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Effects of bacterial inoculants on the microbial community, mycotoxin contamination, and aerobic stability of corn silage infected in the field by toxigenic fungi

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Abstract

This study aimed to evaluate the effects of inoculants on the microbial community and mycotoxins contamination of corn silage during aerobic exposure. Whole-crop corn infected with or without mycotoxigenic fungi were ensiled with *Lentilactobacillus buchneri* (LB, 1.0×10^6 cfu/g fresh weight (FW)), *Lactiplantibacillus plantarum* (LP, 1.0×10^6 cfu/g FW), or LBLP at 1.0×10^6 cfu/g FW each. The concentration of acetic acid (AcA) ($P < 0.05$) in LB and LBLP silages was higher than in control (C) and LP of non-fungal infection (NFI) silages. The fungal infection resulted in a larger increase of zearalenone (ZEN, $P = 0.01$), fumonisin B_1 (FUB_1, $P = 0.02$), and fumonisin B_2 (FUB_2, $P = 0.02$). The relative abundance (RA) of *Issatchenkia* in NFI was higher ($P < 0.001$) than FI silages, whereas the RA of *Kazachstania* ($P < 0.001$), *Zygosaccharomyces* ($P = 0.047$), and *Candida* ($P = 0.025$) in NFI were lower than these of FI silages. The aerobic stability was improved by the application of LB and LBLP as compared with the C of NFI silages. The LB and LBLP had the potential to improve aerobic stability and alleviate mycotoxins contamination of non-fungal infected corn silages.

Keywords: *Lentilactobacillus buchneri*, *Lactiplantibacillus plantarum*, Fungal infection, Mycotoxins

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Graphical Abstract

Introduction

Whole-crop corn silage is currently the predominant conserved forage in dairy systems worldwide, however, it is prone to aerobic spoilage during the feed-out phase [1]. Molds, together with yeasts, are believed to be responsible for the aerobic spoilage of silages. Although most yeast and molds were inhibited during the ensiling, the dominant fermented organic acids showed more fungistatic rather than fungicidal effects [2]. Thus, some acid-tolerant fungi can revive and aggressively proliferate when silage was exposed to air, resulting in poor aerobic stability and mycotoxin contamination. Mycotoxin contamination in food and feed is an ongoing global concern because it is currently recognized as a production constraint and a major animal health risk [3]. Fungal infection and mycotoxins contamination are impossible to entirely avoid during growing and storing forages for cattle [4]. The oxygen-rich microenvironment was usually distributed on the surface and edge of the silo where visible green-gray mold indicative of mycotoxin production, including aflatoxins and several *Fusarium* mycotoxins, were usually visible [5, 6].

Inoculating *L. buchneri* is a common practice to improve the bio-preservation and aerobic stability of corn silage because it can delay the development of spoilage yeasts and molds [7]. Apart from improvement in fermentation profiles and aerobic stability, some LAB had the potential to reduce mycotoxins contamination in silages [8]. Ma et al. [9] reported that inoculation of *L. plantarum* or *L. buchneri* decreased linearly the aflatoxin B_1 (AFB_1) concentration within 3 d of ensiling. However, it is not clear whether their antifungal and anti-mycotoxigenic activities could last to the feed-out phase of silages. *L. buchneri* has been used widely to enhance the aerobic stability of silages because of its heterofermentative nature [10]. Most of the studies aimed at assessing the effects of *L. buchneri* on the aerobic stability of corn silages under normal conditions and a few studies have been conducted on suboptimal conditions (i.e., corn infected by mold in the field) [7].

We hypothesized that infected toxigenic fungi could revive and proliferate during aerobic exposure, while inoculant LAB could mitigate the potential negative effect of fungal infection on aerobic stability and hygienic quality of corn silage. Therefore, the study aimed to evaluate the effects of *L. buchneri* and *L. plantarum* on the aerobic stability of corn silage, which was artificially infected by toxigenic fungi in the field.

Materials and methods

Crop and ensiling

Two toxigenic fungi *Aspergillus flavus* and *Fusarium graminearum* were cultured on potato dextrose agar (PDA, Shanghai Bio-way Technology Co., Ltd) medium at 30 °C under aerobic conditions to fully sporulate. Spores were harvested with sterile glass slides and diluted with 0.1% Tween-80 (V/V) fortified sterile distilled water, followed by filtering with sterile 4-layer gauze to obtain a

spore suspension. Then spore suspension was adjusted to a final concentration of approximately 10^6 cfu/mL. Corn was grown in the experimental field (total of 10 plots) of Nanjing Agricultural University (32.04 °N, 118.88 °E, Nanjing, China). At the silking stage of corn, 5 plots were randomly selected, and the prepared fungal spore mixture (5 mL per plant) was sprayed on ears, husks, silk, and leaves with a vacuum hand sprayer for artificial infection. After 2 weeks, the same plots of corn were artificially infected with the same procedure again.

Fungal infection (FI) and non-fungal infection (NFI) corn at the 1/2 milk line stage were harvested and chopped to a theoretical length of 2–3 cm. The 5 plots were harvested separately to obtain 5 replicates, and the fresh corn of each plot was divided into 4 piles. Both FI and NFI corn was treated either without (C, untreated), or with *L. plantarum* (LP, applied at 1.0×10^6 cfu/g fresh weight (FW)), *L. buchneri* (applied at LB, 1×10^6 cfu/g FW), and combination of *L. plantarum* and *L. buchneri* (LBLP, *L. plantarum* and *L. buchneri* applied at 0.5×10^6 cfu/g FW each). Then about 3.5 kg of treated corn (DM, 336 ± 6.4 g/kg FW) was filled into a plastic silo (5 L), which was sealed and stored for 90 d at an ambient temperature (24 ± 2 °C).

