KSÜ Tarım ve Doğa Derg 26 (2), 450-458, 2023
KSU J. Agric Nat 26 (2), 450-458, 2023
https://doi.org/10.18016/ksutarimdoga.vi.1052345

Effect of Adding Flaxseed Essential Oil in Alfalfa Ensiling Process on Ruminal Fermentation Kinetics

Maghsoud BESHARATI[1], Valiollah PALANGI[2], Masomeh NIAZIFAR[3], Tugay AYAŞAN[4]
Abdelfattah ZM. SALEM[5]
[1,3]University of Tabriz, Ahar Faculty of Agriculture and Natural Resources, Department of Animal Science, 51666, Tabriz, Iran, [2]Department of Animal Science, Agricultural Faculty, Ataturk University, 25240, Erzurum, Türkiye, [4]Osmaniye Korkut Ata University, Kadirli Faculty of Applied Sciences, Osmaniye, Türkiye, [5]Department of Pharmacology, Anesthesia and Analgesia, Faculty of Veterinary Medicine and Animal Science, Autonomous University of the State of Mexico, Toluca, State of Mexico, Mexico
[1]https://orcid.org/0000-0002-5233-6425, [2]https://orcid.org/0000-0001-6470-2608, [3] https://orcid.org/0000-0003-0672-4846
[4]https://orcid.org/0000-0001-7397-6483, [5]http://orcid.org/0000-0001-7418-4170
✉: mbesharati@tabrizu.ac.ir ; tayasan@gmail.com

ABSTRACT
The experiment's purpose was to evaluate the effect of adding different levels of essential flaxseed oils (FEO) on the chemical composition and *in vitro* degradability of alfalfa silage. Treatments were alfalfa silage with no additive (control) or treated with 60 or 120 ml/kg DM of essential flaxseed oils (FEO60, FEO120, respectively). Whole plant alfalfa was ensiled for 60 d in triplicate laboratory-scale tubes. Dry matter (DM) content was more significant for FEO120 than control. Compared with the control, neutral detergent fiber (NDF) concentration was decreased in FEO120. Adding essential oils to the silage significantly decreased silage pH compared with untreated silage ($P < .0001$). Interestingly increased (DM and OM digestibility) for all the silages containing essential oil compared with untreated silage. The addition of flaxseed essential oil to alfalfa silage increased the rate of disappearance of organic matter (OM) and dry matter (DM) in all treatments compared to the control treatment. The degradability potential of alfalfa silage has increased in treatments containing FEO (120 mg/kg DM). In general, the obtained data show that the FEO had a positive effect on the quality of alfalfa silage and its nutritional characteristics.

Animal Science

Research Article

Article History
Received : 02.01.2022
Accepted : 30.06.2022

Keywords
Essential oils
In vitro degradability
Secondary metabolite
Silage

Yonca Silolama Işlemine Keten Tohumu Esansiyel Yağı İlavesinin Rumen Fermantasyon Kinetiğine Etkisi

ÖZET
Yonca, dünya çapında yaygın olarak yetiştirilen ruminant hayvanların beslenmesinde en önemli yem kaynaklarından biridir. Bununla birlikte, kurutma ve depolama işlemi sırasında mekanik işlemlerden kaynaklanan birçok besin kaybı olmaktadır. Bu nedenle yoncanın silolanması kurutma kayıplarını önlemenin alternatif bir yoludur. Bu çalışmanın amacı, farklı seviyelerde keten tohumu esansiyel yağlarının (KEY) eklenmesinin yonca silajının kimyasal bileşimi ve *in vitro* sindirilebilirliği üzerindeki etkisini değerlendirmektir. Muameleler, katkı maddesi içermeyen yonca silajı (kontrol) veya 60 veya 120 ml/kg KM keten tohumu uçucu yağları (sırasıyla KEY60, KEY120) olacak şekilde düzenlenmiştir. Tüm bitki yonca silajları, üç tekerrür olacak şekilde laboratuvar ölçekli tüplerde 60 gün boyunca silolanmıştır. KEY120 için kuru madde (KM) içeriği kontrolden daha büyük olarak bulunmuştur. Kontrol grubu ile karşılaştırıldığında, KEY120'de nötr deterjan fiber (NDF) konsantrasyonu azalmıştır. Uçucu yağların eklenmesi ve silajın kombinasyonu, işlem görmemiş silaja kıyasla silaj pH'ını önemli ölçüde azaltmıştır ($P<.0001$). Kontrol ile karşılaştırıldığında, uçucu yağ içeren tüm silajlarda ilginç bir şekilde artmıştır. Yonca silajına keten tohumu esansiyel yağı ilavesi, kontrol uygulamasına kıyasla tüm uygulamalarda organik madde (OM) ve kuru madde (DM) sindirilebilirlik oranını artırmıştır. Yonca silajının

Zootekni

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 02.01.2022
Kabul Tarihi : 30.06.2022

Anahtar Kelimeler
Esansiyel yağlar
İn vitro sindirilebilirlik
İkincil metabolit
Silaj

yıkılabilirlik potansiyeli, KEY (120 mg/kg KM) içeren gruplarda artmıştır. Genel olarak elde edilen veriler, KEY'in yonca silajının kalitesine ve besleyici özelliklerine olumlu etkisi olduğunu göstermektedir.

