Journal of the Korean Society of Grassland and Forage Science 43(3): 148~155, 2023
https://doi.org/10.5333/KGFS.2023.43.3.148

ISSN 2287-5824 (Print)
ISSN 2287-5832 (Online)

Research Article

Application Effects of Bacterial Inoculants Producing Chitinase on Corn Silage

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ABSTRACT

This study was aimed to isolate bacterial inoculants producing chitinase and evaluate their application effects on corn silage. Four corn silages were collected from four beef cattle farms to serve as the sources of bacterial inoculants. All isolates were tested against *Fusarium graminearum* head blight fungus MHGNU F132 to confirm their antifungal effects. The enzyme activities (carboxylesterase and chitinase) were also measured to isolate the bacterial inoculant. Based on the activities of anti-head blight fungus, carboxylesterase, and chitinase, *L. buchneri* L11-1 and *L. paracasei* L9-3 were subjected to silage production. Corn forage (cv. Gwangpyeongok) was ensiled into a 10 L mini silo (5 kg) in quadruplication for 90 days. A 2 × 2 factorial design consists of *F. graminearum* contamination at 1.010^4 cfu/g (UCT (no contamination) vs. CT (contamination)) and inoculant application at 2.1×10^5 cfu/g (CON (no inoculant) vs. INO (inoculant)) used in this study. After 90 days of ensiling, the contents of CP, NDF, and ADF increased ($p<0.05$) by *F. graminearum* contamination, while IVDMD, acetate, and aerobic stability decreased ($p<0.05$). Meanwhile, aerobic stability decreased ($p<0.05$) by inoculant application. There were interaction effects ($p<0.05$) on IVNDFD, NH3-N, LAB, and yeast, which were highest in UCT-INO, UCT-CON, CT-INO, and CT-CON & INO, respectively. In conclusion, this study found that mold contamination could negatively impact silage quality, but isolated inoculants had limited effects on IVNDFD and yeast.

(Key words: Aerobic stability, Bacterial inoculant, Chitinase, Corn silage)

Ⅰ. INTRODUCTION

Corn silage (CS) is a high-energy forage that is used in almost every country to supply the nutritional requirement for ruminants (McDonald et al., 1991). It is most commonly grown as a summer crop, but due to its high sugar content, it decomposes faster than other crops and is more prone to mold formation. In the anaerobic fermentation process of silage, lactic acid bacteria (LAB) decompose sugar to produce lactate, which quickly lowers the pH and inhibits the growth of harmful bacteria, thereby improving the storage stability of the forage (McDonald et al., 1991). In general, the fermentation quality of silage is affected by the harvest time of forage, moisture content, cutting length, filling and pressing, and additives. When these factors are not adequate, the growth of undesirable bacteria (mold, clostridia, etc.) could increase, resulting in poor fermentation quality (McDonald et al., 1991). Bacterial inoculants containing LAB are representative additives for improving the fermentation quality of silage (Weinberg et al., 1993; Adesogan et al., 2004). Paradhipta et al. (2020) reported that the isolated LAB-producing carboxylesterase with antifungal activity confirmed their effects on corn silage, which improved aerobic stability and nutrient digestibility. The ensiling process of forages generally improves the preservation, but yeast initiates aerobic spoilage at the feed-out phase. After that, molds, *Fusarium*, *Aspergillus*, and *Penicillium* genera can start to grow and produce mycotoxins such as aflatoxins, ochratoxins, fumonisins, and so on (Kung and Shaver, 2001). These mycotoxins are well-known to have fatal adverse effects on livestock. Paradhipta et al. (2020 and 2023) suggested that

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corn silage applied LAB-producing carboxylesterase with antifungal activity can be used as a probiotic to the total mixed ratio (TMR), in the viewpoint of inhibitions of undesirable microbes and improvement of nutrient digestibility.

Meanwhile, chitin is an N-acetyl-glucosamine polymeric polysaccharide found in insects, mollusk shells, and fungi. Chitinase, a hydrolytic enzyme of chitin, is classified into various forms according to the mode of action and is reported to have vitality even at pH 4-7. It's in similar pH ranges with silages and can inhibit fungal growth (Liang et al., 2014). However, none of the studies with the microbes producing chitinase were tested on silage.

