Ankara Üniv Vet Fak Derg, **67**, 185-192, 2020
DOI: 10.33988/auvfd.623821

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

**Buğra GENÇ[1,a,✉], Mustafa SALMAN[2,b], Bora BÖLÜKBAŞ[2,c], İsmail KAYA[2,d], Mustafa AÇICI[3,e]**

[1]Ondokuz Mayıs University, Faculty of Veterinary Medicine,Department of Laboratory Animals, Samsun; [2]Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Samsun; [3]Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Parasitology, Samsun, Turkey.
[a]ORCID:0000-0002-7561-4993; [b]ORCID:0000-0003-0828-5998; [c]ORCID:0000-0002-0732-0192; [d]ORCID:0000-0002-2570-0877; [e]ORCID:0000-0002-8406-9739

✉Corresponding author: bugragenc@omu.edu.tr
Received date: 24.09.2019- Accepted date: 24.12.2019

**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 ($IVTD_{As\ fed}$), dry matter ($IVTD_{DM}$), organic matter ($IVTD_{OM}$), neutral detergent fiber ($IVTD_{NDF}$) 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 $IVTD_{OM}$ increased significantly ($P<0.05$), with the same effect observed for *Q. cerris* ($P<0.01$). However, for the addition of 0.1% FA, $IVTD_{As\ Fed}$, $IVTD_{DM}$ and $IVTD_{NDF}$ 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 protozoan counts decreased for both organic acid applications compared to counts in the fresh rumen contents. Because 0.1% fumaric acid increased the $IVTD_{OM}$ 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 fumaric (FA) ve malik (FA) asitin ruminant alternatif yem kaynağı olarak *Robinia pseudoacacia* (yalancı akasya), *Prunus laurocerasus* (karayemiş), *Quercus cerris* (meşe), ve *Camellia sinensis* (fabrika atığı, TFW) yapraklarının sindirilebilirlik değerleri üzerine olan etkilerini incelemektir. Araştırmada materyallerin yem bazında ($IVTD_{As\ fed}$), kuru madde bazında ($IVTD_{DM}$), organic maddede ($IVTD_{OM}$), nötral deterjen fiber ($IVTD_{NDF}$) *in vitro* gerçek sindirilebilirlikleri ve protozoa sayıları parametreleri üzerine çalışılmıştır. Sindirilebilirlik parametreleri DAISY inkübatör sistemi ile gerçekleştirilmiştir. Kontrol grubuna FA ve MA eklenmezken deneme grupları ayrı ayrı ve sırasıyla %0,1, %0,2, %0,3 FA ve MA içerecek şekilde düzenlenmiştir. Her örnek 6 kez tekrarlı olacak şekilde araştırmaya 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 linear değişimi de önemli ($P<0,01$) bulunmuştur. Fumarik asit TFW materyaline %0,1 düzeyinde uygulandığında $IVTD_{OM}$ önemli derecede ($P<0.05$) yükselmiştir. Aynı etki *Q. cerris* için de görülmüş ($P<0,01$) ancak $IVTD_{As\ Fed,}$ $IVTD_{DM}$ ve $IVTD_{NDF}$ değerlerinin önemli seviyede ($P<0,01$) düştüğü saptanmıştır. Malik asit bu araştırmada *in vitro* gerçek sindirim 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 atıkları ve *Q. cerris* yapraklarına, %0,1 düzeyinde fumarik asit ilavesinin $IVTD_{OM}$ değerlerini artırdığından dolayı, *C. sinensis* fabrika atıkları ve *Q. cerris* yapraklarının ruminantlar için potansiyel alternatif yem kaynağı olarak değerlendirilebileceği kanısına varılmıştır.
**Anahtar sözcükler:** Fumarik asit, gerçek sindirilebilirilik, *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 cellobiose and monensin. 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 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 leave samples were weighed as fresh and then dried at 65 ºC 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 $CO_2$ added. The rumen fluid was collected manually by squeezing two handfulls 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 ºC 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, F57 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),

$$\text{IVTD\%} = \frac{100-(\text{W3}-(\text{W1xC1}))\text{x}100}{\text{W2}}$$

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 transferred 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 $mm^2$) (31).

$$Cell\ number\ per\ CMM = Number\ of\ cells\ counted\ X \frac{1}{Area\ counted\ (mm^2)} X \frac{1}{Depth\ (mm)} X\ Dilution$$