Atıf Şekli: Besharati, M., Palangi, V., Niazifar, M., Ayaşan, T. & Salem, A.Z.M. (2023). Yonca Silolama Işlemine Keten Tohumu Esansiyel Yağı Ilavesinin Rumen Fermantasyon Kinetiğine Etkisi. *KSÜ Tarım ve Doğa Derg 26* (2), 450-458. https://doi.org/10.18016/ksutarimdoga.vi.1052345

To Cite : Besharati, M., Palangi, V., Niazifar, M., Ayaşan, T. & Salem, A.Z.M. (2023). Effect of Adding Flaxseed Essential Oil in Alfalfa Ensiling Process on Ruminal Fermentation Kinetics. *KSU J. Agric Nat 26* (2), 450-458. https://doi.org/10.18016/ksutarimdoga.vi.1052345.

INTRODUCTION

An essential component in ruminant diets is forage; the leguminous family is of particural importance among the forages. Of this family, alfalfa is more critical. One of the important characteristics of alfalfa is its high nutritional quality for livestock. Forage storage is essential for long-term use in many areas when fresh forage is unavailable for ruminant diets. In countries where the growing season is limited, silage and hay play an important role in the diet of ruminants. Ensiling is a method of preserving a product based on the natural fermentation of lactic acid under anaerobic conditions, which can provide fodder throughout the year as the primary source of nutrition, and provide high nutritional value for ruminants. One of the main problems in preparing quality silage is rapid lowering of the pH of the silo in a short time (Besharati et al., 2021; Besharati et al., 2020a; He et al., 2020). Silo additives can be classified as fermentation stimulants and food spoilage inhibitors (Erb and Kliebenstein, 2020). In addition, corruption inhibitors can be divided into subgroups of inhibitors, fermentation, and aerobic stability enhancers in which plant essential oils (EOs) preserve silage nutrients by inhibiting the growth of undesirable microorganisms. Because of increasing public pressure to decrease antimicrobials in livestock production and the regulations that ban these substances in Europe (More, 2020), scientists and the livestock feed industry have been actively working to find alternative antimicrobials (Grant et al., 2018). EOs and their compounds have attracted much attention because their antimicrobial properties may modulate rumen fermentation (Garcia et al., 2020). EOs from aromatic and medicinal plants have been shown to have selective antimicrobial properties and a high potential for binding to proteins (Ayaz et al., 2017).

Flaxseed oil contains α-linolenic acid (ALA) and linolenic acid, the rich source of ω-3 fatty acids in nature and, has more than twice as much omega-3 fish oils. FEO includes a high level of ALA (approximately 55% of total fatty acids). Besharati et al. (2020a), in another study, showed that FEO had a positive effect on silage quality and compositions. A study found that diets rich in unsaturated omega- ω-3 fatty acids before calving were fed to sheep and cattle, respectively, delayed calving time and increased placental abruption. However, increased in ω-3 unsaturated fatty acids in the diet after childbirth improved cow pregnancy (William et al., 2000).

The specific purpose of this study was to evaluate the effects of supplementing different levels of FEO on chemical composition and *in vitro* degradability parameters in alfalfa silage.

MATERIAL and METHODS

Green flax seeds (about 2 kg) were crushed and sifted with a mill. 200 g of milled flaxseed was immersed by maceration using an n-hexane solvent (Sayyah et al., 2005). The EOs were stored at 4°C until they were used in the experiment (Table 1).

Table 1. Aromatic compounds in flaxseed essential oil (percentage of total compounds)
Çizelge 1. Keten tohumu esansiyel yağındaki aromatic bileşikler (Toplam bileşiklerin %'si olarak)

Compounds	%
75.198	9-Octadecenoic acid
1.640	Decane
0.632	Dodecane
0.613	Hexadecanoic acid
0.375	Linolenic
6.020	Linoleic acid
0.213	Nonane
4.380	Octadecanoic acid
0.526	Octane
0.213	Nonane
5.207	Pentadecene
0.113	Tridecane
0.188	Tetradecane
0.964	Undecane
75.198	9-Octadecenoic acid

Silage preparation and experimental treatments

The alfalfa samples were collected from a field in East Azarbaijan province, then ensiled in laboratory-scale mini silos for 60 days after chopping theoretically at 3-5 cm length. The alfalfa was treated with no additives (control) or treated with EO at the rate of 60 or 120 mg/kg. The EO used was FEO (FEO60 and FEO120). Each treatment was provided in triplicate tubes. Before ensiling, FEO was dissolved in aqueous ethanol

(1 cc) (Chaves et al., 2012) and sprayed onto the chopped alfalfa (3 Kg). The exact amount of ethanol (1 cc) was used for the control. The mini-silos (10 cm diameter and 70 cm height) were sealed and stored at room temperature (25°C to 28°C) for 60 days. After 60 days of ensiling, the silos were opened and used to analyze chemical and fermentation quality.

The chemical compositions of the silage samples were determined immediately after the opening. After the opening of silos, the pH, soluble carbohydrate (WSC) and dry matter (DM) of the samples were determined. After drying and grinding, to determine the dry matter (DM), the silage samples were dried in an oven for 48 hours after leaving the freezer at 65 °C and ground with a 1 mm sieve. DM, crude protein (CP), ash (CA), and ether extract (EE) contents were determined by the procedures given by AOAC (2002). To measure Ash, milled samples were placed in a furnace at 550 °C for 5 hours.

The acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined according to Van Soest et al. (1991) procedures without the use of amylase and sodium sulfite. The aqueous extract of ensiled samples was prepared by mixing 20 g of silage with 180 ml of deionized water and homogenizing this mix for 1 min. Silage pH was determined using a pH meter. The ammonia-N (NH_3-N) concentration of acidified silage extracts was determined using the Kjeldahl method. To measure the WSC content by phenolic sulfuric acid method (Dubius et al., 1956), 10 g of fresh silage was poured into 90 cc of distilled water. After diluting the samples, it was measured at 470 nm with a spectrophotometer. Total volatile fatty acids (tVFA) contents in silages were determined using the method described by Markham (1942). Metaphosphoric acid (1 ml of 25%) was added to filtered extract (5 ml) to determine tVFA. The method of Borshchevskaya et al., (2016) was used to determine lactic acid (LA) contents.

