Effects of Lactobacillus Buchneri Inoculation and Fresh Whey Addition on Alfalfa Silage Quality and Fermentation Properties

Maghsoud BESHARATI1,2*, Valiollah PALANGI2, Mojtaba NEKOO3, Tugay AYAŞAN4

1,2University of Tabriz, Ahar Faculty of Agriculture and Natural Resources, Department of Animal Science, 51666, Tabriz, Iran, 3Department of Animal Science, Agricultural Faculty, Ataturk University, 25240, Erzurum, Turkey, 4Osmaniye Korkut Ata University, Kadirli Academy of Applied Sciences, Osmaniye, Turkey

*https://orcid.org/0000-0002-5323-6425, https://orcid.org/0000-0001-6470-2578

ABSTRACT
This study was aimed to determine the effects of different levels of fresh whey and Lactobacillus buchneri inoculation on the nutrient properties and aerobic stability of alfalfa silage. Experiment was conducted with 8 treatments and 3 replicates for 90 days. Treatments included wilted alfalfa (control), wilted alfalfa + bacterial inoculant (AB), wilted alfalfa + 30 g whey/kg (AW1), wilted alfalfa + 30 g whey + bacterial inoculant (AW1B), wilted alfalfa + 60 g whey per kg (AW2), wilted alfalfa + 60 g whey per kg + bacterial inoculant (AW2B), wilted alfalfa + 90 g whey per kg (AW3) and wilted alfalfa + 90 g whey + bacterial inoculant (AW3B). Results showed that, the pH values of treatments were found less than 4.86 but the control pH was higher in comparison with other treatments (P<0.05). The addition of fresh whey and complementary bacterial supplement to alfalfa caused a significant increase in total fatty acids. There was no significant difference between neutral detergent fiber (NDF) and acid detergent fiber (ADF) in all treatments compared to control group. The least resistance to erosion was related to wilted alfalfa + bacterial additive (AB), and the highest amount was related to wilted alfalfa + 60 g whey (AW2: P<0.05). The results showed that fresh whey and bacterial inoculation with the fermentable carbohydrates leads to rapid reduction of pH, limiting the proliferation of yeasts, increased aerobic stability and improved silage quality.

Key words: Alfalfa silage, Fresh whey, Lactobacillus buchneri, Aerobic stability, Silage quality

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Fresh whey
Lactobacillus buchneri
Aerobic stability
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ÖZET
Bu çalışmada, farklı seviyelerde taze peynir altı suyu ve Lactobacillus buchneri aşılamasının yonca silajının besin özellikleri ve aerobik stabelite üzerindeki etkilerinin belirlenmesi amaçlanmıştır. Deneme, 90 gün boyunca 8 muamele ve 3 tekerrür ile gerçekleştirilmiştir. Denemede şu gruplar oluşturulmuştur: Pörsütülmüş yonca (kontrol), Pörsütülmüş yonca + bakteri aşılaması (AB), Pörsütülmüş yonca + 30 g taze peynir altı suyu / kg (SYP), Pörsütülmüş yonca + 30 g taze peynir altı suyu + bakteri aşılaması (SYP1B), Pörsütülmüş yonca + 60 g/kg taze peynir altı suyu + bakteri aşılaması (SYP2B), Pörsütülmüş yonca + 90 g/kg taze peynir altı suyu + bakteri aşılaması (SYP3B). Araştırma sonunda, muamelelerin pH değerlerinin 4.86’nın altında olduğu, ancak kontrol pH’un diğer uygulamalarla kıyasla daha yüksek olduğu saptanmıştır (P<0.05). Yoncaya taze peynir altı suyu ve bakteri kültürü eklenmesinin, toplam yağ asitlerinin ortadan kaldırılmasında önemli bir artışa yol açtığı görülmüştür (P<0.05). Tüm muamelelerde kontrol grubuna göre nötr deterjan lif (NDF) ve asit deterjan lif (ADF) bakımından önemli bir fark bulunmamıştır. Erozıona karşı en az
INTRODUCTION

Ensiling is a momentous procedure in tropical regions to make good use of components with the highest nutritive value (Besharati et al., 2020a; He et al., 2020; Ren et al., 2020). Alfalfa is viewed as a forage with high nutritive value, low water soluble carbohydrates, high buffering capacity and highly degradable crude protein (NRC, 2001; Schmidt et al., 2009; Jiang et al., 2020; Palangi et al., 2020). McDonald et al. (1991) investigated that, quickly reduction of silage pH, causes to decrease on clostridia growth, minimize the proteolysis and heterolytic fermentation, and silage palatability could worsen. Therefore, to solve this problems, the methods of adding sugar substances (such as fermentative sugars, molasses, whey, juice waste), or cultivating alfalfa with wheat (such as barley, corn, cluster corn, silage of a mixture of pulp or beet leaves, or corn with alfalfa), use fermented supplements (such as organic acids, fibrolytic enzyme or minerals) were recommended (Besharati et al., 2020b; Besharati et al., 2020c; Calabro et al., 2020; Rinne et al., 2020).

