Evaluating the Effects of Cellulolytic Enzymes and *Lactobacillus bulgaricus* on Mycotoxins Production and the Quality of Maize Silage

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Fungal spoilage and mycotoxin contamination are two of the greatest hazards of silage. The present work was carried out to evaluate the impact of *Lactobacillus bulgaricus* and cellulolytic enzymes on the maize silage (MS) quality. Fungal analysis of different MS samples showed different mycotoxigenic fungi. The highest frequency (62.8%) was associated with *Fusarium* spp. Four species with different relative densities were found: *F. graminearum* (71.1%), *F. culmorum* (15.2%), *F. proliferatum* (11.2%), and *F. oxysporum* (2.50 %). High-performance liquid chromatography analysis showed the presence of trichothecene, nivalenol, zearalenone, and fumonisins mycotoxins in MS inoculated by *F. graminearum*. The inhibition % of trichothecene, nivalenol, and zearalenone synthesis was 50.2%, 47.5%, and 23.5%, respectively, in MS inoculated by *Lactobacillus bulgaricus* after a 30 d incubation period. *Trichoderma harzianum* succeeded in producing cellulolytic enzymes, *i.e.*, carboxymethyl cellulase, manganase peroxidase, and laccase, with a maximum production of 350 µg/mL, 5.47 µg/mL, and 16.0 µg/mL, respectively, after 21 d using MS as the substrate. Treatment by the extracted cellulolytic enzyme with *L. bulgaricus* enhanced unfavorable conditions for MS fungal contamination, *i.e.*, the production of lactic acid, a lowered pH, and increased *L. bulgaricus* colony-forming units, compared to the addition of enzyme extract or *L. bulgaricus* alone.

Keywords: Cellulolytic enzymes; Maize silage; Lactobacillus bulgaricus; Mycotoxins

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INTRODUCTION

In the last decade, silage production has attracted the attention of most of the livestock farmers in the world due to its high nutritional protein and carbohydrate values. Silage production depends on the fermentation of a chopped fresh crops, *e.g.*, maize, wheat, and rice, followed by storing the prepared silage in the absence of oxygen. Corn silage has become one of the most important food sources for most animals, especially cows and buffaloes, as it was found to increase the amount of meat as well as increase milk productivity (Khatun *et al.* 2016; Miller *et al.* 2020). Certain requirements are essential for silage production in order to avoid its spoilage *via* harmful and toxin-producing microbes, *e.g.*, anaerobic conditions are important for silage preservation. However, these conditions also promote the proliferation of lactic acid bacteria. Anaerobiosis and a lower pH represent an unfavorable habitat for mold proliferation (Motta *et al.* 2020).

Mold growth appears if these conditions are disrupted, particularly after starting to use the corn silage. Whitlow and Hagler (2005) mentioned that the fungi can colonize the silage if oxygen is available. Other environmental conditions, *e.g.*, a high temperature and

