The effects of fumaric and malic acids on the *in vitro* true digestibility of some alternative feedstuffs for ruminants

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Abstract: The aim of this study was to determine the effects of the addition of different amounts of fumaric acid (FA) and malic acid (MA) to the leaves of *Robinia pseudoacacia* (Black locust, acacia), *Prunus laurocerasus* (cherry laurel), *Quercus cerris* (oak), and *Camellia sinensis* (tea factory wastes, TFW), to improve their value as alternative feeds for ruminants. The parameters examined were the *in vitro* true digestibility of feed (IVTDₐₙₙ), dry matter (IVTDₐₙ₈), organic matter (IVTDₐₙ₈₉₈), neutral detergent fiber (IVTDₐₙ₈₉₈₉₈) and count of protozoans. The digestibility experiments were performed with a DAISY incubator system. Organic acids were not added in the control group and 0.1%, 0.2% or 0.3% FA or MA were added to the experimental groups. Each treatment was replicated 6 times. Samples were incubated for 48 hours. Fumaric acid significantly reduced (P<0.01) all digestibility values of *R. pseudoacacia* leaves. When FA was applied at 0.1% to *C. sinensis* factory wastes, the IVTDₐₙ₈ decreased significantly (P<0.05), with the same effect observed for *Q. cerris* (P<0.01). However, for the addition of 0.1% FA, IVTDₐₙ₈ₙ₉₈, IVTDₐₙ₈₉₈ and IVTDₐₙ₈₉₈₉₈ values decreased significantly (P<0.01). Separately, malic acid did not have a significant effect on the *in vitro* true digestibility values determined in this study (P>0.05). Rumen protozoa counts decreased for both organic acid applications compared to counts in the fresh rumen contents. Because 0.1% fumaric acid increased the IVTDₐₙ₈₉₈ values of both *C. sinensis* factory wastes and *Q. cerris* leaves they can be considered potential alternative feed sources for ruminants.

Keywords: Fumaric acid, *in vitro*, malic acid, true digestibility

Ruminantlar için fumarik ve malik asitlerin bazı alternatif yem maddelerinin *in vitro* gerçek sindirilebilirliği üzerine etkileri

Özet: Bu araştırmanın amacı farklı düzeylerdeki fumarik (FA) ve malik (FA) asitlerin ruminant alternatif yem kaynağı olarak *Robinia pseudoacacia* (yalancı akasya), *Prunus laurocerasus* (karayemiş), *Quercus cerris* (meşe), ve *Camellia sinensis* (fabrika atı, TFW) yapraklarının sindirilebilirlik değerlerini üzerine etkilerini incelemektir. Araştırıldığında materyallerin yem bazında (IVTDₐₙₙ), kuru madde bazında (IVTDₐₙ₈), organic maddede (IVTDₐₙ₈₉₈), nötral deterjen fiber (IVTDₐₙ₈₉₈₉₈) *in vitro* gerçek sindirilebilirlikleri ve protozoa sayıları parametreleri üzerine çalışılmıştır. Sindirilebilirlik parametreleri DAISY inkubatör sistem ile gerçekleştirilmiştir. Kontrol grupında FA ve MA eklenmemiş deneme grupları ayrı ayrı ve sırasıyla %0.1, %0.2, %0.3 FA ve MA eklecek şekilde düzenlenmiştir. Her örnek 6 kez tekrarlı olarak şekilde araştırılmaya dahil edilmiştir. Örnekler 48 saat inkubasyona tabi tutulmuştur. Fumarik asit *R. pseudoacacia* yapraklarında tüm sindirilebilirlik değerlerini önemli (P<0.01) derecede azaltırken değerlerin doza bağlı lineer değişimi de önemli (P<0.01) bulunmuştur. Fumarik asit TFW maddesine %0.1 düzeyinde uygulandığında IVTDₐₙ₈ önemli derecede (P<0.05) yükselmiştir. Ayrıca etki *Q. cerris* için de görülmüş (P<0.01) ancak IVTDₐₙ₈ₙ₉₈, IVTDₐₙ₈₉₈ ve IVTDₐₙ₈₉₈₉₈ değerlerinin önemli seviyede (P<0.01) düştüğü saptanmıştır. Malik asit bu artırma *in vitro* gerçek sindirimin değerleri üzerine önemli bir etki yaratmamıştır (P>0.05). Rumen protozoa sayıları her iki organik asit uygulamasında da taze rumen içeriğine göre azalmıştır. Sonuç olarak, *C. sinensis* fabrika atı ve *Q. cerris* yapraklarına, %0.1 düzeyinde fumarik asit ilavesinin IVTDₐₙ₈₉₈₉₈ değerlerini artırdığından dolayı, *C. sinensis* fabrika atı ve *Q. cerris* yapraklarının ruminantlar için potansiyel alternatif yem kaynağı olarak değerlendirebileceği kamsına varılmıştır.

