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Silage Additives Usage in Improving Fermentation Quality of Alfalfa Silage: A Review

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Abstract: Alfalfa is difficult to ensile due to its high protein content, low water-soluble carbohydrate level, low dry matter content, and high buffering capacity. Therefore, alfalfa has recently increased its efforts to make silage with additives. Silage additives have been used to enhancing the silage quality of alfalfa in recent years. This review covers research studies that have investigated the efficacy of silage additives on fermentation quality, the bacterial diversity of alfalfa silage using the Next-Generation Sequencing (NGS) technique, the effects of microbial inoculant on these bacterial communities, silage's antioxidant enzymes, and unsaturated fatty acid levels. In recent studies, more clear information about bacterial communities in silage fermentation has begun to be obtained with the use of NGS technique. In studies using these techniques, it was reported that lactic acid bacteria (LAB) organic acid additions to alfalfa silage caused Lactobacillus, Pediococcus and Sporolactobacillus species to increase relatively in silage environment, the ratio of unwanted species decreased and silage quality increased. Although the amount of alpha-tocopherol, and beta carotene decreased by half in pure silage compared to fresh alfalfa, it was observed that LAB inoculation increased the ratio of alpha tocopherol, beta carotene and also polyunsaturated fatty acids. Future silage additives are expected to determine effects fermentation quality, the bacterial community, the antioxidant enzymes, and unsaturated fatty acid levels.

Keywords: Alfalfa Silage, Fermentation, Silage Additives, Silage Quality, Next-Generation Sequencing Technique

1. Introduction

The main way to obtain quality and healthy animal products is through animal nutrition. Quality roughage production is of great importance for the development of animal production. Roughages, which are the most important and cheapest feed source for livestock, is the main part of ruminant rations and are low-energy feeds grown under natural conditions. Also, forages are suitable for the nutritional physiology of ruminant animals and are of great importance due to their mechanical satiety in animals [1, 2]. Alfalfa is a perennial fodder legume with an economic life of 7 years, known as the queen of forages. It is especially rich in protein, vitamins, and minerals (beta carotene, vitamin B). Therefore, it can provide the nutrient requirements of herbivores. Alfalfa is difficult to ensile due to its high protein content, low water-soluble carbohydrate level, low dry matter content, and high buffering capacity [3]. Alfalfa, which is difficult to ensile, has recently increased its efforts to make silage with additives [4].

When the studies carried out with alfalfa in recent years are examined, it has been seen that there are generally studies to increase the silage quality of alfalfa [5-14]. In some studies, the effects of additives on fermentation quality, bacterial diversity, antioxidant enzymes, and unsaturated fatty acid levels in alfalfa silage have been conducted [5-7, 15-18]. Classical microbiological plating techniques were performed in the identification of microbial species of silage, however, many cultures could not be determined. Polymerase chain reaction-based techniques were the first molecular methods used to study silage microbial communities. Next-generation sequencing (NGS) methods have also been used to thoroughly characterize the structure and diversity of silage microbial communities. Although the Sanger sequencing method is still used routinely in most laboratories, a small number of samples are being studied. However, when studying complex microbial ecosystems that contain millions of microorganisms, such as the digestive system, it will be necessary to cloning and sequencing thousands of PCR amplicons one by one to obtain a representative view of the microbial composition, which will not be a commonly applicable practical approach. In the NGS technique, sequencing is performed by amplifying with PCR and very similar sequences are grouped as an operational taxonomic unit (OTU). Then the diversity and abundance of microorganisms is determined [19]. With this method, it is possible to obtain more clear information about bacterial communities formed as a result of silage fermentation of many feed materials.