humidity, increase the chance of fungi spreading during silage storage (Egal *et al.* 2005; Richard *et al.* 2007; Ogunade *et al.* 2018). Often, the existence of fungi in the silage can be attributed to the colonization of corn in the field or throughout silage storage. However, not only the presence of fungi, but also the presence of mycotoxins, are considered potential threat factors for animals and humans during the daily handling of spoiled silage. Unfortunately, scientific studies have primarily concentrated on the occurrence of mycotoxins in cereal crops, which is likely due to the fact that they are consumed in larger amounts by humans compared to other feed types (Bhat *et al.* 2010). However, numerous mycotoxins have been detected in a range of livestock feed comprising silages (Keller *et al.* 2013). Incidents of mycotoxins in silage were recorded by O'Brien *et al.* (2008) and Cheli *et al.* (2013); therefore, Wambacq *et al.* (2016) stated that proper silage management is important to minimize mycotoxin contamination of dairy animal feeds through the use of chemical additives or microbial inoculants that can reduce the spoiled levels. Many *Fusarium* mycotoxins were discovered in silages, *e.g.*, moniliformin, enniatins (ENN), beauvericins, and fusaric acid; another *Fusarium* mycotoxin, deoxynivalenol (DON), was found in 86% of the collected corn silage samples (McElhinney *et al.* 2015; Kosicki *et al.* 2016). Previously, Eckard *et al.* (2011) reported that maize silage was a good reservoir for *Fusarium* spp. growth and its mycotoxins included numerous trichothecenes, *e.g.*, fusarenon X, diacetoxyscirpenol, nivalenol (NIV), and acetylated DON. Recently, NIV and DON were the most widespread mycotoxins, being found in 97.7% of the collected maize silages samples, followed by ENN B (Vandicke *et al.* 2021). The phenomenon of the spread of fungi and mycotoxins in maize silage deserves more attention than currently given, due to the seriousness of the situation. This can be addressed by following the correct methods of making silage or by adding additives that limit the presence of fungi. Bioadditives, *e.g.*, lactic acid bacteria, were documented to improve the quality of silage and prevent mold growth (Xu *et al.* 2017). Improving the aerobic stability of silages was observed *via* the addition of *Lactobacillus buchneri*, through its conversion of lactic acid to acetic acid (Li *et al.* 2016). Recently, Selwet (2020) studied the addition of different *Lactobacillus* strains on the quality of maize silage. Remarkable increases in concentration of lactic acid, acetic acid and propionic acid were observed, in addition to extended the silage aerobic stability. These acids rapidly acidify silage, thereby repressing the undesirable bacteria growth. Furthermore, acetic and propionic acids serve as fungi-static agents.

Lactobacillus plantarum and *Pediococcus acidilactici*, in conjunction with amylase enzyme, enhanced the fermentation characteristics and aerobic stability of maize silages by promoting bacterial production of volatile fatty acids, *e.g.*, acetic and propionic acids, which repress molds growth (Koc *et al.* 2008). Additional additives, *e.g.*, hydrolytic enzymes, are used extensively in silage production; these enzymes have the ability to release monosaccharaides *via* the degradation of plant cell walls (Muck *et al.* 2018). According to Silva *et al.* (2016) enzyme preparations can be applied as mentioned in silage production, but they are highly expensive and it is more problematic to formulate them. Therefore, a search for alternative economical source such as microbial producing enzymes is needed. An increase in lactate levels and a reduction in ammonia-N levels were observed by Shepherd and Kung (1996) in silage treated with hydrolytic enzymes, *e.g.*, amylase, cellulase, and pectinase. Recently, a combination of lactic acid bacteria and fibrolytic enzymes, *e.g.*, cellulose and hemicellulose, was applied to improve the nutritional availability of silage (Hu *et al.* 2021). Therefore, more data on maize silage is needed to avoid fungal contamination and mycotoxin production by using safe bacterial inoculants, which represents the aim of this study.

EXPERIMENTAL

Collection of Maize Silage for Fungal Analysis

Nine maize silage samples (*Zea mays* L.) were taken from a silo at the top, center, and bottom (three samples for each position) after 40 d of production; the level of each sample was approximately 20 cm in length and 20 cm in width. Then, 500 g of each sample were mixed with a homogenizer to get a homogeneous sample under aseptic conditions (To avoid contamination from other sources such as air, dust, *etc*.) for fungal analysis. Next, 50 g of each sample was shaken for 30 min in 250 mL of sterile water containing 0.15 g of sodium dodecyl sulfate. In a plastic petri plate containing malt extract agar medium supplemented with chloramphenicol (0.05%, w/v), 1 mL of each suspension was sprayed onto the surface of the medium, followed by incubation at a temperature of 28 °C for 6 d. The fungal colonies that appeared were purified and identified. The identification was based on macroscopical and microscopical characterization (Raper and Fennell 1973; Domsch *et al.* 1980; Klich 2002). The isolate frequency (Fr) and relative density (RD) of the species were calculated according to Eq. 1 and Eq. 2, respectively,

$$\text{Fr}\ (\%) = \frac{\text{Number of samples with a species or genus}}{\text{Total number of samples}} \times 100 \quad (1)$$

$$\text{RD}\ (\%) = \frac{\text{Number of isolates of species or genus}}{\text{Total number of isolated fungi in samples}} \times 100 \quad (2)$$

as outlined in Gonzalez *et al.* (1995).