Aerobic stability test

After 90 d of ensiling, all silages of each silo were emptied and mixed thoroughly, followed by filling into a new large plastic silo (capacity 15 L) without compaction and uncovered, which was stored at ambient temperature (24 ± 2 ℃) for 6 d. The silage was sampled on d 0, 2, 4, and 6 d of aerobic exposure. The room and silage temperatures were measured half-hourly by a data logger. Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased by 2 °C above the ambient temperature [11].

Sample preparation and chemical analyses

The silages were split into four subsamples. One subsample (20 g) was extracted with 60 g of distilled water at 4 °C for 24 h, followed by filtering through four layers of medical gauze. The pH of the silage filtrate was immediately determined using a pH meter (HANNA pH 211, Hanna Instruments Italia Srl, Padova, Italy). Subsequently, one aliquot of silage extract was centrifuged at 10,000×*g* for 10 min at 4 °C, and the supernatant was reserved for lactic acid (LA), acetic acid (AcA), and ethanol (EOL) analyses, which were quantified following the protocol of Romero et al. [12].

One subsample of silage was oven-dried at 65 °C to constant weight for determining DM content. Dried samples were ground to pass through a 1-mm screen for water-soluble carbohydrate (WSC) and mycotoxins analyses. The WSC content was analyzed by colorimetry after reaction with anthrone reagent. The concentrations of aflatoxins (AFs), zearalenone (ZEN), deoxynivalenol (DON), and fumonisins were quantified using an ultra-performance liquid chromatography–tandem mass spectrometry system with a Sciex QTRAP® 5500 triple quadruple tandem mass spectrometer (UPLC–MS/MS) (Sciex, Foster City, CA, USA) [13].

Microbial community analyses

One subsample (10 g) was shaken with 90 mL sodium chloride sterile saline (0.85%) at 150 rpm. One aliquot of the solution was tenfold serially diluted to count the microorganisms, and another aliquot of the solution was filtered through four layers of medical gauze for microbial DNA extraction. The LAB was determined on de Man, Rogosa, and Sharpe agar after 48 h of anaerobic incubation at 37 °C. The number of yeasts and molds was counted on the PDA medium after 48–72 h of aerobic incubation at 28 °C. The microbial data were obtained and transformed to log10 for presentation and statistical analysis.

Three replicates of silages sampled from d 0 and 6 of aerobic exposure were randomly chosen for bacterial and fungal community analyses. Microbial DNA from various solutions was extracted with Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). The Universal primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify bacterial 16S rRNA V3–V4 regions, while fungal ITS was amplified with primers ITS1F (5′-CTTGGTCAT TTAGAGGAAGTAA-3′) and ITS2aR (5′-GCTGCGTTC TTCATCGATGC-3′). Then purified PCR amplicons were paired-end sequenced using the Illumina MiSeq PE300 platform (Illumina Inc., San Diego, CA, USA). Paired-end reads were merged and checked by FLASH (Version 1.2.11), and the primers were trimmed using QIIME (Version 1.7.0). Operation taxonomic units (OTUs) were done using open reference clustered with a 97% similarity cutoff using UPARSE (Version 7.1 http://drive5.com/uparse/). Then the chimeric sequences were identified and removed using UCHIME. Alpha-diversity estimates (numbers of observed OUT, Chao1, and Shannon) and beta-diversity evaluation, based on principal coordinate analysis (PCoA), were performed using the Phyloseq and Vegan packages on R. Bacterial and fungal communities at the phylum and genus levels were analyzed based on Silva database with a confidence threshold of 70%. The high-throughput sequencing data were analyzed on the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com). All DNA sequences have been deposited in the NCBI Short Read Archive database under BioProject PRJNA823478.

Statistical analyses

Data were analyzed using the GLM procedure of SAS (Version 9.3, SAS Institute, Cary, NC, USA). The fermentation profiles and microbial community diversity were analyzed as a split plot in a randomized complete block design with fixed effects of fungal infection (F), inoculation (I), aerobic duration (D), and their interaction. The treatments and aerobic duration were considered as main- and sub-plots, respectively. Data on aerobic stability and mycotoxins concentrations were analyzed as a completely randomized design with fungal infection and inoculation as the main factors. Tukey's test was employed to compare the differences among treatments, and significance was declared at $P<0.05$.

Results

Fermentation profiles of corn silages during aerobic exposure

There was an F × I × D interaction for pH ($P=0.002$), LA ($P<0.001$), and AcA ($P=0.008$) concentrations (Fig. 1 and Table 1). The pH of FI silages increased faster and was higher than those of NFI silages over 6 d of aerobic exposure ($P=0.008$). For NFI silages, the pH in LBLP began to lower ($P<0.05$) than other silages from d 4. For FI silages, the inoculants decreased pH value compared with C on d 4 ($P<0.05$), however, their effects disappeared on d 6. The LA concentration of FI silages decreased faster than that of NFI silages. For FI silages, the LP and LBLP silages showed lower ($P<0.05$) LA concentration than C and LB on d 4. For NFI silages, the higher ($P<0.05$) LA concentration in inoculants treated silage than C was only observed on d 6. FI silages had lower AcA concentrations ($P<0.05$) than NFI silages over 6 d of aerobic exposure. For FI silages, the higher ($P<0.05$) concentration of AcA in LB and LBLP silages than in C and LP lasted until d 2 and 4, respectively.

