The aim of the study was to investigate the effects of whey powder (WP) on the fermentation quality, nutritive value, and digestibility of ensiled alfalfa \( (Medicago sativa \text{ L.}) \). Alfalfa treated with different doses of WP (0, 2, and 4% fresh matter silage) was ensiled in plastic drums for 60 days. The results of the study revealed that the physicochemical composition and fermentation quality of the alfalfa silage improved and that mold growth was inhibited in the groups treated with 2 and 4% WP in comparison to that in the control. Production of CO\(_2\) (day 7) was much lower in silages treated with 2 and 4% WP (3.77 and 1.85 g/kg dry matter (DM), respectively) than in the control group (21.36 g/kg DM). In addition, in vivo dry matter digestibility (DMD) was much higher in the group treated with 4% WP (76.45%) than in the control one (55.82%). In this treatment group, all apparent digestibility of coefficients in vivo from crude nutrient contents and cell wall fractions significantly increased and hence raised the net lactation energy value from 1.18 to 1.31 Mcal/kg DM. However, although the in vitro DMD was higher in the silages treated with WP than in the control one and the dose was significant, there was no strong correlation between in vivo and in vitro values. According to our results, WP could provide an advantage for the conservation of alfalfa silage. In addition, WP could be evaluated as a sustainable silage additive.

**Key Words:** alfalfa, digestibility, net energy, silage, whey powder

**Introduction**

Alfalfa \( (Medicago sativa \text{ L.}) \) is a perennial legume with a high yield which is also rich in vitamins and minerals. It can be used as animal feed in the form of green herbage, hay, or silage. Because grazing on fresh alfalfa can cause tympani, it is very limited (Jonker and Yu, 2016). Although the dried form is generally preferred as animal feed, the leaves are easily broken and lost during this process when drying to the appropriate storage moisture of 12%, also the botanical fraction balance (leaf:stem ratio) shifts toward the cellulosic structure. This decreases protein content, digestibility, and quality of the forage, reduces the alfalfa nutritional value, and increases the cost of feed. On the other hand, it could be possible to obtain successful silage by using additives that stimulate fermentation, such as water-soluble carbohydrates (Keener, 2019).

Whey is a byproduct resulting from the processes of converting milk into cheese. It has high water content (>90%) and its major constituent is lactose (77% of the total solids). Whey powder (WP) is the dried form of it. It contains high content of lactose (69–76%), low content of water (<7%) and is rich in protein (β-lactoglobulin and α-lactalbumin), vitamins (B\(_2\), B\(_5\), and C) and minerals (EWPA, 2016). Each year, more than 200 mln t of whey is generated globally, and this value is increasing by ~2% each year (Mariotti et al., 2020).
However, a large part of whey is processed to dried form to be used in the food and feed industry (Królczyk et al., 2016; El-Tanboly et al., 2017). In February 2022 the price of WP used as animal feed ranged between 1.154–1.340 €/t in Europe and USA (Anonymous, 2022). So that, WP could be more affordable than other silage additives like inoculants or organic acids, if its use becomes more widespread. It is well known that WP used economically in milk replaces is healthy for young animals. This by-product has been evaluated for increasing calf growth (El-Shewy, 2016). Some studies have revealed that whey has the potential as a silage additive (Castaño and Villa, 2017; Keener, 2019). But the studies on dried form of whey as silage additive are limited. However, WP can also have a stimulating effect on alfalfa with low water-soluble carbohydrates during fermentation because of its high lactose content (El-Shewy, 2016). Some studies have revealed that whey has the potential as a silage additive (Castaño and Villa, 2017; Keener, 2019). But the studies on dried form of whey as silage additive are limited. However, WP can also have a stimulating effect on alfalfa with low water-soluble carbohydrates during fermentation because of its high lactose content and can be more economical than molasses. So, the aim of the present study was to investigate the effects of WP at different doses (0, 2, and 4%) on the fermentation quality, nutritive value, and digestibility of ensiled alfalfa.

