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RESEARCH PAPER

The Investigation of the Fermentative, Chemical and Microbial Effects of Grape and Tangerine Pomace Added to High Moisture Alfalfa Silage

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Abstract

This study investigated the impact of grape pulp (GRP) and tangerine pulp (TNP) supplementation (10% each) on alfalfa silage quality. The control silage received no additives. After a two-month fermentation period, silage samples were analyzed for chemical composition, fermentation quality, microflora, and organic acids. According to our results, both silage additives effectively lowered pH levels and isobutyric acid, while increased Fleig score and lactic acid levels compared to control group. However, GRP supplementation significantly increased the levels of crude protein (CP), yeast-mold colony count, and butyric acid levels. Conversely, TNP supplementation resulted in higher levels of water-soluble carbohydrates (WSC), valeric acid and lower acetic acid levels in the alfalfa silage samples. In conclusion, both GRP and TNP supplements have distinct effects on the chemical composition, silage quality, microflora, and organic acid profiles of alfalfa silage. These findings provide valuable insights into optimizing alfalfa silage production and its utilization in animal nutrition. Further research could explore optimal inclusion rates and potential synergistic effects with other additives to enhance silage quality.

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important feed crops globally because of its abundant nutritional availability and high protein content (Guo et al., 2019; Boga and Ayasan, 2022; Şengül *et al.*, 2022). This plant is remarkable for its ability to be harvested three to four times per year (Guo *et al.*, 2019). Withered forms of alfalfa are used more often than silage in dairy nutrition; however, silage may be preferable under wet conditions, such as harvesting in autumn (Gül *et al.*, 2015). Nevertheless, preserving alfalfa in silage form poses problems owing to the limited content of water-soluble carbohydrates (WSC) (Canbolat *et al.*, 2010). The use of additives such as fruit pulp in the ensiling process has been explored as a potential solution to improve silage fermentation characteristics and overall quality (Besharati *et al.*, 2020).

Grapes are among the most extensively cultivated fruits in the world, finding their way into both fresh consumption and the thriving wine and cider industries (Li *et al.*, 2017). As a result of food processing, approximately 15-20% of grapes are left as pulp, contributing to the by-product stream of this versatile fruit (Canbolat *et al.*, 2010). Grape pulp is a valuable source of WSC and various polyphenols including anthocyanins, flavanols, hydroxybenzoic and hydroxycinnamic acids, and stilbenes (Li *et al.*, 2017). Polyphenols are recognized for their beneficial effects on silage fermentation processes, wherein they play a pivotal role in preventing degradation and actively participate in the reduction of silage pH (Ke *et al.*,

2015).

Tangerine is a prominent citrus fruit cultivated in Türkiye, accounting for nearly 5% of the world's total tangerine production (Ertek *et al.*, 2020). Tangerine pulp, on the other hand, is a by-product of tangerine fruit processing and has been shown to contain high levels of antioxidants, carotenoids, and dietary fiber (Rodrigo *et al.*, 2015). Citrus waste materials are susceptible to rapid spoilage and can potentially cause environmental pollution, causing challenges for their proper storage and handling, mainly because of their low dry matter content (Büyükkılıç Beyzi *et al.*, 2018). Therefore, these by-products can be utilized in animal nutrition either in their fresh form or as ensilaged products. Alternatively, they could be used as supplements to enhance the quality of other grass silage materials (Ülger *et al.* 2020).

Although there are studies on the use of grape and citrus by-products in different grass silages, no study has compared the effects of grape pulp and tangerine pulp supplementation in alfalfa silage on the chemical composition, silage quality, microflora of silage, and organic acid profiles.

Materials and Methods

The alfalfa was harvested using hand clippers, ensuring a precise cut of approximately 5 cm above ground level, in the fields of Eskil district in Aksaray Province, Türkiye, during late November 2022. The harvested material was chopped into pieces measuring 2-4 cm and divided into three portions. One portion of the chopped material was ensiled without supplementation (Control), whereas the other two portions were ensiled with the addition of 10% grape pulp (GRP) and 10% tangerine pulp (TNP). All samples were placed in polyethylene vacuum bags and the experiment was conducted with four replicates for each treatment. The chemical composition of fresh alfalfa is shown in Table 1.