2. Effects of Silage Additives on Silage Fermentation

Li et al. (2019) [16] used NGS to examine the fermentation and shifts in the microbial communities in alfalfa silage. They reported that it is important to use alfalfa and ryegrass silage in meeting ruminant feed needs due to excessive monsoons in the southern regions of China. They conducted a study to determine the effect of lactic acid bacteria (LAB) (10^6 cfu/g of fresh weight) and formic acid (3 mL/kg of fresh weight) treatment on silage fermentation and the bacterial community for 60 d of alfalfa and ryegrass, which are mixed separately and in certain proportions. They reported that the addition of LAB and formic acid caused the Lactobacillus, Pediococcus or Sporolactobacillus species to increase relatively in the during ensiling, the ratio of undesirable microorganisms decreased and the silage quality increased. In this study, in which 5 different feed groups and 2 additives were tested, ryegrass was used in the ratio of 0, 25, 50, 75 and 100% instead of alfalfa silage. As a result of NGS analysis, it was determined that fresh forages showed high bacterial richness, and silage additives reduced bacterial diversity. For example, 78590 reads were obtained in the control group and 111 OTU values were determined, while 85075 reads were obtained in the inoculant group and 75 OTU values were obtained. Again, in the group that added formic acid, 88641 reads were obtained and OTU was determined as 78 ($P<0.05$). Bacterial diversity decreased with LAB inoculant and formic acid treatment. However, the reason for the significant differences in acetic acid levels in the mixed groups could not be explained. It has been reported that the use of inoculants caused an 11% decrease in ammonia nitrogen level compared to control silage. Some plants that contain significant amounts of energy, fat, amino acids, sugar and crude protein, as well as high bioactive substances such as phenolic compounds, flavonoids, unsaturated fatty acids and vitamins, are used as additives for alfalfa silage, which are thought to increase lactic fermentation. For this purpose, in a study by Chen et al. (2020) [5] added wet sea buckthorn pomace (5% fresh matter basis) and LAB (10^5 cfu/g fresh matter basis) to improve the fermentation of alfalfa silage; compared to the control group, the sea buckthorn group decreased the pH and NH_3-N, increased the lactic acid and propionic acid contents. In NGS analysis, it was determined that it reduced the number of bacterial species, richness and diversity index. It has been determined that the sea buckthorn additive increases the growth of *Lactobacillus plantarum*, and *Lactobacillus brevis* and thus improves the silage quality. They suggested the use of a combination of 5% sea buckthorn plant and 10^5 cfu/g *Lactobacillus plantarum* to improve the silage quality of alfalfa. Bai et al. (2020) [6] evaluated the effects of antibacterial peptide-producing *Bacillus subtilis*, *Lactobacillus buchneri* or their combination on fermentation, proteolysis, aerobic stability and microbial communities during the ensiling and aerobic exposure stages of alfalfa silage. It has been reported that *Bacillus subtilis* treated silage produced a higher lactic acid concentration, less proteolysis, and higher aerobic stability than control silage. Both *Lactobacillus buchneri* and *Bacillus subtilis* inoculations increased the number of Lactobacillus and Ascochyta and decreased the number of Enterococcus and Sporormiacea after 60 days of fermentation. *Lactobacillus buchneri* and *Bacillus subtilis* also inhibited the growth of Enterococcus after 3 days of aerobic exposure, but similarly to control silage. *Bacillus subtilis* silage reported that Candida and Pichia dominated the fungal community after 9 days of aerobic exposure. Therefore, inoculation of *Bacillus subtilis* has been reported to improve silage fermentation quality, aerobic stability and bacterial community during silage and after 3 days of aerobic exposure.

Wang et al. (2020) [7] conducted a study in order to determine the effect of alfalfa silage on fermentation and bacterial community by mixing whole-plant corn and alfalfa in different ratios. Alfalfa-corn ratio was designed as 100, 80-20, 60-40, 100 in groups. When the results of silage fermentation parameters were examined, pH, lactic acid (g/kg dry matter) and NH_3-N results were found at the end of 35 days in the groups, respectively, pH 4.69, 4.19, 3.99 and 3.72 ($P<0.001$); lactic acid 64.6, 96.1, 85.7, 59.2 g/kg dry matter ($P<0.001$), NH_3-N 108, 67.6, 41.1, 34.5 g/kg, total nitrogen ($P<0.01$). The OTU values in the groups were determined as 130, 145, 157 and 158 at the end of 35 days, respectively ($P<0.001$). Generally, a fermentation consisting of Lactobacillus, Weisella and Pediococcus bacterial community developed in silages. It was concluded that the corn additive was better on alfalfa silage fermentation than the alfalfa group made alone.

