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Effect of additives to Italian ryegrass (*Lolium multiflorum* Lam.) silage on nutrient composition and rumen parameters *in vitro*

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Abstract

In this study, Italian ryegrass ensiled in laboratory silos was examined *in vitro* to evaluate the nutrient composition of silage and rumen parameters. In this study, four experimental treatments were included, which included a control group (Italian ryegrass silage), the control group supplemented with 4.5% molasses, the control group supplemented with 4.5% locust bean fracture (*Ceratonia siliqua*), and the control group supplemented with 4.5% locust pods (*Gleditsia triacanthos*). A kitchen-type vacuum machine was used to vacuum the silage samples and store them at 25 °C for 60 days. The use of experimental additives had substantial effects on the nutrient value. The results demonstrated that the molasses-added group had a higher Fleig score while the locust pod-added group showed the lowest pH. The locust pod-added group had higher gas and methane production. The molasses-added group had a higher dry matter digestibility (DMD), relative feeding value (RFV), relative feed quality (RFQ), and total digestible nutrient content (TDN). As a result, the experimental additives were more effective than the control groups. Based on their potential to increase rumen fermentation, these additives are recommended.

Keywords: feed quality, Italian ryegrass silage, locust bean fracture, locust pods, molasses
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Introduction

Italian ryegrass (*Lolium multiflorum* Lam.) is widely distributed in temperate and subtropical regions in the spring season with high productivity (Yan *et al*., 2019). Italian grass, also known as ryegrass, is an easy-to-digest plant that is sometimes planted as an annual or biennial forage. It is also referred to as milk grass because it increases milk yield. In addition to its high protein content, it has a high energy value and can be used both as silage and as dry grass (Ozkul *et al*., 2012). Silage is a source of roughage used in the nutrition of ruminant animals, which is formed as a result of fermentation of water-rich feeds by leaving them exposed to the activity of lactic acid bacteria in an air-free environment. In animal feeding, there is an urgent need for cheap feed sources. In light of the fact that the majority of animal feed is imported and that rising feed prices make it hard to maintain a high level of feed quality and value, low-cost feed sources are in greater demand. Various additives are added to the resulting feed mixture to improve silage quality and ensure fermentation (Eseceli *et al*., 2020; Esen *et al*., 2022). Given the recent scientific advances in increasing the genetic and productive capacity of dairy cows, providing them with the necessary nutrients is essential. Therefore, it is inevitable that feeds with higher nutritional value will be used, and the most appropriate feed additive should be identified.

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Molasses is a source of carbohydrates that is used in silage production and features in the storage life of silage (Uygur, 2007). Carob fruit is used in human nutrition and attracts attention due to its high level of antioxidants and fibre. Studies on the use of carob pulp, fruit, and substances in animal nutrition have been done (Atalay, 2015; Pazir & Alper, 2016). The locust pod is a deciduous tree; in addition to the carbohydrate sources required in silage, research is also underway to explore the potential of utilizing the inexpensive fruit of the tree, which has a high sugar content. According to Canbolat *et al*. (2013), locust pod fruit at 80–100 g/kg DM added to alfalfa silage as a carbohydrate source improves results.

Converting seasonal feed into silage is an effective method of preserving feed and addressing the disparity between year-round livestock production and seasonal fluctuations in available forage. Hence, we hypothesized that treating ryegrass silage with 4.5% molasses, 4.5% locust bean fracture, and 4.5% locust pods could increase fermentation dynamics and nutritive value. Therefore, this study aimed to evaluate the efficiency of supplementary molasses, locust bean fracture, and locust pods on fermentation dynamics and the nutritional value of Italian ryegrass silage.

Materials and Methods

Rumen fluid was obtained from animals that had been humanely slain according to the method reported by Palangi *et al*. (2022). The ethics committee's consent was not necessary for this investigation as the animals had already been slaughtered.