Table 1 Chemical analysis of fresh alfalfa before ensiling.

Parameters (%)*	Fresh Alfalfa	GRP	TNP
Dry Matter	18.69	24.73	20,49
Crude Protein	25.98	9.43	6.22
Ether Extract	3.17	5.94	2.93
Crude Ash	13.55	2.51	3.33
WSC	3.11	4.57	5.12
ADF	25.11	41.28	13.87
NDF	31.30	51.28	16.06

Results are given on a dry matter basis, *GRP: Grape Pulp, TNP: Tangerine Pulp, WSC: Water Solvable Carbohydrates, ADF: Acid Detergent Fiber, NDF: Neutral Detergent Fiber,

The silages were stored for two months. Subsequently, upon opening the bags, 40 g of silage samples were taken from each bag and diluted with 360 mL of distilled water. The samples were then filtered through Whatman No.1 filter papers. The pH of the filtrate was assessed using a pH glass electrode (HI 1230 B, Hanna Instruments). The filtrate was diluted at a ratio of 1/100 for both the WSC and organic acid analyses. To facilitate the analysis of organic acids, we added 1.5 milliliters of 1M orthophosphoric acid to the filtrate. The mixture was then centrifuged at 10,000 rpm for 10 min. The filtrate was stored at − -20°C until analysis. A gas chromatograph equipped with a flame ionization detector (GC-FID) was used for butyric, acetic, and propionic acid analyses. An autosampler (Thermo AI-1310, Thermo Scientific, USA) was used for this process. Lactic acid levels were determined according to the protocol described by Barnett (1951). Water-soluble carbohydrate levels in silages were determined following the method described by Dubois *et al.* (1951). The WSC values were multiplied by 10 and divided by DM. The obtained values were multiplied by 10 and divided by the DM of the silage samples to determine the lactic acid content as a percentage of DM. A seven-day aerobic stability test was conducted on the samples following the method developed by Ashbell *et al.* (1991). The Fleig score of the silage was calculated using the following equation: 220 + (2 × dry matter percentage - 15) - (40 × pH).

The chemical compositions of the fresh material and silage were determined according to AOAC (2000). After the alfalfa silage samples were dried at 105°C for 24 h to determine their DM content, the remaining samples were ground and further dried in an oven at 60°C until a constant weight was achieved (approximately 48 h). The ground samples were carefully placed in Ziplock bags for subsequent chemical analyses. For ash analysis, the samples were ashed in a 550°C ash furnace

(NÜVE) to determine the crude ash (CA) content. The nitrogen (N) content of the samples was assessed using the Kjeldahl method, and the resulting N values were multiplied by a conversion factor of 6.25 (N × 6.25) to obtain the crude protein (CP) content. The determination of crude oil values in the samples involved extraction of ether extract (EE) using petroleum ether in a Soxhlet apparatus. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the method described by Van Soest *et al.* (1991) using an Ankom 2000 fiber analyzer (Ankom Technology, Fairport, NY, USA).

After unsealing the silage samples, 10-gram sample was mixed with 90 mL of peptone water for microbiological cultivation using the spread plate technique. The resulting mixture was poured into Petri dishes according to the manufacturer's instructions. Subsequently, the yeast mold (Sırakaya & Büyükkılıç Beyzi, 2022), *Enterobacteria* (ISO 21528-2, 2018), C*lostridia* (ISO 7937, 2004), and lactic acid bacteria (ISO 15214, 1998) counts were determined. The yeast-mold was counted using Potato Dextrose Agar (Merck, Darmstadt, Germany) medium, which was then incubated for 5 days at a controlled temperature of 25±1°C. *Enterobacteria* were counted using Violet Red Bile Agar W/Glucose (Condalab, Madrid, Spain) medium and were subjected to incubation at a precisely controlled temperature of 37±1°C for a duration of 24 h. Similarly, MRS Agar (Merck, Darmstadt, Germany) was used to determine the population of lactic acid bacteria, which were then incubated at a controlled temperature of 37±1°C for an extended period of 72 h. *Clostridia* were enumerated using Reinforced Clostridial Agar (Condalab, Madrid, Spain) medium, which was then incubated for 48 h at a controlled temperature of 35±2°C. For *Clostridia*, the poor-plate method was used, and incubation was performed in an oxygen-free environment.

