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Treatment of alfalfa silage with chitosan at different levels to determine chemical, nutritional, fermentation, and microbial parameters

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ABSTRACT. Chitosan is a biodegradable, antimicrobial, antifungal polysaccharide, thus used as an additive in different industries. Effects of chitosan supplementations on silage quality traits of alfalfa were investigated in the present study. Chitosan was supplemented into alfalfa silages at four different levels: 0% – CONT group; 0.5% – CHTS0.5 group; 1% – CHTS1.0 group; and 2% – CHTS2.0 group. Neutral detergent fibre (NDF) and neutral detergent insoluble crude protein (NDICP) values increase in CHTS 2.0 group ($P < 0.05$). Lactic acid and butyric acid contents decreased ($P < 0.05$), acetic and propionic acid contents increased with chitosan treatments. On the day of opening the silages, mould production was not encountered, and yeast production decreased in chitosan-supplemented groups. Excessive yeast production was encountered, but mould development was not seen in chitosan-supplemented groups 5 days after opening the silages. Silage pH values increased with chitosan treatments ($P < 0.05$). So, it can be concluded that chitosan negatively influenced fermentation quality of alfalfa silage, but reduced mould and clostridial development.

Introduction

Alfalfa is superior to other forages in terms of protein and energy and thus it is generally used for feeding dairy cattle with high milk yields. Alfalfa is generally used in the form of fodder, but in some cases (e.g. due to climate) when growers are not able to reach the status of quality alfalfa fodder, ensilage offers a reliable way of storage to overcome these problems. Fermentation of alfalfa is quite a difficult process because of the low water-soluble carbohydrate content and high buffering capacity of alfalfa (Undersander et al., 2011). About 75% of the carotene and protein content in alfalfa is encountered in leaves. Thus, drying under improper conditions lead leaf pruning because of mechanical impacts (harvest, baling, transportation), then the loss of nutrients. Ensilage is also used to prevent such losses (Acar and Bostan, 2016). Additives are used most of the time in silage to ensure sufficient fermentation and quality final product. In this sense, soluble carbohydrate sources (molasses, whey, cereal grains, commercial inoculants etc.) are used as additives.

Chitosan is a natural polycationic polysaccharide produced from chitin. Besides antimicrobial and filmogenic effects, chitosan is also a biocompatible and biodegradable material. When used in foodstuffs, chitosan was reported to prevent microbial

degradation and contaminations, improve feed quality and increase shelf life (Martínez-Camacho et al., 2010). Chitosan is among the most abundant biopolymers worldwide and is obtained through deacetylation of the insects and chitin in the outer skeleton of the shells. Biodegradation, non-toxic and antimicrobial characteristics have made chitosan a remarkable product (Araújo et al., 2015). Chitosan is a weak base able to dissolve in weak aqueous acid solutions (pKa < 6.3) (Goy et al., 2009). Since chitosan is gelatinized in acidic ambient, it may have a good effect in the acidic environment of silage. Without excessive accumulation, about 100 mln t of chitosan may biologically be synthesized and degraded. This is a natural cycle and plays an important role in the preservation of the ecosystem, therefore it should attentively be used without destruction of ecological chitin and chitosan cycle (Hirano, 1996). There are different studies about the potential of chitosan in animal feeds (Fadel El-Seed et al., 2003; Goiri and Oregui, 2010; Araújo et al., 2015; Henry et al., 2015). Chitosan supplementation of 6 g/kg dry matter (DM) to sugarcane silage had a positive effect on silage fermentation, reducing fermentative losses, and improving chemical composition and degradation (Del Valle et al., 2018). Also, Gandra et al. (2018) reported that 5 g/kg of fresh forage chitosan supplementation had small positive effects on gas losses however, improved *in vitro* degradation and decreased mould and yeast in whole-soybean plant silage. But these products are fermented more easily than alfalfa. Therefore, in the present study, the effects of chitosan were examined in alfalfa with a low self-fermentation capability. In other studies, chitosan was not used alone, and it was used together with the other inoculant sources. In the present study, chitosan was used alone at different doses.

