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The investıgation of possibility of propolis as additives in alfaalfa silages

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Abstract

This study was carried out to determine chemical, fermentation, microbiological and sensory properties of alfalfa silages of propolis added at different levels as additive. In the study, propolis was added to alfalfa silages at 0 % (control), 0.5, 1.0 and 2.5 levels. The ensiling period continued for 75 days. There was no statistically significant difference between dry matter, crude ash, crude protein, NDF ADF contents and lactic acid bacteria count (P>0.05). pH and propionic acid content decreased with the addition of propolis to the silages (P<0.01). The content of acetic acid decreased with the 1.0% and 2.5% propolis (P<0.01). *Listeria* spp., ammonia nitrogen and butyric acid were not found in silages. The Fleig Score of the groups containing 0.5% and 1.0% propolis was higher than the other groups (P<0.05). Sulphite reducing anaerobes, mold and *Enterobacteriaceae* were found to be below the detection limit and yeast was observed in the propolis group 0.5% and in only one sample. At the end of the study, it was concluded that the addition of 1.0 % propolis could increase the quality of silages.

Key words: Propolis, alfaalfa, silage, additive, quality.

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INTRODUCTION

Propolis comes from the Ancient Greek words pro (front, entrance) and polis (city) and it was defined as " a sticky material with very strong antiviral, antibactrial and antifungal effect collected by honey bees from the cones and barks of trees and from the buds and shoots of plants and composed of the mixture of various oils, pollens, special resins and waxy substances (Kumova et al., 2002). According to another definition, Propolis is the common name of resinous substance collected by honey bees from various plantive sources and also called as "bee glue" (Chemid, 1996).

Colour and physical features of propolis vary according to the plant source and it is used by bees for several purposes (Şahinler 1999; Hepşen et al.,1996). Physical and chemical structure of propolis vary according to the region, plant source and season (Bonvehi and Coll 2000; Oruç et al., 2014). There are more than 300 different compounds in propolis. So far, more than 180 compounds, mostly polyphenols, have been identified as the component of propolis (Castaldo and Capasso 2002).

There are a lot of bioactive substances forming the chemical structure of propolis. These are phenolic compounds (flavonoids and phenolic acids) and their esters, alcohols, aldehydes, ketones, terpenes, coumarins, steroids, aminoacids, elements such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe, vitamin B_1, B_2, B_6, C and E and lots of fatty acids and enzymes (Ghisalberti 1979). Bioactive matters in propolis have protective and curing effects for various bacterial, viral and tumoral diseases depending on their amounts (Velazquez et al., 2007; Szliszka et al., 2009; Oruç et al., 2014). Majority of these effects derive from flavonoids and pheonolic acids that are among the pheonolic compounds in propolis (Grange and Davey, 1990).

In this study it was aimed to investigate the effects of propolis added to alfaalfa in different quantities on chemical, fermentational and microbiological properties of silages.

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MATERIAL AND METHODS

Silage materials

Alfaalfa, the main silage materials used in this study, were obtained from a producer in the territory. Alfaalfa were collected fresh from the field one day after the harvest and they were ensiled at the day that they were collected. The propolis used as silage additives was collected from the hives belonging to Usak University Faculty of Agriculture and Natural Sciences Deparment of Animal Science. The obtained propolis was kept in a deep freezer and then ground. 80 g of the ground propolis was taken and it was mixed with 920 ml of 70 % of ethanol. By keeping this mixture waiting in a dark room for a week, it was stirred three times a day for a while and at the end of this period it was filtered by a filter paper. The obtained filtrate was kept at +4 °C until it was used. Propolis used as a silage additive was added to the alfaalfa silages at 0 % (control), 0.5 %, 1.0 % and 2.5 % rates. Silage samples were prepared in 1 lt of anaerob glass jars as 4 parallels. Ensiling period lasted for 60 days.

Chemical Analyses

The method reported by Kılıç (1986) was used in calculating silage quality classes and Fleig scores. At the end of ensiling period, dry matter contents were determined after the silage samples were dried in circulated incubator at 65 ºC for 48 hours (AOAC, 1999). Samples were ground with 1 mm of sieve diameter after they were dried and crude ash content as indicated in AOAC (1999) and crude protein contents through Kjeldahl distilation method were determined. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) analyses were made using the method reported by Van Soest (1982). When the silages were opened, in order to determine the pH values of the samples 100 ml of distilled water was added to 25 g of silage sample and pH of the liquid obtained after stirring by a shaker was measured using pH digital pH meter (Polan et al., 1998). In addition, as soon as the silages were opened, 40 g of silage sample was taken and it was shaken by adding 360 ml of distilled water. Following this procedure, the this mixture was filtered with Whatman (no:1) paper and ammonia nitrogen (NH_3-N) was determined using Kjeldahl distilation method by taking 100 ml of obtained filtrate (Broderick and Kang, 1980). In order to determine volatile fatty acid (VFA) and lactic acid (LA) contents, 2 ml was taken from the same filtrate and it was kept in a deep freezer at -18 °C until the day of analysis. VFA (acedic acid, propionic acid and butyric acid) and lactic acid analyses of the silages taken out of the deep freezer on the day of analysis were made in HPLC device**.** (Properties of HPLC: Column: C18, 5 µm, 4.6 x 250-mm; Mobile Phase: Isocratic; 25-mM K-phosphate buffer; pH 2.4; Flow Rate: 1.5 mL/min.; Column Temperature: 30 ºC; UV Sensor: Wavelength: 210 nm; Injection Volume: 20 µL).