Material and methods

Alfalfa and whey powder

Alfalfa was harvested in the early bloom of the fifth cutting and was chopped to ~1.5–2.0 cm and allowed to wilt until the dry matter (DM) content was ~29% before ensiling (Table 1). Whey protein was purchased from Maybi (Malkara-Tekirdağ,Turkey) and was obtained from sweet, fresh, pasteurized whey. The whey had been spray-dried without using any preservatives or additives and demineralized. Whey powder containing less protein and more lactose was preferred in the present study to be better carbohydrate source for alfalfa silage fermentation.

Silage preparation

Alfalfa treated with different doses (0, 2, 4% fresh matter silage) of WP was ensiled in 120-l plastic drums. All groups were ensiled again in 2 kg jars for aerobic stability. The ensiling was determined according to silage-making techniques (Kılıç, 1986). Three replicates were used for each group in a completely randomised design and 60 days of storage. Four analyses from each replicate drum was performed to identify chemical and microbiological properties, and in vitro DM digestibility.

Table 1. Chemical composition of alfalfa before ensiling, % dry matter (DM)

<table>
<thead>
<tr>
<th>Alfalfa chemical composition</th>
<th>DM*</th>
<th>NFC</th>
<th>17.55</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM</td>
<td>84.62</td>
<td>46.92</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>15.38</td>
<td>37.91</td>
<td></td>
</tr>
<tr>
<td>AIA</td>
<td>4.64</td>
<td>7.26</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>17.45</td>
<td>9.02</td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>2.70</td>
<td>30.65</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>29.65</td>
<td>4.70</td>
<td></td>
</tr>
<tr>
<td>NIE</td>
<td>34.83</td>
<td>73.78*</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Whey powder chemical composition</th>
<th>DM</th>
<th>EE</th>
<th>pH</th>
<th>6.83</th>
</tr>
</thead>
</table>


Chemical analyses

Samples were dried at 65 °C for 48 h and were ground in a grinder with 1 mm sieve. All samples were analyzed using the following methods: crude nutrients (DM, crude protein (CP), ether extract (EE), and crude fibre (CF)) using the Weende analysis (Menke and Huss, 1975), cell-wall components (neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL)) using the fibre bag system as modified by Goering and Van Soest (1970), organic acids (acetic acid (AA), butyric acid (BA), lactic acid (LA)) using the distillation method adapted from Lepper (Naumann and Bassler, 1993), water-soluble carbohydrates using a spectrophotometer and an anthrone-thiourea method (Anonymous, 1986), ammonia nitrogen (NH3-N) rates using the microdistillation method (Anonymous, 1986), and aerobic stability using the method of Ashbell et al. (1991). The nitrogen-free extract (NfE) was calculated as %NfE = 100% – (CP% + CF% + ash% + fat%) and the nonfibrous carbohydrate (NFC) was calculated as NFC = 100 – (NDF% + CP% + fat% + ash%) (all nutrients are in dry matter) (NRC, 2001). Hemicellulose (HEM) content was calculated from NDF – ADF, and cellulose (CEL) content from ADF – ADL. Before ensiling, the fresh material buffer capacity was detected using the method of Playne and McDonald (1966). All pH values of the samples were measured using a desktop pH meter (Hanna HI2211-02; Chennai, Tamil Nadu, India). The physical characteristics of all silages were determined using three different parameters of colour, odour, and structure.
The Flieg score:

\[ \text{Flieg score} = 220 + (2 \times \text{DM\%} - 15) - 40 \times \text{pH}, \]

was calculated according to silage dry matter (DM) content and pH value (Kılıç, 1986; DLG, 1987).

The relative feed value (RFV) was calculated according to Rohweder et al. (1978) from the equation:

\[ \text{RFV} = \frac{\text{DMI} \times \text{DDM}}{1.29}, \]

where: DMI, % of body weight (BW) = 120 / (neutral detergent fibre (NDF), % of dry matter (DM)); and DDM, % of DM = 88.9 – 0.779 × (acid detergent fibre (ADF), % of DM).

**Microbial analyses**

The colonies of mold-yeast were counted using Tournas et al. (1998) and counts were expressed as colony-forming units per g (CFU/g). Malt extract agar was used for the enumeration of mold/yeast and the Petri plates were incubated at 25 °C for 3–5 days under aerobic conditions.