According to Daniels et al. (1983), whey is a cheap by-product obtained from cheese production enterprises: straw and bran are added to silage. De Lurdes et al. (1998) reported that dehydrated whey and molasses are used as additives to support fish by-products (Ventura-Canseco et al., 2012). Cheese whey is a valuable source of lactose so it could be used as a silage additive (El-Shewy, 2016; Yüksel et al., 2019; Mariotti et al., 2020).

Exposure of silage to the air at the time of feeding causes erosion of the silage, yeasts that are able to metabolize lactic acid; are the first cause of corruption with increases the pH (Kleinschmit and Kung, 2000), which is also a stimulant for the growth of other harmful microorganisms in the silage (Besharati et al., 2017; Mariotti et al., 2020), ultimately reduces livestock production due to feed depreciation or poisoning. The use of Lactobacillus buchneri has been reported to improve the aerobic stability of silage (Kleinschmit and Kung, 2000). Lactobacillus buchneri, by converting lactic acid to acetic acid, increased the resistance of silage to aerobic erosion (Besharati et al., 2017; Schumcher et al., 2019).

According to the latest statistics, in Iran about 2,000,000 tons of fresh whey is produced annually, which is containing approximately 227,000 tons of dry matter. The use of this material in livestock feeding, will solve the environmental problems, and on the other hand, it will be economically useful by reducing the price of feeds that needed for livestock. Therefore, fresh whey can be used as valuable waste and also, because of the good source of fermentable of carbohydrates (lactose), which can be used to ensiling alfalfa in order that increases the quality.

The hypothesis of this study was that in bacterial inoculation with the fermentable carbohydrates causes to improve the silage quality. The purpose of this experiment was to investigate the effects of adding Lactobacillus buchneri and fresh whey to alfalfa silage on the chemical characteristics and fermentation properties.

MATERIALS and METHODS

The experiment was conducted in the Arasbaran area in West-North of Iran. The alfalfa was produced during the second cut growing season in June 2016 at the Ahar town (39°05'10" latitude N. 47°33'10" longitude E) in East Azerbaijan Province. Second crop alfalfa was collected and wilted for 24 h at comfortable ambient temperature. The wilted samples were cut into pieces to achieve approximately 2 cm theoretical length of cut. The fresh whey was also prepared from Factory in Tabriz and used as needed in experimental treatments.

Experimental treatments were Wilted Alfalfa (without additives, control), Wilted Alfalfa + bacterial inoculant (AB), Wilted Alfalfa +30 g whey/kg (AW1), Wilted Alfalfa + 30 g whey + bacterial inoculant (AW1B), Wilted Alfalfa + 60 g whey per kg (AW2), Wilted Alfalfa + 60 g whey per Kg + bacterial inoculant (AW2B), Wilted Alfalfa + 90 g whey per kg (AW3) and Wilted Alfalfa + 90 g whey + bacterial inoculant (AW3B). Bacterial additive used at level of 3×108 cfu per g of alfalfa wet weight. Bacterial additive, after dissolved in water, sprayed onto the treatments and for control only water was sprayed. Treatments were ensiled in laboratory mini silos (tripplicate) for 90 d at ambient temperature (15 to 18 °C) in a room.

At the opening time, about 100 g from the top of each silo were throw away and the content of each silo were...
thoroughly mixed, and samples were randomly obtained for chemical analysis. For extraction of silage juice, 10 g of silage were homogenized with 100 ml of distilled water (22 °C) for 3 times (each time 30s) and strained through double layers of cheesecloth. The pH of silage was forthwith ascertained using pH-meter. Dry matter (DM) of silage samples were determined after being oven-dried for 72 h at 60 °C. After drying process, the samples were milled through a 1 mm screen using a Wiley mill. Organic matter (OM) was determined by incinerating it at 550°C for 5 h. The crude protein (CP) (1030 Micro Kjeltec Auto Analyzer, method 984.13; AOAC 2000), NDF and ADF (Van Soest et al., 1991) were analyzed by using the above methods. Concentration of total volatile fatty acids (VFA) of silages were measured according to the method of Filya (2003).