Microbiological analyses

Using the method reported by Stanley et al. (1971) sülfite-reducing anaerobes, using the method reported by Harrigan (1998) number of lactic acid bacteria, whether there exists *Enterobacteriaceae*, *Listeria* spp., yeast and mold or not were determined in each silage.

Statistical analyses

The obtained results were analyzed in SPSS Package Programme according to One-Way Anova procedure and Duncan Multiple Comparison Test was implemented for the differences of the groups. In addition, microbiological features of the silages were analyzed and evaluated in SPSS Package Programme according to Frequency procedure (SPSS, 2007).

RESULTS

The effect of propolis treatment on the chemical properties of alfalfa silages are given in Table 1.

Table 1. The effect of propolis on the chemical properties of alfaalfa silages

Parameters	Control	0.5 % Propolis	1.0 % Propolis	2.5 % Propolis
DM, %	34.82±0.58	34.68±0.08	34.87±0.15	34.35±0.57
CA, % DM	11.46±0.19	11.59±0.15	11.47±0.10	11.71±0.31
CP, % DM	28.94±0.33	28.94±0.56	29.03±1.14	28.79±0.33
NDF, % DM	24.18±1.00	23.73±0.27	23.07±0.44	23.90±0.34
ADF, % DM	17.35±0.70	16.88±0.57	17.40±1.31	17.86±0.37

DM: Dry matter; CA: Crude ash; CP: Crude protein; NDF: Neutral detergent fiber; ADF: Acid detergent fiber.

Propolis did not affect dry matter, crude ash, crude protein, NDF and ADF contents of alfalfa silages (P>0.05). The effect of propolis additive to alfaalfa silages at different levels on fermentation properties of the silages was presented in Table 2.

Table 2. The effect of propolis on the fermentation properties of alfaalfa silages

Parameters	Control	0.5 % Propolis	1.0 % Propolis	2.5 % Propolis
pH**	4.86±0.04[a]	4.53±0.01[c]	4.56±0.07[c]	4.60±0.03[b]
LA, %*	2.95±0.81[a]	2.58±0.74[a]	1.16±0.15[b]	1.30±0.47[b]
AA, %**	0.35±0.11[a]	0.26±0.07[a]	0.10±0.03[b]	0.12±0.05[b]
PA, %**	0.42±0.12[a]	0.15±0.05[b]	0.08±0.01[b]	0.02±0.01[c]
BA, %	ND	ND	ND	ND
NH_3-N	ND	ND	ND	ND
Fleig Score*	79.94±1.54[c]	93.10±0.63[a]	91.49±2.39[a]	88.43±1.32[b]
	Good	Excellent	Excellent	Excellent

* [a-c:] The differences between the averages in the same column are significant (P<0.05).
** [a-c:] The differences between the averages in the same column are significant (P<0.01).
LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid. ND: Not determined

Accordingly, it was found that the pH of the silages especially including 0.5 % and 1.0 % of propolis was significantly lower than the other groups (P<0.01). It was determined that lactic acid (P<0.05) and acetic acid contents of the silages statistically reduced with 1.0 % and 2.5 % of propolis additives (P<0.01). Especially 2.5 % of propolis additive reduced the propionic acid content of the silages (P<0.01). No butyric and ammonia nitrogen contents were detected in experiment silages. While the quality class of the control group was "good", the quality class of the silages with propolis procedure increased to "excellent". The effect of propolis additive to alfaalfa silages on microbiological properties was shown in Table 3.

Table 3. The effect of propolis on the microbiological properties of alfaalfa silages

Parameters	Control	0.5 % Propolis	1.0 % Propolis	2.5 % Propolis
LAB (Log(cfu/g)	6.07±0.13	5.79±0.23	6.08±0.08	5.54±0.22
SRA (cfu/g)	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
Listeria spp.	ND	ND	ND	ND
Mold (cfu/g)	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$	$<1.0x10^2$
Yeast	$<1.0x10^2$ cfu/g	2 (1 sample) (Log(cfu/g)	$<1.0x10^2$ cfu/g	$<1.0x10^2$ cfu/g
Enterobacteriaceae (cfu/g)	$<2.0x10^2$	$<2.0x10^2$	$<2.0x10^2$	$<2.0x10^2$

LAB: Lactic Acid Bacteria; SRA: Sulphite Reducing Anaerobes ND: Not determined

The differences among silages in terms of lactic acid bacteria (LAB) content were not found as significant (P>0.05). The lowest LAB was found in 2.5 % of propolis, the highest LAB was found in 1.0 % of propolis. Sulphite reducing anaerobes, mold and *Enterobacteriaceae* were determined under the detection limit; however, *Listeria* spp. could not be identified in all experiment silages. Yeast was found in 0.5 % of propolis and only one sample.

