feng (2022). Transcriptome and protein networks to elucidate the mechanism underlying nitrite degradation by *Lactiplantibacillus plantarum.* Food Res. Int. 156: 111319. https://doi.org/10.1016/j.foodres.2022.111319

Mwai, L. M., A. M. Kingori and M. K. Ambula (2021). Mulberry leaves as a feed source for livestock in Kenya: a review. Int. J. Agr. Res. Innov. Technol. 11(2): 1-9. https://doi.org/10.3329/ijarit.v11i2.57249

Ma, T., D. D. Chen, Y. Tu, N. F. Zhang, B. W. Si and Q. Y. Diao (2017). Dietary supplementation with mulberry leaf flavonoids inhibits methanogenesis in sheep. Anim. Sci. J. 88(1): 72-78. https://doi.org/10.1111/asj.12556

Marco, M. L., D. Heeney, S. Binda, C. J. Cifelli, P. D. Cotter, B. Foligne, M. Ganzle, R. Kort, G. Pasin, A. Pihlanto, E. J. Smid and R. Hutkins (2017). Health benefits of fermented foods: microbiota and beyond. Curr. Opin. Biotech. 44: 94-102. https://doi.org/10.1016/j.copbio.2016.11.010

Muirhead, M. and T. Alexander (1997). Poisons: recognition, treatment and control. In Managing pig health and the treatment of disease. 1st Ed., 5M Enterprises Limited, Sheffield (UK). 423-

440 p.

Obour, A., J. D. Holman and D. B. Mengel (2018). Nitrogen application effects on forage sorghum biomass production and nitrates. Kansas Agr. Exp. Station Res. Rep. 4: 5. https://doi.org/10.4148/2378-5977.7586

Oswell, N. J., H. Thippareddi and R. B. Pegg (2018). Practical use of natural antioxidants in meat products in the U.S.: a review. Meat Sci. 145: 469-479. https://doi.org/10.1016/j.meatsci.2018.07.020

Peng, W., Y. J. Liu, M. B. Hu, M. M. Zhang, J. Yang, F. Liang, Q. W. Huang and C. J. Wu (2019). *Toona sinensis*: a comprehensive review on its traditional usages, phytochemisty, pharmacology and toxicology. Rev. Bras. Farmacogn. 29(1): 111-124. https://doi.org/10.1016/j.bjp.2018.07.009

Qiao, H. T., G. Y. Shi, J. Y. Xu and Z. Y. Sun (2016). Changes of nitrate reductase activity and nitrite conten of *Toona sinensis* buds in different harvesting times. Shangdong Agr. Sci. 48(1): 51-53. https://doi.org/10.14083/j.issn.1001-4942.2016.01.011

Sample, R. D., Z. J. Delisle, J. M. Pierce, R. K. Swihart, J. N. Caudell and M. A. Jenkins (2023). Selection rankings of woody species for white-tailed deer vary with browse intensity and landscape context within the central hardwood forest region. Forest Ecol. Manag. 537: 120969. https://doi.org/10.1016/j.foreco.2023.120969

Schrenk, D., M. Bignami, L. Bodin, J. K. Chipman, J. Del Mazo, B. Grasl-Kraupp, L. R. Hoogenboom, J. C. Leblanc, C. S. Nebbia, E. Nielsen, E. Ntzani, A. Petersen, S. Sand, T. Schwerdtle, C. Vleminckx, H. Wallace, V. Bampidis, B. Cottrill, M. J. Frutos, P. Furst, A. Parker, M. Binaglia, A. Christodoulidou, P. Gergelova, I. M. Guajardo, C. Wenger and C. Hogstrand (2020). Risk assessment of nitrate and nitrite in feed. EFSA J. 18(11): e06290. https://doi.org/10.2903/j.efsa.2020.6290

Setchell, B. P. and A. J. Williams (1962). Plasma nitrate and nitrite in chronic and acute nitrate poisoning in sheep. Aust. Vet. J. 38(2): 58-62. https://doi.org/10.1111/j.1751-0813.1962.tb08721.x

Sharifi, M., A. Taghizadeh, A. Hosseinkhani, H. Mohammadzadeh, V. Palangi, M. Macit, A. Z. M. Salem and S. Abachi (2022). Nitrate supplementation at two forage levels in dairy cows feeding: milk production and composition, fatty acid profiles, blood metabolites, ruminal fermentation, and hydrogen sink. Ann. Anim. Sci. 22(2): 711-722. https://doi.org/10.2478/aoas-2021-0044

Sikora, M. C., R. D. Hatfield and K. F. Kalscheur (2021). Impact of long-term storage on alfalfa leaf and stem silage characteristics. Agronomy. 11(12): 2505. https://doi.org/10.3390/agronomy11122505

Su, S., L. J. Wang, C. Y. Feng, Y. Liu, C. H. Li, H. Du, Z. Q. Tang, Y. J. Xu and L. S. Wang (2016). Fingerprints of anthocyanins and flavonols of *Vaccinium uliginosum* berries from different geographical origins in the Greater Khingan Mountains and their antioxidant capacities. Food Control. 64: 218-225. https://doi.org/10.1016/j.foodcont.2016.01.006

Su, S., L. J. Wang, J. W. Ni, Y. H. Geng and X. Q. Xu (2020a). Differences and correlations of morphological characteristics and fatty acid profiles of seeds of *Toona sinensis*. Chem. Biodivers. 17(11): e2000553. https://doi.org/10.1002/cbdv.202000553