**In vitro digestibility**

In vitro DM digestibility (IVDMD) of the silages was determined using the enzyme technique, which is based on incubating forages with pepsin (2000 FIP-U/g) and cellulase enzyme (Onozuka R 10 from *Trichoderma viride*; Merck, Darmstadt, Germany). The incubation time and temperature of each enzyme were 24 h at 38 °C, respectively. After incubation, the forages were washed, dried at 105 °C for 24 h, and incinerated in a muffle furnace at 550 °C for 4 h (De Boever et al., 1986).

**In vivo digestibility**

All procedures concerning animal usage were approved by the Ethics Committee of Ege University, Izmir, Turkey (No: 2016-001).

Nine Kivrıç rams, 1–1.5 years old with similar physical characteristics and 45.0 ± 3.0 kg body weight were used in the study. They were fed twice/day at 8:30 and 16:30 and had *ad libitum* access to drinking water and lick blocks during the trial. The study was conducted using a completely randomised design (3 rams per each treatment).

To evaluate in vivo digestibility, animals were maintained in individual pens for 12 days (7 days of adjustment period + 5 days of sampling). Feed intake was calculated as 1.2 fold animal maintenance requirements and feed (3.5 kg of fresh matter silage). The manure was collected in the morning before feeding during the sampling period. The manure picked up from each group was weighed and 100–150 g of it was kept into a jar by adding 3–5 drops of chloroform. There were three jars of the manure from one animal. Each jar with manure was analysed four times. All samples were kept in the refrigerator at −20 °C until analysis. The apparent digestibility of coefficient (ADC) of the groups was calculated according to GfE (1991):

\[ \text{ADC} (%) = \left( \frac{\text{feed intake} - \text{throw out with manure}}{\text{feed intake}} \right) \times 100. \]

Total digestible nutrients (TDN) were calculated according to NRC (2001) and Küçük (2019):

\[ \text{TDN} (%) = \left( \frac{\text{CP} \times \text{ADC of CP}}{100} + \frac{\text{CF} \times \text{ADC of CF}}{100} + \frac{\text{NfE} \times \text{ADC of NfE}}{100} + \frac{\text{EE} \times \text{ADC of EE} \times 2.25}{100} \right), \]


Net energy for lactation (NE₅) was calculated according to NRC (2001):

\[ \text{NE}_5 \text{(Mcal/kg)} = 0.0245 \times \text{TDN} - 0.12, \]

where: TDN – total digestible nutrients.

**Statistical analyses**

All data were conducted to one-way analysis of ANOVA by employing the procedure SPSS version 22.0 (IBM Corp., Armonk, NY, USA) package software (SPSS, 2013). A completely randomised design was used according to the following model:

\[ y_{ij} = \mu + \alpha_i + \epsilon_{ij}, \]

where: yij – dependent variable, μ - overall mean, αi – fixed effect of treatment (i = 1 to 3), εij – random error.

Duncan multiple comparison test was used to compare the differences among the mean values (P < 0.05).

**Results**

**Chemical composition**

In comparison with the control group (Table 2), the addition of WP to alfalfa silage led to an increase in the content of DM of 4.42% in the samples with 4% WP and of 1.63% in the samples with 2% WP (P < 0.05). No effect of WP addition on OM, CP, and EE (P > 0.05) was observed.

The addition of WP also increased the NfE content in the silage in comparison to control samples (P < 0.05), but there was no dose-depending effect (P > 0.05). On the other hand, NFC values in silages ranged from 17.06 to 21.92%, and the difference between the groups was important (P < 0.05). The addition of WP also affected RFV values in the silages. According to the data, in the groups treated
with WP higher RFV values than in the control one were noted, and this effect increased with the WP addition (*P < 0.05*).

The addition of 4% WP was more effective on NDF fraction – the lowest NDF value was found in this group (*P < 0.05*). Similarly, only treatment with 4% WP had a significant effect on the ADF fraction in the silage and lowered its value (*P < 0.05*). However, the both doses of WP had no effect on ADL, NFC and HEM in the silages treated with 2% WP; however, the NH$_3$N in the silage treated with 4% WP decreased in comparison to that in the control group.

At day 7 it was observed that the addition of WP reduced the CO$_2$ content in the silages and that the WP dose had a crucial effect (*P < 0.05*). According to the data, the lowest level of CO$_2$ was found in the 4% WP group, and aerobic deterioration was the highest in the control group. Hence, the highest improvement of aerobic stability was determined in the group with 4% WP. In addition, it was observed that mold growth was prevented in the groups treated with WP.