As shown in Fig. 2, WSC concentration decreased ($P<0.001$) over 6 d of aerobic exposure regardless of fungal infection. FI silages showed lower ($P<0.001$) WSC concentration than NFI silages. The WSC concentrations in C and LP were lower ($P<0.01$) than those of LB and LBLP for NFI silages after d 6. There was an F × I × D interaction for the concentration of EOL ($P<0.001$). For NFI silages, the EOL concentration gradually ($P>0.05$) decreased and there was no significant difference ($P>0.05$) among NFI silages over 6 d of aerobic exposure. For FI silages, there was a slight increase in EOL concentration within 2 d followed by a rapid drop, and LP and LBLP silages had lower EOL concentrations than C and LB on d 4 ($P<0.05$).

The F × D and I × D interactions ($P<0.001$) were detected for LAB counts (Fig. 3). The LAB counts in both NFI and FI silages increased slightly during the initial 2 d of aerobic exposure, followed by a gradual decrease in FI silages until d 6, while that in NFI rapidly decreased after 4 d of aerobic exposure. The LBLP of NFI silages had the lowest LAB counts on d 6, while there was no significant difference in LAB counts among FI silages during aerobic exposure. The I × D and F × D interactions were observed for yeast and mold counts. The count of yeasts and molds in FI silages increased faster and remained at higher levels than that of NFI silages. The inoculants decreased ($P<0.05$) the counts of yeasts and molds compared with C of FI silages after 6 d of aerobic exposure, however, there was no significant difference ($P>0.05$) in yeasts and molds counts among NFI silages during the 6 d of aerobic exposure.

Bacterial and fungal communities of corn silages during aerobic exposure

A total of 2,294,390 and 2,948,299 reads were obtained by high-throughput amplicon sequencing of bacterial 16S rRNA and fungal ITS genes, respectively. The Chao1 and Shannon indices of bacterial and fungal communities decreased after 6 d of aerobic exposure, and inoculants reduced the Chao 1 and Shannon indices in NFI silages but did not affect those of FI silages (Additional file 1: Figs. S1 and S2).

Changes in bacterial and fungal communities at the genus level during aerobic exposure are shown in Fig. 4A. *Acetobacter* (57.1%), *Lactobacillus* (27.4%), *Acinetobacter* (2.95%), *Klebsiella* (1.93%), and *Enterobacter* (1.31%) were the dominant bacterial genus in corn silages. The relative abundance (RA) of *Acetobacter*, *Lactobacillus*, *Klebsiella*, and *Enterobacter* were neither affected ($P>0.05$) by fungal infection nor inoculation. During 6 d of aerobic exposure, the RA of *Acetobacter* in silage increased ($P<0.001$), while the RA of *Lactobacillus* ($P<0.001$), *Klebsiella* ($P=0.003$), and *Enterobacter* ($P<0.001$) significantly decreased. There was an F × I interaction ($P=0.007$) for the RA of *Acinetobacter* because it was the greatest ($P<0.05$) in C among NFI silages, but it was the lowest in C among FI silages.

The fungal genera including *Issatchenkia* (52.3%), *Kazachstania* (18.1%), *Zygosaccharomyces* (9.78%), *Candida* (7.14%), *Aspergillus* (2.23%), and *Pichia* (1.96%) were the dominant fungal genera in corn silages during aerobic exposure. The RA of *Issatchenkia* was higher ($P<0.001$) in NFI than in FI silages, whereas the RA of *Kazachstania* ($P<0.001$), *Zygosaccharomyces* ($P=0.047$), *Candida* ($P=0.025$), and *Pichia* ($P=0.013$) in NFI were lower than in FI silages. There was an F × D interaction for the RA of *Issatchenkia* ($P=0.013$), *Candida* ($P<0.001$), and *Pichia* ($P<0.001$) because they were significantly ($P<0.01$) decreased for FI silages, but there were no marked changes for NFI silages