In Vitro Degradability

Rumen fluid was collected in a slaughterhouse using a four-layer cheesecloth and transferred rapidly to the laboratory in a 39° C water flask. Approximately 300 mg of silages was added to serum bottles. The 20 mL of buffered rumen fluid with McDougall's buffer was pipetted into each serum bottle. The degradability was recorded after 2, 4, 8, 12, and 24 h of incubation. The degradability values are expressed as % of DM (Besharati et al., 2008).

Rate and extent of degradability was determined for each treatment by fitting data to the non-linear function $Y = a + b\,(1 - e^{-ct})$,

Where, y is the volume of degradability at time t, $a+b$ the fermentation of soluble and insoluble fraction (% of DM), and c the constant fractional rate of fermentation (Ørskov and McDonald, 1979).

$$ED = a + b \times c/(c + 0.05)$$

Where, effective degradability is 24 h fermentation (% of DM); a, b and c are soluble part (%), insoluble with degradability (%) and rate of degradability (DM/h), respectively (Ørskov & McDonald, 1979).

Statistical analysis

Data obtained from chemical composition and degradability were subjected to analysis of variance as a completely randomized design by the GLM procedure of SAS (2000). The significance of differences among treatments was tested using Duncan test. Differences were declared as significant at $p \leq 0.05$.

RESULTS and DISCUSSION

The DM, OM, CP, WSC, ADF and NDF contents of alfalfa before ensiling were 22.20, 88.40, 19.60, 3.74, 17.00 and 24.80%, respectively. The effects of EO on the chemical composition of silages are shown in Fig 1. Adding FEO increased DM and CP contents of silages and decreased pH and NDF, and ADF contents. The effects of EOs on DM can depend on the source of the EO. Interactions or the habituation of microbial populations to plant EOs varies. The increase in DM content is probably due to the restriction of growth and development of a certain group of microorganisms in silage and, as a result, less loss of silage nutrients. Relative to the control, CP concentration was increased with treatments supplemented with EOs ($P<0.001$). Turan et al. (2018) did not observe any change in crude protein by adding plant EOs, probably related to the amount of use and the type of silage. Some previous studies have reported the inhibitory effects of some plant EOs, including peppermint, on Clostridia growth (IVergis et al., 1990). This may justify the increase in CP in EO-containing silage. The FEO60 and FEO120 silages had lower NDF concentrations compared with the control ($p<0.001$). The ADF content decreased in all treatments compared to the control silage.

The addition of EOs to alfalfa silage decreased the pH value ($p<0.001$) compared with the control. This decrease in pH is due to lactic acid bacteria, which increase lactic acid production in silos and reduce acetic and butyric acid (Rowghani et al., 2018). On the other hand, lowering the pH reduces the action of proteolysis and plant enzymes or respiratory enzymes, that of silage corruption, and prevents the conversion of protein to non-protein nitrogen. The effects of EOs on DM can depend on the source of the EO; interactions or the habituation of microbial populations to plant EOs varies. The increase in DM content is probably due to the restriction of growth and development of a certain group of microorganisms in silage and, as a result, less loss of silage nutrients. Palangi & Macit (2021) stated that the percentage of plant dry matter depends on many factors such as species, growth stages of different parts of the plant, soil moisture, and

rainfall. The chemical composition of forage plants under natural conditions is influenced by topographic features, climatic conditions, harvest time, and folds.