The study was conducted in one field located within the borders of Erzincan Province, Turkey. In the 2021 season, annual grass (*Lolium multiflorum* L.) samples were taken by mowing during the flowering period. After being slightly dried, the fodder plant was broken up into 2–3-cm long pieces. A total of 12 silage samples (3 replicates × 4 treatments) were prepared: 4.5% molasses added, 4.5% locust fracture added, 4.5% locust pod added, and a control group (no additives). The chemical content of Italian ryegrass, molasses, locust bean fracture, and locust pod additives is given in Table 1. Dry matter (DM), crude protein (CP), crude ash (CA), crude fat (CF), and NH_3 values were determined according to the reported methods. The analysis of fibrous substances insoluble in acid solvents (ADF) and fibrous substances insoluble in neutral solvents (NDF) was performed using the ANKOM 2000 Fibre Analyzer (Ankom Technology, Macedon NY), and lignin insoluble in acid solvents (ADL) was determined according to the method of Van Soest *et al*. (1991). The prepared silage samples were vacuum-sealed in vacuum bags (25 × 35 cm) in three replications in a kitchen vacuum machine (Lavion DZ-100SS, Xiamen Yeasincere Industrial Corporation, China) and stored at 25 ± 2 ºC for 60 days.

Table 1 Chemical content of Italian grass, molasses, locust bean fracture, and locust pod additives

Chemical composition	Italian ryegrass	Molasses	Locust pod	Locust bean fracture
DM (%)	34.02	84.39	92.11	97.21
CA (%)	8.74	11.35	3.60	3.69
EE (%)	2.28	0.18	2.16	2.11
CP (%)	11.24	11,69	34.94	4.71
NDF (%)	73.08	0	17.67	32.78
ADF (%)	4421	0	11.07	16.67
CT (%)	0	0	5.19	8.78
Sugar (%)	-	56.65	15.04	41.36

DM: dry matter, CA: crude ash, EE: ether extract, CP: crude protein, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin, CT: condensed tannin

The silages were opened 60 days after they had been made. For pH analysis, 250 ml of distilled water was added to 25 g of silage sample. The pH value of the filtrate obtained after shaking for 30 min was measured using a digital pH meter (HI 2211 PH /ORP METER). The Fleig score (FP) of the silage samples was calculated using Equation (1):

FP = [220 + (ratio of 2 × DM - 15)] - 40 × pH (Goel *et al*., 2008) (1)

Relative feeding value (RFV) was calculated using the formula developed by Van Dyke & Anderson (2000), which is based on the estimation of the energy value that the feed will provide using the animal's consumption potential:

%DMD (Dry matter digestion) = 88.9 - (0.779 × %ADF) (2)

%DMI (Dry matter intake) = 120/NDF (3)

RFV = %DMD × %DMT × 0.775 (4)

The buffer solutions necessary for calculating the *in vitro* digestibility parameters supplied by the Ankom Daisy incubator were made in accordance with the instructions provided by the Ankom Daisy *in vitro* fermentation system. The relative feed quality of silage (RFQ) was determined using Equation (5) with the values of true dry matter digestion (TDMD), true NDF digestion (TNDFD), true organic matter digestion (ROMD), DMI, and total digestible nutrients (TDN) obtained using the Daisy incubator for 48 h:

RFQ = (DMI, %DM) × (TDN, % DM)/1.23 (Ward & Ondarza, 2008) (5)

Metabolic energy of feed raw materials (ME) and net energy lactation (NE_L) values were determined using the equation of Menke & Steingass (1988):

OMD (organic matter digestion, %) = 15.38 + 0.8453 × GP + 0.0595 × CP + 0.0675 × CA (6)

ME (MJ/kg DM) = 2.20 + 0.1357 × GP + 0.057 × CP + 0.002859 × CF^2 (7)

NE_L (MJ/kg DM) = 0.101 × GP + 0.051 × CP + 0.112 × CF (8)

where GP is net gas production at the end of the 24-h incubation period of a 200 mg dry feed sample; CP is %crude protein; CF is % crude fat; and CA is %crude ash.

Within approximately 5 min after slaughter, the rumen of each animal was opened and the liquid was taken from the rumen and brought to 39 °C, placed in a thermos containing CO_2 (carbon dioxide), and taken to the feed analysis laboratory. Approximately 500 mg feed samples were incubated for 24 hours in a water bath at 39 ˚C in 100 ml glass syringes with 40 ml of buffered rumen fluid (Menke *et al*., 1979). After 24 hours of fermentation, the amount of methane (%) in the total gas produced was determined using an infrared methane analyser (Sensor Europe GmbH, Erkrath, Germany) (Goel *et al*., 2008). After the gas measurements were made, the rumen fluid and feed samples remaining in the syringe were boiled for 1 hour in the NDF solution prepared as described by Van Soest *et al*. (1991). Following boiling, the true amount of digested dry matter, the true digestion, the division factor, the generation of microbial proteins, and the efficiency values were calculated in accordance with the approach and equations published by Blümmel *et al*. (1997):