Effect of *Lactobacillus bulgaricus* on Mycotoxins Production by *Fusarium graminearum*

Freshly prepared maize silage (50 g), from the same samples used for the fungal analysis, was moistened in a 250 mL conical flask using 100 mL of distilled water, followed by autoclaving for 30 min at a temperature of 121 °C. Two discs (1 mm radius) from the active margin of a *F. graminearum* colony (species with the highest RD) growing on potato dextrose agar medium and 2 mL of a *L. bulgaricus* (obtained from the researchers in El-Taher *et al.* (2012) suspension were inoculated in the silage followed by incubation at a temperature of 30 °C for up to 30 d. At the end of the incubation period, a filtrate of the metabolized medium was extracted *via* chloroform and methanol (a 2 to 1 ratio) for mycotoxins analysis *via* high-performance liquid chromatography (HPLC). (Series 1100, Agilent Technologies, USA; Column C18 Inertsil: 4.6x250mm, 5µm). Silage without inoculation by *F. graminearum* was used as a control with 15 d and 30 d of incubation.

Enzyme Preparation

Freshly prepared maize silage (10 g), from the same samples used for the fungal analysis, was moistened in a 250 mL conical flask using 20 mL of distilled water, followed by autoclaving for 30 min at a temperature of 121 °C. Two discs (1 mm radius) from the active margin of a *Trichoderma harzianum* (from the Regional Center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt) colony growing on Czapek dox agar medium were inoculated in the silage and incubated at a temperature of 30 °C for different incubation periods, up to 28 d. At the end of the incubation period, 50 mL of distilled water was added to the substrate and agitated *via* shaking (at 500 rpm) for 25 min, then clarified *via* muslin cloth, and then centrifuged for 5 min at 10×10^3 rpm for further filtration. The supernatant containing the enzymes was collected and used as crude enzymes for assaying

its carboxymethyl cellulase (CMCase), manganase peroxidase (MnPase), laccase, and soluble protein contents.

Carboxymethyl Cellulase, Manganase Peroxidase, and Laccase Assay

The CMCase activity was determined by a reaction mixture containing 1.0 g of carboxymethyl cellulose (CMC) as an enzyme substrate in 100 mL of sodium acetate buffer (with a pH of 5.0). Then, 1 mL of the supernatant containing enzymes was tested to break down the enzyme substrate in a tube containing the reaction mixture, then incubated for 30 min at a temperature of 63 °C. The amount of liberated reducing sugars was calculated using a spectrophotometer at 540 nm *via* the 3,5-dinitrosalicylic acid (DNS) protocol regarding to the standard curve of glucose, by reading the absorbance at 540 nm (Miller 1959). The analysis found that 1 unit of CMCase is the micromoles of glucose liberated per 1 mL of supernatant containing enzyme per min (Wang *et al.* 1988). The reaction mixture for determining the MnPase activity, according to Garzillo *et al*. (2001), included 100 µL of the supernatant containing enzyme, 1 mM of $MnSO_4$ in a Mcllvaine buffer (with a pH of 5.0), and 2 mM of 2,2'-azino-bis(3-ethyl benzo-thiazoline-6-sulforic acid) (ABTS) (1 mL), followed by the addition of 4 mM of H_2O_2 as a substrate for peroxidase activity in the reaction mixture. The MnPase activity was determined in IU by checking the spectrophotometer absorbance change at 420 nm (ABTS), ϵ = 36 $Mm^{-1}cm^{-1}$. For the laccase assay, the reaction mixture comprised of 100 µL of the supernatant containing enzyme and 2 mM of ABTS (1 mL) adjusted to a pH of 5 using Mcllvaine buffer. The activity of the enzyme was recorded in IU *via* a spectrophotometer at 420 nm (ABTS), ϵ = 36 $Mm^{-1}cm^{-1}$ (Garzillo *et al*. 2001). Distilled water was used instead of the enzymes for the blank sample.