In the present study, alfalfa was preferred since it has a low self-fermentation capacity and is not sufficiently available for silage in terms of composition. Desired fermentation levels are not achieved in alfalfa silage without the use of various additives. Therefore, for quality alfalfa silage, different additives are used. Previously unused chitosan was used in alfalfa silage and effects of chitosan on silage quality traits were investigated.

Material and methods

Silage preparation

In the present study, Italian origin prosementi alfalfa (*Medicago sativa L.*) cultivar was used. Samples were supplied from a grower field in Aksaray province located in the Central Anatolia Region of Turkey between 38°21' N and 34°00' E with an altitude of 980 m. Alfalfa samples were harvested before flowering as to leave 4–5 cm straw on the ground. Harvested samples were wilted for 1 day under laboratory conditions with a temperature of 27 ± 2 °C and humidity of 30 ± 2%. Wilted plants were chopped into 2–3 cm pieces and randomly separated into four groups. Chitosan (Sigma Aldrich, Saint Louis, USA) was applied as a weight basis to fresh alfalfa samples for each chitosan level. Additives were not used in the first group and assessed as the control (CONT) group. The second group was supplemented with 0.5% (5 g chitosan + 995 g fresh alfalfa) chitosan in weight (CHTS0.5), the third group was supplemented with 1.0% (10 g chitosan + 990 g fresh alfalfa) chitosan (CHTS1.0), and fourth group was supplemented with 2.0% (20 g chitosan + 980 g fresh alfalfa) chitosan (CHTS2.0). Nutritional composition of alfalfa at harvest is provided in Table 1. In the study, chitosan with catalogue number 448869 (Sigma Aldrich, Saint Louis, USA), low molecular weight and distillation degree 75–85% was used. The molecular weight was approximately 50 000–190 000 Daltons based on viscosity. The density was 0.15–0.30 g/cm^3. The nitrogen value of chitosan was determined as 69.9 g/kg DM (AOAC International, 2006).

Table 1. Chemical composition of alfalfa at harvest, dry matter (DM) basis

Chemical analysis, g/kg		Calculated values	
DM	249.7	Non-fibre carbohydrates, g/kg	201.5
Crude protein	230.7	Digestible DM, g/kg	628.9
Ether extract	20.3	DM intake, %*	2.77
Crude ash	114.6	Relative feed value	135.20
Starch	26.3	TDN, g/kg	537.9
Acid detergent fibre	333.9	Metabolizable energy, Mcal/kg	2.11
Neutral detergent fibre	433.0	NE_L, Mcal/kg	1.15
Crude fibre	233.1	NE_M, Mcal/kg	1.25
Acid detergent lignin	87.0	NE_G, Mcal/kg	0.68

*as a percentage of body weight; TDN – total digestible nutrients, NE_L – net energy lactation, NE_M – net energy maintenance, NE_G – net energy gain

For each group, 1 kg alfalfa sample and chitosan were homogeneously mixed in 3 replicates (in total 12 samples, 4 groups × 3 replicates). Mixed samples were placed into 30 × 35 cm polyethylene vacuum bags (Caso Professional Vacuum Rolls, Arnsbeg, Germany) and ensiled with a vacuum sealer (DZ-260/PD, SELES vacuum-package device, Bursa, Turkey). Firstly, silage material was filled in the bag

by hand. Then the bags were heat-sealed (without melting the plastic bag) and air evacuated. After the sealing, the vacuum-machine automatically cut the plastic bag 5 mm above the seal remaining. Silage bags then were left for fermentation at 20–25 °C for 60 days.

Chemical analyses

Following 60-day fermentation period, samples were opened in the laboratory environment. Silage samples were dried and grounded in a laboratory mill (IKA MF.10, Staufen, Germany) to pass 1 mm sieve for chemical analyses. Crude protein (CP) was determined with the use of Dumas method (AOAC International, 2006). Ether extract (EE) was determined with the use of extraction method and petroleum ether was used as a solvent (AOAC International, 2005). For crude ash (CA), samples were ashed in an ash oven at 550 °C (AOAC International, 2005). Analyses of crude fibre (CF), acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined according to the Van Soest et al. (1991). For acid detergent insoluble crude protein and neutral detergent insoluble crude protein (ADICP and NDICP) analyses, CP analysis was conducted on residues of ADF and NDF analyses based on the above-specified method. Starch analysis was conducted with the use of the polarimetric method (ISO 10520, 1997).