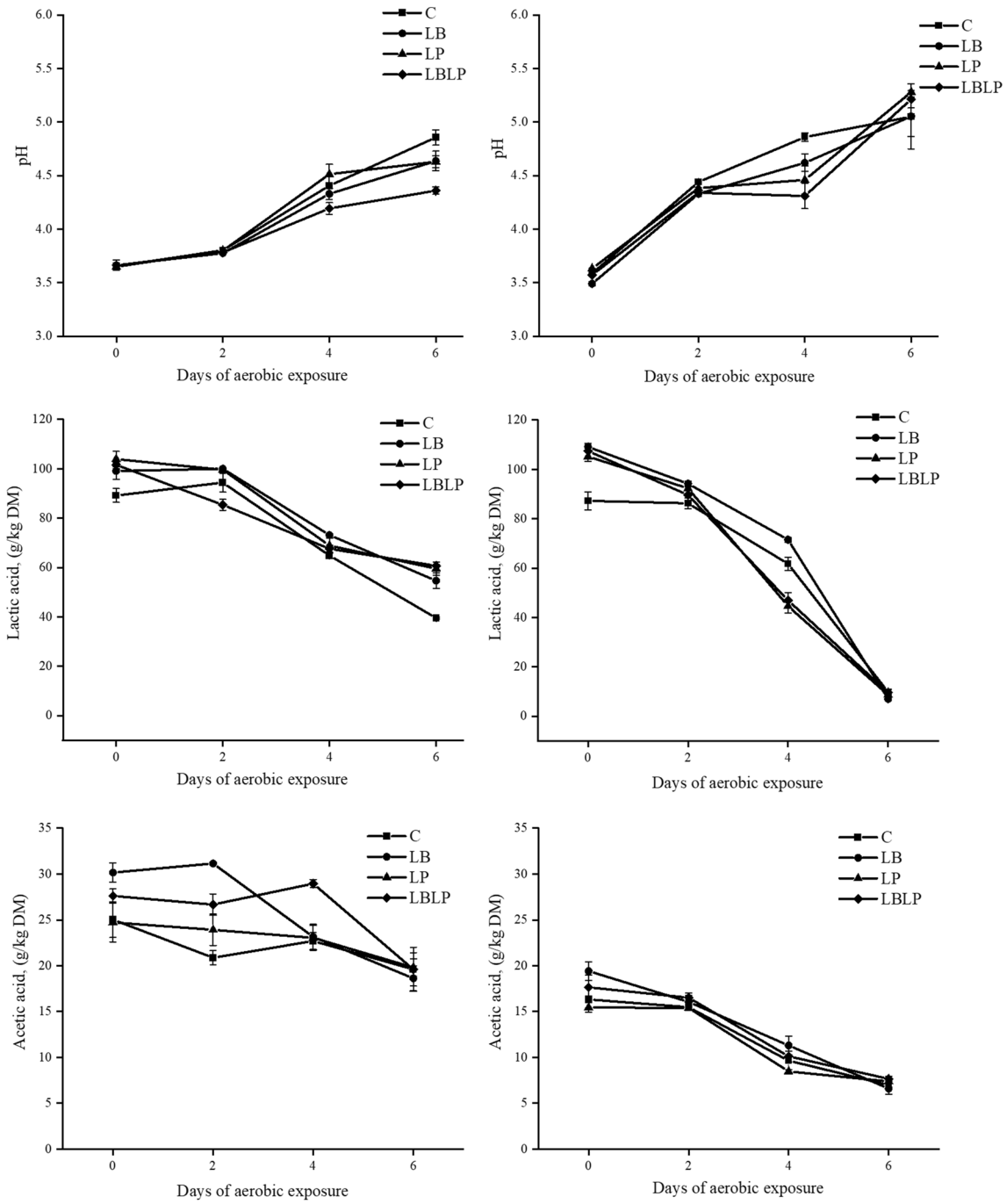

Fig. 1 Changes in pH, lactic acid, and acetic acid concentration in non-fungal infection (left) and fungal infection (right) of corn silage during aerobic exposure. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW; DM, dry matter

during aerobic exposure. The F × I interaction was also found for RA of *Kazachstania* ($P < 0.001$) and *Zygosaccharomyces* ($P < 0.001$). The RA of *Kazachstania* and *Zygosaccharomyces* in FI silages significantly ($P < 0.01$) increased during aerobic exposure, but there were no significant changes except C for NFI silages.

Table 1 Probability of the effects of fungal infection, inoculants, days of aerobic exposure, and their interactions on chemical composition, fermentation products, and microbial populations of corn silage ($n = 5$)

Item	Main effect			Interaction effect[a]			
	F	I	D	F × I	F × D	I × D	F × I × D
pH	< 0.001	< 0.001	0.002	0.727	< 0.001	0.126	0.002
Lactic acid	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Acetic acid	< 0.001	< 0.001	< 0.001	0.463	< 0.001	0.002	0.008
Ethanol	0.009	< 0.001	< 0.001	0.003	< 0.001	< 0.001	< 0.001
WSC	< 0.001	< 0.001	< 0.001	0.073	0.460	0.408	0.289
Y&M	< 0.001	< 0.001	< 0.001	0.428	< 0.001	0.011	0.710
LAB	0.780	< 0.001	< 0.001	0.120	< 0.001	0.010	0.099

F, fungal infection; I, inoculants; D, days of aerobic exposure, WSC, water-soluble carbohydrates; LAB, lactic acid bacteria; Y&M, yeasts and molds