Protein and Reducing Sugar Assays

First, 1 mL of DNS reagent was added to 1 mL of sugar solution in a tube, followed by placing it in a boiling water bath for 10 min. After cooling the tube containing the reaction mixture to 30 °C, 5 mL of distilled water was added. The developed color was analyzed *via* a spectrophotometer at 540 nm, with glucose being used as the standard (Miller 1959). Assessing the total soluble protein in the supernatant was achieved according to the methodology outlined in Lowry *et al.* (1951). The blank sample was recorded using 1 mL of distilled water.

Effect of Enzymes Extract and *Lactobacillus bulgaricus* on Chemical Characteristics of Silage

Freshly prepared maize silage (50 g), from the same samples used for the fungal analysis, was moistened in a 250 mL conical flask using 100 mL of distilled water, followed by autoclaving for 30 min at a temperature of 121 °C. Then, 2 mL of the *L. bulgaricus* suspension and enzyme extract from the previous enzyme production experiment at optimal conditions were added to the silage, followed by incubation at a temperature of 30 °C for up to 30 d. At the end of the incubation period, the filtrate of the metabolized medium was extracted to determine the lactic acid content *via* HPLC. In addition, the dry and organic matter content, pH, and the number of *L. bulgaricus* cells were determined.

Statistical Analysis

The standard deviation mean (± SD) of the three readings of obtained results was calculated using SPSS software (version 22.0, IBM, Armonk, NY).

RESULTS AND DISCUSSION

Fungal Identification of the Contaminated Maize Silage

Fungal spoilage was observed in the maize silage samples after 60 d of production (Fig. 1).

Fig. 1. Typical mold growth in dry silage

The identification methods confirmed the presence of five filamentous toxigenic fungi, *i.e.*, *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., and *Penicillium* spp., which all had different Fr values (Fig. 2). *Fusarium* spp. was identified with the highest Fr (62.8%), followed by *Aspergillus* spp., with *Mucor* spp. having the lowest Fr (10.2%). The presence of mycobiota in the maize silage was comparable to the species present in the same type of silage used in many countries, since *Fusarium* was found to be the primary isolate. In addition, in the Netherlands, Asselt *et al*. (2012) recorded an elevated incidence of *Fusarium* species and their mycotoxins in maize silage. However, Keller *et al*. (2013) demonstrated that *Aspergillus*, followed by *Penicillium*, were the most frequently isolated genera. A report by O'Brien *et al*. (2008) showed the presence of *Penicillium* spp. as a major contaminant in various types of silages. The presence of different fungi is considered unattractive in maize silage, as this suggests a high likelihood that the silage may be contaminated with numerous mycotoxins.

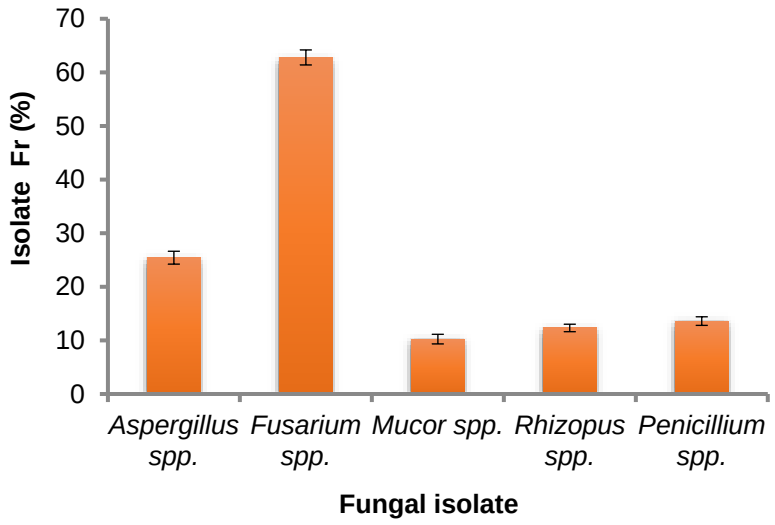

Fig. 2. Isolate Fr of the fungal isolates from maize silage (Note: the error bars referred to the SD of the three treatment values)