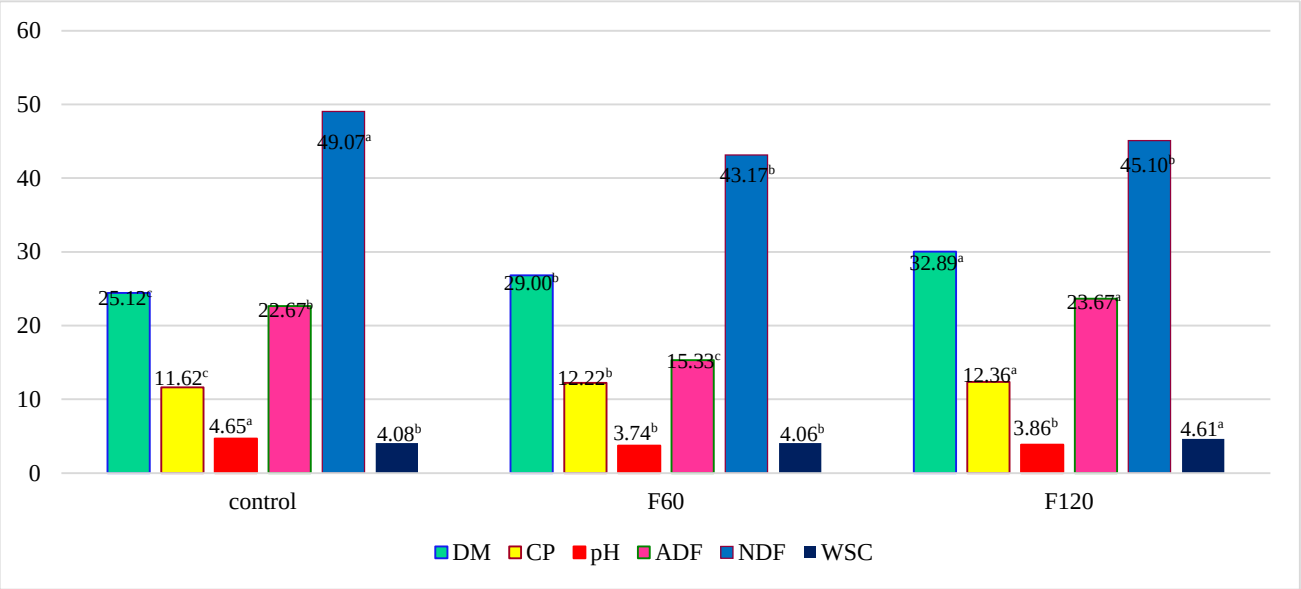

Figure 1. Effect of FEO on chemical composition of silage after 60 d of silage (DM, %)
Şekil 1. KEY'in 60 günlük silajdan sonraki silajın kimyasal kompozisyonu üzerine etkisi (%KM)
Trt: control: silage without additives, F60: silage with 60 ml FEO/kg, F120: silage with 120 ml FEO/kg. Chemical composition[2]: DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; CA, crude ash; WSC: water soluble carbohydrate; ADF, acid detergent fiber. The means without common letter(s) differ ($p<0.05$)

In vitro Degradability

The results of degradability values and degradability parameters are shown in Table 2. Adding FEO to alfalfa silage increased DM, OM degradability and the rate of degradation of OM and DM in all treatments compared to the control ($p<0.05$). After 24 h of incubation, the treatment FEO120 had the highest ($64.45^{a}\pm0.040$), and the treatment control had the lowest OM degradability ($61.84^{c}\pm0.037$) ($p<0.05$). According to the presented results, 2 h after incubation, the highest rate of CP degradability was for control, and the lowest rate of CP degradability was for treatment FEO60. In 24 h after incubation, treatment FEO60 had the highest degradability rate. The concentration of EOs in plants is affected by factors such as species, subspecies, geographical location, the harvest time of the plant, and the part used for EO collection. In addition, factors such as light, heat, and moisture stress also affect their concentration in the plant.

The effects of EOs on rumen microbial populations are dose-dependent (Macheboeuf et al., 2008). The results of this experiment agree with the findings of Chaves et al. (2012) and Hodjatpanah et al., (2016). In another experiment, the processing of alfalfa silage with thyme EOs decreased *in vitro* degradation (Amini Pour et al., 2017). The experiment of Hodjatpanah et al. (2016) examined the EOs of mint, oregano, thyme, cumin, and cinnamon to change silage fermentation and gas production that the degradability of EO treatments in 24 hours of incubation increased significantly compared to the control. Salam et al. (2011) reported a decrease in the digestibility of dry matter and organic matter due to the addition of EOs. In an experiment conducted by Newbold et al. (2004), adding a mixture of active compounds of plant EOs to the diet of adult sheep did not affect the degradability of dry matter. Adsogan et al. (2004) reported that inoculation with EOs increased the digestibility of OM, DM, NDF, and ADF.

Degradation Parameters

The fermentation properties of CP, DM, NDF, and OM of alfalfa silage are presented in Table 3. The results show that FEO60 significantly increases the effective degradability (ED), increasing the soluble fraction of DM (a) ($56.89^{c}\pm 0.58$, $61.88^{a}\pm0.56$, $54.7^{b}\pm0.67$).

The degradability potential (PD) of alfalfa silage in the FEO120 increased, which is statistically significant with the control ($p<0.05$) ($63.75^{b}\pm0.31$, $64.56^{ab}\pm0.37$, $64.57^{a}\pm0.37$). Decreased of insoluble part (b) degradability of alfalfa silage by adding FEO60, which was significant compared to the control. Increasing or decreasing the degradability of the insoluble part depends on the dose used at the silage level. The constant DM degradability rate, FEO60, was significantly different from the control and was numerically higher than theirs. According to the reported results, the organic matter solution (a) was significantly different, statistically significant from the

control treatment. FEO60 treatment significantly increased the soluble fraction of OM compared to the control. The ED of organic matter was the highest for FEO60. According to the reported results, the rate of degradability of the rapidly decomposing part (a) of crude protein, FEO60, has significantly increased with the control. The results of this experiment showed that the addition of EOs to alfalfa silage caused a significant decrease in the insoluble fraction (b) of CP ($p < 0.05$). In terms of effective degradability (ED) of crude alfalfa silage protein, the highest amount is associated with the control, and the lowest amount of ED is related to F120 at a passing rate of 2% per hour, which shows a significant decrease compared to all treatments ($p < 0.05$). However, more tests are needed to determine the appropriate dose of EO; different species of EOs can alter the binding and colonization of ruminal microbes relative to plant materials entering the rumen and are likely to affect the segregation of insoluble protein sources as opposed to soluble protein sources (Moselhy et al., 2016). Due to the low amount of soluble carbohydrates, alfalfa makes the fermentable substrate a limiting factor in changing silage compositions. In this case, adding compounds such as EOs with cell wall digestion provides the amount of substrate available for fermentation (Besharati et al., 2019). High cell wall digestibility positively affects dry matter intake, while cell wall concentration negatively correlates with the amount of feed cell wall (Duodu et al., 2003).