Therefore, this study was conducted to isolate bacterial inoculants-producing chitinase and evaluate their application effects on corn silage.

Ⅱ. METERIALS AND METHODS

1. Collection of corn silage samples

Four corn silage samples (1 kg) were sub-sampled from the bale silo produced at the commercial beef cattle farms in Sancheong, South Korea, to harvest microbial isolates. The sub-sampled corn silage was mixed with sterile distilled at a 1:100 ratio and shaken at 220 rpm for 1 h to make silage extraction. Then, silage extraction was serially diluted (10^{-3} to 10^{-8}) with sterile distilled water. Each dilution (100 μL) were spread on de Man, Rogosa, and Sharpe (MRS; Difco, Detroit, USA) agar medium and incubated at 30℃ for 5 d in a CO_2 incubator (Thermo Scientific, Waltham, USA).

2. Isolation and identification of bacterial inoculant

The activities of antifungal and enzymes (carboxylesterase and chitinase) were tested to isolate bacterial inoculants. All isolates were tested against *Fusarium graminearum* head blight fungus MHGNU F132 to confirm antifungal activity, according to a previous report (Paradhipta et al., 2020). Carboxylesterase enzyme activity from each isolate was conducted according to the protocol described by Pérez-Martín et al. (2013) with some modifications. According to the manufacturer's instructions, chitinase hydrolysis was performed in an acidic environment (pH ~4.8) at 37°C (Sigma-Aldrich, St. Louis, USA). Enzymatic hydrolysis liberates *p*-nitrophenol, and adding base stop solution ionizes *p*-nitrophenol to form the yellow *p*-nitrophenylate ion (Frändberg and Schnürer, 1994). The absorbance of *p*-nitrophenylate ion was measured at 405 nm (Tronsmo and Harman, 1993). The polymerase chain reaction (PCR) was used to amplify a partial 16S rRNA gene region, using Premix (Bioneer, Seoul, South Korea) and universal primers 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGYTACCTTGTTACGACTT-3). The PCR was performed according to a previous report (Paradhipta et al., 2020).

3. Silage production

Corn (Gwangpyeongok) forage was harvested at 1/2 milk line stage (26.2% DM) from the Animal Research Unit at Gyeongsang National University, Jinju, South Korea, cut into 3-5 cm lengths, and separated into four groups. The corn forage (500 g) was sub-sampled just before ensiling for the chemical compositions. As the bacterial inoculant, the mixture of *L. paracasei* L9-3 and *L. buchneri* L11-1 at a 1:1 ratio was used in this study. The 2 × 2 factorial design consists of the contamination by *F. graminearum* at 1.0×10^4 cfu/g (UCT (no contamination) vs. CT (contamination)) and inoculant application at 2.1×10^5 cfu/g (CON (no inoculant) vs. INO (inoculant)). The corn forage in each group was ensiled into a 10 L bucket silo (5 kg) with four replications for 90 days. After silo open, silages were sub-sampled for the analyses of chemical compositions and *in vitro* digestibility (500 g), fermentation characteristics (20 g), and aerobic stability (1 kg), respectively.

4. Chemical compositions and *in vitro* digestibility

The sub-sampled corn forage and silage (10 g) were dried at 105℃ for 24 h to measure dry matter (DM) content. Additionally, the sub-samples (100 g) were dried at 55℃ for 48 h, then ground using a cutting mill (Shinmyung Electric Co., Ltd, South Korea) and sieved through a 1 mm screen for the analyses of chemical compositions and *in vitro* digestibility. The contents of crude protein (CP), ether extract (EE), and crude ash (CA) were analyzed using the Kjeldahl method, the Soxhlet method, and a muffle furnace (550℃, 4 h), respectively AOAC (2005). The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were established using an

Ankom^{200} fiber analyzer (Ankom Technology, Macedon, USA) following the method of Van Soest et al. (1991). For the rumen content donator, the animal care procedures were approved by the Animal Ethical Committee of Gyeongsang National University, Jinju, South Korea (GNU-191011-E0050). Rumen fluid was collected from two cannulated Hanwoo heifers before morning feeding (08:00). According to the method of Tilley and Terry (1963), the mixture of rumen buffer with ground sample (0.5 g) was incubated for 48 h in Ankom Daisy^{II} incubators (Ankom Technology, Macedon, USA) for *in vitro* digestibility of DM (IVDMD). Additionally, NDF content of the residue was analyzed to calculate NDF digestibility (IVNDFD).