In recent years, antioxidant additives have been widely used in livestock feed, and it has been proven that they extend storage time, protect nutrients and increase feed safety. It is suggested that antioxidant additives have positive effects on improving meat flavor, animal production and survival of newborn livestock [18]. A study was conducted in which a new strain of *Lactobacillus plantarum* (24-7), reported to has a high antioxidant effect, was inoculated into alfalfa silage with two different dry matter content (30% and 40% dry matter). Higher levels of acetic acid and non-protein nitrogen and lower concentrations of lactic acid, propionic acid and water-soluble carbohydrates were detected in alfalfa silage with low dry matter compared to silage with high dry matter. They determined lower silage pH, non-protein nitrogen, neutral detergent fiber, acid detergent fiber and higher water soluble carbohydrate concentrations with the 24-7 incubation of *L. plantarum* compared to control silage. Although the amount of alpha-tocopherol and beta carotene decreased by an average of half in the control silage compared to the fresh material, the researchers reported that *L. plantarum* 24-7 inoculation increased the ratio of alpha tocopherol, beta carotene and also polyunsaturated fatty acids (PUFA) in both dry matter silages. These values were found to be higher in low dry matter silage than high dry matter silage. Different results in some silage studies have been attributed to silage with different characteristics. While this inoculation increased the total antioxidant capacity, glutathione peroxidase and catalase activities of alfalfa silage, it decreased superoxide dismutase activity. They found the decrease of superoxide dismutase surprising and explained that this could be caused by the closure of the aerobic pathway by an anaerobic fermentation and could cause a rapid drop in pH in the presence of LAB. The researchers reported that *L. plantarum* inoculated into 24-7 alfalfa silage improved the fermentation quality and antioxidant status of alfalfa silage compared to control silage [18].

It is known that PUFA can pass into meat and milk in ruminants if the PUFA value in feeds is high. PUFA is higher in fresh feeds than in silage. It has been reported that during silage silage the activity of lipoxygenase decreases due to the biohydrogenation of unsaturated fatty acids [15]. Investigation of the effects of additives that improve alfalfa fermentation on PUFA is among the latest studies. Indeed, Zhang et al. (2020) [17] investigated the effect of inoculation of *L. plantarum* MTD-1 and *Pediococcus acidilactici* J17 strain on alfalfa silage on antioxidant, alpha tocopherol, carotene concentrations and fatty acid composition. *Pediococcus acidilactici* J17 strain with high antioxidant effect significantly increased the total antioxidant capacity, catalase activity, alpha tocopherol and beta carotene concentrations of alfalfa silage with 30% and 40% dry matter content compared to silage inoculated with control and *L. plantarum* MTD-1. However, both inoculants decreased the superoxide dismutase level. *Pediococcus acidilactici* J17 strain has a lower effect on fatty acid composition in alfalfa silage. There was no significance between the groups.

In another study, it has been investigated that the effect of alfalfa (*Medicago sativa L.*), sainfoin (*Onobrychis viciifolia Scop.*), and ryegrass (*Lolium multiflorum Lam*) crops used alone and in different proportions on silage and feed quality. As plant material in study; Kayseri alfalfa variety, Lütfübey sainfoin variety and Karamba ryegrass variety were used. Alfalfa was harvested at 10% flowering, sainfoin at 50% flowering, and ryegrass at the same time during flowering. The mixing ratios of alfalfa, sainfoin and ryegrass alone are 100% (ryegrass, sainfoin, alfalfa), 75:25 (ryegrass+alfalfa, ryegrass+sainfoin, sainfoin+alfalfa), 50:50 (ryegrass+alfalfa, ryegrass+sainfoin, sainfoin+alfalfa), 25:75 (ryegrass+alfalfa, ryegrass+sainfoin, sainfoin+alfalfa). Silages were left to a 40-day fermentation. The lowest pH value of the silages is in plain Karamba Italian grass (4.40), the highest dry matter rate is in sainfoin silage made alone (40.90%), the highest crude protein rate is in alfalfa silage made alone (24.43%), the highest flieg value alone made from ryegrass (105.26). The lowest acid detergent fiber (24.73%) and neutral detergent fiber (33.10%) ratio and the highest digestible dry matter (69.63%), dry matter intake (3.63%) and relative feed values (195.85) values were also obtained from alfalfa silage made alone. Due to these findings, they reported that the quality of silage obtained from alfalfa was superior to the other Karamba, ryegrass and mixtures made alone [20].