Calculated parameters

Chemical analysis data were used to calculate digestibility and energy parameters including non-fibre carbohydrate (NFC), digestible dry matter (DDM), dry matter intake (DMI, % body weight), relative feed value (RFV), total digestible nutrients (TDN), metabolic energy (ME), net energy maintenance (NE_M), net energy gain (NE_G) and net energy lactation (NE_L). These parameters were calculated with the use of equations specified in Nutrient Requirements of Dairy Cattle (NRC, 2001).

Organic acid analyses

Following 60-day fermentation, samples were supplemented with 20% distilled water, mixed in a blender, filtered through filter paper and pH of the resultant filtrate was measured with the use of a pH meter. For organic acid composition 40 g silage sample was supplemented with 360 ml distilled water and mixed in a blender. The mixture was filtered, 40 ml of filtrate was completed to 400 ml with distilled water. The resultant liquid was filtered through Whatman 54 filter paper and centrifuged. Samples were kept at −20 °C until analyses. The Lepper method was used for lactic acid analysis (Akyıldız, 1984). Acetic, propionic, and butyric acid analyses were conducted with the use of a gas chromatography (GC 2010+ Shimadzu Corporation, Kyoto, Japan) device.

Microbiological analyses

Right after opening silage samples, yeast-mould counts were made, the remaining portion of samples was left for aerobic degradation and yeast-mould counts were made again. Yeast-mould counts were made by spread plate technique with the use of Potato Dextrose Agar (PDA) nutrient medium (Merck, Darmstadt, Germany). About 10 g sample was homogenized with 90 ml peptone water, dilutions were prepared from 10^{-1} to 10^{-4} and sown on Petri dishes. The Petri dishes were incubated in an oven (25 °C ± 1) for 5 days and then yeast-mould counts were performed.

Aflatoxin B1 analyses

About 20 g silage sample was mixed with 70% methanol and filtered through Whatman filter paper. About 5 ml filtrate was analysed in ELISA reading device (BioTek ELx800, Vermont, USA) in accordance with the instructions specified in ELISA kit (Bio-Shield Es, Larissa, Greece) procedure.

In vitro gas production

For analyses of *in vitro* gas production, metabolizable energy (ME), NE_L and *in vitro* organic matter digestibility (IVOMD), rumen fluids were taken from 3 fistulated goats feed diet composed of alfalfa hay (60%) and concentrated feed (40%, consisting of: wheat bran 30%, maize 36%, barley 19%, soybean meal 12%, vitamin-mineral premix 2% and salt 1%). Rumen fluids were collected before morning feeding and were strained through four layers of cheesecloth under CO_2 and heating conditions. About 0.200 ± 0.005 g dried silage samples were placed into 100 ml special glass syringes (Model Fortuna, Häberle Labortechnik, Lonsee-Ettlenschieβ, Germany) in three parallels and samples were then supplemented with 10 ml rumen fluid and 20 ml buffer solution mixture prepared in accordance with the method specified by Menke and Steingass (1988). Sample tubes were incubated in a water bath at 39 °C and gas productions were measured at certain intervals.

Statistical analysis

Experimental data were subjected to fully randomized one-way ANOVA with the use of Minitab 16.1 software (Minitab Ltd., Coventry, United

Kingdom). Significant means were compared with the use of Tukey's multiple comparison tests at 95% confidence interval. All data were expressed in mean ± standard deviation.