5. Fermentation characteristics, microbial enumerations, and aerobic stability

The sub-sampled corn forage and silage (20 g) with distilled water (180 mL) were blended for 30 s and filtered through two layers of cheesecloth for fermentation characteristics and microbial enumerations. Silage pH and ammonia-N were measured using a pH meter (SevenEasy, Greifensee, Switzerland) and the colorimetric method (Chaney and Marbach, 1962), respectively. Silage extract was centrifuged at 5,645 × g for 15 min, and the upper layer was collected for the analysis of volatile fatty acid (VFA) content using HPLC (Hitachi, Tokyo, Japan) fitted with a UV detector (L-2400; Hitachi) and a column (Metacarb 87H; Varian, USA) described by Adesogan et al. (2004). The silage extraction was continued into diluted 10^{-4} to 10^{-6} of dilution series to analyze LAB using the lactobacilli MRS agar media (MRS; Difco, Detroit, USA), while yeast and mold using PDA (Difco, Detroit, USA). The MRS agar plates were placed in a CO_2 incubator (Thermo Scientific, USA) at 30℃ for 48 h, while PDA plates were incubated at 30℃ for 72 h in a standard incubator (Johnsam Corporation, Korea). Visible colonies were counted from the plated, and the number of cfu per gram of silage was expressed. For aerobic stability, silage (1 kg) was located in a polystyrene box with aerobic condition at room temperature (15 ± 1℃) for 10 days. The temperature change of each silage at the geometric center was recorded every 30 min by a thermocouple sensor (MORGAN TR-60CH, Hong Kong, China). The aerobic stability was calculated by a 2℃ increase in silage temperature to the ambient temperature (Adesogan et al., 2004).

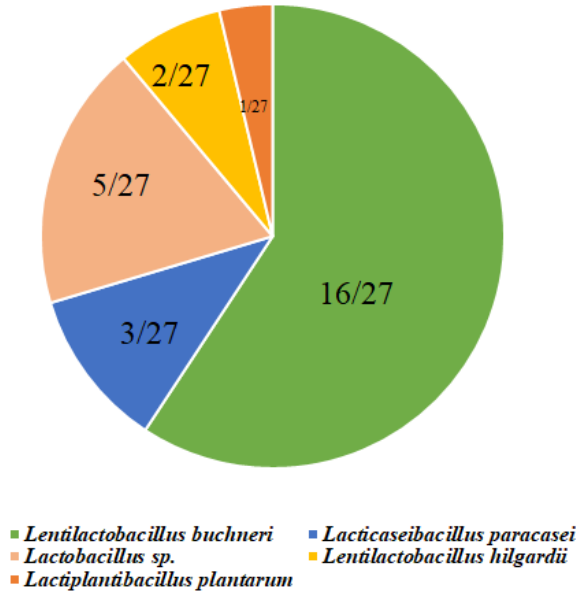
6. Statistical analysis

The standard error values were used to declare the differences in antifungal and enzyme activities among isolates. All data were analyzed as a 2 (*F. graminearum* contamination, UCT vs. CT) × 2 (inoculant application, CON vs. INO) factorial design by PROC MIXED of the statistical analysis system (SAS), package program, version 9.4 (SAS, 2013). The model was $Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + eijk$, where $Yijk$ = response variable, μ = overall mean, αi = effect of contamination treatment, βj = effect of inoculant treatment, $(\alpha\beta)ij$ = the interaction effect of contamination and inoculant, and $eijk$ = error term. Mean separation and differences were performed using Tukey's test at $p<0.05$.