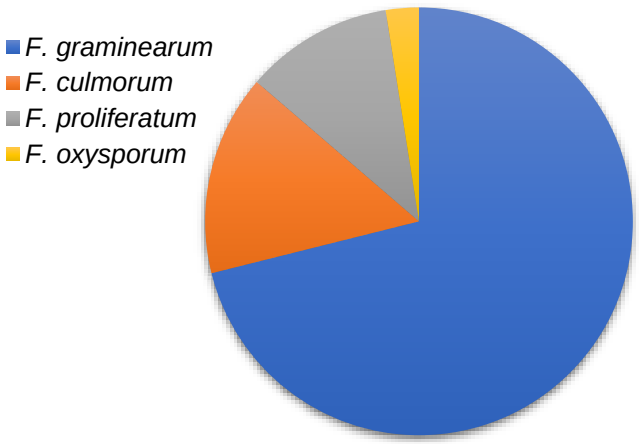

Fig. 3. RD (%) of the *Fusarium* species isolated from the maize silage samples

The highest occurrence of fungal isolates was observed at the upper and lower sections (Fig. 1A and 1B), while the middle section showed a lower level of fungal contamination (Fig. 1C). Fungal analysis of the highest Fr. isolate (*Fusarium* spp.) indicated the presence of four species with different RD values, *i.e.*, *F. graminearum* (71.1 %), *F. culmorum* (15.2%), *F. proliferatum* (11.2%), and *F. oxysporum* (2.50 %). The species with the highest RD value (*F. graminearum*) was used for further study (Fig. 3).

Mycotoxin production

Four mycotoxins, *i.e.*, trichothecene, nivalenol, zearalenone, and fumonisins, were produced by *F. graminearum* using maize silage as a substrate (as shown in Table 1 and Fig. 4). These mycotoxins were found to be *F. graminearum* metabolites by Tralamazza *et al.* (2016). As mentioned by Wu *et al.* (2017), *Fusarium* mycotoxins are considered the most common toxin in crops globally. The synthesis of mycotoxins is a complicated metabolic pathway that is regulated by genetic factors, which can be affected *via* several environmental and nutritional stimuli, *e.g.*, temperature, pH, nutrient composition, and the availability of substrates (Abdel-Ghany 2008; Xue *et al.* 2014). The obtained results reflected the effect of *L. bulgaricus* on *F. graminearum* mycotoxins production in maize silage after 15 and 30 d of incubation (as shown in Table 1). The production of trichothecene and nivalenol were more affected by the addition of *L. bulgaricus*, particularly after 30 d of incubation, while zearalenone production was less affected. Unfortunately, fumonisins productivity increased as a result of the addition of *L. bulgaricus*. The inhibition % of trichothecene, nivalenol, and zearalenone synthesis, regarding the control at 30 d, reached 50.24%, 47.50%, and 23.54%, respectively, while at 15 d reached 24.58%, 19.92%, and 2.68%, respectively. The pH was considered the critical factor for regulating the synthesis of mycotoxins outside of improving the silage quality. The lowering of the pH of the silage was due to the production of lactic acid in the silage. As such, a sharp decrease in the pH of the maize silage treated with lactic acid bacteria was observed, which was accompanied by organic acids production (Santos *et al.* 2019).

Table 1. Mycotoxin Production by *F. graminearum* Using Different Treatments

Treatment	Mycotoxin Concentration (µg/mL)			
	Trichothecene	Nivalenol	Zearalenone	Fumonisins
Control (30 d)	60.79 ± 0.02	51.21 ± 0.02	32.94 ± 0.351	16.15 ± 0.05
Control (15 d)	40.24 ± 0.01	44.27 ± 0.12	29.84 ± 0.01	17.49 ± 0.20
L. bulgaricus (15 d)	30.35 ± 0.02	35.45 ± 0.33	29.04 ± 0.05	28.98 ± 0.05
L. bulgaricus (30 d)	30.25 ± 0.33	26.89 ± 0.02	22.22 ± 0.02	20.15 ± 0.02
*I% Control/ *L. bulgaricus* at 30 d	50.24	47.50	23.54	00.00
*I% Control/ *L. bulgaricus* at 15 d	24.58	19.92	2.68	00.00
Retention time	6.01	7.30	8.60	8.80
Note: *I is the inhibition % of mycotoxins *via* the *L. bulgaricus* treatment with regards to the control				