[a] Two-way and three-way interaction among main factors

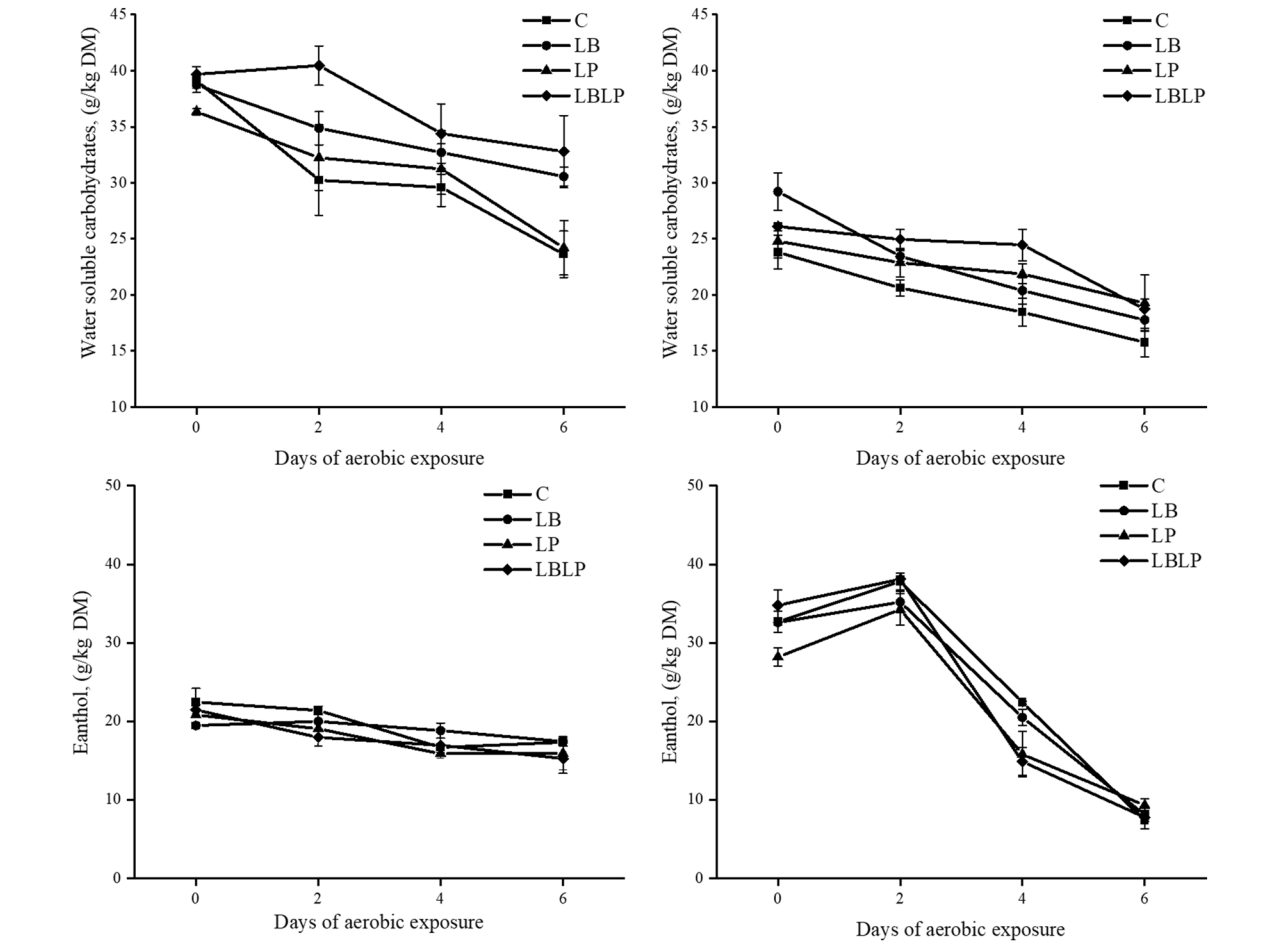

Fig. 2 Changes in water-soluble carbohydrates and ethanol concentrations in non-fungal infection (left) and fungal infection (right) of corn silage during aerobic exposure. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW; DM, dry matter

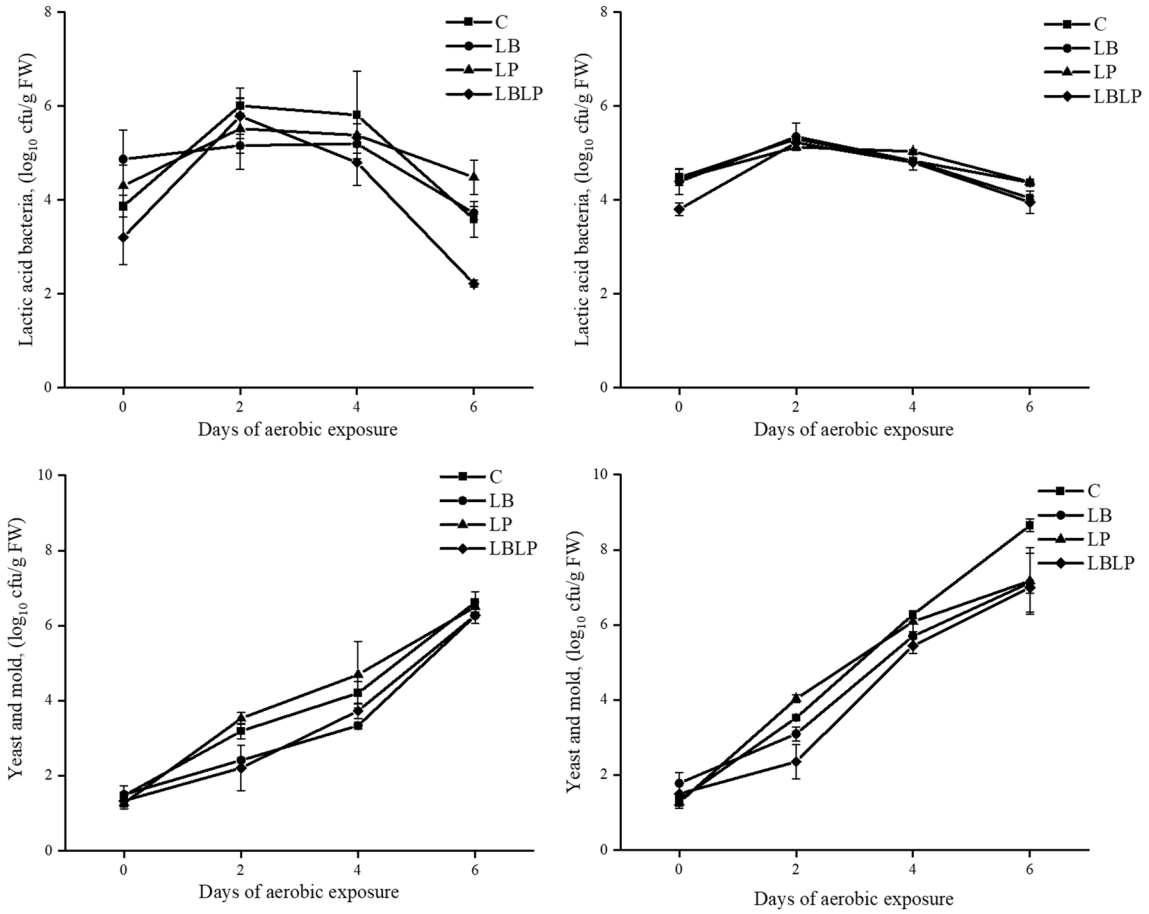

Fig. 3 Changes in lactic acid bacteria, yeasts and mold in non-fungal infection (left) and fungal infection (right) of corn silage during aerobic exposure. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW; FW, fresh weight

Correlation relationship between microbial community and fermentation profiles

For NFI silages, redundancy analysis (RDA) indicated that the microbial community of silage was greatly affected by pH, LA, EOL, WSC, and AcA (Fig. 5A). *Lactobacillus* was positively correlated with WSC, LA, and AcA, while negatively correlated with pH in NFI silages. Positive correlations were found between *Issatchenkia* and WSC, LA, and AcA, while a negative correlation between *Issatchenkia* and pH was observed. For FI silages (Fig. 5B), the WSC, EOL, LA, and AcA concentrations were positively correlated with *Lactobacillus*, *Issatchenkia*, and *Candida*, while negatively correlated with *Kazachstania*, *Zygosaccharomyces*, and *Acetobacter*. The pH was positively correlated with *Kazachstania*, *Zygosaccharomyces*, and *Acetobacter* for FI silages.