Anahtar sözcükler: Fumarik asit, gerçek sindirilebilirlik, *in vitro*, malik asit.
Introduction

Organic acids have been used for many years for the purpose of improving the effectiveness of beneficial microorganisms and hence of rumen fermentation in ruminants. Organic acids are described as “Generally Recognized As Safe” (32) for addition to animal feed. The rumen has both a complex biota and ecology. Different species of eukaryotes, prokaryotes, archaeans and bacteriophages play important roles in the functioning of the rumen (20). Dicarboxylic organic acids such as fumaric acid (FA) and malic acid (MA) have been used to manipulate the microbial ecology of the rumen (10, 19). Organic acids can pass through the intestinal mucosal barrier by diffusion and are then involved in the Krebs cycle (32). The use of FA and MA increased the growth rate and number of the bacterium Selenomonas ruminantium (25, 32) which uses lactate produced by ruminal bacteria as a source of energy (25). These acids have beneficial effects on fermentation and microbial populations when used in combination with cellulose and mornesin. Organic acids have also been shown to increase the rate of protein hydrolysis (32).

The plant materials used in this research can be found in many geographies and are easy to obtain and naturally contain tannins. The search for alternative feed sources for ruminants has increased the interest in leaves containing tannins as potential resources. Tannins are water-soluble, polyphenolic compounds usually found in plants with high fibre levels. Although there can be adverse effects from tannins, improved feed consumption, feed efficiency and growth have been reported (35). Furthermore, tannins have been included in animal feed for research purposes due to their antioxidant effects against free radicals, metal binding properties and lipid peroxidation inhibition properties (18). However, Tieman et al. (36) reported that plants with high tannin content have low cellulose digestibility. In spite of some anti-nutritional properties, plant sources containing tannins have been used in the feeding of different kinds of animals because they are easy to access and cheap (35).

Against that background, the aim of this study was to use in vitro methods to determine the effects of the addition of FA and MA on the actual digestibility parameters and count of protozoans associated with four alternative roughage sources.

Materials and Methods

For this study, ethics committee approval was not needed because the rumen samples were collected only from slaughtered animals.

The tree leaves (Robinia pseudoacacia (black locust, acacia), Prunus laurocerasus (cherry laurel), Quercus cerris (oak)), used in the study were collected according to the methodology described in British Columbia Ministry of Forests (5) from Samsun Canik Town forest (41°15' 29" N, 36°21'56" E and 41° 15' 48" N, 36° 22' 05" E), which is located approximately 150 m above sea level. The tea (Camellia sinensis) factory waste was obtained from tea factories located in Rize Province at the eastern end of the Black Sea region in Turkey. All tree leaves and tea factory waste samples were collected in May 2019. The FA (≥99% purity) and MA (≥95% purity) were obtained from Sigma Aldrich® (Istanbul, Turkey).