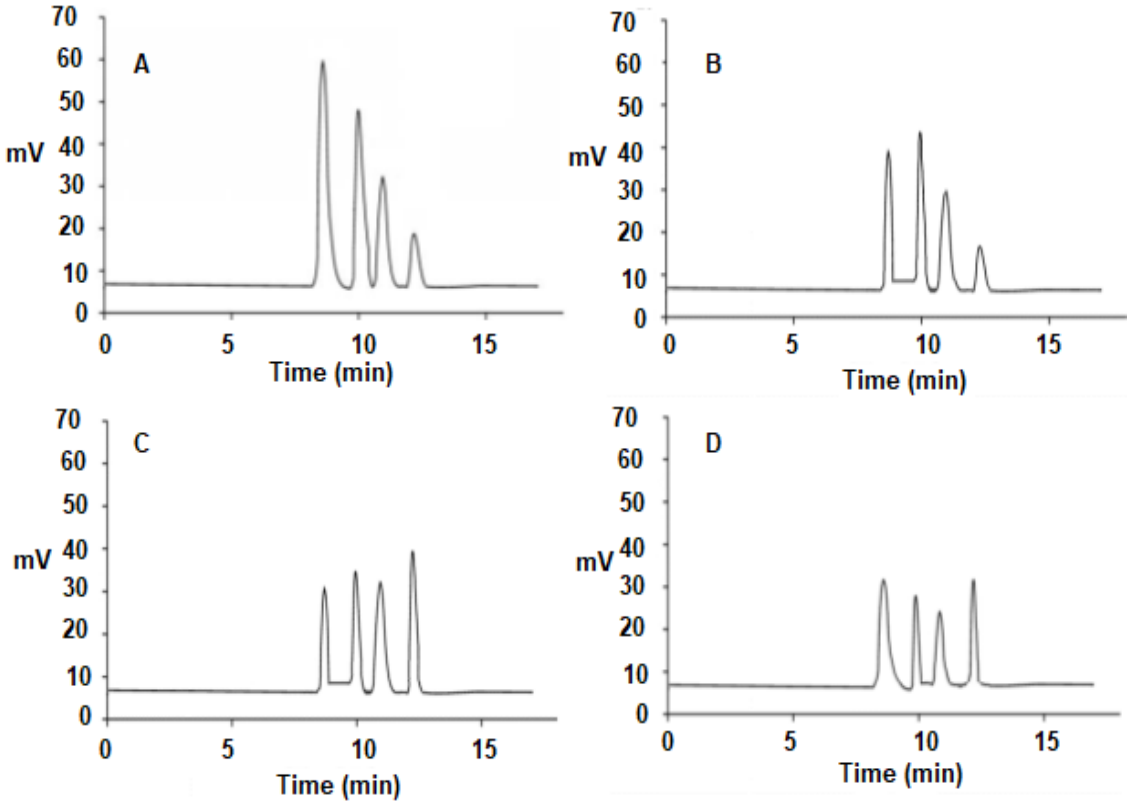

Fig. 4. The HPLC chromatogram of mycotoxin production by *F. graminearum* for different treatments: A) control (30 d); B) control (15 d); C) *L. bulgaricus* (15 d); and D) *L. bulgaricus* (30 d)

Enzyme production

Due to the richness of the maize silage with lignocellulolytic compounds, in addition to other nutritional compounds, the production of cellulolytic enzymes by *T. harzianum* using maize silage increased as the incubation period increased, up to 21 d (accompanied by increased protein liberation), but it decreased after 28 d (as shown in Table 2). The maximum productivity of CMCase, manganase peroxidase, and laccase was reached after 21 d, *i.e.*, 350.50 µg/mL, 5.47 µg/mL, and 16.04 µg/mL, respectively. In addition to the biosafety of *T. harzianum*, its ability to produce multiple cellulolytic enzymes using different agricultural residues has been recognized and documented and it is often chosen as a good enzyme producer (Lee *et al.* 2017; Abdel-Ghany and Bakri 2019; Asis *et al.* 2021).

