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EFFECT OF *Lactobacillus plantarum* AND *Lactobacillus buchneri* ON COMPOSITION, AEROBIC STABILITY, TOTAL LACTIC ACID BACTERIA AND E. COLI COUNT OF ENSILED CORN STOVER WITH OR WITHOUT MOLASSES SUPPLEMENTATION

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This study was conducted to evaluate the molasses and bacterial inoculant effects on ensiled corn stover chemical composition, physical characteristics, aerobic stability and total microbial cell count. Corn stover (CS) was ensiled into 24 laboratory silos with molasses or without molasses (3 and 0%), with inoculant (10 and 0 ml/kg of CS) and air ingression (air ingressed or no air ingressed) for 60 days. On day 30 of ensiling, air ingressed treatments were exposed to air. A significant interaction was observed among molasses, inoculant and air ingressed for chemical composition of ensiled CS ($P<0.05$). Highest crude protein contents were present in non-air ingressed, molasses, without inoculant treated ensiled CS ($P<0.05$). The molasses and inoculant interaction showed lowest neutral detergent fibre contents in molasses and inoculant treated CS ($P<0.05$). The lowest acid detergent fibre contents were found in molasses treated CS compared to CS without molasses treatment ($P<0.05$). The molasses, inoculant and air ingressed interaction showed the lowest ash contents in air ingressed CS ensiled with inoculant and without molasses ($P<0.05$). While, this interaction showed highest ash contents by ensiled CS treated with molasses, without inoculant and air ingressed ($P<0.05$). The highest total lactobacillus cell load was expressed by Molasses with inoculant with air ingression (MIA), MA (Molasses with air ingression) treatments than MN (Molasses with non-air ingression), IN (Inoculant with non-air ingression), IA (Inoculant with air ingression), N (non-air ingression), A (air ingression) and MIN (Molasses with inoculant with non-air ingression) treatments ($P<0.05$). Lowest cell count of E.coli was expressed by IN treatments than MN, MA, MIA, IA, N, A and MIN treatments ($P<0.05$). Based on findings, it is concluded that bacterial inoculants (*L. buchneri 40788* and *L. plantarum MTD-1*) along with molasses supplementation improves the physical characteristics, nutritional profile and aerobic stability of ensiled CS.
Keywords: Corn stover; molasses; Ensiling; Aerobic stability

INTRODUCTION

The rapidly increasing human population has decreased the fodder cultivation area. Improper supply of quality fodder is one of the constraints for livestock production (Muhammad *et al.*, 2016; Xia *et al.*, 2018a; Xia *et al.*, 2018b; Xia *et al.*, 2018c). For improved livestock production, steady supply of quality fodder is important. For this purpose, use of crop residue as corn stover (CS) is an important strategy (Aziz ur Rahman *et al.*, 2017; Aziz ur Rahman *et al.*, 2019). Corn stover contributes about 35% of total crop residues production (Li *et al.*, 2014). It is estimated that 50% dry matter of corn crop is CS and normally after harvesting of maize grain CS is destroyed (Aziz ur Rahman *et al.*, 2017; Aziz ur Rahman *et al.*, 2019). It is reported that after harvesting of maize, CS is available for livestock as a roughage source. However, feeding of CS for long time is not done because of dry matter and nutrient loss.

Therefore, CS can be used in ruminant feed for a long time by ensiling CS in anaerobic conditions (Perlack and Turhollow, 2003). Ensiled CS is considered as low quality silage due to poor digestibility of nutrients. It mainly consists of cellulose, hemicelluloses and lignin, which prevents the better utilization of CS nutrients (Kleinschmit *et al.*, 2007). Different additives and supplements are used to stimulate and inhibit the fermentation, and to increase the nutrition of CS silage (Olson *et al.*, 1966, Morais *et al.*, 2017). Major additives and supplements which are being used to improve the silage quality are microbial inoculants and molasses. Molasses is added in the silage to modify the fermentation (Baytok *et al.*, 2005) and level of fermentable products of corn silage during fermentation process (Kung *et al*., 2003). Growth and level of detrimental microorganisms (e.g., spoilage aerobic bacteria and molds) is inhibited by LAB during the fermentation (Li and Nishino, 2011). Molasses is the sources of soluble carbohydrates (Olson *et al.*, 1966, Morais *et al.*, 2017) to microbes for their growth and optimum microbial activities. Molasses are mainly added in silage preparation to improve silage quality (Whiter and Kung, 2001). Because, molasses improves the nutritional, preservative and physical properties of CS silage (Wattanaklang *et al.,* 2016).