A study conducted by Soycan Onenc et al. (2019) [21] on the effects of the addition of oregano and cumin essential oil as antimicrobial additives to the final form of alfalfa on fermentation quality, aerobic stability, and feed value. Alfalfa was harvested at the beginning of flowering and withered for 8-10 hours. The research was carried out in 4 replications in 3 groups, which were formed by adding 650 mg/kg oregano and cumin essential oils to the control without any additives. Oregano and cumin essential oils were equally sprayed and mixed into 8 kg of fresh material. The silage material is packed in 2 kg plastic bags and compressed. After being vented by vacuum, it was wrapped in stretch film for 10-12 times and finally covered with a layer of tape. Ensiling process was carried out in a closed warehouse (8±2°C), chemical and microbiological analyzes were performed on the silages opened on the 120th day. At the same time, aerobic stability test was applied for 5 days. The addition of oregano and cumin essential oil significantly reduced the pH, water-soluble carbohydrate and ammonia nitrogen amounts ($P<0.01$), while the dry matter and lactic acid contents increased ($P<0.01$). In the study, total mesophilic aerobic bacteria, enterobacter, yeast and mold counts decreased with the addition of oregano and cumin essential oil ($P<0.01$), while LAB counts increased ($P<0.01$). According to the results of the study, the addition of oregano and cumin essential oils to alfalfa at the level of 650 mg/kg promoted silage fermentation while at the same time improving aerobic stability. But oregano essential oil in mold development; cumin has become more prominent as a preventive measure in yeast development. In addition, the addition of oregano and cumin increased the content of organic matter dissolved in the enzyme, which caused an increase in the metabolic energy content of silages.

Arslan Duru (2019) [8] investigated effects of lavender (*Lavandula angustifolia*) flower and stem part added to alfalfa at different levels on silage quality. Lavender is preferred due to its high quality essential oil content. It is also known to reduce the formation of methane gas in the rumen. In the study, the flower and stem of lavender were mixed in equal levels (50%-50%) to the alfalfa taken to the silo the day after it was harvested. Alfalfa silages; lavender was added at levels of 0.5, 1.0, 1.5 and 2.0, and a control group without lavender was formed. It was determined that the pH value of the control group was significantly lower than the other groups except the 2% lavender added group ($P<0.05$). Acetic acid, butyric acid, *Enterobacteriaceae*, *Listeria* spp, sulfite reducing anaerobes, mold and yeast were not found in alfalfa silages with lavender added. The LAB count of alfalfa silages with 2% lavender added was significantly higher than the other groups ($P<0.01$).

Yakisir and Aksu (2018) [9] studying the effects of adding 5% and 10% dried sugar beet pulp with molasses (MDSBP, containing 2% molasses) to fresh alfalfa material for the production of quality alfalfa silage. MDSBP was preferred because it is thought to contribute to fermentation with its high carbohydrate content. In this study, it was prepared by adding MDSBP in different proportions (0, 5 and 10%) to alfalfa harvested at the beginning of the flowering period. When the physical and chemical properties of alfalfa silages were examined, it was determined that silage with the addition of 10% MDSBP to alfalfa increased the quality of alfalfa silage.

Malhatun Cotuk and Soycan Onenc (2017) [10] studied the effects of pudding (with expired shelf life), which is a food industry waste, on fermentation quality, aerobic stability, in vitro digestibility and relative feed value of alfalfa silage. The study was carried out in three groups consisting of alfalfa harvested at the beginning of flowering, control without additives, 100 g/kg bran and 50 g/kg pudding. It was opened on the 60^{th} day and evaluated physically, chemically and microbiologically. The aerobic stability test was applied for 7 days, and the amount of organic matter dissolved in the enzyme, metabolic energy content and relative feed value were determined. As a result of the physiological evaluation, the control group silage was evaluated as good quality, while bran and pudding added silage groups were evaluated as good. When bran and pudding were added to alfalfa, neutral detergent fiber, acid detergent fiber and acid detergent lignin values among cell wall contents decreased ($P<0.05$). The treatment carried out positively affected the dry matter ratio and decreased the losses due to this. The pH and water-soluble carbohydrate contents of the studied silages and the amount of NH_3-N decreased compared to the control, and the lactic acid content increased in the pudding group ($P<0.05$). With the addition of bran and pudding, the number of LAB increased ($P<0.05$), while the numbers of Enterobacter, yeast and mold decreased compared to the control ($P<0.05$). The addition of pudding in the aerobic period decreased dry matter loss, pH, the release of CO_2, but could not stop the growth of yeast and mold ($P<0.05$). Adding bran and pudding to alfalfa silage increased the amount of organic matter dissolved in enzyme, in vitro metabolic energy content, digestible dry matter, dry matter intake and relative feed value of silages ($P<0.05$). The study has shown that ensilage alfalfa by adding 50 g/kg of pudding is more advantageous than traditional alfalfa silage, especially in the spring when there is a lot of rain and there is no drying opportunity, in regions where the food industry is developed. In future studies, it was stated that 50 g/kg pudding level should be accepted as the upper limit and the usage levels should be investigated by supporting in vitro and in vivo digestion trials.