Mycotoxins concentrations in corn silages

As shown in Fig. 6, there were F × I interactions for AFB_1 ($P = 0.03$) and AFB_2 ($P = 0.02$), because the increase of AFB_1 in C was larger ($P < 0.05$) than other treatments for NFI silages but there was no significant difference in AFB_1 concentration among FI silages. The change of AFB_2 was similar among all silages for NFI silage, while the decline of AFB_2 was only ($P < 0.05$) found in LB of FI silages. The FI silages showed larger increases of ZEN ($P = 0.01$), FUB_1 ($P = 0.02$), and FUB_2 ($P = 0.02$) than NFI silages. The increase of ZEN in LP was the smallest ($P < 0.05$) among all FI silages. The concentrations of FUB_1 ($P = 0.15$) and FUB_2 ($P = 0.89$) were not affected by inoculation. Neither inoculation nor fungal infection affected the concentration of DON.

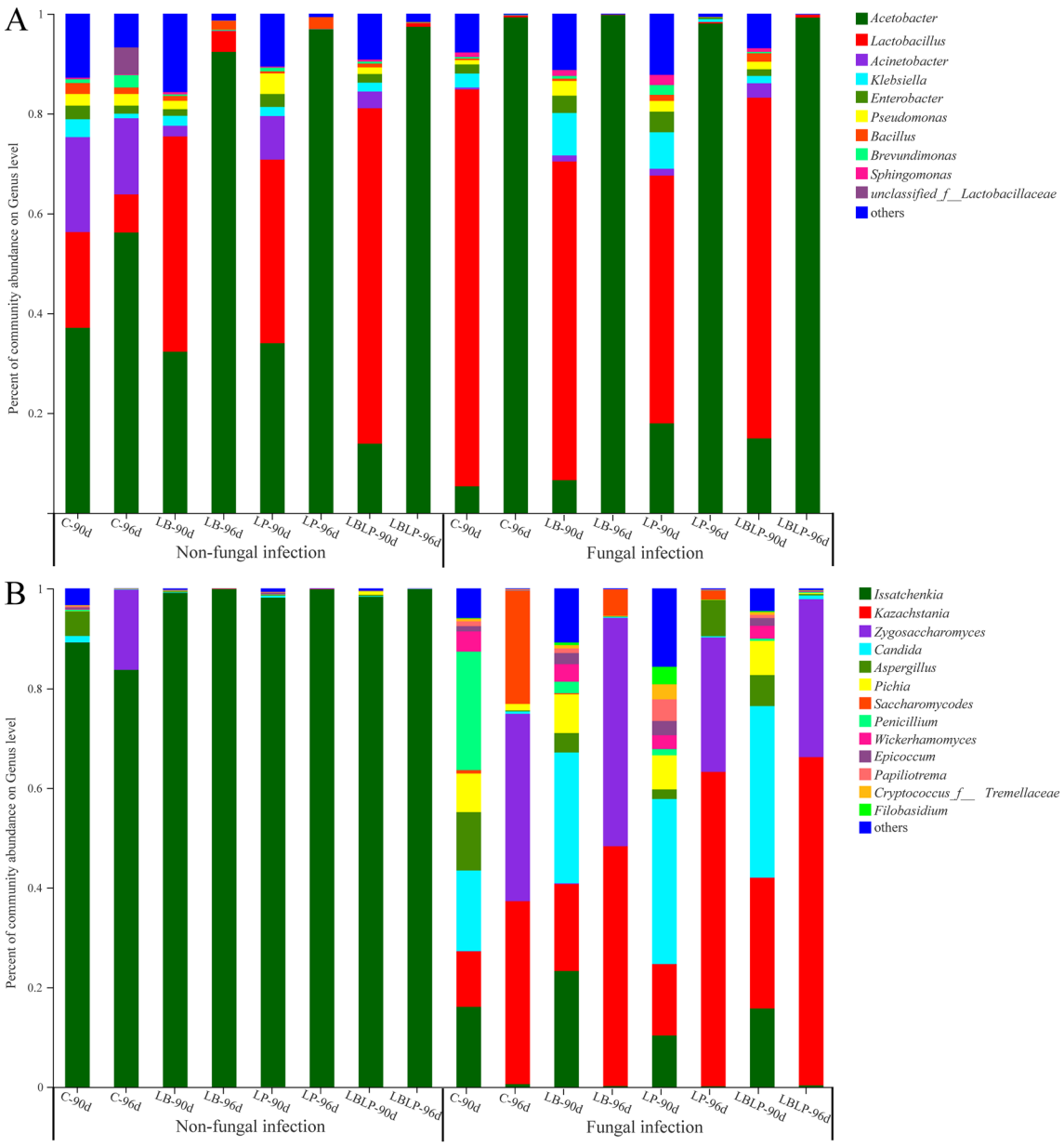

Fig. 4 Bacterial (**A**) and fungal (**B**) community at the genus level in non-fungal infection and fungal infection of corn silage during aerobic exposure. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW; Arabic number indicating days of aerobic exposure; FW, fresh weight

Aerobic stability of corn silages

There was a F × I interaction ($P < 0.001$) for the aerobic stability (Fig. 7). The aerobic stability of LB (82 h) and LBLP (85 h) was longer than that of C (62 h) for NFI silages, however, there was no significant difference ($P > 0.05$) in aerobic stability among FI silages. The fungal infection enhanced aerobic deterioration compared with NFI silages ($P < 0.001$).