Table 2. The effect of FEO on fermentation properties of alfalfa silage (% of DM).
Çizelge 2. Keten tohumu esansiyel yağının yonca silajının fermentasyon özelliklerine olan etkisi (%KM)

Incubation times (h)					Trt[1]
24	12	8	4	2	**DM(%)**
61.88^{c}±0.53	51.24^{c}±0.37	46.64^{b}±0.63	34.99^{c}±0.35	24.93^{c}±0.48	Control
65.82^{a}±0.55	57.97^{a}±0.37	52.26^{a}±0.63	45.42^{a}±0.35	34.28^{a}±0.36	FEO60
63.88^{b}±0.60	52.94^{b}±0.39	46.42^{b}±0.67	35.70^{b}±0.35	32.44^{b}±0.36	FEO120
0.319	0.702	0.728	0.355	0.605	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*P-value*
					OM(%)
61.84^{c}±0.037	52.52^{b}±0.15	38.33^{c}±0.24	27.67^{c}±0.68	27.67^{b}±0.57	Control
62.63^{b}±0.049	59.92^{a}±0.15	54.26^{a}±0.24	43.99^{a}±0.53	32.29^{a}±0.41	FEO60
64.45^{a}±0.040	52.97^{b}±0.15	47.43^{b}±0.24	32.70^{b}±0.37	31.45^{ab}±0.57	FEO120
0.084	0.073	0.041	0.218	0.051	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*p-value*
					CP(%)
56.52^{a}±0.22	45.32^{a}±0.14	34.44^{a}±0.22	23.16^{ab}±0.33	17.59^{a}±0.21	Control
35.18^{c}±0.21	28.99^{c}±0.18	23.07^{c}±0.23	17.01^{b}±0.32	13.47^{c}±0.25	FEO60
40.54^{b}±0.22	33.05^{b}±0.11	30.30^{b}±0.22	24.64^{a}±0.32	14.05^{b}±0.21	FEO120
0.269	0.186	0.202	0.383	0.156	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*p-value*
					NDF(%)
47.43^{a}±0.45	36.76^{a}±0.33	31.57^{a}±0.14	26.67^{a}±0.15	24.32^{a}±0.24	Control
42.17^{c}±0.45	32.60^{b}±0.33	26.55^{c}±0.13	22.20^{c}±0.24	20.06^{b}±0.28	FEO60
45.10^{b}±0.46	32.77^{ab}±0.33	29.03^{b}±0.12	24.11^{b}±0.17	21.96^{ab}±0.29	FEO120
0.437	0.380	0.147	0.081	0.125	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*p-value*
47.43^{a}±0.45	36.76^{a}±0.33	31.57^{a}±0.14	26.67^{a}±0.15	24.32^{a}±0.24	Control

Trt[1]-control: silage without additives, FEO60: silage with 60 ml FEO/kg, FEO120: silage with 120 ml FEO/kg. Means within same column with different superscripts differ ($P<0.05$).

Ruminal Metabolites

The effect of adding EOs in this study on the concentration of tVFA and NH_3-N and pH alfalfa silage in 24 hours after incubation is shown in Table 4. After 24 hours of incubation, ammonia nitrogen in FEO60 increased, and the control decreased ($p < 0.05$). Ammonia-producing bacteria may show different sensitivities to the addition of EOs. EOs and their compounds have been shown to affect ruminal nitrogen metabolism in dose-dependent methods. The results show that the addition of FEO120 caused a significant increase in tVFA ($p < 0.05$). The lowest amount of tVFA was related to the treatment control. However, decreased ruminal ammonia N concentration and decreased tVFA concentration indicate that fermentation in the diet as a whole has decreased. The pH changes 24 hours after incubation show that the pH of all treatments increased slightly.

Table 3. The effect of different levels of FEO on fermentation properties parameters of alfalfa silage
Çizelge 3. Farklı düzeylerdeki keten tohumu esansiyel yağının yonca silajının fermentasyon özelliklerine olan etkisi

Items								Treatment
K(0.08)	*K(0.05)*	*K(0.01)*	*ED*	*PD*	*c*	*b*	*a*	**DM**
44.63^{c}±0.22	50.46^{c}±0.24	60.13^{c}±0.37	56.89^{c}±0.58	63.75^{b}±0.31	0.125^{b}±0.02	49.01^{a}±0.62	14.73^{b}±0.68	Control
51.07^{a}±0.19	54.87^{a}±0.22	61.83^{b}±0.37	61.88^{a}±0.56	64.56^{ab}±0.37	0.17^{a}±0.03	40.84^{c}±0.66	23.23^{ab}±0.63	FEO60
47.93^{b}±0.16	53.67^{b}±0.52	67.00^{a}±0.39	54.7^{b}±0.67	64.57^{a}±0.37	0.075^{c}±0.02	47.89^{b}±0.61	24.73^{a}±0.64	FEO120
0.231	0.416	0.337	0.955	0.46	0.010	1.017	1.094	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	*P-value*
								OM
21.93^{c}±0.06	23.03^{b}±0.08	26.33±0.50	23.09^{c}±0.15	28.18±0.64	0.040^{b}±0.003	9.65±0.32	18.53^{a}±0.06	Control
22.54^{a}±0.05	23.50^{a}±0.06	26.31±0.28	23.43^{a}±0.11	27.93±0.24	0.078^{a}±0.004	9.02±0.16	18.11^{b}±0.09	FEO60
22.30^{b}±0.03	23.00^{b}±0.02	25.42±0.67	23.29^{b}±0.28	26.73±0.39	0.052^{ab}±0.002	7.605±0.52	18.65^{a}±0.03	FEO120
0.028	0.058	0.295	0.035	0.257	0.003	0.372	0.131	SEM
<.0001	0.0021	0.396	0.0005	0.230	0.038	0.107	0.005	*P-value*
								CP
46.30^{a}±0.77	35.50^{b}±0.49	42.67^{b}±0.08	56.87^{a}±0.51	62.53^{a}±0.31	0.090^{b}±0.001	54.70^{a}±0.28	7.83^{c}±0.13	Control
35.30^{b}±0.72	39.57^{a}±0.28	49.73^{a}±0.09	40.47^{b}±0.58	53.47^{b}±0.37	0.11^{a}±0.002	43.66^{b}±0.37	9.80^{a}±0.13	FEO60
24.33^{c}±0.74	27.93^{c}±0.50	39.23^{c}±0.15	28.37^{c}±0.54	39.53^{c}±0.62	0.08^{c}±0.001	31.32^{c}±0.68	8.21^{b}±0.11	FEO120
0.846	0.601	0.087	2.574	1.059	0.0104	1.086	0.364	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	*P-value*
								NDF
35.93^{a}±0.50	40.43^{a}±0.62	56.03^{a}±0.76	40.48^{a}±0.67	65.30^{a}±0.54	0.036^{c}±0.002	42.58^{b}±0.74	22.71^{a}±0.64	Control
30.33^{c}±0.54	35.13^{c}±0.79	51.83^{b}±0.77	35.15^{c}±0.66	62.74^{b}±0.50	0.041^{b}±0.001	46.52^{a}±0.62	16.22^{c}±0.64	FEO60
33.20^{b}±0.51	37.77^{b}±0.78	46.10^{c}±0.74	37.82^{b}±0.68	60.17^{c}±0.62	0.046^{a}±0.003	41.39^{c}±0.62	18.80^{b}±0.64	FEO120
0.795	1.016	1.051	0.767	0.858	0.0012	0.778	0.644	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	*P-value*