All data were subjected to analysis of variance (ANOVA) using the General Linear Model of SPSS (version 25.0; IBM Corp., Armonk, NY, USA). Significant differences between individual means were identified using Tukey's multiple-range test. Differences were considered statistically significant at $P<0.05$.

Results

The effects of GRP and TNP supplementation on the nutritional composition of the alfalfa silage are presented in Table 2. These results suggest that the silage additives evaluated in this study did not produce statistically significant differences with respect to EE, CA, CF, ADF, NDF, and ADL content in alfalfa silage compared with Control ($P>0.05$). The CP values in the silage samples supplemented with GRP exhibited a notable increase compared to both Control and TNP-supplemented samples ($P<0.05$). However, WSC levels were statistically higher in the TNP-treated silage than in the GRP-treated silage and Control ($P<0.05$).

Table 2 Effects of GRP and TNP supplementation on the chemical composition of alfalfa silage

Parameters (%)*	Silage Additives			P
	Control	GRP	TNP	
CP	21.93±0.96^{ab}	23.82±1.43^{a}	21.72±0.90^{b}	0.022
EE	3.80±0.48	4.57±0.89	4.88±1.81	0.368
CA	13.06±0.61	12.68±1.79	11.25±1.45	0.133
WSC	1.68±0.08^{b}	2.06±0.15^{b}	2.59±0.46^{a}	0.001
ADF	19.42±1.08	18.82±1.16	19.32±0.87	0.632
NDF	27.45±1.96	29.07±1.62	28.22±0.96	0.300
ADL	6.98±1.60	7.46±1.86	5.05±0.85	0.058
CF	12.44±2.65	11.35±2.65	14.27±0.47	0.144

Results are given on a dry matter basis, *GRP: Grape Pulp, TNP: Tangerine Pulp, CP: Crude Protein, EE: Ether Extract, CA: Crude Ash, WSC: Water Solvable Carbohydrates, ADF: Acid Detergent Fiber, NDF: Neutral Detergent Fiber, ADL: Acid Detergent Lignin, CF: Crude Fiber.
a–b Means in the same row followed by different superscript letters are significant difference ($P<0.05$).

The effects of GRP and TNP supplementation on alfalfa silage quality parameters are presented in Table 3. The present study revealed that DM and CO2 values were unaffected by silage additives ($P>0.05$). Conversely, the use of silage additives resulted in a significant reduction in pH levels compared with Control ($P<0.001$). Additionally, the silage additives led to a noteworthy enhancement in the Fleig score values when compared to the alfalfa silage samples in

Control (P<0.001).

Table 3 Effect of GRP and TNP supplementation on DM, pH, Fleig Score and CO2 values of alfalfa silage.

Parameters*	Silage Additives			P
	Control	GRP	TNP	
DM %	19.75±0.51	19.30±0.29	19.13±0.34	0.067
pH	6.06±0.16[a]	5.23±0.11[b]	5.36±0.17[b]	0.000
Fleig Score	2.15±5.65[b]	34.6±3.92[a]	29.0±6.58[a]	0.000
CO_2, g/Kg	31.5±15.9	25.8±10.6	27.2±2.17	0.760

*GRP: Grape Pulp, TNP: Tangerine Pulp, DM: Dry Matter.
a–b Means in the same row followed by different superscript letters are significant difference (P<0.05).