Aerobic stability was determined on all treatments. About 55 g of each replicate was placed in container

Table 2 shows the effect of additives on chemical properties and their interaction in alfalfa silage. According to the reported results, the amount of dry matter in the bacterial inoculant treatments decreased significantly compared to the control (P<0.05), and fresh whey treatments in the treatment of 30 g fresh whey had a significant increase compared to the control treatment (P<0.05), but in the treatment of 60 g of water. The fresh whey was significantly less than the control treatment, and a significant increase was observed in the treatment of 90 g of fresh whey.

The amount of ash content in bacterial inoculant silages increased significantly compared to control, but compared to fresh whey treatments, 30, 60 and 90 g of fresh whey treatments were observed unreasonably. The amount of NDF, ADF and EE in bacterial inoculated treatments and fresh whey treatments at 30, 60 and 90 g levels were not significantly different from that of control (P>0.05). There was no significant difference in pH value in bacterial inoculant treatments, but in fresh whey treatments, the lowest amount of pH was observed for 90 g fresh cheeses and the highest amount was observed for control treatment. Also, 30 and 90 g fresh whey treatments were significantly reduced in terms of pH value compared to control (P<0.05).

The amount of ammonia nitrogen in bacterial inoculated treatments was increased significantly, but in fresh whey treatments at 30, 60 and 90 g, was decreased significantly compared to control. The total amount of total volatile fatty acids in the bacterial inoculant treatments increased significantly compared to the control, but in fresh whey treatments with 30 and 60 g were unreasonable, but in treatment containing 90 g fresh whey significant decrease was observed (Table 2).

The effect of fresh whey and bacterial inoculation on the nutritional value of alfalfa molded silage is given in Table 3. The results of this experiment showed that adding a carbohydrate source such as fresh whey to alfalfa silage reduced the pH of the silage compared to the control (P<0.05).

The results of this experiment were consistent with the results of other researchers (Asadi et al., 2004; Touqir et al., 2007; Hashemzadeh et al., 2011). The data of this experiment corresponded to the experimental data used for different levels of whey to alfalfa silage (grams per kilogram of dry matter) (Repetto et al., 2011). Other researchers reported the use of fresh whey in reducing the pH of feed materials from the beginning of the period. Bacterial additive also significantly decreased pH compared to control (P<0.05). In the experiment (Asadi et al. 2004), the effect of Lactobacillus plantarum additive on millet forage was studied and the results showed that the pH decreased in the treatment containing bacterial additive.

In experiment of Adesogan et al. (2004), the effect of bactericidal supplementation of pseudococcus pentoseto (105) and lactobacillus buccaneer (4×105) on Bermuda fodder was used. The pH of the whole
### Table 2. The chemical composition of wilted alfalfa, fresh whey and bacterial inoculant silage after 90 d (DM% basis)

| Nutrients | Bacterial Inoculation | SEM | fresh whey (g/kg fresh alfalfa weight) | SEM | P value |
|-----------|-----------------------|-----|--------------------------------------|-----|---------|
|           | 0                     |     | 3×10⁶cfu                              |     |         |
|          |                       |     |                                      |     |         |
| DM        | 25.01±0.34            | 0.28| 24.51±0.44                           | 0.23|        |
| Ash       | 10.66±0.12            | 0.48| 10.91±0.66                           | 0.35|        |
| NDF       | 42.81±0.75            | 0.58| 43.65±0.87                           | 0.64|        |
| ADF       | 34.25±0.66            | 0.50| 34.81±0.45                           | 0.50|        |
| EE        | 12.93±0.21            | 0.42| 13.23±0.89                           | 0.84|        |
| CP        | 14.2±0.05             | 0.01| 13.83±0.09                           | 0.02|        |
| pH        | 4.72±0.04             | 0.05| 5.01±0.11                            | 0.10|        |
| N-NH3     | 67.6±0.54             | 1.54| 76.33±0.96                           | 3.08|        |
| VFA       | 11.08±0.03            | 0.59| 14.33±0.82                           | 1.18|        |

1DM=Dry matter, NDF=Insoluble fiber in neutral detergent, ADF=Insoluble fiber in acid detergent, EE=Ether extract, CP=Crude protein; SEM: Standard error of means

A Differences between the averages indicated by different letters in the same line are important.