Discussion

Effect of inoculant and fungal infection on the fermentation profiles and mycotoxins of corn silages during aerobic exposure

Once the silage is opened, aerobic microorganisms that survived during the ensiling process, e.g., bacilli, yeast, and acid-tolerant bacteria, can rapidly proliferate and metabolize residual sugars and organic acids to CO_2,

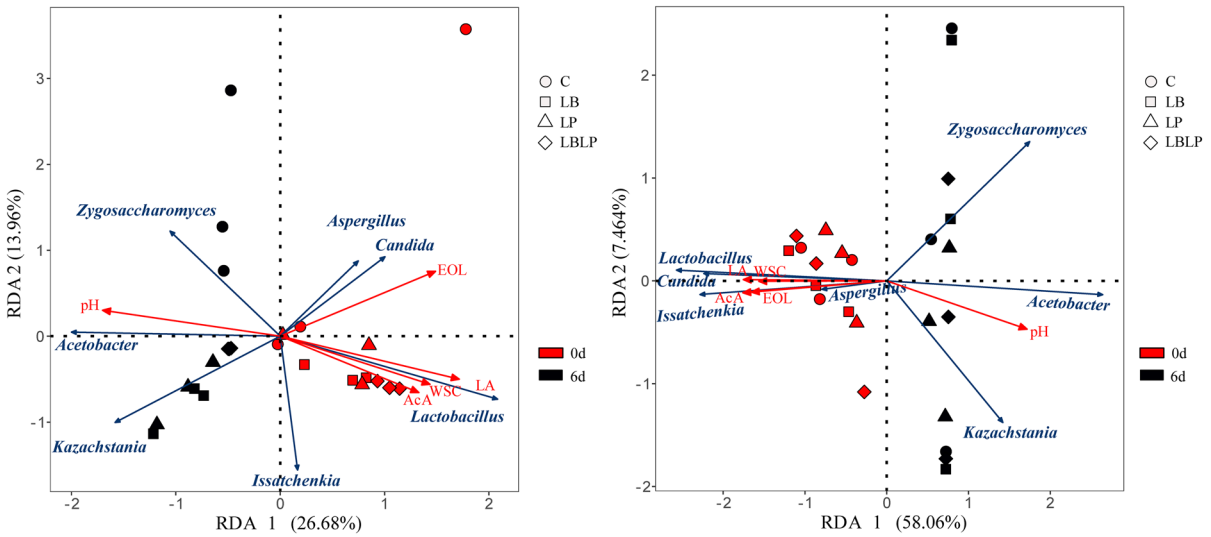

Fig. 5 Redundancy analysis (RDA) of the effect of compost parameters (abiotic factors: red arrows; biotic factors: blue arrows) on bacterial and fungal taxonomic distribution in non-fungal infection (**A**) and fungal infection (**B**) of corn silage during aerobic exposure. LA, lactic acid; AcA, acetic acid; EOL, ethanol; WSC, water-soluble carbohydrates; C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW. 0 d, after silo opening; 6 d, after 6 days of aerobic exposure; FW, fresh weight

H_2O, and heat [14, 15]. Consequently, silage temperature increases and the silage mass becomes aerobically unstable. The consumption of acids by aerobic microorganisms is accompanied by the increase in silage pH, thus, the variation of silage pH is used as the criteria for aerobic deterioration [16]. During the initial 2 d of aerobic exposure, the pH of NFI silages showed a slight increase followed by a marked increase, while that in FI silages rapidly increased. This might be attributed to the rapid revival of aerobic microorganisms including infected toxigenic fungi [16]. This hypothesis was confirmed by the changes in LA: the LA in FI sharply decreased once aerobic exposure, while the rapid drop of LA was following a transient stable (2 d) in NFI silages. Kung, et al. [17] also reported that the increase in silage pH was accompanied by a decline of LA in corn silages during aerobic exposure. The faster and larger changes of pH and LA in FI than in NFI silages were related to its lower AcA concentration. The AcA has been proven to be responsible for enhancing aerobic stability because it acts as an inhibitor of spoilage organisms. Danner, et al. [18] stated that the silage aerobic stability increased exponentially with acetic acid concentration. Queiroz, et al. [19] also found that fungal infection negatively affected the fermentation of corn silage and resulted in a lower AcA concentration, which could explain the poor aerobic stability of FI silages in the present study.

Although other modes of action may exist, the production of AcA has been the most accepted explanation of how organisms from the *Lb. buchneri* group of bacteria increases the aerobic stability of silages [7]. In the study, the higher AcA concentrations in LB and LBLP than in other treatments of NFI silages lasted to d 2 and 4, respectively, while no significant difference in AcA concentrations was observed among FI silages. This indicated that the fungal infection might disturb the microbial community and fermentation of silages, diminishing the effect of LB on AcA production. The decline in WSC contents along with the aerobic exposure progress was attributed to the extensive proliferation of aerobic microorganisms, which can oxidize WSC to CO_2 and H_2O [20]. The FI silages always had lower WSC concentrations than NFI silages. It can be conjectured that infected fungi consumed more WSC during the initial transient aerobic stage of ensiling, resulting in the lower residual WSC being in FI silages than NFI silages. For NFI silages, the concentration of WSC in LB and LBLP was consistently higher than that of C and LP over the 6 d of aerobic exposure. This was attributed to the more AcA produced by *L. buchneri*, which inhibited the proliferation of undesirable microorganisms, preserving more residual WSC [21]. Kung, et al. [22] reported that higher dissociation constants (pKa) of AcA (4.75) than LA (3.86) contributed to the higher antimicrobial activity of AcA in surroundings where the pH values are low (around pH 4). In the study, there was a transient increase in the ethanol concentration of FI silages, which was possibly due to the metabolism of yeast within the initial 2 d of aerobic exposure. The FI silages had a higher number of yeasts