Silage feeding exhibit the major issue of aerobic stability, even after its proper ensiling (Muck, 2010). Ensiling conditions such as air ingress during the ensiling period affects the fermentation characteristics, aerobic stability and yeast count (Weiss *et al.*, 2016). Kleinschmit *et al*. (2006) stated that inoculation of heterofermented (*Lactobacillus buchneri*) bacteria makes the corn silage aerobically more stable. The aerobically unstable silage affects the productive and reproductive performance of dairy animals (Weiss *et al*., 2016). *Lactobacillus buchneri* under anaerobic conditions, produces acetic acid from lactic acid (Olveiria *et al*., 2017).

The objective of this study was to evaluate the effect of *Lactobacillus plantarum* and *Lactobacillus buchneri* inoculation on composition, quality, aerobic stability and microbial cell count of CS with or without molasses.

MATERIALS AND METHODS

Corn stover and ensiling conditions: The CS was harvested at 38.4 % dry matter (DM). Corn stover was chopped at 0.92-4 cm theoretical length (Chea *et al.*, 2015; Chen *et al.*, 2020) by chopper machine. Within half hour the chopped CS was ensiled into 24 laboratory silos (20 × 30 cm plastic jars of 1 kg capacity) with molasses (3%) or without molasses, with two levels of bacterial inoculant (0 and 1×10^{5} cfu/g of fresh CS) and two levels of air ingress (either air ingress or no air ingress) for 60 days in 2 × 2 × 2 factorial arrangement. A product of Poineer Hybride of *L. buchneri 40788* with *L. plantarum MTD-1* was applied at 1×10^5 cfu/g of CS. The solution of inoculant was made in 200 mL deionized water and then it was sprayed and mixed thoroughly in chopped CS piles which were decided by plan as inoculant treated treatments. The ensiling duration (60 days) and ambient temperature (18-25 ˚C) were kept same for all treatments. On day 30 of ensiling, air ingress treatments were made air ingress by making hole of 6 mm diameter in lid as well in the body of all selected jars, 5 cm above the bottom of each jar. There were eight treatments: 1) Molasses plus Inoculant treated Non-air ingressed (MIN), 2) Molasses plus Inoculant treated Air ingressed (MIA), 3) Molasses treated Non-air ingressed (MN), 4) Molasses treated Air ingressed (MA), 5) Inoculant treated Non-air ingressed (IN), 6) Inoculant treated Air ingressed (IA), 7) Non-air ingressed (N), 8) Air ingressed (A) and each treatment was triplicated.

Sample Collection and Analyses: Triplicate samples of the chopped fresh CS were collected prior to ensiling. Each sample was subjected to forced-air oven at 60 °C for 48 h to determine the DM contents as described in literature (Su *et al.*, 2013; Li *et al.*, 2014; Zhang *et al.*, 2015; Wang *et al.*, 2016). Dried samples were ground with a Udy Cyclone sample mill (1-mm screen, Udy Corp., Fort Collins, CO). Samples were analyzed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) by the methods of Van Soest (1970) with some modification as described in recent studies (Niu *et al.*, 2017; He *et al.*, 2018; Hussain *et al.*, 2018; Qiu *et al.*, 2018; Sharif *et al.*, 2018; Dong *et al*, 2019; Li *et al.*, 2019). Crude protein (CP) was determined by Kjeldahl method as described in literature (Arshad *et al.*, 2020; Bajwa *et al.*, 2020; Hussain *et al.*, 2020; Kamran *et al.*, 2020; Muhmmad *et al.*, 2020; Shahid *et al.*, 2020).