### Table 3. The chemical composition of wilted alfalfa with whey and bacterial inoculant silage after 90 d (DM% basis)

| Nutrients | Silage treatment | SEM | P value |
|-----------|-----------------|-----|---------|
|           | CON             | AB  |         |
| DM        | 25.41±0.33      | 23.60±0.54 | 0.28 | 0.072 |
| pH        | 5.16±0.05       | 4.86±0.04 | 0.09 | 0.001 |
| TVFA      | 12.00±0.39      | 16.66±0.50 | 0.59 | 0.001 |
| NDF       | 44.30±0.79      | 36.00±0.50 | 0.58 | 0.001 |
| ADF       | 34.56±0.50      | 35.06±0.50 | 0.50 |         |
| CP        | 13.81±0.13      | 13.82±0.16 | 0.10 |         |

CON: Wilted Alfalfa (Without additives). AB: Wilted Alfalfa + bacterial cfu 3×10⁶g. AW1: Wilted Alfalfa +30 g kg wet weight whey. AWB1: Wilted Alfalfa +30 gr of whey + bacterial 3×10⁶g cfu. AW2: Wilted Alfalfa +60 grams of whey kg wet weight. AWB2: Wilted Alfalfa +60 g whey +bacterial 3×10⁶g cfu. AW3: Wilted Alfalfa +90 g whey kg wet weight. AWB3 Wilted Alfalfa +90 g whey + bacterial 3×10⁶g cfu. SEM: standard error of means

Within a row, means followed by different letters differ (P<0.05).
fermentation period was significantly lower in the bacterial inoculated treatment. In experiment of Nadeau et al. (2006), the effect of bacterial supplementation of Lactobacillus plantarum and Pseudococcus cerevisiae on alfalfa and arched grass with DM of 22 and 32% was observed that the bacterial additive in alfalfa containing cellulose caused a slight decrease but also a meaning it was in pH, but this was not observed in arched grass forage. Other studies have reported similar results (Curtis, 1996; Whiter and Kung, 2001; Nishino et al., 2004; Touqir et al., 2007; Hashemzadeh et al., 2011). In general, the reason for pH reduction in comparison with control treatment is the high soluble carbohydrate in other treatments and the availability of a higher nutrient for dominating the population of bacteria producing lactic acid and production of lactic acid.

The amount of dry matter was not affected by the bacterial inoculation (P>0.05). Other studies also reported similar results with this study (Driehuis et al., 2001; Filya, 2003; Kung et al., 2003; Filya et al., 2007; Hashemzadeh et al., 2014).

According to Table 3, total volatile fatty acids in AB and AWB2 treatments increased significantly compared to control (P<0.05). In the experiment (Hashemzadeh et al., 2011), adding molasses as carbohydrates to alfalfa fodder increased total fatty acids relative to treatment was monitored. The addition of fresh whey with bacterial inoculation with lower levels of fresh whey cheese (AW1, AWB1) and higher levels (AW3, AWB3) was not significantly different, but significantly increased compared to the two treatments (AW2, AW3).

The results of this experiment were consistent with the results of other researchers (McAllister et al., 1998; Baytok et al., 2005; Kleinschmit et al., 2006). In an experiment with 14 types of bacterial inoculant on forage hay in China, all additives increased esophageal fatty acids (Filya et al., 2007). This increase in the total amount of volatile fatty acids can be attributed to the high population of lactic acid bacteria and the availability of carbohydrate source for the production of lactic acid and acetic acid in experimental treatments relative to the control treatment. Adding fresh whey with bacterial inoculant to alfalfa fodder resulted in a small increase in the amount of NDF, which was not statistically different (P>0.05) (Table 3). This slight increase in NDF levels can be attributed to higher acetic acid production due to higher soluble carbohydrates in the treatment. The bacterial inoculant in all four levels with fresh whey with different levels did not change the NDF compared to the control.

In addition, AWB2 had significant increase with other treatments. Many studies also reported similar results to the current test results (Gordon et al., 1999; Ranjit and Kung, 2000; Kung and Ranjit, 2001; Kızılçımışek et al., 2007; Hashemzadeh et al., 2014). A study was conducted to investigate 14 different types of bacterial inoculant on first-line hay fodder, in the 6 additive increases in NDF, 5 additives unchanged and 3 additives reductions in NDF levels (Filya et al., 2007). There was no significant difference between ADF and AW1, AW3, AWB2 and AB treatments. However, ADF levels in AWB1, AW2 and AWB3 treatments were significantly decreased in control silage (P<0.05) (Table 3).