Fresh tree leaf samples were weighed as fresh and then dried at 65 °C for 48h. The dried samples were ground in a mill and then passed through a sieve with a mesh diameter of 1 mm for chemical analysis. Dry matter (DM) content of each sample was determined in air circulation drying oven at 105 °C for 4 hours and the ash content was determined by burning the dried material in an ash oven at 550 °C for 4 hours. The Kjeldahl method was used to determine the crude protein (CP) percentage. Ether extract (EE) was performed according to the methods of AOAC (2). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of the materials that form the cell wall components of the feeds used in this study were determined in accordance with the method reported by Van Soest et al. (37) and were analyzed in a ANKOM 200 Fiber Analyzer (ANKOM Technology Corp. Fairport, NY, USA). The in vitro true digestibility (IVTD) analysis was performed with the ANKOM Daisy Incubator (ANKOM Technology Corporation), according to the methodology described in ANKOM (1). The rumen fluid was collected post-mortem from the rumens of four Holstein x Yerlikara hybrid cattle aged three years that were slaughtered in a commercial abattoir in Samsun, Turkey. Animals were fed twice daily with a diet containing grass hay and maize silage (60%) and concentrates (40%). A thermos was used to store and transport the rumen fluid, which was preheated to 39°C and had CO2 added. The rumen fluid was collected manually by squeezing two handfuls of ruminal contents from each animal’s rumen into the same thermos. In the laboratory, the rumen fluid was filtered through 4 layers of gauze. The F57 bags to be used in the analysis were rinsed with acetone (99.5%) for 3 minutes and then the acetone was evaporated at room temperature. All the bags were marked with both acid and alkaline resistant pen. The bags were dried in a drying cabinet at 105 °C for 2 hours. The tare weights of the bags were recorded. Samples of plants weighing 0.5 g were transferred to separate F57 bags which were then closed. The buffer solution to be used in the analysis was prepared according to the Ankom Daisy in vitro fermentation system described in ANKOM (1). Four digestion units, each with a volume of 2 L, were used in this test. The buffer solution was heated to 39 °C and 1.6 L was poured into each digestion unit. Four hundred mL of rumen fluid was added to each unit. A total
of 24 feed samples were used in each digestion unit, and 6 replicates were formed from each feed sample at the same time. The tests with FA and MA were carried out separately but with otherwise identical procedures. While no acid was added to the control group units, 0.1, 0.2 or 0.3% FA or MA was added to the experimental group units. A total of 24 feed samples were added to each of the control and experimental group units, that is, 57 bags, with 6 replicates of each feed sample. The samples were incubated for 48 hours. After the incubation period, all the liquid in the digestion units was removed and the bags were washed under running water. NDF analysis was performed as per the method outlined in ANKOM (1) by placing the bags in the Ankom Fiber Analyzer device. After analysis, the bags were retained in the drying cabinet until they reached a constant weight at 105 °C. The IVTD values of all samples were calculated with the formula reported in ANKOM (1),

\[
\text{IVTD}\% = \frac{100 - (W3 - (W1 \times C1)) \times 100}{W2}
\]

where:
- W1 = Bag tare weight
- W2 = Sample weight
- W3 = Final bag weight after in vitro process and sequential ND treatment
- C1 = Blank bag correction (final oven-dried weight/original blank bag weight)

For the protozoan count, a mixture of 0.6 g methyl green, 8 g sodium chloride (NaCl) and 100 ml 37% formaldehyde was prepared (31). The mixture volume was made up to 1,000 ml with distilled water. One milliliter of the mixture and 1 mL of the liquid containing protozoans that had been taken from the digestion unit were mixed and 1 mL of the liquid containing protozoans was added to a Fuchs Rosenthal counting chamber apparatus. For the protozoa count, fresh rumen fluid and samples taken from each digestion unit after the incubation period were studied in parallel. The protozoans were counted on an object slide under a light microscope (Nicone eclipse 80i) with a Fuchs Rosenthal counting chamber (depth: 0.2 mm, small square area: 0.0625 mm²) (31).

Statistical analysis: The Kolmogorov-Smirnov Test was used to check for normal distribution of the data, and for homogeneity of variance, the data were evaluated with the Levene Test. All traits on digestibility in the study were summarized as the mean of the group and standard error of means (SEM). For the determination of the differences among the groups, the one-way ANOVA model was fitted to the data for chemical composition, IVTDAs, IVTDOM, IVTDOM and IVTNDOM. ANOVA equation is:

\[
Y_{ij} = \mu + a_i + e_{ij} (1),
\]

Where \(Y_{ij}\) is the value for i. group and j. observation; \(\mu\) is the population mean; and \(e_{ij}\) are the individual error terms distributed as N-(0, 1).

To evaluate the differences among the three concentrations of the organic acids, second degree orthogonal polynomial contrasting was used (13). One-way ANOVA and the other statistical tests and calculations were executed with SPSS Software (34).