After 60 days of ensiling, each laboratory silo was opened and mixed thoroughly. The physical properties of all ensiled treatments were observed and documented. Visual test of each treatment sample was done to detect the fungus growth. After the observation, sample was taken from each replication for chemical analysis. The DM contents were determined on fresh basis. The samples were dried and ground with a Udy Cyclone sample mill (1-mm screen, Udy Corp., Fort Collins, CO) for CP, NDF, ADF, ash content and ether extract (EE) determination. The pH was measured by soaking 20g sample of each replication in 20 ml distilled water for 30 minutes. Procedure of proximate analysis was followed to determine the EE, CP, NDF, ADF, and ash contents (AOAC 1984). After the collection of samples, reaming silage of each replication was subjected to aerobic stability test. Silage from each silo was placed in a plastic bucket and kept in a room having temperature about 24˚C. For the DNA preparation, primer designing of *E. coli* and quantitative real time polymerase reactions were performed according to recent studies (Stevenson *et al.*, 2006; Qiu *et al.*, 2019a; Qiu *et al.*, 2019b). The data was analyzed using GLM procedure of SAS under Complete Randomized Design. Means were compared by Turkey's test, where applicable.

RESULTS

Physical characteristics: Physical characteristics of CS silages has been presented in Table 1. The color of MIN and IN treatments were greenish. Non-air ingressed control treatments had green to brown color while, the color of MN was brown to yellowish. The color of all A treatments was gray to black. The smell of all N treatments was desirable. The A treatments were possessed undesirable bad smell. From all N treatments, MIN treatment had more acidic smell. The

Table 1. Physical characteristics evaluation of corn stover silage at 60th day of ensiling

Parameter	Replicate	Treatments							
		MIN	**MIA**	**MN**	**MA**	**IN**	**IA**	**N**	**A**
Color	R1	Greenish	Brown Grey	Brown-yellowish	Grey-Blackish	Greenish	Grey	Brown	Blackish
	R2	Green to Brownish	Brown Grey	Brown-yellowish	Grey-Blackish	Greenish	Grey	Brown	Blackish
	R3	Green to Brownish	Brown Grey	Brown-yellowish	Grey-Blackish	Greenish	Grey	Brown	Blackish
Smell	R1	desirable	Desirable	desirable	Undesirable	desirable	Undesirable	Undesirable	Undesirable
	R2	desirable	Desirable	desirable	Undesirable	desirable	Undesirable	Undesirable	Undesirable
	R3	desirable	Desirable	desirable	Undesirable	desirable	Undesirable	Undesirable	Undesirable
Fungus	R1	Absent	Moderate	Absent	Present	Absent	Present	Present	Present
	R2	Absent	Moderate	Absent	Present	Absent	Present	Present	Present
	R3	Absent	Present	Absent	Present	Absent	Present	Absent	Present

MN=Molasses + non-air ingression, MA= Molasses +air ingression, IN=Inoculant + non-air ingression, MIN=Molasses+ Inoculant + non- air ingression, MIA=Molasses+ Inoculant + air ingression, N= only non-air ingression, A= only air ingression R1=Replicate 1, R2=Replicate 2 and R3=Replicate 3.

Table 2. Compositional characteristics of corn stover silage

	M				**M$_O$**											
	I		**I$_0$**		**I**		**I$_0$**			***P-value***						
Parameters	**N**	**A**	**N**	**A**	**N**	**A**	**N**	**A**	**SEM**	**M**	**I**	**A**	**MxI**	**MxA**	**IxA**	MxIxA
DM %	23.4	21.7	22.4	20.7	23.0	23.8	25.05	23.8	0.560	*	NS	*	*	NS	NS	NS
CP %	8.4	6.5	8.6	7.1	5.4	6.4	5.6	5.3	0.171	*	NS	*	*	*	NS	*
EE %	1.9	1.1	1.4	1.2	1.8	1.5	1.4	1.5	0.152	NS	*	NS	NS	*	NS	NS
Ash %	10.3	9.9	8.9	11.2	8.5	7.9	8.4	8.2	0.309	*	NS	NS	NS	*	NS	*
NDF %	61.3	63.1	61.6	64.7	69.6	72.1	66.7	67.9	0.880	*	NS	NS	*	NS	NS	NS
ADF %	31.2	31.9	30.4	30.9	37.7	41.1	41.03	40.0	0.349	*	NS	NS	NS	NS	NS	NS
pH	4.36	4.87	4.52	4.61	4.76	5.07	5.11	6.17	0.131	*	*	*	*	*	*	*