### In vitro and in vivo digestibilities

As shown in Table 4, the addition of WP to silages influenced IVDMD (*P < 0.05*). In all groups values between 62.25 and 67.87% were noted; in the control group IVDMD was the lowest whereas in the 4% WP group – the highest. However, there was no strong correlation between in vitro and in vivo values for DMD.

Treatment with 4% WP had a significant effect on the digestibility of all nutrients in alfalfa silage.
(P < 0.05). The greatest apparent digestibility coefficient (ADC) values were found in the silages with 4% WP (P < 0.05). No effect on ADC digestibility in silage samples with 2% WP was noted (P > 0.05). However, it was observed that WP addition influenced carbohydrate digestibility. In silages treated with WP higher ADC of CF and NfE contents were noted, and this effect increased with the dose (P < 0.05). Treatment with WP improved the digestibility of silages, and dose greatly affected ADC of NDF and ADF. The greatest ADC of NDF was in the 4% WP silage (P < 0.05). Accordingly, the lowest ADC of ADF caused it to stimulate N efficiency and improved rumen microorganisms.

Whey powder treatment improved silages by decreasing their NDF and ADF contents. These results are in line with the results of previous studies on the effect of WP on the cellulose content in the silage (Dash et al., 1974; Fallah, 2019). According to chemical analyses data, it could be stated that WP used as a fermentation stimulant had a significant effect on the carbohydrate structure of the ensiling material by increasing the rate of NFC versus decreasing the rates of NDF and ADF.

**Fermentation quality, aerobic stability and microbiology.** The pH value is an important indicator reflecting silage fermentation toward dominant microorganisms either desired or not desired. In the present study WP improved the silage fermentation but not as enough to lower the pH to < 5. Bijelić et al. (2015) have reported that DM has a direct effect on pH. Also, Kung (2010) stated that legume silages have a pH value of more than 4.6 to 4.8 due to low DM value of fresh material (< 30%) that causes

### Discussion

**Chemical composition.** It was found that DM and NFC contents of the silages increased along with the increasing dose of WP. It could be associated with the high DM content (95%) in WP, and so the ability of WP to improve silage fermentation. On the other hand, NFC could play a critical role as a fermentation stimulant in the rumen because of the carbohydrate. It was shown that NFC as a carbohydrate source balancing with nitrogen (N) as a protein source improved rumen microorganisms and N efficiency in the rumen (Ma et al., 2015). In the present study, slight increase of NFC in the groups treated with WP (with high lactose content) had a positive effect on alfalfa silage digestibility in the rumen because it stimulated N efficiency and improved rumen microorganisms.

**Table 4. Effect of whey powder (WP) on in vitro dry matter digestibility (IVDMD), in vivo digestibility, total digestible nutrients (TDN) and net energy lactation (NE\textsubscript{L}) in alfalfa silage, in dry matter (DM)**

<table>
<thead>
<tr>
<th>Indices</th>
<th>WP treatment, %</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVDMD, %</td>
<td>0 (n = 3)</td>
<td>2 (n = 3)</td>
</tr>
<tr>
<td>Crude nutrients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>55.82 ± 2.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.83 ± 2.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OM</td>
<td>62.18 ± 1.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.02 ± 1.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CP</td>
<td>65.70 ± 1.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70.37 ± 1.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EE</td>
<td>68.53 ± 2.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.38 ± 1.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CF</td>
<td>69.87 ± 1.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70.37 ± 1.44&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>NfE</td>
<td>58.21 ± 2.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>66.28 ± 1.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell wall fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>68.23 ± 1.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.00 ± 1.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADF</td>
<td>59.38 ± 2.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.86 ± 1.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TDN, %</td>
<td>53.19 ± 0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.37 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NE\textsubscript{L}, Mcal/kg</td>
<td>1.18 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.31 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