Treatment[1]-control: silage without additives, FEO60: silage with 60 ml FEO/kg, FEO120: silage with 120 ml FEO/kg. Means within same column with different superscripts differ (P<0.05).

Table 4. The effect of different levels of FEO on tVFA, NH_3-N and pH of experimental treatments
Çizelge 4. Farklı düzeylerdeki keten tohumu esansiyel yağının tVFA, NH_3-N ve pH'a olan etkisi

Incubation times (h)					Treatments[1]
24	12	8	4	2	pH
6.49^{c}±0.02	6.60^{b}±0.02	6.77^{b}±0.03	6.83^{b}±0.01	6.93^{b}±0.03	control
6.50^{b}±0.02	6.57^{c}±0.01	6.63^{c}±0.02	6.72^{c}±0.01	6.86^{c}±0.06	FEO60
6.51^{a}±0.01	6.83^{a}±0.02	6.90^{a}±0.02	6.94^{a}±0.02	6.99^{a}±0.01	FEO120
0.0411	0.0192	0.019	0.053	0.0275	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*p-value*
					NH_3-N (mmol/L)
142.33^{c}±0.31	135.33^{ab}±0.123	112.00^{c}±0.47	100.33^{c}±0.19	84.00^{c}±0.42	control
161.00^{a}±0.30	149.33^{a}±0.27	137.67^{a}±0.46	114.33^{b}±0.18	102.67^{b}±0.42	FEO60
149.33^{b}±0.31	137.67^{b}±0.28	130.67^{b}±0.48	116.67^{a}±0.16	107.33^{a}±0.49	FEO120
0.367	0.245	0.410	0.132	0.440	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*p-value*
					Ruminal VFA (mmol/L)
24.67^{c}±0.40	35.00^{c}±0.36	40.33^{c}±0.14	46.33^{c}±0.13	61.00^{c}±0.35	control
41.67^{a}±0.37	49.00^{b}±0.49	57.33^{b}±0.12	86.33^{a}±0.17	96.33^{a}±0.33	FEO60
45.67^{b}±0.32	52.00^{a}±0.40	62.67^{a}±0.12	77.33^{b}±0.15	93.33^{b}±0.32	FEO120
0.451	0.401	0.212	0.125	0.342	SEM
<.0001	<.0001	<.0001	<.0001	<.0001	*p-value*

Treatment[1]-control: silage without additives, FEO60: silage with 60 ml FEO/kg DM, FEO120: silage with 120 ml FEO/kg DM. Means within same column with different superscripts differ (P<0.05)

The highest significant increase was related to the treatment FEO120 compared to the control (p <0.05). Kolling et al. (2018) reported no significant effect of adding EOs on ruminal ammonia concentration (Kolling et al. 2018). Puupponen-Pimiä et al. (2011) also stated that different levels of lemon did not have a significant effect on NH_3-N. The effects of EOs on ruminal nitrogen metabolism are probably due to high bacterial ammonia production, resulting in reduced amino acid deamination and ammonia N production.

The discrepancy between these studies and some of the treatments of the present study can be attributed to the differences in the doses used.

Numerous *in vitro* studies have been performed to determine the effects of EOs and their main Compounds on N metabolism in the rumen. Basquit et al. (2005) stated that the EOs of cinnamon, cloves, oregano, green tea, garlic oil, cinnamaldehyde, caracrol, and eugenol increased the pH of the abdomen during 24 hours of incubation. In studies (Kolling et al., 2018; Foskolos et al., 2016), an increase in pH was associated with a reduction in the concentration of tVFA, which indicates a decrease in the fermentability of the diet due to the antimicrobial activity of phenolic compounds. In general, the differences between the results of the present study and other studies can be related to differences in the type, dose, and chemical composition of the EO used the composition of the basic diet, and test conditions (*in vivo* versus *in vitro*, the duration of the test). Natural additives could improve animal performance through modulating rumen fermentation (Valero, 2014).

CONCLUSION

The obtained data show that FEO had a positive effect on alfalfa silage quality and its fermentation properties. Based on the obtained results, it can be concluded that the use of EO as silage additive has the potential to improve its nutritional value and silage quality.

Statement of Conflict of Interest

Authors had no conflict of interest

Author's Contributions

MB and MN: have designed the study and collected the data and wrote the article. MN: executed the experiment. VP, TA: reviewed the article and AZMS: critically reviewed.