Results

Chemical composition of the four alternative feeds are stated in Table 1. The IVTDAs, IVTDOM, IVTDOM and IVTNDOM values for all doses of FA applied to R. pseudoacacia leaves were significantly different from the control (P<0.01) (Table 2). Fumaric acid negatively affected in vitro digestion across all parameters in R. pseudoacacia leaves. In contrast, MA did not significantly affect in vitro digestion in any of the examined digestion parameters (P>0.05) in R. pseudoacacia leaves. However, when MA was administered at 0.1%, the values of all parameters for in vitro digestion were numerically higher in R. pseudoacacia leaves.

Fumaric acid was found to be significantly (P<0.05) effective only on IVTDOM and the highest increase was seen in 0.1% dosing in TFW (Table 3). On the other hand, in vitro digestion was found to be numerically higher to all parameters when administered at doses of 0.1% and 0.2% in TFW. Malic acid did not significantly (P>0.05) affect in vitro digestion of TFW. However, it was observed that in vitro digestion values increased numerically to all parameters although it was not statistically (P>0.05) significant depending on the doses in TFW.

Table 1. Nutrient composition (g/100g DM) of alternative feed sources for ruminants.

| Feed sources | DM     | Ash   | CP     | EE     | ADF    | NDF    |
|--------------|--------|-------|--------|--------|--------|--------|
| C. sinensis  | 93.5   | 4.8   | 18.2   | 1.16   | 34.6   | 40.5   |
| Q. cerris    | 95.7   | 4.3   | 10.1   | 3.2    | 32.2   | 40.1   |
| R. pseudoacacia | 92.3 | 5.5   | 27.5   | 2.4    | 15.8   | 18.2   |
| P. laurocerasus | 90.7 | 10.8  | 8.5    | 1.1    | 11.3   | 23.1   |

DM: Dry matter, CP: Crude protein, EE: Ether extract, ADF: Acid detergent fiber, NDF: Neutral detergent fiber.
Table 2. Effects of the addition of different concentrations (%) of organic acids to *R. pseudoacacia* leaves (n=6) on *in vitro* true digestibility values (Mean±SEM).

| Fumaric acid | IVTD<sub>As Fed</sub> | IVTD<sub>DM</sub> | IVTD<sub>OM</sub> | IVTD<sub>NDF</sub> |
|-------------|---------------------|----------------|----------------|------------------|
| Control 0%  | 60.98±0.01          | 60.66±0.01     | 60.81±0.03     | 17.46±0.02       |
| 0.1%        | 60.24±0.31          | 59.85±0.34     | 60.02±0.34     | 16.22±0.53       |
| 0.2%        | 58.13±0.27          | 57.58±0.29     | 57.73±0.20     | 12.72±0.45       |
| 0.3%        | 57.94±0.02          | 57.37±0.02     | 57.55±0.01     | 12.41±0.03       |
| P           |                     | 0.001          | 0.001          | 0.001            |
| Combined    |                     | 0.002          | 0.002          | 0.002            |

| Malic acid  | IVTD<sub>As Fed</sub> | IVTD<sub>DM</sub> | IVTD<sub>OM</sub> | IVTD<sub>NDF</sub> |
|-------------|---------------------|----------------|----------------|------------------|
| Control 0%  | 59.75±0.62          | 59.32±0.68     | 59.43±0.69     | 15.40±1.05       |
| 0.1%        | 60.80±0.16          | 60.46±0.17     | 60.52±0.15     | 17.16±0.26       |
| 0.2%        | 60.86±0.39          | 60.53±0.42     | 60.69±0.44     | 17.27±0.66       |
| 0.3%        | 57.93±0.96          | 57.35±1.04     | 57.39±0.91     | 12.38±1.60       |
| P           |                     | 0.077          | 0.077          | 0.077            |
| Combined    |                     | 0.943          | 0.943          | 0.943            |
| Linear      |                     | 0.057          | 0.057          | 0.061            | 0.062            |

IVTD<sub>As Fed</sub>: *In vitro* true digestibility as fed, IVTD<sub>DM</sub>: *In vitro* true digestibility of dry matter, IVTD<sub>OM</sub>: *In vitro* true digestibility of organic matter, IVTD<sub>NDF</sub>: *In vitro* true digestibility of neutral detergent fiber.