Ⅲ. RESULTS AND DISCUSSION

1. Screening of bacterial inoculant

The LAB isolated from corn silages based on the 16S rRNA gene are shown in Fig. 1. A total of 27 bacteria were isolated, including 16 of *L. buchneri*, 5 of *L.* sp., 3 of *L. paracasei*, 2 of *L. hilgardii*, and 1 of *L. plantarum*. The bacteria numbers were higher in the order of *L. bucheneri*, *L.* sp., and *L. Paracasei*. The inhibition area of *F. graminearum* and the activities of esterase and endo-chitinase activity by LAB isolates are shown in Fig. 2. A total of 27 LAB isolates were collected from 4 corn silages. Two isolates, L9-3 and L11-1, were shown strong antifungal activity than the other isolates. The isolates, L4-3 and L9-3 and L11-1, were classified as higher esterase producing bacteria. These isolates showed a relatively higher esterase activity than the mean value and the other isolates. The isolates, L2-1, L8-1, L9-3, and L11-1, were showed high normalized exo-chitinase activity. As a result, L9-3 and L11-1 isolates exhibited high in both activities of antifungal and carboxylesterase. In general, the mold can aerobically infected to corn forage at the corn farm and corn silage in the feed-out phase at the animal farms. Also, dry weather and stress of forage become factors that increase of mold infection in corn farm (Scudamore and Livesrty, 1998). An antifungal-producing inoculant may have beneficial effects



L2-1	*Lacticaseibacillus paracasei*
L2-2	*Lentilactobacillus hilgardii*
L2-3	*Lentilactobacillus hilgardii*
l4-2	*Lentilactobacillus buchneri*
L4-3	*Lentilactobacillus buchneri*
L5-2	*Lentilactobacillus buchneri*
L6-1	*Lentilactobacillus buchneri*
L6-2	*Lentilactobacillus buchneri*
L7-2	*Lentilactobacillus buchneri*
L8-1	*Lacticaseibacillus paracasei*
L8-2	*Lactobacillus* sp.
L8-3	*Lactobacillus* sp.
L8-4	*Lentilactobacillus buchneri*
L9-1	*Lentilactobacillus buchneri*
L9-2	*Lentilactobacillus buchneri*
L9-3	*Lacticaseibacillus paracasei*
L9-4	*Lactobacillus* sp.
L9-5	*Lentilactobacillus buchneri*
L9-6	*Lactiplantibacillus plantarum*
L10-1	*Lentilactobacillus buchneri*
L10-3	*Lentilactobacillus buchneri*
L11-1	*Lentilactobacillus buchneri*
L11-2	*Lactobacillus* sp.
L12-1	*Lentilactobacillus buchneri*
L12-2	*Lentilactobacillus buchneri*
L13-1	*Lentilactobacillus buchneri*
L13-2	*Lactobacillus* sp.

Fig. 1. Lactic acid bacteria isolated from corn silage based on the 16S rRNA gene.