Hisman Akca et al. (2019) [11] evaluated the effect of sodium diacetate (SDA) addition on fermentation characteristics, chemical composition, and aerobic stability of alfalfa silages. SDA, an organic acid salt, is used as an antibacterial agent to extend the feeding period of silages. Alfalfa (*Medicago sativa L.*) used in the study was harvested at the beginning of flowering (about 10% flowering). SDA at the level of 0 (control), 3.0, 5.0 and 7.0 g/kg dry matter was added to the fresh material prepared. 20 ml of tap water, equivalent to the other treatment groups, was added to the control group. After the additives were added, approximately 500 g of the sample was placed in plastic bags, compressed and air removed by vacuum. A total of 40 packages of silage, 10 for each group, were left to fermentation for 45 days under laboratory conditions (25-30°C). Aerobic stability test was applied for 7 days to silages opened on 45th day. In the study, on the 45^{th} day of fermentation, the dry matter levels of SD5 and SD7 silages were determined to be higher than the control group silage ($P<0.05$). In the study, adding SDA to alfalfa significantly decreased the pH of silages compared to the control group ($P<0.01$). The addition of SDA caused an increase in crude protein content ($P<0.001$), reduced the amount of NH_3-N in total nitrogen ($P<0.001$) and prevented proteolysis. Parallel to the increase in the dose of SDA used in the study, the fermentation properties of alfalfa silages had a positive effect on their aerobic stability. By showing antibacterial effect, SDA decreased the number of harmful microorganisms (yeast and mold) in alfalfa silages and at the same time increased the number and efficiency of Lactobacilli. Accordingly, the conversion of sugars to lactic acid has increased, lactic acid, which is present in the environment at a high rate, has inhibited the enzymes that break down proteins by lowering the pH and reduced the rate of protein breakdown into ammonia. Propionic acid and butyric acid contents have decreased due to the increased SDA in alfalfa silage. Mold was detected among alfalfa silages only in the control group. The data obtained as a result of the study showed that silage of alfalfa by adding SDA improved the fermentation properties and aerobic stability, especially in periods when rainfall is abundant and drying is not possible, and the most effective dose is 7 g/kg.

It is known that LAB, which constitute the bacterial flora of kefir, reduce nutrient losses, especially on silage fermentation and have positive effects on aerobic stability [12]. Koc et al. (2020) [12] conducted a study to evaluate the effects of adding different doses of natural and commercial kefir culture on the fermentation development and aerobic stability of alfalfa silages. Alfalfa was harvested at the beginning of 10% flowering in June and withered for about 24 hours. Natural and commercial kefir culture was used as an additive in the study. Alfalfa treated with control and additives was sealed in plastic bags with a capacity of 500 g, 10 replicates for each application. Chemical and microbiological analyzes were performed on all silages opened 45 days after silage. Aerobic stability test was applied to all silages for 7 days at the end of the silage period. In the study, the highest dry matter content was detected in natural kefir and commercial kefir applications on the 45th day of fermentation, while the lowest dry matter level was found in the control group silage (P<0.001). Adding kefir to the alfalfa plant significantly reduced the pH of the silages and the difference between treatment groups was found to be statistically significant (P<0.001). The addition of kefir caused the HP content to increase, while the amount of NH_3-N in the total nitrogen decreased (P <0.001). The pH, acetic acid, butyric acid, propionic acid contents of kefir silage added to alfalfa silage decreased dry matter loss and increased lactic acid content compared to the control group (P<0.001). As a result of the research, the use of kefir positively affected the fermentation and microbiological composition properties of alfalfa silages. Considering the evaluation results regarding aerobic stability; it is noteworthy that kefir silages have less CO_2 production, and yeast contents. In this study, while LAB isolated in natural kefir culture applications have heterofermantative properties, homofermantative LAB have been isolated in commercial kefir culture applications. In this sense, it has been stated that kefir can be used as a LAB source, but it should not be forgotten that especially LAB types in natural kefir culture applications will differ depending on the kefir culture.