DISCUSSION

The studies to increase both silage qualities and the yield of animals without harming both human and animal health by adding natural additives to especially hard ensiled fresh material like alfalafa have been increased in number recently. Propolis is also thought to be one of these additives. Propolis has strong antibacterial, antifungal and antiviral features (Bonkava, et al., 2000). It is also reported that especially propolis extracts have antibacterial activities against Gram

negative (-) and Gram positive (+) bacteria and also antifungal activities against the fungi (Matsuno et al., 1997; Aksoy and Dığrak, 2006; Menensez et al., 2009; Gallez et al., 2014). Propolis is used in many different fields. However, according to us, there is no study on the use of propolis as silage additives.

It was determined in this study that propolis procedure had no effect on chemical contents of the silages. No dry matter lost in silages with propolis additive to alfalafa. Accoding to these findings, it can be said that propolis inhibits dry matter losses via its strong antimicrobial effect by preventing the development of harmful microorganisms. In also the studies with different silage additives Ke et al. (2015), Acar and Bostan (2016) and Pour et al. (2017) obtained similar results.

Decreasing pH and quantity and compositions of organic acids in fermentation stage are important criteria in determining the quality of silages. pH of silages reduced with propolis procedure. It can be said that the fact that the pH level of hard ensiled fodders like alfalafa which are water-soluble, poor in carbonhydrade and rich in protein is reduced by ensiling is due to the antibacterial and antifungal activities of propolis. Morover, these activities reduced the lactic acid content that is necessary for especially a good silage. However, it was reported that lower amount of lactic acid content lower than the values indicated in literature developed poor fermentation in silages, but did not cause dry matter losses (Baytok et al., 2005). No dry matter losses, suppression of acedic acid and propionic acid formation and no butyric acid content in experiment silages support these findings. Liu et al. (2016) reported that lactic acid, acetic acid and butyric acid levels significantly reduced with Lactobacillusplantarum inoculant additive to alfaalfa silages. There are studies indicating that different additives to alfalafa silages reduced the pH (Filya et al., 2006; Guo et al., 2008; Wen et al., 2017).

Ammonia concentration indicates the fragmentation rate of proteins by butyric acid bacteria. It was reported that ammonia nitrogen content of a quality silage was required to be lower than 80 g/kg of total N (Petterson, 1988). It is also stated that deamination occurs in aminoacids in case of high acedic acid content in silages and as a result the ammonia nitrogen level in silages increases (McDonald et al., 1991). With propolis additives to alfalafa lactic acid fermentation developed in silages and therefore pH reduced and the development of undesired butyric acid bacteria and the formation of ammonia nitrogen which is the final product of proteolisis were completely blocked.

The quality classes of the silages increased to “very good” with propolis additive. Fleig Scoring method is a scoring method based on dry matter and pH values of silages. pH value is an important criterion which numerically reveals the quality class of silages and whether the silages are sufficiently fermented or not. It is stated that high dry matter content not only reduces pH but also affects the lactic acid fermentation negatively and therefore, reduces the quality of silages (Kılıç, 1986). When the pH and dry matter content criteria used in the calculation of Fleig Scores are considered, it can be said that low pH level of the groups with propolis treatment in the study also reflects to Fleig Scores of the silages.

When the experiment silages are appreciated in terms of microbiological features, we can see that lactic acid bacteria reproduced in sufficient amounts; however, harmful microorganisms did not generate. Only 2 log (cfu/g) rate of yeast reproduced in 0.5 % of propolis. Many problems can be introduced while ensiling leguminosae, herbs and their mixtures. During the ensiling, these fresh materials may include both aerobic and anaerobic microorganisms and various bacteria and fungi affecting the quality of silages (Muck, 2010). The existence of low pH and sufficient lactic acid bacteria in good quality silages prevents the reproduction of these microorganisms which reduce the quality of silages. The fact that lactic acid bacteria are the dominant flora and their pH level is low, butyric acid and ammonia nitrogen can not be detected and there are almost no undesirable microorganism activity is considered as an indicator of the formation of acidic environment in experiment silages. Filya et al. (2001) and Koç et al. (2017) obtained similar findings in alfalafa silages.

CONCLUSION

It was aimed to determine the possibility to use propolis as silage additives. As a result of the study, no dry matter losses in silages, reduction in pH of silages, production of lactic acid up to protect the silages, undesirable harmful microorganisms in silages and no butyric acid and ammonia nitrogen contents indicate that propolis can be used as an additive in silages. It was concluded that especially 1.0 % of propolis may have a positive effect to protect silages.

CONFLICT AND INTEREST

Authors declare no conflict and interest.

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