Results and discussion

Chemical composition. Addition of chitosan to alfalfa silage did not affect DM content (P = 0.732, Table 2). Since silage was made in vacuum bags, there were not any leakages, thus DM was not significantly different. The bags lost their tight appearance when the first package was made, and gas formation occurred at a level that would not burst the bags. It was reported that 1.0% chitosan supplementation increased DM of sugarcane silage, but the ensiling technique was different in that study (Gandra et al., 2016). Similar to the present study, the effects of chitosan supplementations on silage DM of sugarcane and soybean silages were not found to be significant (Del Valle et al., 2018; Gandra et al., 2018).

might have also alter the relative effect of chitosan. Chitosan is a weak base soluble in weak aqueous acid solutions and may convert glucosamine ($-NH_2$) units into soluble protonated forms ($-NH^+_3$) (Goy et al., 2009). There was a significant decrease in EE contents in CHTS1.0 and CHTS2.0 groups (P = 0.001). Such a case may be related to the negative effects of chitosan supplementation on silage fermentation. With increasing chitosan supplementation levels, the formation of an anaerobic environment might have been delayed and thus a decrease might have been seen in fat oxidation (Del Valle et al., 2018; Gandra et al., 2018).

In the present study, the CA was increased between CHTS 0.5 and CHTS 1.0 and 2.0 groups (P = 0.009), but no difference in comparison to the control group was noticed. In previous studies, 1.0% chitosan supplementation decreased CA of sugarcane silage, but in another study, the CA was increased only with 0.5% chitosan supplementation (Gandra et al., 2016; Del Valle et al., 2018). Also, 0.5% chitosan supplementation

Table 2. Effects of chitosan supplementation on the chemical composition of alfalfa silage, g/kg dry matter (DM)

Indices	Treatment groups				SEM	*P*-value
	CONT	CHTS0.5	CHTS1.0	CHTS2.0		
DM	252.3 ± 0.67	254.3 ± 0.38	252.1 ± 0.68	255.9 ± 0.86	6.710	0.732
Crude protein	221.4 ± 0.52	224.6 ± 0.93	225.7 ± 0.09	229.7 ± 0.84	6.800	0.237
Ether extract	15.9^{a} ± 0.20	15.6^{a} ± 0.30	11.2^{b} ± 0.20	11.9^{b} ± 0.09	2.101	0.001
Crude ash	119.6^{ab} ± 0.06	119.4^{b} ± 0.16	121.4^{a} ± 0.09	121.3^{a} ± 0.13	1.160	0.009
Starch	25.7^{b} ± 0.25	30.4^{a} ± 0.17	24.3^{b} ± 0.21	19.3^{c} ± 0.11	1.915	0.000
Acid detergent fibre	332.1 ± 1.25	326.1 ± 0.29	325.9 ± 0.28	321.3 ± 0.88	7.910	0.165
Neutral detergent fibre	382.5^{b} ± 1.37	386.2^{b} ± 0.80	397.5^{ab} ± 1.26	409.2^{a} ± 1.19	11.74	0.003
Acid detergent insoluble crude protein	18.1^{b} ± 0.09	20.0^{ab} ± 0.10	18.9^{ab} ± 0.17	20.3^{a} ± 0.11	1.226	0.020
Neutral detergent insoluble crude protein	19.9^{d} ± 0.06	26.0^{c} ± 0.07	31.4^{b} ± 0.38	42.1^{a} ± 0.21	2.207	0.000
Crude fibre	273.1 ± 1.38	271.0 ± 0.98	280.9 ± 1.30	295.1 ± 2.15	15.16	0.049
Acid detergent lignin	67.1^{ab} ± 0.33	66.5^{b} ± 0.16	71.3^{a} ± 0.17	68.5^{ab} ± 0.35	2.643	0.024

CONT – control group (0% chitosan), CHTS0.5 – group supplemented with 0.5% chitosan, CHTS1.0 – group supplemented with 1% chitosan, CHTS2.0 – group supplemented with 2% chitosan; SEM – standard error of the mean; ^{abc} means with different superscripts within the row are significantly different at $P < 0.05$