REFERENCES

Adesogan, A.T., Krueger, N., Salawu, M.B., Dean, D.B. & Staples, C.R. (2004). The Influence of Treatment with Dual Purpose Bacterial Inoculants or Soluble Carbohydrates on the Fermentation and Aerobic Stability of Bermudagrass. *Journal of Dairy Science 87*, 3407–3416.

Association of Offical Analytic Chemists (AOAC) 2002. Official method of Analytic. Vol. 1. 17 thed. AOAC, Arilington VA. P, 120-155.

Ayaz, M., Sadiq, A., Junaid, M., Ullah, F., Subhan, F. & Ahmed, J. (2017). Neuroprotective and Anti-

Aging Potentials of Essential Oils from Aromatic and Medicinal Plants. *Frontiers in Aging Neuroscience 9*, 168.

Benchaar, C., McAllister, T.A., Petit, H.V. & Chouinard, P.Y. (2014). Whole Flax Seed and Flax Oil Supplementation of Dairy Cows Fed High-Forage or High-Concentrate Diets: Effects on Digestion, Ruminal Fermentation Characteristics, Protozoal Populations and Milk Fatty Acid Profile. *Animal Feed Science and Technology 198*, 117–129.

Besharati, M., Palangi, V., Nekoo, M. & Ayaşan, T. (2021). Effects of Lactobacillus Buchneri Inoculation and Fresh Whey Addition on Alfalfa Silage Quality and Fermentation Properties. *KSÜ Tarım ve Doğa Dergisi 24* (3), 671–678. https://doi.org/10.18016/ksutarimdoga.vi.777031.

Besharati, M., Karimi, M., Taghizadeh, A., Nemati, Z. & Kaygısız, A. (2020a). Improve Quality Of Alfalfa Silage Ensiled With Orange Pulp And Bacterial Additive. *KSÜ Tarım ve Doğa Dergisi 23* (6), 1661-1669. https://doi.org/10.18016/ksutarimdoga.vi.673623.

Besharati, M., Palangi, V., Niazifar, M. & Nemati, Z. (2020b). Comparison Study of Flaxseed, Cinnamon a Lemon Seed Essential Oils Additives on Quality and Fermentation Characteristics of Lucerne Silage. *Acta Agriculturae Slovenica 115*(2), 455–462.

Besharati, M., Shafipour, N. & Nemati, Z. (2019). Effect of Supplementation of Alfalfa Silage with Lactobacillus Buchneri Additive, Orange Pulp and Molasses on Dry Matter, Crude Protein and Organic Matter Degradability by Nylon Bags. *Research on Animal Production 10*(23), 45–52.

Besharari, M., Taghizadeh, A., Jan Mohammadi, H. & Moghadam, G. (2008). Determination of degradability of grape by products by using gas production method and nylon bags. *Journal of Agricultural Science* 18(3), 173–185. (In Persian).

Busquet, M., Calsamiglia, S., Ferret, A., Carro, M.D. & Kamel, C. (2005). Effect of Garlic Oil and Four of its Compounds on Rumen Microbial Fermentation. *Journal of Dairy Science 88*, 4393-4404.

Chaves, A.V. (2012). Effects of Cinnamon Leaf, Oregano and Sweet Orange Essential Oils on Fermentation and Aerobic Stability of Barley Silage. *Journal of Science Food and Agriculture 92*(4), 906-915.

Dubios, A., Giles, M.K.A., Hamilton, J.K., Ronerts, P.A. & Smith, F. (1956). Colorimetric Method for Determination of Sugars And Related Substances. *Analytical Chemistry 28*, 350-356.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. & Hamaker, B.R. (2003). Factors Affecting Sorghum Protein Digestibility. *Journal of Cereal Science 38*(2), 117–131

Erb, M. & Kliebenstein, D.J. (2020). Plant Secondary Metabolites as Defenses, Regulators and Primary Metabolites: The Blurred Functional Trichotomy. *Plant Physiology 184*(1), 39-52.

Garcia, F., Colombatto, D., Brunetti, M.A., Martínez, M.J., Moreno, M.V., Scorcione Turcato, M. & Martínez Ferrer, J. (2020). The Reduction of Methane Production in The in Vitro Ruminal Fermentation of Different Substrates is Linked with The Chemical Composition of The Essential Oil. *Animals 10*(5), 786.

Grant, A.Q., Gay, C.G. & Lillehoj, H.S. (2018). Bacillus spp. As Direct-Fed Microbial Antibiotic Alternatives To Enhance Growth, Immunity, And Gut Health in Poultry. *Avian Pathology 47*(4), 339-351.

He, L., Lv, H., Xing, Y., Wang, C., You, X., Chen, X. & Zhang, Q. (2020). The Nutrients in Moringa Oleifera Leaf Contribute to The Improvement of Stylo and Alfalfa Silage: Fermentation, Nutrition and Bacterial Community. *Bioresource Technology 301*, 122733.

Hojat Panah, M. & Ranjbar Kohan, Z. (2014). A Study of Relationship Between Sexual Satisfaction, Marital Satisfaction and Life Satisfaction in Couples. *Cheshmandaz Aminal Applied Psychology 1*(1), 62.

Jahani-Azizabadi, H., Danesh-Mesgaran, M., Vakili, A.R. & Rezayazdi, K. (2014). Effect of Some Plant Essential Oils on in Vitro Ruminal Methane Production and on Fermentation Charac-Teristics of A Mid-Forage Diet. *Journal of Agricultural Science and Technology 16*, 1543-1554.