OM – organic matter, CP – crude protein, EE – ether extract, CF – crude fibre, NfE – nitrogen-free extract, NDF – neutral detergent fibre, ADF – acid detergent fibre; <sup>a</sup>–<sup>c</sup> means within the row with different superscripts are significantly different at P < 0.05.
clostridial fermentation. Clostridia is undesirable bacteria in silages as it causes protein degradation, DM loss, and production of toxins. However, butyric acid is a marker for growing clostridia and it was not identified in the silages treated with WP in this study. The second reason for such low pH values is the high buffering capacity of legumes which might be a barrier. On the other hand, pH values of the silages treated with WP were compatible with some previous studies (Şengül and Aydın, 2019; Kang et al., 2021). Nevertheless, the addition of 2 and 4% WP was enough to improve other fermentation characteristics, such as aerobic stability and the microbial population in the alfalfa silages, without changing the pH.

Lactic acid is the primary acid responsible for the decrease in silage pH, and the ratio of LA should constitute 65–70% of the total acids in silages that have undergone successful fermentation. Acetic acid is the second most commonly found volatile acid in silages, and BA should not be >0.5% in well-preserved silages because it induces ketosis in lactating cows. High ratios of BA in silage also indicate that feed proteins are broken down and the silage begins to spoil (McDonald et al., 2010; Kung et al., 2018). When the profile of the volatile acids in the silage groups was examined, we observed that the silages treated with WP had higher LA and AA but lower BA than the control. Hence, it is possible that WP could be a good source for improving end products of legume silages. It was found that the AA ratio was slightly higher in the WP groups than in the control one. This moderate increment could be beneficial for improving aerobic stability. Even so, raising the LA ratio and reducing the BA ratio supported the fermentation to occur at the desired level and that the Lactobacillus species were dominant in the fermentation environment in the present study. Furthermore, AA showed a protective property for improving other fermentation characteristics, such as reducing the CO2 ratio (Kung et al., 2018). Danner et al. (2011) have shown that lactic acid concentration, incubation time, and washing type have a significant effect on enzymatic DMD. During incubation, enzymatic activity or enzyme type might have affected and altered the results of the study. Similarly, it has been reported that as the incubation time increases when using the enzymatic method, while other methods, such as in situ, may decrease it (Olowu and Yaman Firincioglu, 2019). Additionally, this inconsistency between in vitro and in vivo results could be related to the unpredicted effects of feed additives such as WP on rumen health. Promoting to grow rumen microbiota in a living organism might be an unpredicted effect of 4% WP addition.

Previous studies have generally shown that WP addition to silage improves the fermentation process (Castaño and Villa, 2017; Fallah, 2019). In some studies, it was also observed that addition of WP significantly increases the digestibility of the alfalfa silage, which is believed to be a result of its high lactose content (Dash et al., 1974; Keener, 2019). Because WP has a stimulating effect as a carbohydrate on fermentation during ensiling, it is presumed that the silages treated with it are of high quality. In the present study, we observed that the WP groups had higher in vivo digestibility resulting from the successful fermentation process. Hence, the increase in DMD in the treated silages was associated with the increased DM content resulting from the addition of WP. This increment in DM rate supported in vitro and in vivo DMD in the group treated with 4% WP. On the other hand, silage treated with 4% WP had the NE6 value closest to that of alfalfa hay (NRC, 2001).

Conclusions

Chemical composition and fermentation quality of alfalfa silage was improved by the treatment with whey powder (WP). The additive provided a crucial improvement in aerobic stability, and prevent the mold growth. Also, 4% WP addition significantly increased in vivo nutrient digestibility. Whereas the addition of both 2 and 4% WP affected the digestibility of crude fibre and acid detergent fibre. Accordingly, it is recommend that silage additives such as WP could be successfully used with legumes as alfalfa, that are difficult to ensilage, and in order to avoid losses in silage digestibility. Also, using whey in ruminant nutrition could prevent environmental pollution caused by industrial wastes and support investments for ecological recycling facilities in cheese factories pulverizing whey to powder.
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SÖ: original draft, conceptualization, investigation, data collection, formal analysis, and writing;

HÖ: original draft, conceptualization, project administration, supervision, validation, and editing;

HHİ: in vitro digestibility and the correlation between in vitro and in vivo parameters and contributed to writing this article. The authors also thank TÜBİTAK for financial support.

Conflict of interest

The authors declare that there is no conflict of interest.

References