Table 2. Enzyme Production by *Trichoderma harzianum* Using Maize Silage

Incubation Period (Day)	Enzyme Activity (µg/mL)			Protein (µg/mL)
	Carboxymethyl cellulase	Manganase peroxidase	Laccase	
7	55.23 ± 0.58	2.52 ± 0.01	12.94 ± 0.13	400 ± 0.76
14	200.43 ± 1.53	4.34 ± 0.02	15.84 ± 0.06	470 ± 0.50
21	350.50 ± 1.15	5.47 ± 0.29	16.04 ± 0.58	600 ± 0.29
28	300.35 ± 0.02	4.61 ± 0.04	15..22 ± 0.58	578 ± 1.53

The role of L. bulgaricus and enzyme extract

Cellulolytic enzymes play a vital role in numerous industrial applications, and in the current study, cellulolytic enzymes were applied in order to enhance conditions that prevented the growth of undesirable fungi and their mycotoxins in maize silage indirectly through encouraging the proliferation of *L. bulgaricus* (Ganash *et al.* 2021). Li *et al.* (2020) mentioned that some lactic acid bacteria (LAB) might be able to minimize the lignin content in silage when amended with cellulase enzyme. The addition of extract containing a high amount of enzyme (after 21 d of incubation) to maize silage inoculated with *L. bulgaricus* increased the growth of *L. bulgaricus*, as well as increased the dry matter (DM) and organic matter (OM) contents, and the lactic acid production, when compared to the untreated silage or the silage inoculated with only enzymes or *L. bulgaricus* (Table 3), as well as lowering the pH. Recently, the enhancement of maize silage quality using microbial diversity and rumen degradation proportions were found *via* the addition of *Lactobacillus* and cellulase (Hu *et al.* 2021; Zhao *et al.* 2021). According to Khota *et al.* (2017), LAB are incapable of directly decaying lignocellulolytic residues of plants. Therefore, it is necessary to add other microbes that are able to break down lignins during the production of silage. Moreover, Kaewpila *et al.* (2021) found that cellulases or *T. harzianum* attractive inoculates enhanced the ensiling features of maize silage. In addition, lactic acid production increased when a mixture of enzyme extract and *L. bulgaricus* was added (Table 3), which demonstrated that the combined enzyme and *L. bulgaricus* treatment may be more effective compared to the enzyme extract treatment alone; these results were in agreement with a previous study by Li *et al.* (2018). The obtained results were recorded recently in alfalfa silage, since the LAB amplified the enzyme activity, thus accelerating the fermentation process and increasing lactic acid productivity (Hu *et al.* 2021). Further and coming studies is necessary to evaluate the other productivity of enzyme hydrolysis to silage components and its beneficial effects on consumers.

Table 3. Chemical and *L. bulgaricus* Analysis of Maize Silage Extracts After 40 d of Fermentation Treated by Crude Enzyme and *L. bulgaricus*

Treatment	*L. bulgaricus* (log CFU/g)	pH	DM (%)	OM (%)	Lactic Acid (g/kg DM)
Control*	0.0 ± 0.00	4.9 ± 0.02	26.25 ± 0.76	71.56 ± 0.2	2.6 ± 0.02
L. bulgaricus	6.6 ± 0.01	4.6 ± 0.03	33.54 ± 0.50	79.11 ± 0.62	12.56 ± 0.05
L. bulgaricus + Crude Enzyme**	8.9 ± 0.02	4.4 ± 0.06	37.33 ± 0.33	88.06 ± 0.2	14.67 ± 0.10
Crude Enzyme**	0.0 ± 0.00	4.8 ± 0.01	26.45 ± 0.20	72.12 ± 0.25	6.3 ± 0.05

Control*, Fresh maize silage un-treated with *L. bulgaricus* or Crude enzyme; Crude Enzyme**, Supernatant containing enzymes

CONCLUSIONS

1. Bioadditives, *e.g.*, *Lactobacillus bulgaricus*, or lowering the pH through organic acids production, will help to support safe storage conditions and prevent mycotoxin production in maize silage.

2. Proving the existence of multiple mycotoxigenic fungi was the highlight of this study; as such, the presence of various mycotoxins in the prepared maize silage was confirmed.
3. A combined enzyme extract and *Lactobacillus bulgaricus* treatment may be more valuable than either treatment alone, which provides suitable justification for its application in maize silage production.

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