TDD (true digestion degree, %) = ((Incubated DM (mg) – Remaining DM (mg))/ Incubated DM (mg)) × 100 (9)

TDDM (true digestible dry matter, mg) = Incubated DM (mg) – Remaining DM (mg) (10)

Partition factor (PF) = TDDM / GP (gas production) (11)

Microbial yield (MY, mg) = TDDM – (GP (24 hours) × 2.2 mg/ml) (12)

Microbial Protein (MP, mg/g DM) = TDDM – (GP × 2.2 mg/ml) (13)

Effectiveness of Microbial Protein Synthesis (EMPS) = (TDDM – (GP × 2.2 mg/ml))/TDDM (14)

To compare the data obtained from the study, Duncan's comparison test was applied to compare the groups using variance analysis in SPSS 24 software (MacInnes, 2016).

Results and Discussion

There was an effect of additives on the nutrient content of Italian grass silage (P <0.05). Based on the extracted crude levels of ash from the examined parameters (Table 2), the CA level was highest in the group where locust bean fracture was added to Italian grass, whereas molasses reduced the CA level (P <0.05). The reason molasses lowers the CA content in silage is that the CA content of molasses is lower than that of Italian grass. Sakalar & Kamalak (2016) found that molasses reduces the CA value of silage, but on the contrary, Canbolat *et al*. (2019) reported that molasses increased the value of silage CA.

The crude fat level caused a statistical difference between the groups; the CF level was found to be the highest in the control group at 2.25%. In the group where molasses was added to the Italian grass, the CF was found to be the lowest at 1.97%. Similarly, Canbolat *et al*. (2019) stated that molasses reduced the CF content in silage. Ates & Atalay (2022) found that locust bean fracture increased the content of CF in silage.

In the current study, the crude protein level, which was 10.91% in the control group, was found to be 10.57% in the molasses-added group, 11.45% in the locust bean fracture-added group, and 11.18% in the locust pod-added group (P >0.05). While the additives used in silage did not statistically affect the CP ratio, molasses and locust bean fracture have been reported to decrease the CP content in some studies (Canbolat *et al*., 2019; Ates & Atalay, 2022).

Table 2. The effect of different additives on the nutrient content of Italian grass silage

	Control	Molasses	Locust bean fracture	Locust pods	P
CA (%)	8.83^{b}	7.86^{c}	9.40^{a}	8.93^{b}	0.000
CF (%)	2.25^{a}	1.97^{c}	2.08^{b}	2.10^{b}	0.000
CP (%)	10.91	10.57	11.45	11.18	0.286
NDF (%)	71.97^{b}	65.69^{c}	78.23^{a}	72.95^{b}	0.000
ADF (%)	43.93^{b}	39.94^{c}	46.86^{a}	43.34^{b}	0.000
ADL (%)	15.39^{a}	12.82^{b}	12.23^{b}	8.47^{c}	0.000

^{a,b,c} The differences between the averages, which are indicated by different letters in the same column, are significant. P <0.05, CA: crude ash, CF: crude fat, CP: crude protein, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin

The NDF content, which is one of the criteria for determining the quality of coarse feed, varied between 65.69% and 78.23% in the groups. The effect of different additives on the NDF level was found to be significant. While the NDF ratio was found to be highest in the locust bean fracture group, the molasses addition decreased the NDF level.

Acid detergent fibre (ADF) was statistically different between the groups; the locust bean fracture group was found to be the highest with 46.86%; molasses addition caused a decrease in the ADF content. The low content of ADF indicates that the feed within this group is easily digestible and feeds with this condition are of higher quality. ADF is the part of the fibre that is difficult to digest, or indigestible, such as cellulose and lignin. While the addition of locust pods decreased the ADL level, it was found to be the highest in the control group (P <0.05). The main reason why the addition of molasses reduces the NDF, ADF, and ADL of silage is that molasses does not contain components of the cell wall components (NDF, ADF and ADL), and the molasses assists in the degradation of components of the plant cell wall as a result of increased bacterial activity of water-soluble sources in the silo such as molasses (Canbolat *et al*., 2010). The use of molasses in silage has been shown to reduce cell wall components (Canbolat *et al*., 2019). The use of locust pods reduces the content of NDF and ADF (Guven & Kamalak, 2021), and the use of locust bean fracture does not affect the content of NDF but reduces the content of ADF (Ates & Atalay, 2022). It is thought that the difference in the nutrient contents from the results of the current study using the same additives is due to the different forage plant that was ensiled.