The fermentation profiles of the alfalfa silages enriched with GRP and TNP are presented in Table 4. According to these findings, silage additives did not exert a statistically significant influence on propionic acid levels in the alfalfa silage samples (P>0.05). In contrast, butyric, valeric, and isobutyric acid levels exhibited a notable response to GRP and TNP treatments in the alfalfa silage (P<0.05). Similarly, acetic acid levels in the GRP- and TNP-treated groups were significantly lower, whereas lactic acid levels were higher in the same groups than in Control (P<0.001). TNP supplementation led to the most pronounced increase in lactic acid levels compared with Control and GRP-supplemented silage samples. Furthermore, the inclusion of TNP resulted in the lowest butyric acid levels among all treatments in this study.

Table 4 Effect of GRP and TNP supplementation on fermentation profiles of alfalfa silages.

Parameters (%)*	Silage Additives			P
	Control	GRP	TNP	
Butyric Acid	0.17±0.04[a]	0.21±0.05[a]	0.08±0.01[b]	0.003
Propionic Acid	0.07±0.02	0.06±0.01	0.05±0.01	0.141
Acetic Acid	1.08±0.23[a]	0.30±0.05[c]	0.73±0.17[b]	<0.001
Lactic Acid	6.01±0.20[b]	6.80±0.45[a]	7.07±0.21[a]	<0.001
Valeric Acid	0.040±0.000[b]	0.041±0.001[a]	0.040±0.000[b]	0.007
Isobutyric Acid	0.057±0.003[a]	0.052±0.002[b]	0.049±0.001[b]	0.002

Results are given on a dry matter basis, *GRP: Grape Pulp, TNP: Tangerine Pulp
a–b Means in the same row followed by different superscript letters are significant difference (P<0.05).

The effects of GRP and TNP supplementation on the microbiological values of the alfalfa silage are presented in Table 5. The results indicated that *Clostridium* and lactic acid bacterial populations were not significantly affected by supplementation (P>0.05). In contrast, GRP (3.13) and TNP (0.00) supplementation resulted in fewer *Enterobacteria* colonies in the medium than Control (7.65) (P<0.001). Furthermore, yeast-mold colonies were significantly reduced in the alfalfa silage samples supplemented with TNP compared to both Control and GRP-treated silage samples (P<0.05).

Discussion

In the present study, the silages supplemented with GRP exhibited the highest levels of CP. Similarly, Bulut *et al.* (2023) also observed an increase in CP levels in sorghum-sudan grass silages supplemented with grape pulp, albeit not statistically significant, but evident in numerical terms, at various inclusion rates ranging from 10% to 40%. Similarly, in a study conducted by Li *et al.* (2017), the CP content of sweet sorghum silage treated with a combination of 10-15% grape pulp and lactic acid bacteria inoculant was higher than that of groups that were not supplemented with grape pulp. These results could be attributed to the inhibitory effect of polyphenols present in grapes on the proteolytic enzyme activity of bacteria in silages. This inhibition may lead to an improvement in the utilization of silage nitrogen content, resulting in higher levels of crude protein in the treated samples (Ke *et al.*, 2015; Li *et al.*, 2017). Moreover, WSC levels were significantly

higher in TNP-treated silage and GRP-treated alfalfa silage, not statistically but numerically. Similar to our findings, other researchers have also observed that by-products of grape pulp (Canbolat *et al.*, 2010) and citrus fruits (Tao *et al.*, 2021) were added to their products to increase the WSC values. It has been previously reported that citric acid suppresses the growth of undesirable microorganisms, which may affect the utilization of WSC by these bacteria (Tao *et al.*, 2021).

Table 5 Effect of GRP and TNP supplementation on microbiological values of alfalfa silage.