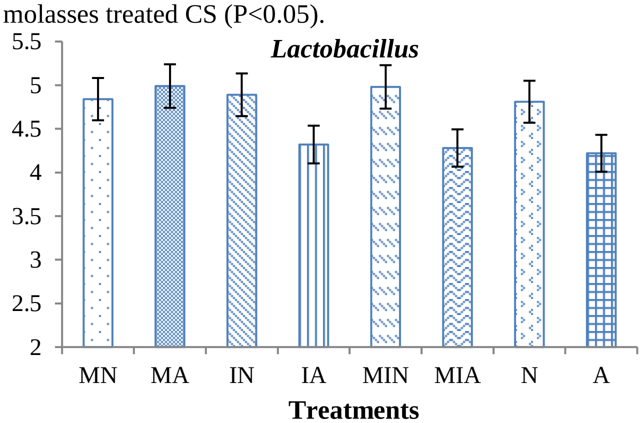
M=Molasses added, M$_0$= without Molasses, I= Inoculant treated, I$_0$= without Inoculant, N=Non air ingressed at 30th day of ensiling, A= Air ingressed at 30th day, NS= Non-significant difference (P>0.05), SE= Standard error DM= Dry matter, CP=Crude protein, EE=Ether extract, NDF= Neutral detergent fiber, ADF=Acid detergent fiber and *= Significant (p< 0.05) difference

MIA, MA, IA and A treatments showed visual fungus growth while in MIN, MN, IN and N treatments visual fungus was not detected.

Compositional characteristics: Data of DM is presented in Table 2. Molasses and air ingression levels had significant effect on DM losses in ensiled CS (P<0.05). The interaction of molasses and inoculant was found for DM losses of CS (P<0.05). Molasses treated CS showed more DM losses than without molasses added CS treatments (P<0.05). Similar trend of DM losses was observed in air ingressed treatments than non-air ingressed treatments (P<0.05). The molasses and inoculant interaction revealed that higher DM losses were present in molasses without inoculant enisled CS as compare to the other treatments (P<0.05). While, molasses and inoculant interaction showed lowest DM losses in CS ensiled without molasses and inoculant (P<0.05). Levels of molasses and air ingression had effects on CP contents of ensiled CS (P<0.05). While, interaction of molasses with inoculant, molasses with air ingressed and molasses with inoculant and air ingression were also observed for CP of CS silages (P<0.05). The results are presented in Table 2. Increased CP contents were found in molasses treated CS compared to without molasses ensiled CS (P<0.05). Similar trend of CP increment was observed in non-air ingressed treatments than air ingressed treatments (P<0.05). The molasses and inoculant interaction revealed that higher CP was present in molasses without inoculant enisled CS (P<0.05). While, molasses and inoculant interaction showed lowest CP contents in CS ensiled without molasses and inoculant (P<0.05). The molasses and air ingressed interaction showed higher CP contents in molasses without air ingressed enisled CS (P<0.05).