Table 3. Effects of addition of different concentrations (%) of organic acids (fumaric acid, malic acid) to *C. sinensis* (n=6) factory waste product (TFW) on *in vitro* true digestibility values (Mean±SEM).

| Fumaric acid | IVTD<sub>As Fed</sub> | IVTD<sub>DM</sub> | IVTD<sub>OM</sub> | IVTD<sub>NDF</sub> |
|-------------|---------------------|----------------|----------------|------------------|
| Control 0%  | 50.96±0.90          | 49.21±0.96     | 49.73±0.96     | 12.19±1.27       |
| 0.1%        | 53.21±0.10          | 51.63±0.11     | 52.13±0.10     | 15.36±0.14       |
| 0.2%        | 51.26±0.35          | 49.54±0.38     | 50.12±0.37     | 12.67±0.51       |
| 0.3%        | 50.06±0.36          | 48.25±0.38     | 48.73±0.36     | 10.94±0.53       |
| P           |                     | 0.051          | 0.051          | 0.054            |
| Combined    |                     | 0.057          | 0.057          | 0.061            |
| Linear      |                     | 0.943          | 0.943          | 0.943            |

| Malic acid  | IVTD<sub>As Fed</sub> | IVTD<sub>DM</sub> | IVTD<sub>OM</sub> | IVTD<sub>NDF</sub> |
|-------------|---------------------|----------------|----------------|------------------|
| Control 0%  | 50.20±0.23          | 48.41±0.25     | 48.89±0.24     | 11.17±0.35       |
| 0.1%        | 51.38±0.87          | 49.67±0.93     | 50.26±0.94     | 12.79±1.24       |
| 0.2%        | 51.97±0.75          | 50.30±0.81     | 50.91±0.80     | 13.65±1.03       |
| 0.3%        | 52.98±0.37          | 51.38±0.40     | 51.97±0.42     | 15.06±0.56       |
| P           |                     | 0.128          | 0.128          | 0.117            |
| Combined    |                     | 0.536          | 0.536          | 0.525            |
| Linear      |                     | 0.536          | 0.536          | 0.527            |

IVTD<sub>As Fed</sub>: *In vitro* true digestibility as fed, IVTD<sub>DM</sub>: *In vitro* true digestibility of dry matter, IVTD<sub>OM</sub>: *In vitro* true digestibility of organic matter, IVTD<sub>NDF</sub>: *In vitro* true digestibility of neutral detergent fiber.

Fumaric acid produced only a numerical increase (P>0.05) in the *in vitro* digestion parameters of the leaves of *P. laurocerasus* (Table 4) when applied at a dose of 0.3%, however MA had no significant (P>0.05) effect on any of the parameters.

It was found that *in vitro* digestion levels were significantly (P<0.005) reduced for IVTD<sub>As Fed</sub>, IVTD<sub>DM</sub> and IVTD<sub>NDF</sub> parameters by adding FA to *Q. cerris* (Table 5) leaves however, IVTD<sub>OM</sub> digestion was found to be significantly (P<0.005) higher when 0.1% dose was applied. At the same time dose-dependent changes of the differences were also significant (P<0.05). It was found that MA had an enhancing effect on the *in vitro* digestion of *Q. cerris* leaves at a dose of 0.1% for all parameters, but the increase was not statistically significant (P>0.05).

The effects of different concentrations of organic acids on the total count of rumen protozoans are stated in Table 6. Compared to the fresh rumen content group, a
decrease was observed in the experimental groups. Fumaric acid at all level of 0.1% and 0.3% increased the count of protozoans numerically in comparison to the control group and in contrast MA numerically reduced the count of protozoa inversely proportional to increasing dose.