Kolling, G.J., Stivanin, S.C.B., Gabbi, A.M., Machado, F.S., Ferreira, A.L., Campos, M.M., Tomich, T.R., Cunha, C.S., Dill, S.W., Pereira, L.G.R. & Fischer, V. (2018). Performance and Methane Emissions in Dairy Cows Fed Oregano and Green Tea Extracts as Feed Additives. *Journal of Dairy Science 101*, 221–4234.

Makkar, H.P.S. (2010). In Vitro Screening of Feed Resources for Efficiency of Microbial Protein Synthesis. In: In Vitro Screening of Plant Resources for Extra-nutritional Attributes in Ruminants: Nuclear and Related Methodologies. Springer p. 107–144.

Markham, R. (1942). A Steam Distillation Apparatus Suitable for Micro-Kjeldahl Analysis. *Biochemical Journal 36*(10-12), 790-791.

Marković, J., Radović, J., Lugić, Z. & Sokolović, D. (2007). The Effect of Development Stage on Chemical Composition of Alfalfa Leaf and Stem. *Biotechnology in Animal Husbandry 23*(5-6-2), 383–388.

Mcdougall, E.I. (1948). The Composition and Output of Sheep's Saliva. *Biochemical Journal 43*(1), 99–109.

Moghaddasi, M.S. (2011). Linseed and Usages in Humanlife. *Advances in Environmental Biology 5*(6), 1380-1392.

More, S.J. (2020). European Perspectives on Efforts to

Reduce Antimicrobial Usage in Food Animal Production. *Irish Veterinary Journal 73*(1), 2.

Newbold, C.J., McIntosh, F.M., Williams, P., Losa, R. & Wallace, R.J. (2004). Effects of A Specific Blend of Essential Oil Compounds on Rumen Fermentation. *Animal Feed Science and Technology 114*, 105–112.

Ørskov, E.R. & McDonald, P. (1979). The Estimation of Protein Digestibility in The Rumen From Incubation Measurements Weighed According to Rate of Passage. *The Journal of Agricultural Science 92*, 499-503.

Palangi, V. & Macit, M. (2021). Indictable Mitigation of Methane Emission Using Some Organic Acids as Additives Towards a Cleaner Ecosystem. *Waste and Biomass Valorization 12*(9), 4825-4834. https:// doi.org/10.1007/s12649-021-01347-8

Pawar, M.M., Kamra, D.N., Agarwal, N. & Chaudhary, L.C. (2014). Effects of Essential Oils on In Vitro Methanogenesis and Feed Fermentation with Buffalorumen Liquor. *Agricultural Research 3*, 67–74.

Pour, H.A., Naserian, A.A., Vakili, A.R. & Tahmasbi, A.M. (2017). Effect of Essential Plant Oil Used as an Additive to Alter Silage Fermentation in Ruminant by In Vitro. *Biosciences Biotechnology Research Asia 14*(1), 145-152.

Przybylski, R. (2005). Bailey's Industrial Oil and Fat Products. Sixth Edition. Vol:6, John Wiley & Sons, Inc. 281-301.

Saeed, A.A. & Abo-Ellul, M.H. (2020). Effect of Addition of Different Levels of Commercial Inoculant of Lactic Acid Bacteria and Date Juice on Nutritive Value of Wheat Straw Silages. *Euphrates Journal of Agriculture Science 12*(1), 153-163.

Sallam, S.M.A., Abdelgaleil, S.M.A., Bueno, I.C.S., Nassera, M.E.A., Araujo, R.C. & Abdalla, A.L. (2011). Effect of Some Essential Oils on In Vitro Methane Emission. *Archives of Animal Nutrition 65*, 203–214.

Silva, V.P., Pereira, O.G., Leandro, E.S., Da Silva, T.C., Ribeiro, K.G. & Mantovani, H.C. (2016). Effects of Lactic Acid Bacteria with Bacteriocinogenic Potential on The Fermentation Profile and Chemical Composition of Alfalfa Silage in Tropical Conditions. *Journal of Dairy Science 99*(3), 1895–1902.

Tomkins, N.W., Denman, S.E., Pilajun, R., Wanapat, M., McSweeney, C.S. & Elliott, R. (2015). Manipulating Rumen Fermentation and Methanogenesis Using Anessential Oil and Monensin in Beef Cattle Fed a Tropical Grass Hay. *Animal Feed Science and Technology 200*, 25–34.

Turan, A. & Önenç, S.S. (2018). Effect of Cumin Essential Oil Usage on Fermentation Quality, Aerobic Stability and In Vitro Digetibility of Alfalfa Silage. *Asian-Australasian Journal of Animal Science 31*(8), 1252.

Valero, T. (2014). Editorial (thematic Issue: Mitochondrial Biogenesis: Pharmacological Approaches). *Current Pharmaceutical Design 20*(35), 5507-5509.

Van Soest, P.J., Robertson, J.B. & Lewis, B.A. (1991). Methods For Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science 74*, 3583.

Vergis, J., Gokulakrishnan, P., Agarwal, R.K. & Kumar, A. (2015). Essential Oils as Natural Food Antimicrobial Agents: A Review. *Critical Reviews in Food Science and Nutrition 55*(10), 1320-1323.

Yang, W.Z., Benchaar, C., Ametaj, B.N. & Beauchemin, K.A. (2010). Dose Response to Eugenol Supplementation in Growing Beef Cattle: Ruminal Fermentation and Intestinal Digestion. *Animal Feed Science and Technology 158*(1-2), 57–64.

Yildiz, S., Kaya, I., Unal, Y., Aksu Elmali, D., Kaya, S., Cenesiz, M., Kaya, M. & Oncuer, A. (2015). Digestion and Body Weight Change in Tuj Lambs Receiving Oak (Quercus Hartwissiana) Leaves with and without PEG. *Animal Feed Science and Technology 122*, 159-172.