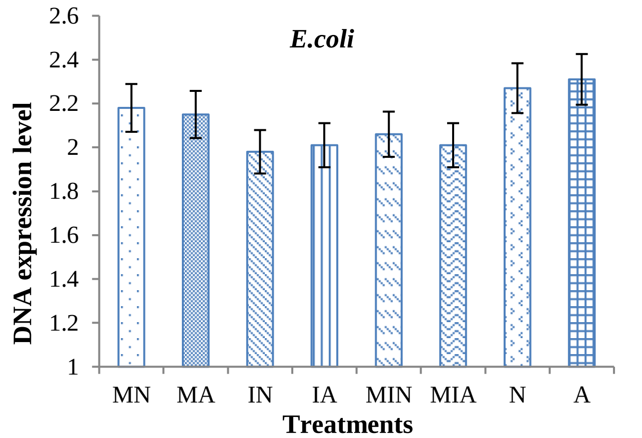
While, molasses and air ingressed interaction showed CP contents in air ingressed CS ensiled without molasses (P<0.05). The molasses, inoculant and air ingressed interaction showed lowest CP contents in air ingressed CS ensiled without molasses and inoculant. Highest results on this interaction were showed by non-air ingressed CS molasses treated without inoculant (P<0.05). Data of EE is documented in table 2. Inoculant levels had changed the EE contents of ensiled CS (P<0.05). Similarly, interaction of molasses and air ingress had altered the EE contents of ensiled CS (P<0.05). The molasses and air ingressed interaction showed the lowest EE contents in molasses treated air ingressed enisled CS (P<0.05). Results of ash contents are presented in table 2. Molasses levels had changed ash contents of ensiled CS (P<0.05). Results showed interactions of molasses and air ingressed (P<0.05). An interaction was also present for inoculant with air ingressed (P<0.05). There was also an interaction of molasses, inoculant and air ingression for ash contents of ensiled CS (P<0.05). The highest ash contents were found in molasses treated CS than without molasses added CS (P<0.05). The molasses and air ingressed interaction showed lowest ash contents in without molasses treated air ingressed enisled CS (P<0.05). While, molasses and air ingressed interaction showed the higher ash contents in molasses treated air ingressed enisled CS (P<0.05).

The molasses, inoculant and air ingressed interaction showed the lowest ash contents in air ingressed CS ensiled with inoculant and without molasses. Highest ash contents were showed by interaction of CS silage treated with molasses, without inoculant and air ingressed (P<0.05). The results of NDF are documented in table 2. Molasses levels have changed NDF contents of ensiled CS (P<0.05). While, interaction of molasses and inoculant was observed for NDF contents of ensiled CS (P<0.05). The lowest NDF contents were found in molasses treated CS than without molasses treated CS (P<0.05). The molasses and inoculant interaction results showed lowest NDF contents in molasses and inoculant treated CS (P<0.05). While, molasses and inoculant interaction showed the highest NDF content with inoculant and without molasses treated CS (P<0.05). Data of ADF is documented in table 4.2. Molasses levels have changed ADF contents of ensiled CS (P<0.05). Inoculant and air ingression levels didn't show any-effects on ADF contents of ensiled CS and no interaction effects were observed (P>0.05). The lowest ADF contents were found in molasses treated CS than without molasses treated CS (P<0.05).



MN = Molasses + non-air ingression, MA = Molasses +air ingression, IN = Inoculant + non-air ingression, MIN = Molasses+ Inoculant+ non-air ingression, MIA = Molasses+ Inoculant+ air ingression, N = only non-air ingression, A = only air ingression

Figure 1. Percentage of total *Lactobacillus* DNA detected of corn stover silage at 60th day of ensiling



MN = Molasses + non-air ingression, MA = Molasses +air ingression, IN = Inoculant + non-air ingression, MIN = Molasses+ Inoculant+ non-air ingression, MIA = Molasses+ Inoculant+ air ingression, N = only non-air ingression, A = only air ingression

Figure 2. Percentage of total *E.coli* DNA detected at 60th day of corn stover ensiling

Total Lactobacillus cell load in ensiled corn stover: The highest total lactobacillus cell load was expressed by MIA and MA treatments than MN, IN, IA, N, A and MIN treatments (P<0.05) as shown in Fig. 1.

***E. coli total bacterial DNA expression*:** The lowest cell count of *E. coli* was expressed by IN treatments than MN, MA, MIA, IA, N, A. and MIN treatments (P<0.05) as shown in Fig. 2.

DISCUSSION

According to our study, fungus was present in ensiled CS treated with A, MA and IA, showed undesirable smell and blackish to greyish colour. However, fungus was absent in ensiled CS treated with IN, MN, showed desirable smell and greenish to brown-yellowish colour. Similarly, molasses with MIN and MIA showed desirable smell and colour (greenish to brown-grey). However, fungus was present in MIA treated ensiled CS. It was concluded that presence of molasses and inoculant showed better results. These finding are similar to Bostami *et al*. (2008). Cai *et al*. (1999) also suggested that silage quality could be improved by the addition of inoculant and molasses. Man and Wiktorsson, (2003) reported that molasses addition improves the smell, color, and has inhibitory effects on fungal growth. It was also reported that molasses could enhance the acceptance and DM intake of roughages due to sweet taste and optimum flavor (Aregheore and Perera, 2004).