More recently, Mut et al. (2020) [13] investigated the effects of the quality content of silage obtained by growing Hungarian vetch, turnip and oats in the form of a mixture with alfalfa. Alfalfa and companion crops were planted plain and in 3 different mixing ratios (75:25, 50:50, 25:75). First, companion plants were planted as 20 cm between the row lengths and 4 rows. Then, alfalfa was planted by hand on the field whose trial area was determined. The seed amount was calculated as 3 kg/da in alfalfa, 10 kg/da in Hungarian vetch, 1 kg/da in feed turnip and 20 kg/da in oats. For this study, the harvest time was carried out in the period of milk death in lean oats, flowering in turnip, in the period when sub pods are formed in lean Hungarian vetch, and in the flowering period of 10% in lean alfalfa. After the shredding process, the materials were left to fermentation for 45 days at 25±2°C by closing the mouths in 2 kg jars in a way that it would not be airtight. The effect of mixing ratios on dry matter, pH and flieg scores was found to be very significant (P<0.01). The highest lactic acid ratio was obtained from silage of 6.57%, 75% alfalfa+25 oats and the lowest 0.78% from 75% alfalfa+25 Hungarian vetch silage. It was determined that alfalfa was insufficient in terms of silage quality, but the addition of oats and feed turnips positively affected the quality of silage. The protein content of silages made from a mixture of alfalfa and Hungarian vetch was higher than other mixtures. However, since the lactic acid content of these silages was lower than the desired level, it was determined that these two legumes were not suitable for silage. Accordingly, when the flieg scores of silages and crude protein ratios are evaluated together; it was concluded that all mixtures of alfalfa with forage turnips and silage of alfalfa with oats 75+25% are more suitable.

3. Conclusion and Recommendations

As a result; the cheapest and easiest way to store green forages is silage production. Silage of forage crops such as corn, alfalfa, vetch, wheat, barley is made alone or in a mixture. Alfalfa, which is a difficult feed to silage, has recently increased its efforts to make silage with additives. As it is known, alfalfa silage is rich in protein and poor in water-soluble carbohydrates, but it is very difficult to silo because of its high buffering capacity. Therefore, it is necessary to add regulatory additives to alfalfa silages. In the studies conducted, it is generally aimed to investigate additives that will improve fermentation. In recent studies, more clear information about bacterial communities in silage fermentation has begun to be obtained with the use of NGS technique. In studies using these techniques, it was reported that LAB organic acid additions to alfalfa silage caused Lactobacillus, Pediococcus or Sporolactobacillus species to increase relatively in silage environment, the ratio of unwanted species decreased and silage quality increased. Although the amount of alpha-tocopherol and beta carotene decreased by half in pure silage compared to fresh alfalfa, it was observed that LAB inoculation increased the ratio of alpha tocopherol, beta carotene and also polyunsaturated fatty acids.

According to the studies examined, considering the additives and their rates, especially *L. brevis* and *L. plantarum* inoculation (average 10^5 or 10^6 cfu/g fresh feed) [4-5], 650 mg/kg thyme and cumin essential oils [21], 3% sweet chestnut extract [14], 7 g/kg sodium diacetate [11], 50 g/kg pudding [10], 10% molasses dry sugar beet pulp [9], 2% lavender flowers [8], 1% sucrose, barley and molasses [22] were reported to positively affect silage fermentation of alfalfa, while alfalfa silages made with ryegrass and sainfoin [20], 100 g/kg bran [10], and silages with a mixture of alfalfa-hungarian vetch [13] have not been reported to have a positive effect.

In recent years, NGS has been used to identify bioprospecting studies and to better understand the microbial populations in silages. Future silage additives have the potential to improve fermentation quality, aerobic stability of silages, the bacterial community, silage safety, antioxidant enzymes, and unsaturated fatty acid levels.

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