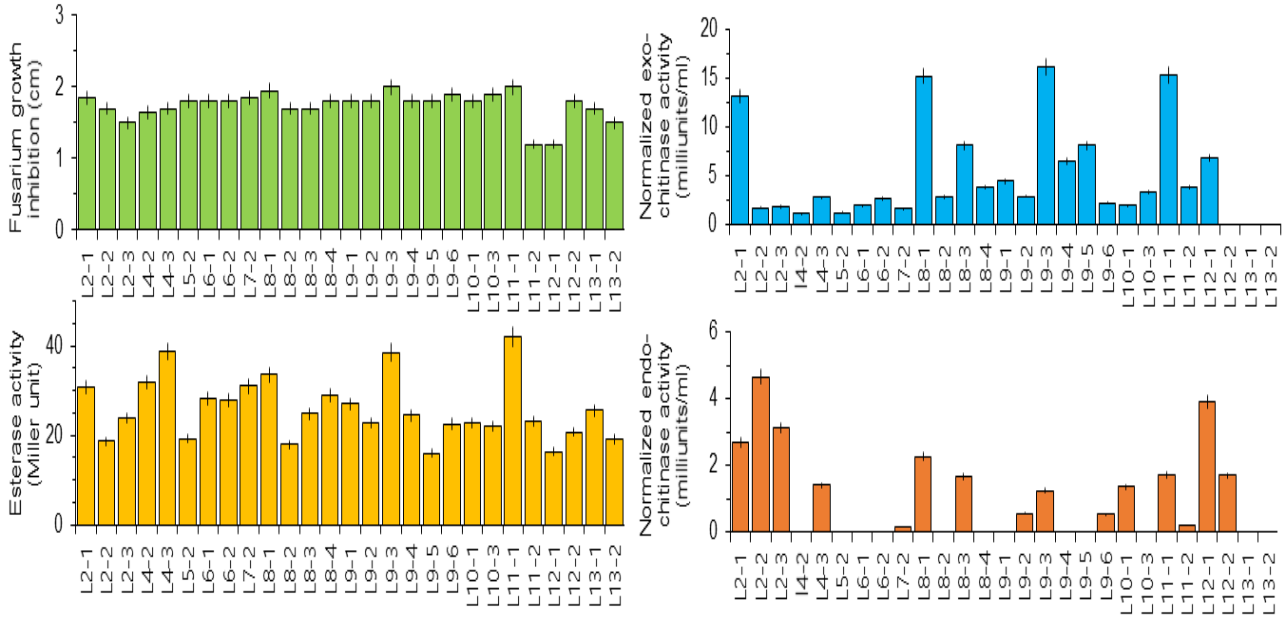


Fig. 2. The inhibition area against *Fusarium graminearum*, and esterase, exo-chitinase, and endo-chitinase activity of isolated lactic acid bacteria.

L2-1, L8-1, and L9-3 = *L. paracasei*; L2-2, L2-3 = *L. hilgardil*; L4-2, L4-3, L5-2, L6-1, L6-2, L7-2, L8-4, L9-1, L9-2, L9-5, L10-1, L10-3, L11-1, L12-1, L12-2, and L13-1 = *L. buchneri*; L8-2, L8-3, L9-4, L11-2, and L13-2 = *L. sp.*; L11-2 = *L. plantarum*.

against the mold growth in the corn silage. Several strains of LAB, such as, *L. buchneri*, *L. brevis*, *L. paracasei* etc. can produce antifungal substances and fibrinolytic enzymes, resulted the improvement of silage quality (Kang et al., 2009; Dalie et al., 2010; Paradhipta et al., 2020). In the present study, both L11-1 and L9-3 were selected and would be applied in the corn silage to confirm their activities. Based on 16S rRNA gene sequence results, L11-1 and L9-3 isolates were identified as *L. buchneri* and *L. Paracasei*, respectively.

2. Chemical compositions of corn forage

The chemical compositions of corn forage are shown in Table 1. The DM, CP, NDF, and ADF contents of corn forages were 26.2, 7.93, 48.8, and 24.8%, respectively. The previous studies (Lee, 2012; Paradhipta et al., 2020) with same corn hybrid also reported similar results with this study, in which CP, NDF, and ADF contents in corn forage were 7.10-8.64, 47.0-48.3, and 24.2-25.9%, respectively.

3. Chemical compositions and *in vitro* digestibility of corn silage

The effect of inoculant application with *F. graminearum* contamination on the chemical compositions and *in vitro* digestibility of corn silages ensiled for 90 days are shown in Table 2. There were no inoculant effects on chemical compositions and IVDMD, while IVNDFD in INO silages (p=0.012; 19.7 vs. 23.2%) was higher, and showed an interaction effect (p=0.008) between contamination and inoculant. The contents of CP (p=0.038; 10.1 vs. 10.3), CA (p=0.012; 7.11 vs. 7.67), NDF (p<0.001; 45.9 vs. 50.0), and ADF (p<0.001; 24.7 vs. 28.3%) were higher in CT silages than those in UCT silages. The IVDMD (p=0.001; 56.8 vs. 52.6%) was higher in UCT silages

Table 1. The chemical compositions of corn forage before ensiling (%, DM)

	Corn forage	SD
Dry matter	26.2	2.513
Crude protein	7.93	0.055
Ether extract	3.52	0.348
Crude ash	8.67	0.104
Neutral detergent fiber	48.8	2.115
Acid detergent fiber	24.8	0.420

SD, standard deviation.