***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, $IVTD_{As\ Fed}$, $IVTD_{DM}$, $IVTD_{OM}$ and $IVTD_{NDF}$. 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 $IVTD_{As\ Fed}$, $IVTD_{DM}$, $IVTD_{OM}$, and $IVTD_{NDF}$ 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 $IVTD_{OM}$ 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$_{As\ Fed}$** | **IVTD$_{DM}$** | **IVTD$_{OM}$** | **IVTD$_{NDF}$** |
|---|---|---|---|---|
| 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 | | | | |
| Combined | 0.001 | 0.001 | 0.001 | 0.001 |
| Linear | 0.002 | 0.002 | 0.001 | 0.002 |
| **Malic acid** | **IVTD$_{As\ Fed}$** | **IVTD$_{DM}$** | **IVTD$_{OM}$** | **IVTD$_{NDF}$** |
| 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 | | | | |
| Combined | 0.077 | 0.077 | 0.058 | 0.077 |
| Linear | 0.943 | 0.943 | 0.861 | 0.943 |

IVTDAs Fed: *In vitro* true digestibility as fed, IVTD$_{DM}$: *In vitro* true digestibility of dry matter, IVTD$_{OM}$: *In vitro* true digestibility of organic matter, IVTD$_{NDF}$ : *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$_{As\ Fed}$** | **IVTD$_{DM}$** | **IVTD$_{OM}$** | **IVTD$_{NDF}$** |
|---|---|---|---|---|
| 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 | | | | |
| Combined | 0.051 | 0.051 | 0.048 | 0.054 |
| Linear | 0.057 | 0.057 | 0.061 | 0.062 |
| **Malic acid** | **IVTD$_{As\ Fed}$** | **IVTD$_{DM}$** | **IVTD$_{OM}$** | **IVTD$_{NDF}$** |
| 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 | | | | |
| Combined | 0.128 | 0.128 | 0.117 | 0.131 |
| Linear | 0.536 | 0.536 | 0.525 | 0.527 |

IVTDAs Fed: *In vitro* true digestibility as fed, IVTD$_{DM}$: *In vitro* true digestibility of dry matter, IVTD$_{OM}$: *In vitro* true digestibility of organic matter, IVTD$_{NDF}$ : *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$_{As\ Fed}$, IVTD$_{DM}$ and IVTD$_{NDF}$ parameters by adding FA to *Q.cerris* (Table 5) leaves however, IVTD$_{OM}$ 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** | **$IVTD_{As\ Fed}$** | **$IVTD_{DM}$** | **$IVTD_{OM}$** | **$IVTD_{NDF}$** |
|---|---|---|---|---|
| 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 | | | | |
| Combined | 0.706 | 0.706 | 0.747 | 0.706 |
| Linear | 0.736 | 0.736 | 0.719 | 0.736 |
| **Malic acid** | **$IVTD_{As\ Fed}$** | **$IVTD_{DM}$** | **$IVTD_{OM}$** | **$IVTD_{NDF}$** |
| 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 | | | | |
| Combined | 0.634 | 0.634 | 0.659 | 0.634 |
| Linear | 0.787 | 0.787 | 0.750 | 0.787 |

IVTDAs Fed: *In vitro* true digestibility as fed, $IVTD_{DM}$: *In vitro* true digestibility of dry matter, $IVTD_{OM}$: *In vitro* true digestibility of organic matter, $IVTD_{NDF}$ : *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** | **$IVTD_{As\ Fed}$** | **$IVTD_{DM}$** | **$IVTD_{OM}$** | **$IVTD_{NDF}$** |
|---|---|---|---|---|
| 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 | | | | |
| Combined | 0.004 | 0.004 | 0.004 | 0.005 |
| Linear | 0.020 | 0.020 | 0.017 | 0.020 |
| **Malic acid** | **$IVTD_{As\ Fed}$** | **$IVTD_{DM}$** | **$IVTD_{OM}$** | **$IVTD_{NDF}$** |
| 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 | | | | |
| Combined | 0.499 | 0.499 | 0.464 | 0.520 |
| Linear | 0.227 | 0.227 | 0.190 | 0.240 |

IVTDAs Fed: *In vitro* true digestibility as fed, $IVTD_{DM}$: *In vitro* true digestibility of dry matter, $IVTD_{OM}$: *In vitro* true digestibility of organic matter, $IVTD_{NDF}$ : *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 current 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 $IVTD_{DM}$. 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 $IVTD_{DM}$, 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 $IVTD_{DM}$ 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 $IVTD_{DM}$, 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 $IVTD_{DM}$ 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%, $IVTD_{OM}$ 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 protozoans. 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 $H_2$ transfer. Therefore, the author(s) of the present study recommend that the determination of true digestibility should also take into consideration the counts of protozoans, 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.