Also, chitosan supplementation had no effect on CP content in all studied groups (P = 0.237). However, in the study of Del Valle et al. (2018) was shown that 0.6% supplementation of chitosan increase CP level in silage. However, in another study, the CP was decreased with 0.5% chitosan supplementation in soybean silage (Gandra et al., 2018). The additives increasing silage pH levels also increase silage ammonia quantities and high ammonia concentrations may be related to a high protein content of chitosan (Manni et al., 2010). The high protein content of alfalfa (23%) used in the present study

increased the CA in soybean silage (Gandra et al., 2018). There were significant differences in starch content of the experimental groups (P = 0.000), but a gradual change was not seen with supplementation ratios since alfalfa silage starch might have been converted into different organic acids because of fermentation. In the literature there is no information on the starch content in chitosan-supplemented silages. The NDF increased with chitosan supplementation levels (P = 0.003). In previous studies, similar changes were reported in NDF with chitosan supplementations.

In other studies, NDF was decreased with 1.0% and 0.6% chitosan supplementation as compared to the control sugarcane silages (Del Valle et al., 2018; Gandra et al., 2016). Similarly to the present study, 0.5% chitosan addition increased NDF in soybean silage (Gandra et al., 2018). In the present study, slow acidification was encountered with chitosan addition, then the heat generated through microorganism activity increased NDICP, thus NDF values. There were significant differences in ADICP level of the experimental groups (P = 0.020), but such differences do not comply with the chitosan supplementation levels. In the present study, the NDICP increased with increasing chitosan levels. Such a case was parallel to a change in NDF values. It was reported that NDICP values increased (R^2 = 0.892) with the heat up in roughage bales as compared to pre-storage values (Coblentz et al., 2010).

Nutritional composition. The effects of chitosan supplementation on the nutritional composition of alfalfa silages are presented in Table 3. The differences in DDM of the experimental groups were not found to be significant (P = 0.165). DMI decreased with increasing chitosan levels (P = 0.004). Relative feed value is used for the classification of forage based on quality classes and it is a product of DDM and DMI values; and it was not affected by treatments (P = 0.080). Values of TDN decreased with chitosan treatments. Similarly, 1.0% chitosan supplementation increased TDN values of sugarcane silage (Gandra et al., 2016).

In vitro gas production parameters of silage are provided in Table 4. Gas production, organic matter digestibility (OMD), ME and NE_L in alfalfa silage were not affected with chitosan supplementation ($P > 0.05$). In the literature, the effect of chitosan addition to silage on gas production parameters is not described.

Table 3. Effects of chitosan supplementation on the nutritional composition of alfalfa silages

Indices	Treatment groups				SEM	*P*-value
	CONT	CHTS0.5	CHTS1.0	CHTS2.0		
DDM, g/kg DM	630.3 ± 0.98	635.0 ± 0.22	635.1 ± 0.22	638.7 ± 0.67	6.17	0.165
DMI, % body weight	3.14^{a} ± 0.11	3.11^{a} ± 0.07	3.02^{ab} ± 0.10	2.94^{b} ± 0.09	0.09	0.004
RFV, score	153.67 ± 7.61	152.83 ± 3.76	148.67 ± 5.61	145.50 ± 5.47	5.77	0.080
TDN, g/kg DM	568.6^{a} ± 1.04	567.8^{a} ± 0.29	554.2^{b} ± 0.78	556.4^{ab} ± 0.85	7.88	0.006

CONT – control group (0% chitosan), CHTS0.5 – group supplemented with 0.5% chitosan, CHTS1.0 – group supplemented with 1% chitosan, CHTS2.0 – group supplemented with 2% chitosan; SEM – standard error of the mean; DDM – digestible dry matter (DM) (DDM = 88.89 – (0.779 × acid detergent fibre), DMI – dry matter intake (DMI = 120/neutral detergent fibre), RFV – relative feed value (RFV = (DDM×DMI)/1.29), TDN – total digestible nutrients (TDN(%) = tdNFC + tdCP + (tdFA × 2.25) + tdNDF–7); ^{ab} means with different superscripts within the row are significantly different at $P < 0.05$

Table 4. Effects of chitosan supplementation on *in vitro* gas production of alfalfa silage