Table 2. Effect of inoculant application with *Fusarium graminearum* contamination on chemical compositions and *in vitro* digestibilites of corn silage ensiled for 90 days (%, DM)

	UCT		CT		SEM	CE	IE	CE × IE
	CON	INO	CON	INO				
Dry matter	22.8	22.8	22.2	22.1	0.922	0.168	0.939	0.981
Crude protein	10.1	10.0	10.2	10.4	0.163	0.038	0.191	0.124
Ether extract	3.98	4.26	4.42	4.32	0.407	0.238	0.657	0.355
Crude ash	7.18^{ab}	7.04^{b}	7.48^{ab}	7.85^{a}	0.371	0.012	0.542	0.200
NDF	44.6^{b}	47.1^{b}	49.9^{a}	50.1^{a}	1.219	<0.001	0.057	0.087
ADF	24.6^{b}	24.8^{b}	27.8^{a}	28.7^{a}	1.002	<0.001	0.337	0.493
IVDMD	56.0^{ab}	57.6^{a}	52.7^{b}	52.4^{b}	1.975	0.001	0.566	0.347
IVNDFD	18.1^{b}	25.5^{a}	21.2^{ab}	20.9^{ab}	2.425	0.555	0.012	0.008

UCT, no contamination; CT, contaminated by *Fusarium graminearum*; CON, no inoculant; INO, inoculant appliaction; CE, contamination effect; IE, inoculant effect; CE × IE, interaction effect between contamination and inoculant; ND, not detected; NA, not availble; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; IVDMD, *in vitro* digestibility of DM; IVNDFD, *in vitro* digestibility of NDF; SEM, standard error of the mean.

^{a,b}Means in the same row with different superscripts differ significantly (p<0.05).

than those in CT silages. The IVNDFD was higher in INO silages (p=0.012; 19.7 vs. 23.2%), and showed an interaction effect (p=0.008) between contamination and inoculant. In this study, it couldn't be clarified the results of no effects on chemical compositions by inoculant application. However, it could be partially explained by low silage pH (3.83-4.09), which might inhibit nutrient degradation by undesirable microbes in silages. Generally, the growth of undesirable microbes in silages (mold, clostridia, etc.) could lead to poor fermentation quality and energy loss (McDonald et al., 1991). This explanation could support the results of lower IVDMD with higher fiber contents (NDF and ADF) in CT silages. Paradhipta et al. (2020) and Lee et al. (2021a,b) reported that inoculated with LAB, IVDMD and IVNDFD were higher than no inoculated control group. Similarly, IVNDFD improved by applying LAB without *F. graminearum* contamination in this study. However, the reason for no difference on IVNDFD of CT silages is unclear.

4. Fermentation characteristics of corn silage

The effect of inoculant application with *F. graminearum* contamination on fermentation characteristics of corn silage ensiled for 90 days are shown in Table 3. There were no inoculant effects on all measurements. However, NH_3-N (p=0.002; 0.08 vs. 0.06%) and acetate (p=0.005; 7.10 vs. 4.58%) contents were higher in UCT silages than those in CT silages. The NH_3-N contents showed an interaction effect (p=0.012) between contamination and inoculant, in which UCT-INO silage was lower (p<0.05) than UCT-CON silage. The contamination of undesirable microbes in the silages can result in lower fermentation quality, which had shown a similar result (lower acetate content) in this study (McDonald, 1991; Dalie et al., 2010). The application of LAB usually increased organic acid contents (lactate or acetate) in the silages. However, neither lactate nor acetate contents in the silages were changed by inoculant applications in this study. Further study needs to be conducted to clarify their effects.

Table 3. Effect of inoculant application with *Fusarium graminearum* contamination on fermentation characteristics of corn silage ensiled for 90 days

	UCT		CT		SEM	CE	IE	CE × IE
	CON	INO	CON	INO				
pH	3.88	3.83	3.89	4.09	0.198	0.193	0.485	0.217
NH3-N, mg/dL	0.09^{a}	0.07^{b}	0.06^{b}	0.06^{b}	0.009	0.002	0.082	0.012
Lactate, %	19.1	20.5	18.8	18.4	2.344	0.358	0.644	0.503
Acetate, %	7.21^{a}	6.98^{ab}	5.21^{ab}	3.95^{b}	1.480	0.005	0.336	0.503

UCT, no contamination; CT, contaminated by *Fusarium graminearum*; CON, no inoculant; INO, inoculant appliaction; CE, contamination effect; IE, inoculant effect; CE × IE, interaction effect between contamination and inoculant; ND, not detected; NA, not availble; SEM, standard error of the mean.