Table 4. Effects of addition of different concentration (%) of organic acids (fumaric acid, malic acid) to *P. laurocerasus* (n=6) leaves on *in vitro* true digestibility values (Mean±SEM).

| Fumaric acid | IVTD<sub>As Fed</sub> | IVTD<sub>OM</sub> | IVTD<sub>DM</sub> | IVTD<sub>NDF</sub> |
|--------------|----------------------|-----------------|-----------------|-----------------|
| Control 0%   | 59.76±0.58           | 58.90±0.64      | 58.99±0.63      | 14.33±0.94      |
| 0.1%         | 59.49±0.71           | 58.60±0.79      | 58.98±0.86      | 13.89±1.16      |
| 0.2%         | 59.04±1.49           | 58.11±1.64      | 58.45±1.61      | 13.16±2.41      |
| 0.3%         | 60.51±0.11           | 59.73±0.12      | 59.96±0.11      | 15.55±0.18      |
| P            | 0.706                | 0.706           | 0.747           | 0.706           |
| Linear       | 0.736                | 0.736           | 0.719           | 0.736           |

| Malic acid   | IVTD<sub>As Fed</sub> | IVTD<sub>OM</sub> | IVTD<sub>DM</sub> | IVTD<sub>NDF</sub> |
|--------------|-----------------------|-------------------|-------------------|-------------------|
| Control 0%   | 61.17±0.56            | 60.46±0.61        | 60.63±0.61        | 16.62±0.90        |
| 0.1%         | 61.73±0.14            | 61.07±0.15        | 61.23±0.17        | 17.51±0.22        |
| 0.2%         | 61.58±0.01            | 60.91±0.02        | 61.04±0.02        | 17.28±0.03        |
| 0.3%         | 61.17±0.42            | 59.73±0.12        | 60.63±0.47        | 16.61±0.69        |
| P            | 0.634                 | 0.634             | 0.659             | 0.634             |
| Linear       | 0.787                 | 0.787             | 0.750             | 0.787             |

IVTD<sub>As Fed</sub>: *In vitro* true digestibility as fed, IVTD<sub>OM</sub>: *In vitro* true digestibility of organic matter, IVTD<sub>DM</sub>: *In vitro* true digestibility of dry matter, IVTD<sub>NDF</sub>: *In vitro* true digestibility of neutral detergent fiber.

Table 5. Effects of addition of different levels (%) of organic acids (fumaric acid, malic acid) to *Q. cerris* (n=6) leaves on *in vitro* true digestibility values (Mean±SEM).

| Fumaric acid | IVTD<sub>As Fed</sub> | IVTD<sub>OM</sub> | IVTD<sub>DM</sub> | IVTD<sub>NDF</sub> |
|--------------|-----------------------|-------------------|-------------------|-------------------|
| Control 0%   | 46.75±0.14            | 45.47±0.15        | 45.40±0.89        | 12.86±0.14        |
| 0.1%         | 46.64±0.11            | 45.35±0.12        | 46.01±0.12        | 11.88±0.55        |
| 0.2%         | 45.82±0.25            | 44.49±0.26        | 44.94±0.37        | 9.64±0.62         |
| 0.3%         | 45.69±0.29            | 44.36±0.30        | 44.81±0.15        | 9.17±0.07         |
| P            | 0.004                 | 0.004             | 0.004             | 0.005             |
| Linear       | 0.020                 | 0.020             | 0.017             | 0.020             |

| Malic acid   | IVTD<sub>As Fed</sub> | IVTD<sub>OM</sub> | IVTD<sub>DM</sub> | IVTD<sub>NDF</sub> |
|--------------|-----------------------|-------------------|-------------------|-------------------|
| Control 0%   | 46.95±0.09            | 45.68±0.10        | 46.36±0.09        | 11.48±0.12        |
| 0.1%         | 47.52±0.43            | 46.28±0.46        | 46.94±0.46        | 12.33±0.58        |
| 0.2%         | 46.76±0.62            | 45.47±0.66        | 46.08±0.56        | 11.21±0.98        |
| 0.3%         | 46.73±0.75            | 45.44±0.79        | 46.13±0.80        | 11.16±1.15        |
| P            | 0.499                 | 0.499             | 0.464             | 0.520             |
| Linear       | 0.227                 | 0.227             | 0.190             | 0.240             |

IVTD<sub>As Fed</sub>: *In vitro* true digestibility as fed, IVTD<sub>OM</sub>: *In vitro* true digestibility of dry matter, IVTD<sub>DM</sub>: *In vitro* true digestibility of organic matter, IVTD<sub>NDF</sub>: *In vitro* true digestibility of neutral detergent fiber.