Parameters (log10 cfu/g of FM)*	Silage Additives			P
	Control	GRP	TNP	
Clostridia	6.30±0.86	6.52±1.16	6.54±0.65	0.841
Enterobacteria	7.65±0.28[a]	3.13±0.46[b]	0.00[c]	<0.001
Lactic acid bacteria	6.25±0.69	6.95±0.16	6.72±0.70	0.673
Yeast-Mold	+++	+++++	+	

*GRP: Grape Pulp, TNP: Tangerine Pulp, FM: Fresh Matter, +: indicator of growing density

a–c Means in the same row followed by different superscript letters are significant difference (P<0.05

In the present study, it was noted that the incorporation of silage additives led to a reduction in the pH levels of alfalfa silage when compared to Control. An investigation conducted on alfalfa silages demonstrated that pH levels decreased at both 0 and 5 d after air exposure as grape pulp levels increased (Canbolat *et al.*, 2010). Similarly, Li *et al.* (2017) found that the final pH of sweet sorghum silage decreased with both 10% GRP and lactic acid bacteria supplementation compared with Control. Moreover, investigations carried out on Marandu grass silage (Bernardes *et al.*, 2005), untreated lucerne (Besharati *et al.*, 2022), and elephant grass silage (Gomes *et al.*, 2017) demonstrated that the introduction of citrus pulp or its by-products resulted in decreased pH levels compared with Control. Both grapes and citrus fruits contain soluble sugars that act as substrates for lactic acid bacteria. SE On the other hand, in some situations, the buffering capacity of alfalfa might hinder the decrease in pH, even when a sugar source is added. Bulut *et al.* (2023) reported that both white and black grape pulp supplementation increased pH levels in sorghum sudan grass silage compared with silage samples without supplementation.

The Fleig score is an indicator of fermentation quality and preservation of alfalfa silage, and a higher Fleig score is associated with good preservation and enhanced silage fermentation (Gao *et al.*, 2021). In the current study, the untreated alfalfa silage samples had the lowest Fleig score values (2.15±5.65) compared to the silage samples treated with GRP (34.6±3.92) and TNP (29.0±6.58). This result was consistent with that of Gao *et al.* (2021), who documented that the Fleig scores of alfalfa silages were elevated in samples treated with various carbohydrate sources, including pectin, starch, molasses, and fructose. Similarly, another investigation of raw lucerne silages observed an increase in Fleig score values with varying substitutions of lemon pulp (Besharati *et al.*, 2022). The Fleig score exhibits a negative correlation with pH and a positive association with the DM content of the silage (Gao *et al.*, 2021). Lower pH levels in the treatment groups could be the reason for the higher Fleig scores.

Butyric acid is an important indicator of silage quality and the fermentation process (Tao *et al.*, 2021). In the current study, the silage samples in Control exhibited a butyric acid level of approximately 0.17% in DM, whereas samples with GRP addition had a level of approximately 0.21% in DM, and those supplemented with TNP had a level of 0.08% in DM. Moreover, isobutyric acid levels decreased in the treated silages. Butyric acid levels of approximately %0.5 and %1 are acceptable for legume silages with lower than %30 DM (Kung *et al.*, 2018). In our study, the application of TNP significantly decreased butyric acid levels in ensiled alfalfa compared with Control and GRP. Similar to our results, another study conducted on Napier grass silage showed a decrease with the addition of citric acid residue at 45 days of ensiling (Tao *et al.*, 2021). Rapid production of lactic acid could result in the inhibition of clostridial fermentation, specifically the formation of butyric acid in silage, which is attributed to the heightened osmotic pressure and reduced pH levels (Kung *et al.*, 2018). Conversely, the levels of butyric acid were elevated in silage samples supplemented with GRP, in contrast to the TNP group. Grape pulp naturally contains yeasts (Zott *et al.*, 2010). Higher pH levels and an extended fermentation process, resulting from the absence of antifungal organic acids, may prove

inadequate in suppressing yeast proliferation within silage, consequently leading to an increase in silage temperature (Kung *et al.*, 2018). Elevated temperatures may trigger a shift in the end-product from acetate to butyrate during the fermentation process of food and feed (Wang *et al.*, 2020). This observation could potentially explain the higher presence of butyric acid, coupled with an elevated yeast population and reduced acetic acid levels in the alfalfa silages supplemented with GRP, compared to Control and TNP groups.