Addition of molasses improved the fermentation and blocked fungal growth that resulted in production of good quality silage. Previous studies reported that fungal growth could be increased by increasing the ensiling duration (Hiep and Man, 2003).

In current study, the highest DM content was found in non-air ingressed CS ensiled without molasses and inoculant. Lowest DM contents were observed in air ingressed CS ensiled with molasses and without inoculant. These findings were supported by the finding of Nour (1990) who, concluded that ensiling with molasses has reduced the DM content as molasses stimulate the microbial growth. Similar findings were also documented by Otieno *et al*. (1986) and Wiktorsson (2003) on DM contents reductions with increasing the ensiling time due to more microbial activities. In present study, the interactions of MxA showed that DM contents were higher in inoculant added without molasses ensiled CS and molasses added without inoculant treated CS. To support it, Filya (2003) documented that *Lactobacillus plantarum* had significant role in DM contents recovery of corn silage. It may be due to lower rate of fermentation process in without molasses added CS and DM conservational characteristics of *Lactobacillus plantarum*. Lynch *et al.* (2011) stated that *Lactobacillus plantarum* had non-significantly improved the DM recovery of ensiled CS.

The CP content was highest in molasses added CS than without molasses. As molasses shared its nitrogenous contents and increased the CP contents of ensiled CS. Because, Lanari *et al*. (1987) documented that molasses increased the CP content of CS. Previous studies also reported that addition of liquid residue derived from molasses increase the CP content (Andrighelto *et al*. 1988) and (Otieno *et al*., 1986). In the present experiment the CP content was higher in non-air ingressed ensiled CS than air ingressed ensiled CS.

Molasses act as a substrate for the microbial growth. Microbial nitrogen supply increased with increasing the supply molasses (Tolera and Sundstol, 2000). The anaerobic environment promote the microbial growth and resulted in increased microbial protein share. It may be due to the readily available energy from the molasses, which was used by the microorganism for their growth and increased microbial protein in the silage. Molasses, inoculant and air ingression interaction had increased ash contents of ensiled CS. To support it, Duraisam *et al.* (2011) stated that molasses had up to 10 % ash contents. It was due to molasses sharing of its own minerals contents. The major portion (about 80 %) of the total molasses ash contents, consist of potassium and sodium (Riehm and Baron, 1953). In current study, molasses levels had decreased NDF and ADF contents of ensiled CS. This was due to higher rate of cell wall degradation by microbes as molasses having boosting effects on microbial growth that resulted in higher rate of cell wall degradation (Bolsen *et al*., 1996, Bingol *et al*., 2006). Similar findings were documented by Baytok *et al*. (2005) and Baytok and Muruz (2003) on ADF and NDF contents of molasses treated corn silage. It has been reported that feeding such kind of fermented fodder influence the ruminal and fecal microbiota and hence the performance of the animals (Qiu *et al.*, 2020a; Qiu *et al.*, 2020b).

The highest total *Lactobacillus* cell load gene was expressed by MIA and MA treatment than MIN, MN, IN, IA, N and A treatments. These findings were supported by Schmidt *et al*. (2008) and Mari *et al*. (2009). They also reported greater numbers of LAB in corn silages treated with *Lactobacillus buchneri.* The increased number of LAB might be due to well pressing, better fermentation pattern and molasses stimulation to the fermentation process (Baytok *et al*., 2005). The total number of E.coli were lowered in inoculated treatments than non treated in present study. It was may be due to extensive role of *Lactobacillus buchneri* and molasses in ensiled CS fermentation.

Conclusion**:** The quality of corn stover silage could be improved via molasses supplementation and by the use of bacterial inoculant (*L. plantarum* plus *L. buchneri*). Addition of molasses and bacterial inoculation enhanced the degradation rate of water soluble carbohydrates, produced more lactic acid contents, decreased the rate of fermentation losses during whole ensiling period. In addition, molasses and bacterial inoculants improved the aerobic stability of CS silage.

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