## Acknowledgements

The authors would like to thank Mr. Gregory T. Sullivan for editing the English in this manuscript.

## Financial Support

This research received no grant from any funding agency/sector.

## Conflict of Interest

The authors declared that there is no conflict of interest.

## References

1. **ANKOM** (2002): Operator's manual. Ankom 200/220 fiber analyzer. Ankom Technology Corporation, Fairport.
2. **AOAC** (2006): Official Methods of Analysis, 18th edn. Association of Official Analytical Chemists, Inc., Arlington, VA.
3. **Asanuma N, Hino T** (2000): *Activity and properties of fumarate reductase in ruminal bacteria*. J Gen Appl Microbiol, **46**, 119–125.
4. **Bharathidhasan A, Karunakaran A, Pugazhenthi TR, et al** (2016): *The effect of supplemental organic acid on methane reduction to decrease the global warming from dairy cattle*. IJACSA, **3**, 4.
5. **British Columbia Ministry of Forests** (1996): Collection of Specimens. p.2-5. In: Techniques and procedures for collecting, preserving, processing, and storing botanical specimens. Res. Br., B.C. Min. For., Victoria, British Columbia.
6. **Burner DM, Carrier DJ, Belesky DP et al** (2008): *Yield components and nutritive value of Robinia pseudoacacia and Albizia julibrissin in Arkansas*. Agroforest Syst, **72**, 51-62.
7. **Callaway TR, Martin SA** (1997): *Effects of cellobiose and monensin on in vitro fermentation of organic acids by mixed ruminal bacteria*. J Dairy Sci, **80**, 1126–1135.
8. **Canbolat O, Kamalak A, Özköse E, et al (2005):** *Effect of polyethylene glycol on in vitro gas production, metabolizable energy and organic matter digestibility of Quercus cerris* leaves. *LRRD, **17**, 4.*
9. **Carro MD, Ranilla MJ** (2003): *Effect of the addition of malate on in vitro rumen fermentation of cereal grains*. Brit J Nutr, **89**, 181–188.
10. **Castillo C, Benedito JL, Méndez J, et al** (2004): *Organic acids as a substitute for monensin in diets for beef cattle*. Anim Feed Sci Tech, **115**, 101–116.
11. **Chen Y, Zhao Y, Fu ZY et al** (2011): *Chemical composition and in vitro ruminal fermentation*

*characteristics of tetraploid black locust (Robinia pseudoacacia L.).* Asian J Anim Vet Adv, **7**, 706-714