Table 6. Effects of addition of different concentrations (%) of fumaric acid (FA) and malic acid (MA) on the total ruminal protozoa count per mL.

| Additives | Protozoa count in fresh rumen liquid | Protozoa count after 48 hours incubation |
|-----------|-------------------------------------|----------------------------------------|
|           | Control 0%                          | 0.1%                                   | 0.2%                                   | 0.3%                                   |
| FA        | 917262                              | 161938                                 | 186731                                 | 143438                                 | 190625                                 |
| MA        | 950000                              | 198929                                 | 182929                                 | 165954                                 | 161928                                 |
Discussion and Conclusion

Chemical composition (DM, Ash, CP, EE, ADF and NDF) of feedstuffs is known to be important in terms of animal nutrition. For *P. laurocerasus*, the parameters mentioned above have not been investigated previously. Özyılmaz (28) reported that TFW had 93.42% DM, 14.07% CP, 4.69% ash, 1.07% EE, 47.76% NDF and 40.93% ADF levels and that these values varied according to the organic or conventional cultivation of the tea plants and their harvest periods. Parissi et al. (29) and Luginbuhl and Mueller (24) reported that *R. pseudoacacia* leaves had 27.3% CP (g/100g DM) and 28.0% (g/100g DM) respectively, which is consistent with the results of the present study.

For *Q. cerris*, Kaya and Kamalak (15) reported 91.6% DM, 4.3% CP, 23.6% NDF and 18% ADF. Also for *Q. cerris* leaves, Canbolat et al. (8) reported 94.6% DM, 8.4% CP, 5.5% ash, 43.5% NDF and 36% ADF levels. While some of the values obtained in our study are in relative agreement with the results presented for other studies, other results differed markedly. These differences may have been due to the growing of the plants in different environments, different variety of plants and harvesting of leaf samples at different times.

In the present study, the effects of FA and MA on the *in vitro* true digestibility and protozoa count of four alternative feedstuffs for cattle were varied considerably. In a trial (4) in which these acids were used in conjunction with paddy straw, no concentrations had a statistically significant effect on IVTD (P > 0.05). In another study reported that rumen digestion and sodium retention were not affected by adding a salt of MA to corn silage (21). Furthermore, Ebrahimi et al. (12) reported that both FA and MA had no effect on digestibility as measured through DM, OM, NDF and ADF. Similarly, in our study, both FA and MA did not have a significant effect on IVTD values when they were used in combination with *P. laurocerasus* leaves (P>0.05).

In our study, the fact that FA dramatically reduces the *in vitro* digestion values of acacia leaves may be related to its high level of crude protein. As a matter of fact, Chen (11) attributed the low *in vitro* digestion values of *R. pseudoacacia* leaves to higher levels of crude protein compared to carbohydrate levels, despite low levels of NDF and ADF. They also pointed out that the *R. pseudoacacia* leaves had high level of condensed tannin and lectin that could prevent fermentation. A similar description of the *in vitro* digestive properties of *R. pseudoacacia* leaves are described by Burner et al. (6).

Sirohi et al. (33) reported that FA (0, 5, 10 and 15 mM) added to rations that included berseem, sorghum and wheat straw containing different proportions of cellulose had a positive effect on IVTDOM. In this study (33), the highest digestion percentage was seen in the group with the lowest cellulose level and 10 mM concentration of fumaric acid. There was a significant increase (P<0.05) in microbial biomass for all cellulose levels in the berseem group. Furthermore, for the sorghum group, FA at 15 mM concentration significantly (P<0.05) decreased the IVTDOM, proportional to the amount of cellulose. In addition, the count of protozoa was significantly reduced in all sorghum and berseem groups (P <0.05) and this effect was not correlated with the amount of cellulose (33). The fact that the data obtained in our research on IVTDOM is not compatible with the study of Sirohi et al. (33) may be attributed to the different nutrient and cell wall structure of plant materials. Sirohi et al. (33) reported that FA increased the IVTDOM, but the true digestibility values for the groups that received the highest concentrations of FA and MA were low, which is in agreement with the results of the current research. The same researchers reported that the different types of feed may have had different effects on IVTDOM values; this argument is supported by the results of our studies.