One of the methods for determining the quality of silage, FP, is a value calculated with the help of dry matter and pH. The higher the FP, the higher the quality of the feed. In the study, the dry matter was 33.41% in the control group (Table 3); the addition of molasses and locust bean fracture to Italian grass silage increased the DM level and locust pods decreased the DM (P <0.05).

Table 3. Fleig's score of different additives to Italian grass silage and their effect on NH_3

	Control	**Molasses**	**Locust bean fracture**	**Locust pods**	***P***
DM (%)	33.41^{ab}	40.16^{a}	35.75^{ab}	32.38^{b}	0.144
pH	5.19^{a}	4.77^{b}	4.87^{b}	4.58^{c}	0.000
FS	63.95^{b}	94.26^{a}	81.43^{a}	86.56^{a}	0.014
NH_3, %	0.33^{c}	0.67^{b}	1.35^{a}	1.28^{a}	0.000

^{a,b,c} The differences between the averages, which are indicated by different letters in the same column, are significant. *P* <0.05, DM: dry matter, FS: Fleig score, NH_3: Ammonia

Generally, as the dry matter increases, the pH of the silage decreases (Ayasan & Karakozak, 2012). The pH value used to calculate the Fleig score is a criterion that quantifies whether the feed is sour enough. The pH value varied between 4.58–5.19 in the groups. Although the pH value of the feed was low in the locust pod group, the pH was high in the control group (*P* <0.05). The flora of the rumen are highly affected by the pH and chemical composition of feed (Sharifi *et al*., 2022). Ammonia (NH_3) content in silage was different between the groups (P <0.001). The NH_3 content was found to be the lowest in the control group and highest in the locust bean fracture group. The effect of additives of molasses (Canbolat *et al*., 2019), locust pods (Canbolat *et al*., 2013; Guven & Kamalak, 2021), and locust bean fracture (Atalay & Kamalak, 2018) on the pH and FP value of silage was found to be similar in some studies. Contrary to the results of the current study, some studies have shown that locust bean fracture does not affect silage pH and FP (Ates & Atalay, 2022), It has been reported that the addition of molasses and locust pods reduces the value of NH_3 (Canbolat *et al*., 2013; Sakalar & Kamalak, 2016; Canbolat *et al*., 2019). It was observed that the addition of 4.5% was not sufficient to reduce the amount of NH_3 in Italian grass silage. The effect of different additives to Italian grass silage on gas production and fermentation parameters is shown in Table 4.

Table 4. The effect of different additives to Italian grass silage on gas production, methane production, and fermentation parameters

	Control	**Molasses**	**Locust bean fracture**	**Locust pods**	***P***
GP (ml)	75.89^{b}	84.75^{a}	74.06^{b}	84.78^{a}	0.000
Methane (ml)	11.11^{b}	14.10^{a}	10.74^{b}	14.77^{a}	0.000
Methane (%)	14.64^{c}	16.63^{b}	14.50^{c}	17.42^{a}	0.000
PF (mg/ml)	2.39^{c}	2.59^{b}	2.71^{ab}	2.86^{a}	0.001
MP (mg)	14.07^{c}	31.40^{b}	37.42^{b}	56.13^{a}	0.000
MPSE (%)	8.15^{c}	15.19^{b}	18.70^{ab}	23.21^{a}	0.001
ME (MJ/kg)	6.95^{b}	7.41^{a}	6.88^{b}	7.45^{a}	0.000
NEL (MJ/kg)	3.87^{b}	4.18^{a}	3.81^{b}	4.23^{a}	0.000
OMD (mg)	42.28^{b}	45.19^{a}	41.73^{b}	45.32^{a}	0.000
TDDM (mg)	171.00^{c}	206.66^{b}	199.48^{b}	241.66^{a}	0.000

^{a,b,c} The differences between the averages, which are indicated by different letters in the same column, are significant. *P* <0.05, G: Gas production, PF: Partition Factor, MP: Microbial Protein (mg), ME: Metabolic Energy, NEL: Net energy lactation, OMS: Organic matter digestion, TDDM: True digestible dry matter

In vitro testing is one technique used to assess the nutritional value of feeds used in the nutrition of ruminant animals. *In vitro* gas production reached a highest level of 75.89 ml in the control group, 84.75 ml in the molasses-added group, 74.06 ml in the locust bean fracture group, and 84.78 ml in the locust bean fracture group (*P* <0.05). Ozkan *et al*. (2020) stated that gas production also increases as the amount of fermentable carbohydrates increases. The decrease in NDF and ADF with the addition of molasses and the decrease in ADL with the addition of locust pods increased the GP with these additives.