Su, S., L. J. Wang, J. W. Ni, Y. H. Geng and X. Q. Xu (2020b). Diversity of red, green and black cultivars of Chinese Toon [*Toona sinensis* (A. Juss.) Roem]: anthocyanins, flavonols and antioxidant activity. J. Food Meas. Charact. 14: 3206-3215. https://doi.org/10.1007/s11694-020-00560-8

Tabaszewska, M., A. Gabor, G. Jaworska and I. Drożdż (2018). Effect of fermentation and storage on the nutritional value and contents of biologically-active compounds in lacto-fermented white asparagus (*Asparagus officinalis* L.). LWT-Food Sci. Technol. 92: 67-72. https://doi.org/10.1016/j.lwt.2018.02.003

Trif, A., D. Pârvu and V. Curtui (1993). The dynamic of methaemoglobin in sheep in correlation with the level of nitrate and nitrite intake. Lucrari Stiintifice Medicina Veterinara, Timisoara. 100-104.

Valdivié-Navarro, M., Y. Martínez-Aguilar, O. Mesa-Fleitas, A. Botello-León, C. Betancur Hurtado and B. Velázquez-Martí (2020). Review of *Moringa oleifera* as forage meal (leaves plus stems) intended for the feeding of non-ruminant animals. Anim. Feed Sci. Tech. 260: 114338. https://doi.org/10.1016/j.anifeedsci.2019.114338

Wang, B., X. G. Zhao, B. Y. Zhang, Y. M. Cui, M. Nueraihemaiti, Q. F. Kou and H. L. Luo (2022). Assessment of components related to flavor and taste in Tan-lamb meat under different silage-feeding regimens using integrative metabolomics. Food Chem. X. 14: 100269. https://doi.org/10.1016/j.fochx.2022.100269

Wang, C., B. B. Zhang, Y. F. Li, J. Hou, C. D. Fu, Z. H. Wang and J. F. Zhang (2023). Integrated transcriptomic and volatilomic profiles to explore the potential mechanism of aroma formation in *Toona sinensis*. Food Res. Int. 165: 112452. https://doi.org/10.1016/j.foodres.2022.112452

Wang, C. L., Y. Y. Ma, M. H. Chen, Y. R. Wang, S. Lei, F. J. Li and D. W. Liu (2010). Effect of pH on nitrite reduction of pickled Chinese cabbage. 4th Int. Co. Bioinformatics Biomed. Eng., Chengdu (China). https://doi.org/10.1109/ICBBE.2010.5515295

Wang, L., Y. Luo, Y. N. Wu, Y. Liu and Z. Q. Wu (2018). Fermentation and complex enzyme hydrolysis for improving the total soluble phenolic contents, flavonoid aglycones contents and bio-activities of guava leaves tea. Food Chem. 264: 189-198. https://doi.org/10.1016/j.foodchem.2018.05.035

Wang, X. K., H. Liu, Y. X. Xie, Y. C. Zhang, Y. L. Lin, Y. L. Zheng, X. P. Yang, N. W. Wang, K. K. Ni and F. Y. Yang (2021). Effect of sucrose and lactic acid bacteria additives on fermentation quality, chemical composition and protein fractions of two typical woody forage silages. Agriculture. 11(3): 256. https://doi.org/10.3390/agriculture11030256

Weiby, K. V., S. J. Krizsan, M. Eknæs, A. Schwarm, A. C. Whist, I. Schei, H. Steinshamn, P. Lund , K. A. Beauchemin and I. Dønnem (2022). Associations among nutrient concentration, silage fermentation products, in vivo organic matter digestibility, rumen fermentation and in vitro methane yield in 78 grass silages. Anim. Feed Sci. Techn. 285: 115249. https://doi.org/10.1016/j.anifeedsci.2022.115249

Wright, M. J. and K. L. Davison (1964). Nitrate accumulation in crops and nitrate poisoning in animals. Adv. Agron. 16: 197-247. https://doi.org/10.1016/S0065-2113(08)60025-5

Xiong, Y., X. Wang, X. M. Li, L. N. Guo, F. Y. Yang and K. K. Ni (2023). Exploring the rumen microbiota of Hu lambs in response to diet with paper mulberry. Appl. Microbiol. Biotechnol. 107(15): 4961-4971. https://doi.org/10.1007/s00253-023-12614-0

Yan, P. M., W. T. Xue, S. S. Tan, H. Zhang and X. H. Chang (2008). Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. Food Control. 19(1): 50-55. https://doi.org/10.1016/j.foodcont.2007.02.008

Zhang, X. H., F. L. Cao, Z. Y. Sun, W. W. Yu, L. G. Zhao, G. B. Wang and T. Wang (2012). Effect of feeding Aspergillus niger-fermented *Ginkgo biloba*-leaves on growth, small intestinal structure and function of broiler chicks. Livest. Sci. 147(1-3): 170-180. https://doi.org/10.1016/j.livsci.2012.04.018

Zhang, Y., Y. J. Zhang, J. L. Jia, H. C. Peng, Q. Qian, Z. L. Pan and D. Y. Liu (2023). Nitrite and nitrate in meat processing: functions and alternatives. Curr. Res. Food Sci. 6: 100470. https://doi.org/10.1016/j.crfs.2023.100470.