In the present study, both treatments led to elevated levels of lactic acid while concurrently suppressing acetic acid levels in alfalfa silage, compared to Control. The levels of WSC in fruit pulp facilitate the proliferation of lactic acid bacteria, leading to an increase in lactic acid production in the silage (Canbolat *et al.*, 2010). Numerous studies have demonstrated elevated lactic acid levels in various silages treated with citrus (Besharati *et al.*, 2022; Gomes *et al.*, 2017) and grape pulp (Canbolat *et al.*, 2010; Ke *et al.*, 2015). Acetic acid is an important volatile acid in silage owing to its inhibitory effect on yeast proliferation and its capacity to enhance aerobic stability when the silage is exposed to air (Kung *et al.*, 2018). In the present study, acetic acid levels were diminished in the treatment groups compared to Control. Furthermore, a study focusing on various levels of citric acid residue-treated Napier grass silage showed a reduction in the acetic acid content (Tao *et al.*, 2021). Similarly, Bulut *et al.* (2023) reported lower acetic acid levels in Sudan grass silage supplemented with 40% GRP. The higher acetic acid content in Control could be attributed to undesired bacterial activity, potentially stemming from elevated pH levels in these silages (Tao *et al.*, 2021).

In the current study, yeast levels were significantly higher in GRP-treated silage than with GRP compared Control and TNP-treated silage. This observation is supported by the findings of Ke *et al.* (2015), who reported a greater number of yeast colonies in alfalfa silage treated with GRP. Furthermore, the lowest yeast levels in alfalfa silage were observed in TNP-treated silage. Similar to our results, Ke *et al.* (2017) demonstrated that treating alfalfa silage with 0.1% citric acid resulted in a decrease in yeast population. As previously stated, grapes can naturally contain yeasts. Additionally, higher pH and lower acetic acid levels in silage can contribute to the proliferation of yeast (Kung *et al.*, 2018). *Enterobacteria*, along with clostridial, yeast, and mold, constitute another group of undesirable bacteria that can lead to aerobic deterioration and subsequent nutritional losses (Tao *et al.*, 2021). In the present study, both GRP- and TNP-treated alfalfa silage exhibited lower counts of *Enterobacteria* than Control. It has been reported that *Enterobacteria* are sensitive to pH levels below 5.0, which could elucidate the higher counts observed in Control (Bernardes *et al.*, 2005).

Conclusion

Overall, post-ensiling alfalfa silages treated with both GRP and TNP showed improved fermentation quality. This improvement can be attributed to their capacity to lower pH levels by increasing lactic acid content, which may be linked to the WSC content in the fruit pulps. While GRP supplementation exhibited a more pronounced effect in preserving CP content, TNP-treated alfalfa silages demonstrated higher levels of WSC, lower concentrations of butyric acid, and a more effective inhibition of yeast-mold and *Enterobacteria* growth. This study demonstrated the efficacy of fruit pulp supplementation, particularly tangerine pulp supplementation, in improving the nutritional content and fermentation quality of alfalfa silage. These findings have implications for optimizing silage production practices, particularly in regions with abundant access to fruit-processing byproducts. Future research should further explore the nuanced interactions between fruit pulp composition and silage fermentation dynamics for more refined and tailored approaches to silage production.

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Author contributions

First Author: Conceptualization, Formal Analysis, Statistical Analysis, Writing -review and editing; Second Author: Funding Acquisition, Project Administration, Resources; Third Author: Data Curation, Formal Analysis, Investigation, Methodology, Visualization; Fourth Author: Formal Analysis, Supervision, Writing - review and editing; Fifth Author: Formal Analysis, Writing -review and editing. Sixth Author: Formal Analysis, Writing -review and editing.

Conflict of Interest

The authors declare no conflicts of interest.

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