Methane, another gas released during fermentation, presented varying values among the groups. Methane gas was found to be high in the group that had locust pods added to Italian grass silage. In the study, the methane production values of the feeds varied between 14.50–17.42%.

The partition factor (PF) is the most important parameter that indicates the effectiveness of microbial protein synthesis. The partition factor (PF) values obtained in this study were determined to be between 2.39 and 2.86 (mg/ml). These values are between the values reported by various authors (PF: 2.75–4.41 mg/ml) (Ozkan *et al*., 2020; Gursoy *et al*., 2022). The efficiency of microbial protein synthesis of feeds with a high PF in the feed is high. The MP value of the silage feeds varied between 14.07 and 56.13 mg. The microbial protein synthesis activity (MPSA) was also highest in the group with locust pods added (P <0.05).

When the metabolic energy values of the silage feed, the net energy lactation values, the digestibility values of organic matter, and GSKM values were examined, it was found that the locust pod group obtained the highest values with respect to all these parameters (P <0.05). It is thought that the decrease in NDF and ADF with the addition of molasses (as in the case of GP) and the decrease in ADL with the addition of locust pods, increased the ME of silage. Molasses and *Gleditsia* additives increased the GP, OMD, ME, TDDM values, and similar results were obtained from studies using the same additives (Sakalar &Kamalak, 2016; Canbolat *et al*., 2019; Guven &Kamalak, 2021). Similarly, Ates & Atalay (2022) reported that the silage created using locust bean fracture did not statistically affect the GP, whereas Atalay & Kamalak (2018) reported that it improved the GP. This difference is due to forage plants used in the studies.

The effect of different additives to Italian grass silage on relative feeding value, dry matter, relative feed quality, and other parameters is shown in Table 5.

Table 5. The effect of different additives on Italian grass silage on the relative feed value, dry matter digestibility, relative feed quality, and digestibility parameters

	Control	Molasses	Locust bean fracture	Locust pods	*P*
TDD (%)	33.63[d]	41.06[b]	36.73[c]	43.44[a]	0.000
TDDM (%)	16.45[c]	25.19[b]	24.19[b]	34.66[a]	0.001
NDFDD (%)	29.35[b]	39.13[a]	27.93[b]	41.02[a]	0.000
TOMDD (%)	93.90[a]	93.19[b]	93.19[b]	92.89[b]	0.040
DMD (%)	54.68[b]	57.78[a]	52.39[c]	55.13[b]	0.000
RFV	70.66[b]	81.85[a]	62.30[c]	70.31[b]	0.000
TDF (%)	41.30[c]	49.20[a]	36.55[d]	45.76[b]	0.000
DMI (%)	1.66[b]	1.83[a]	1.53[c]	1.64[b]	0.000
RFQ	55.99[c]	**73.08[a]**	45.58[d]	61.20[b]	0.000

[a,b,c,d]. The differences between the averages, which are indicated by different letters in the same column, are significant. P <0.05, TDD: True degree of digestion, TDDM: True digestible dry matter, NDFDD: NDF digestive degree, TOMDV: True organic matter digestive degree, DMD: Dry matter digestion, RFV: Relative feeding value, TDF: Total digestible feed, DMI: Dry matter intake, RFQ: Relative feed quality

The TDD varied between 33.63 and 43.44%; TDDM, 16.45–34.66%; and NDFDD, 27.93–41.02%. The group to which locust pods was introduced had the highest value of all three (P <0.05). TOMDD was observed to have the highest (93.90%) value in the control group, and the additives reduced this value (P <0.05). Silage was also examined in terms of RFV and RFQ, and it was determined that the highest values (81.85 and 73.08) were in the molasses-added group, while the control group had the lowest value (P <0.05). The decrease in ADF and NDF values with the addition of molasses increased the RFV and RFQ values of the silage. Canbolat *et al*. (2019) also reported that molasses addition increased the RFV.

Conclusions

As a result, it has been proven that the addition of molasses, locust bean fracture, and locust pod substances to Italian grass silage produces positive results. According to the mentioned parameters, the molasses and locust pod additives are more effective.

Authors' Contributions

EG conceived the study design, managed the data acquisition, and performed the experiments together with GS; AK conducted the data analysis. GS, AK, and TA proofread the manuscript.

Conflict of Interest Declaration

The authors declare that they have no conflict of interest.

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