In the researches, the findings of the effects of MA administered on different doses and durations on rumen fermentation vary. Carro and Ranilla (9) reported that a 10 mM/L of malate and a 17-hour incubation period was insufficient for the complete fermentation of treatments that included corn, barley, wheat and sorghum separately. Among the feeds used, the most fermentation occurred in the corn group. However, Callaway and Martin (7) reported that the application of malate at a concentration of 7.5 mM/L resulted in complete fermentation in ruminal fluid within 10 to 24 hours. It therefore appear that no any significant (P<0.05) enhancing effects of MA on *in vitro* digestion were observed in the present study for any plants because of the low content of fermentable material they have.

According to Castillo et al. (10), organic acid salts may be more useful in facilitating rumen fermentation due to their buffering properties. Montano et al. (26) reported that MA added to a highly concentrated feed had no effect on ruminal digestion of OM, ADF, starch level, microbial numbers, microbial digestion and protein level. Similar findings were reported for a high roughage ration supplemented with MA (21). The high solubility of this acid contributes to the effects it has on chemical reactions (25). In the study of Kara (14) in which MA was added to corn silage at 0.5%, 1%, and 1.5%, IVTDOM was not affected (P>0.05) by the applications. This finding is supported by the results of our research. Kara (14) suggested that the effects of MA on NDF were due to the increased solubility of the cellulose in silage. Khampa et al. (16) demonstrated that Dimethyl (DL) -malate had no effect on digestion of DM, OM, CP and NDF but increased ADF digestion for cassava (P>0.05). The use of MA at high doses did not have a statistically significant effect on
in vitro digestion in other studies (4, 7, 9, 12). Consistent with these data, in our study, when MA was used at 0.1% dose, it was observed that in vitro digestion levels increased only numerically in *R. pseudoacacia*, TFW and *P. laurocerasus* leaves. Different findings obtained from in vitro digestion studies with different feed materials can be explained by the efficacy of antinutritional factors such as tannin and other polyphenols that affect rumen microbial activity.

In the present study, only 0.1% and 0.3% dose of FA increased protozoa counts numerically and MA showed a reducing effect on protozoa counts with increasing dose. This finding is consistent with the reports of other studies (17, 27, 32) investigating the effects of organic acids on rumen microorganisms. Ok et al. (27) reported that FA and MA have different effects on bacteria, protozoa and other microbial community. They reported that these acids had an increasing effect on rumen bacteria, but decreased the number of methanogenic archaea species that could form complexes with protozoa. Sahoo and Jena (32) reported that MA increases the number of lactate utilizing *S. ruminantium* in rumen, leading to a decrease in lactic acid levels. Therefore the count of ruminal protozoa may also be adversely affected due to the change in pH. This situation may be explained as the anionic effects of organic acids may adversely affect microbial life (32).

Li et al. (22) stated that the numbers of fumarate-utilising bacteria (*Fibrobacter succinogenes, S. ruminantium*) did not change significantly in the presence of FA and also that DM digestibility ratios were not affected by the application. Lopez et al. (23) reported that the application of sodium fumarate to rumen fluid did not change the total number of bacteria during a 48-hour incubation period but increased the number of cellulolytic bacteria three fold (P<0.01).

Partanen (30) stated that the effects of organic acids on rumen bacteria vary according to the chemical properties of the acids. Gram (+) bacteria are sensitive to long chain acids whereas Gram (-) bacteria are sensitive to acids with less than 8 carbon atoms. The authors of the study suggest that these effects should also be investigated with respect to protozoa. In addition, Asanuma and Hino (3) stated that the increasing effects of higher amounts of organic acids on DM digestibility are associated with an increase in the cellulolytic bacteria population, along with an increase in H₂ transfer. Therefore, the author(s) of the present study recommend that the determination of true digestibility should also take into consideration the counts of protozoa, ruminal bacteria and methanogen archaea together.

In this study, it was observed that the digestion of organic matter of *C. sinensis* factory wastes and *Q. cerris* leaves could be increased by treating with 0.1% fumaric acid. It can be concluded that this application may be beneficial in using *C. sinensis* factory wastes which are considered as undesirable material as an economical alternative feed source in ruminant nutrition. Malic acid has no negative effect on in vitro true digestibility values for *R. pseudoacacia, C. sinensis* factory waste, *P. laurocerasus* and *Q. cerris* in this study. On the other hand, in vivo studies with similar organic acids and alternative feed raw materials are needed.

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**Conflict of Interest**

The authors declared that there is no conflict of interest.

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