Items	Treatment groups				SEM	*P*-value
	CONT	CHTS0.5	CHTS1.0	CHTS2.0		
Gas production, ml	37.33 ± 0.33	38.00 ± 1.15	37.00 ± 1.00	34.00 ± 1.53	0.657	0.122
OMD, % dry matter	65.79 ± 0.29	66.51 ± 0.59	65.80 ± 0.89	63.30 ± 0.36	0.554	0.180
ME, MJ/kg	7.40 ± 0.04	7.50 ± 0.16	7.36 ± 0.13	6.95 ± 0.21	0.089	0.122
NE_L, MJ/kg	4.21 ± 0.03	4.27 ± 0.11	4.18 ± 0.10	3.89 ± 0.15	0.063	0.129

CONT – control group (0% chitosan), CHTS0.5 – group supplemented with 0.5% chitosan, CHTS1.0 – group supplemented with 1% chitosan, CHTS2.0 – group supplemented with 2% chitosan; SEM – standard error of the mean; OMD – organic matter digestibility, ME – metabolizable energy, NE_L – net energy lactation

Table 5. Effects of chitosan supplementation on pH and organic acids of alfalfa silages, g/kg

Indices	Treatment groups				SEM	*P*-value
	CONT	CHTS0.5	CHTS1.0	CHTS2.0		
pH	5.88^{b} ± 0.19	6.11^{ab} ± 0.06	6.28^{ab} ± 0.01	6.33^{a} ± 0.06	0.103	0.003
Lactic acid	60.68^{a} ± 0.30	56.62^{b} ± 0.11	55.20^{c} ± 0.14	54.76^{c} ± 0.16	0.709	0.000
Acetic acid	17.80^{d} ± 0.14	19.80^{c} ± 0.14	20.50^{b} ± 0.18	21.26^{a} ± 0.15	0.394	0.000
Propionic acid	0.83^{b} ± 0.02	1.01^{a} ± 0.01	1.01^{a} ± 0.02	1.05^{a} ± 0.04	0.027	0.001
Butyric acid	3.18^{a} ± 0.02	2.39^{b} ± 0.14	2.12^{bc} ± 0.06	1.85^{c} ± 0.15	0.156	0.000

CONT – control group (0% chitosan), CHTS0.5 – group supplemented with 0.5% chitosan, CHTS1.0 – group supplemented with 1% chitosan, CHTS2.0 – group supplemented with 2% chitosanSEM – standard error of the mean; ^{abc} means with different superscripts within the row are significantly different at $P < 0.05$

Silage fermentation. The silages pH and organic acid concentrations are provided in Table 5. Supplementation of 2% chitosan increased pH values in comparison to control and other groups, in this case, chitosan supplementation slowed down acidity development. In the literature, chitosan supplementation decreased (4.22–3.32), increased (3.47–3.55), or did not affect (5.33–5.32) pH levels in silages (Del Valle et al., 2018; Gandra et al., 2018, respectively). In the present study, treatments with 2% chitosan increased pH values of alfalfa silages. The desired pH levels of legume silages (30–35 DM) were reported with pH 4.3–4.5 (Kung and Shaver, 2001). In the present study, 25% DM and 23% protein content increased buffering capacity and pH of the CONT group (pH 5.88).

Alfalfa silage lactic and butyric acid concentrations were decreased with increasing chitosan levels ($P < 0.05$). Acetic and propionic acids concentrations were increased with increasing chitosan levels ($P < 0.01$). Lactic acid is stronger than acetic acid, lactic acid quantity positively influences silage quality and high lactic acid contents are desired in the final product (Muck, 2010). In the present study, chitosan treatments reduced lactic acid and butyric acid concentrations. In similar studies, 1.0% chitosan treatments increased lactic, acetic, and butyric acids concentrations of sugarcane silages in comparison to control group but did not change propionic acid concentration (Gandra et al., 2016). In another study, 0.6% chitosan treatment reduced lactic acid ($P = 0.015$), increased acetic and butyric acid, and did not change propionic acids concentrations in sugarcane silage (Del Valle et al., 2018). In addition, 0.5% chitosan supplementation increased lactic and propionic acids concentrations and reduced acetic and butyric acids concentrations in soybean silages (Gandra et al., 2018). The lactic acid produced through heterofermentative lactic acid bacteria fermentation is dependent on the existence of an electron receptor (Oude Elferink et al., 2001). It may also act as an electron receiver bound to the chitosan chain (Goy et al., 2009). Therefore, lactic acid content might have decreased, and acetic acid content increased (Gandra et al., 2016). Present findings support that case. Butyric acid is an indicator of clostridial fermentation (Bryan, 2019). The potential of clostridial fermentation is high in legume silages with a high moisture content (>70%) and butyric acid content of well-fermented silages is of not detectable levels (Kung et al., 2018). In the present study, clostridial fermentation might have been encountered especially in CONT group since butyric acid content was greater as compared to CHTS groups.