^{a,b}Means in the same row with different superscripts differ significantly (p<0.05).

Table 4. Effect of inoculant application with *Fusarium graminearum* contamination on microbial counts and aerobic stability of corn silage ensiled for 90 days

	UCT		CT		SEM	CE	IE	CE × IE
	CON	INO	CON	INO				
LAB, log cfu/g	4.54^{ab}	4.24^{b}	4.10^{b}	5.15^{a}	0.269	0.501	0.138	0.003
Yeast, log cfu/g	5.38^{ab}	4.43^{b}	6.05^{a}	6.53^{a}	0.557	<0.001	0.425	0.032
Mold, log cfu/g	ND	ND	6.19	5.77	0.726	NA	NA	NA
Aerobic stability, h	91.4^{a}	72.0^{a}	74.0^{a}	58.9^{b}	13.84	0.044	0.034	0.773

UCT, no contamination; CT, contaminated by *Fusarium graminearum;* CON, no inoculant; INO, inoculant appliaction; CE, contamination effect; IE, inoculant effect; CE × IE, interaction effect between contamination and inoculant; LAB, Lactic acid bacteria; ND, not detected; NA, not availble; SEM, standard error of the mean.

^{a,b}Means in the same row with different superscripts differ significantly (p<0.05).

5. Microbial counts and aerobic stability of corn silage

The effect of inoculant application with *F. graminearum* contamination on microbial counts and aerobic stability of corn silages ensiled for 90 days are shown in Table 4. Mold was detected only in the silage contaminated by *F. graminearum* (ND vs. 5.98 log10 cfu/g). There were interaction effects between contamination and inoculant on LAB (p=0.003) and yeast (p=0.032) counts. The LAB was higher in CT-INO silage than in CT-CON silage (p<0.05; 5.15 vs. 4.10 log10 cfu/g), while yeast count was higher in CT-CON and CT-INO silages than in UCT-INO silage (p<0.05; 6.05 and 6.53 vs. 4.43 log10 cfu/g). Aerobic stability was decreased by *F. graminearum* contamination (p=0.044; 81.7 vs. 66.5 h) and inoculant application (p=0.034; 82.7 vs. 65.5 h). Generally, it is well-known the antifungal activity of acetate and propionate in silages (McDonald, 1991; Weinberg et al., 1993; Adesogan et al., 2004). Joo et al. (2020) and Paradhipta et al. (2023) also reported that the silage applied LAB-producing carboxylesterase with antifungal activity had higher acetate content and aerobic stability. This explanation might support the lower aerobic stability by *F. graminearum* contamination (UCT vs. CT) and inoculant application (CON vs. INO) in this study.

Ⅳ. CONCLUSIONS

In the study of bacterial inoculant isolation, *L. buchneri* L11-1 and *L. paracasei* L9-3 were isolated from commercial corn silages based on the results of antifungal and enzyme (carboxylesterase and chitinase) activities. In the study of silage application, the contents of CP, CA, NDF, and ADF, as well as the counts of yeast and mold increased in the silages contaminated by *F. graminearum*, while IVDMD, NH_3-N, acetate, and aerobic stability decreased. With isolated inoculant application, IVNDFD only increased, adversely aerobic stability decreased. Therefore, this study concludes that preventing the contamination of undesirable microbes is crucial during the ensiling process on farms. However, further studies are needed to investigate the effect of LAB-producing chitinase on corn silage.

Ⅴ. ACKNOWLEDGEMENTS

This research was funded (Project No. 821069-03-3-HD020) by IPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), and Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

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(Received : September 20, 2023 | Revised : September 25, 2023 | Accepted : September 25, 2023)