12. **Ebrahimi SH, Datta MM, Heidarian V, et al** (2015): *Effects of fumaric or malic acid and 9,10 anthraquinone on digestiblity, micobial protein synthesis, methane emission and performance of growing calves*. Indian J Anim Sci, **85**, 1000-1005.
13. **Gill JL** (1978): Design and analysis of experiments in the animal and medical sciences. Vol 1. The Iowa State Univ. Press, Ames, Iowa, USA.
14. **Kara K** (2015): *In vitro methane production and quality of corn silage treated with maleic acid.* Ital J Anim Sci, **14**, 3994.
15. **Kaya E, Kamalak A** (2012): *Potential nutritive value and condensed tannin contents of acorns from different oak species.* Kafkas Univ Vet Fak, **18**, 1061-1066
16. **Khampa S, Wanapat M, Wachirapakorn C, et al** (2006): *Effect of levels of sodium DL-malate supplementation on ruminal fermentation efficiency of concentrates containing high levels of cassava chip in dairy steers*. Asian Austral J Anim, **19,** 368-375.
17. **Kluge H, Broz J, Eder K** (2004): *Untersuchungenzum Einfluss von benzoesäureals futterzusatz auf leistungs parameter, nährstoffverdaulichkeit, n-bilanz, mikroflora und parameter des mikrobiellen stoffwechselsim verdauungstrakt von absetzferkeln*. Tagungfür Schweine und Geflügelernährung Halle (Saale) Germany, 42-45.
18. **Koleckar V, Kubikova K, Rehakova Z, et al (2008):** *Condensed and hydrolysable tannins as antioxidants influencing the health*. Mini-Rev Med Chem, **8,** 436-447.
19. **Kolver E, Aspin P** (2006): *Supplemental fumarate did not influence milk solids or methane production from dairy cows fed high quality pasture*. Proc Nz Soc Anim Prod, **66**, 409–415.
20. **Kumar S, Puniya AK, Puniya M, et al (**2009): *Factors affecting rumen methanogens and methane mitigation strategies*. World J Microb Biot, **25**, 1557-1566.
21. **Kung L, Huber JT, Krummrey JD, et al (**1982): *Influence of adding malic acid to dairy cattle rations on milk production, rumen volatile acids, digestibility, and nitrogen utilization*. J Dairy Sci, **65**, 1170–1174.
22. **Li Z, Liu N, Cao Y, et al (**2018): *Effects of fumaric acid supplementation on methane production and rumen fermentation in goats fed diets varying in forage and concantrate particle size*. J Anim Sci Biotechno, **9**, 21.
23. **Lopez S, Valdes C, Newbold CJ, et al (**1999): *Influence of sodium fumarate addition on rumen fermentation in vitro*. Brit J Nutr, **81,** 59–64.
24. **Luginbuhl JM, Mueller JP** (2000): Evaluation of fodder trees for meat goats. 77-79. In: L. Gruner and Y. Chabert (Ed). Nutrition and Feeding Strategies. 7th International Conference on Goats. Tours, France.
25. **Martin SA, Streeter MN, Nisbet DJ, et al** (1999*): Effects of Dl-malate on ruminal metabolism and performance of cattle fed high-concentrate diet*. J Anim Sci, **77**, 1008–15.
26. **Montano MF, Chai W, Zinn-Ware TE, et al (**1999): *Influence of malic acid supplementation on ruminal pH, lactic acid utilization, and digestive function in steers fed high-concentrate finishing diets*. J Anim Sci, **77**, 780–784.
27. **Ok JU, Ha DU, Lee SJ, et al (**2012): *Effects of organic acids on in vitro ruminal fermentation characteristics and methane emission*. J Life Sci, **22**, 1324-1329.
28. **Özyılmaz N** (2019): *Organik ve konvansiyonel yöntemlerle üretilen çayların (Camellia sinensis) fabrika çay atıklarında besin madde içeriği ve in vitro sindirilebilirlik değerlerinin belirlenmesi.* Yüksek Lisans Tezi, Ondokuz Mayıs Üniversitesi Sağlık Bilimleri Enstitüsü, Samsun.
29. **Parissi ZM, Abraham EM, Roukos C, et al** (2018): *Seasonal quality assessment of leaves and stems of fodder ligneous species.* Not Bot Horti Agrobo, **6**, 426-434.
30. **Partanen K** (2001): Organic acids – their efficacy and modes of action in pigs. pp. 201-218. In: Piva E, Knudsen B, Lindberg JE (Ed), Gut environment of pigs. Nottingham University Press, Nottingham, UK.
31. **Ranilla MJ, Carro MD, Valdés C, et al (**1997): *A comparative study of ruminal activity in Churra and Merino sheep offered alfalfa hay*. Anim Sci, **65**, 121-128.
32. **Sahoo A, Jena B** (2014): Organic acids as rumen modifiers. IJSR, **3**,11.
33. **Sirohi SK, Pandey P, Goel N** (2012): *Response of fumaric acid addition on methanogenesis, rumen fermentation, and dry matter degradability in diets containing wheat straw and sorghum or berseem as roughage source*. ISRN Vet Sci, 1-8.
34. **SPSS** (2007): Statistical packages for social science. Version 21, SPSS In., Illinois, USA.
35. **Stukelj M, Valencak Z, Krsnik M, et al (**2010): *The effect of the combination of acids and tannin in diet on the performance and selected biochemical, haematological and antioxidant enzyme parameters in grower pigs*. Acta Vet Scand, **52**, 19.
36. **Tieman TT, Lascano CE, Kreuzer M, et al (**2008): *The ruminal degradability of fibre explains part of the low nutritional value and reduced methanogenesis in highly tanniniferous tropical legumes*. J Sci Food Agr, **88**, 1794–1803.
37. **Van Soest PJ, Robertson JB, Lewis BA** (1991): *Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition*. J Dairy Sci, **74**, 3583-3597.