In the study of the effect of L. buchneri inoculation on corn silage, L. buchneri reduced ADF at low levels of consumption (Ranjit and Kung, 2000). In another study, the same authors of the L. buchneri inoculation at each of three levels reduced the ADF number in 100 days of barley, with a low level of L. buchneri consumption at 5% level. Adding fresh whey and bacterial inoculant to alfalfa forage, in the amount of crude protein in alfalfa treatment with 30 g fresh whey (AW1) and in alfalfa treatments with 90 g fresh cheese and bacterial inoculation (AWB3), a significant increase compared to control (P<0.05). The rest of the treatments were not significantly different from each other (Table 3). The results of this experiment were perfectly consistent with the results of Takasi (1996) and Nkosi and Meeske (2010) which all of these studies reported adding fresh whey to the amount of crude protein. Adding 30 g of fresh whey and bacterial inoculant (AWB1) haylage reduced the amount of crude protein compared to control (P<0.05). Repetto et al. (2011) was reported that the effect of adding fresh whey on alfalfa fodder has a decrease in crude protein content with a rise in fresh whey. Adding fresh whey to different levels of alfalfa silage reduced aerobic stability compared to control and other experimental treatments (Figure 1). The main reasons for accelerating the occurrence of aerobic agglomeration can be due to high levels of soluble carbohydrates and probably lactic acid and the absence of volatile fatty acids (Adesogan et al., 2004).

The level of aerobic stability in treatments (AWB3 and AW1, AW2, AWB2) increased significantly compared to control and AB treatment (P<0.05). The least resistance to corrosion was related to AB treatment and the highest amount was related to AW2 treatment (30 hours and 120 hours, respectively, in the vicinity of the air). The other main reason causing aerobic silage is the low amount of dry matter. Lindgren (1999) reported that the use of L. buchneri supplementation in silage materials improved the acetic acid and increased the aerobic stability during silo storage. Injes et al. (2001) examined three different experiments of the L. buchneri additive alone and in combination with L. plantarum on grass forage in the 90 and 180 days, and reported that experimental treatments were improved aerobic stability of silage.
Figure 1. Effect of fresh whey and bacterial inoculation on aerobic stability

şekil 1. Taze peynir altı suyu ve bakteri aşılamasının aerobik stabilite üzerine etkisi

T1: Wilted Alfalfa (Without additives). T2: Wilted Alfalfa + bacterial cfu 3×10⁸ g . t3: Wilted Alfalfa +30 g/ kg wet weight whey. T4: Wilted Alfalfa + 30 gr of whey + bacterial 3×10⁸ g cfu. T5: Wilted Alfalfa + 60 grams of whey kg wet weight. T6: Wilted Alfalfa + 60 g whey +bacterial 3×10⁸ g cfu. T7: Wilted Alfalfa + 90 g whey kg wet weight. T8: Wilted Alfalfa + 90 g whey + bacterial 3×10⁸ g cfu.

Within a row, means followed by different letters differ (P<0.05).

In the experiment of Filya (2003), the effect of *L. Buchneri* alone and in combination with *L. plantarum* on wheat, corn and sorghum was evaluated. In all treatments, improvement of aerobic stability was reported. Pahlow and Zimmer (1985) found that bacterial inoculant leads to improve aerobic stability of forage silage.

Improving the stability of these silages to a special energy detoxification system, yeasts are attributed to the excessive concentration of organic acids inside the cell. Kautz (1998) reported that, *L. Buchneri* was used to produce lactic acid and acetic acid, which reduced the yeast and improved the aerobic stability step from 26 hours to 400 hours in silages. In general, the effect of *L. Buchneri* on the improvement of aerobic stability can be explained by increasing the amount of acetic acid produced by bacteria, as well as reducing the amount of carbohydrate remaining solution and lactic acid and lower ammonia nitrogen accumulation.

**CONCLUSION**

The addition of fresh whey to alfalfa fodder resulted in a decrease in the final pH of the silage and an increase in total volatile fatty acids, insoluble fiber in acid detergent and, on the other hand, decreased the aerobic stability in control silage. The bacterial inoculation also improved the fermentation parameters and increased the overall quality of the silage. With regard to the above results, it can generally be concluded that the bacterial inoculation and whey alone and together produced a better fermentation quality and the use of a carbohydrate...
source such as whey as sources of bacterial populations. Bacterial inoculations are essential for the preparation of high-quality silage from alfalfa, due to the deficiencies of this valuable plant.

**Conflict of interest**
The authors declare no conflict of interest.

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