Silage microbiology. Effects of chitosan on yeast-mould counts of alfalfa silage are provided in Table 6. The aerobic environment in feed out phase significantly altered yeast-mould development.

Table 6. Effects of chitosan supplementation on yeast-mould counts of alfalfa silage

Items	Treatment groups			
	CONT	CHTS0.5	CHTS1.0	CHTS2.0
Silage opening day				
yeast, CFU/g	72 x 10^3	31 x 10^3	1.4 x 10^3	0.4 x 10^3
mould, CFU/g	4.1 x 10^1	ND	ND	ND
5 days after opening				
yeast, CFU/g	0.4 x 10^4	268 x 10^4	1893 x 10^4	193 x 10^4
mould, CFU/g	67 x 10^1	ND	ND	ND

CONT – control group (0% chitosan), CHTS0.5 – group supplemented with 0.5% chitosan, CHTS1.0 – group supplemented with 1% chitosan, CHTS2.0 – group supplemented with 2% chitosan; SEM – standard error of the mean; ND – not determined

Mould development increased and was not encountered in CONT group, but quite much yeast development was encountered in CHTS groups. It was seen that chitosan prevented mould development, but increased yeast production in feed out phase. In previous studies, yeast and moulds production was decreased in both sugarcane and soybean silages with chitosan treatments (Gandra et al., 2016, 2018). Yeasts develop throughout the fermentation process of the silage at the beginning of the aerobic phase and anaerobe phase and following the opening of silage, they start to reproduce when the silage is exposed to oxygen in feed out phase and degrade lactic acid, thus acting as the primary microorganism resulting in silage spoilage (Inglis et al., 1999). As the pH increases through lactic acid degradation, the development of opportunist bacteria increases and silage quality decreases with the mould development (McDonald et al., 1991). Under both aerobic and anaerobic conditions, yeast activity is not desired for silage quality since it results in loss of nutrients and aerobic degradation (Kızılşimşek et al., 2016).

Table 7. Effects of chitosan supplementation on aflatoxin of alfalfa silages

Items	Treatment groups				SEM	*P*-value
	CONT	CHTS0.5	CHTS1.0	CHTS2.0		
Silage opening day						
aflatoxin B1, ppb	$0.75^{b} \pm 0.04$	$0.75^{b} \pm 0.14$	$0.75^{b} \pm 0.07$	$0.83^{a} \pm 0.06$	0.088	0.005
5 days after opening silage						
total aflatoxin, ppb	19.46	20.60	23.67	16.19		

CONT – control group (0% chitosan), CHTS0.5 – group supplemented with 0.5% chitosan, CHTS1.0 – group supplemented with 1% chitosan, CHTS2.0 – group supplemented with 2% chitosan; SEM – standard error of the mean; [ab] means with different superscripts within the row are significantly different at $P < 0.05$

Silage aflatoxin. Aflatoxin analyses are provided in Table 7. In all groups, aflatoxin B1 levels did not exert a threat on animal health. The lowest total aflatoxin level was observed in CHTS2.0 group five days after opening. There were not sufficient data available to explain the relationships between aflatoxin B1 and total aflatoxin.

Conclusions

It was concluded that chitosan, a biodegradable and non-toxic biological additive with known antifungal effects, could be used as a silage additive alone. Further research is recommended with the use of different additives and *in vivo* experiments.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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