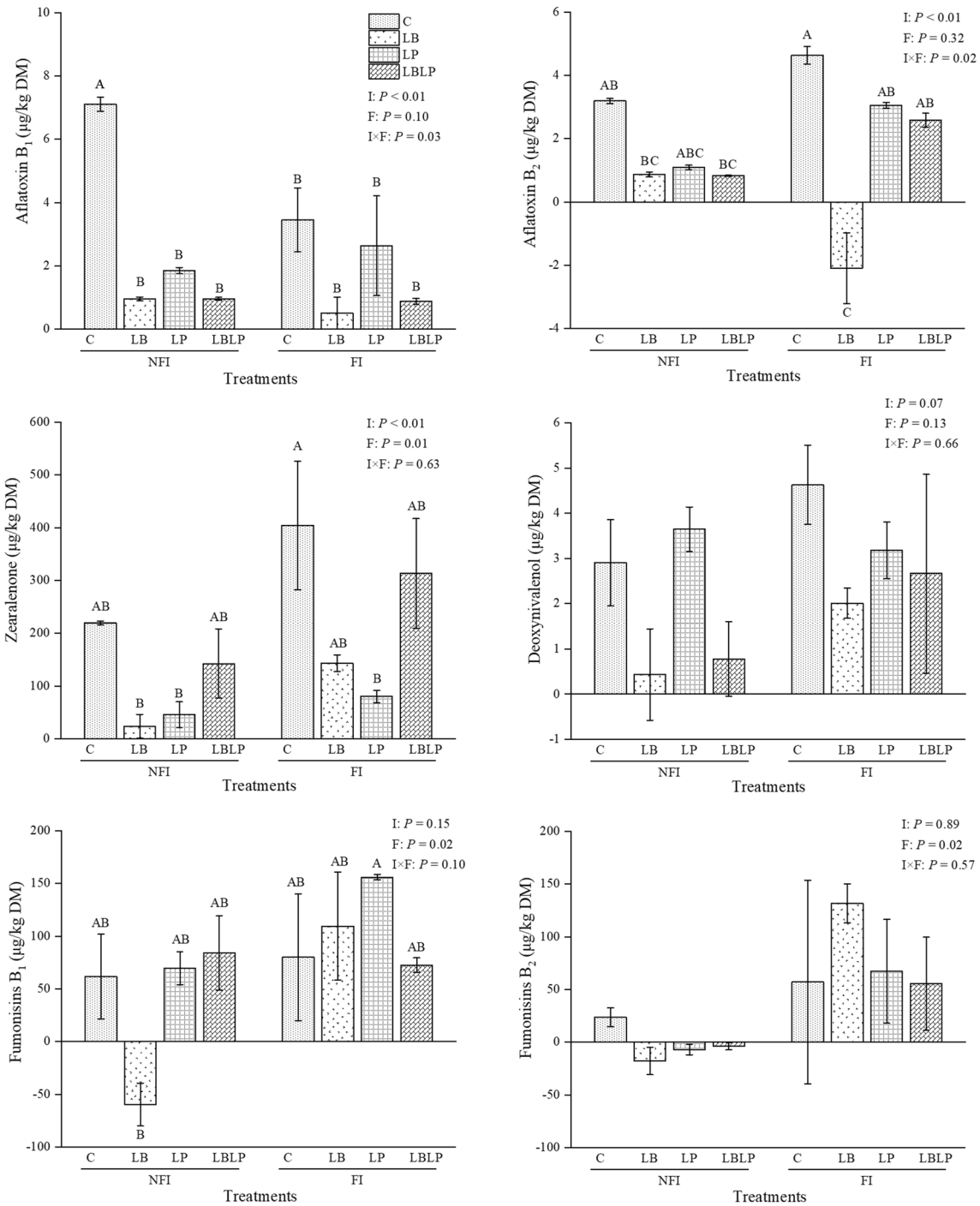

Fig. 6 Changes in relative concentrations of mycotoxins in corn silage during aerobic exposure. NFI, non-fungal infection; FI, fungal infection. I, inoculation; F, fungal infection; C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW; DM, dry matter; FW, fresh weight

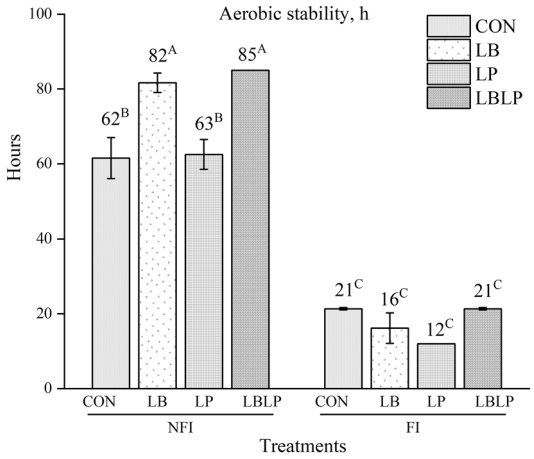

Fig. 7 The aerobic stability (h) of corn silage after 6 days aerobic exposure. Aerobic stability was defined as a 2 °C increase in temperature over the established baseline temperature. SEM = 6.04. NFI, non-fungal infection; FI, fungal infection. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW; FW, fresh weight

and molds than the NFI silages. Irrespective of fungal infection, the lower numbers of yeast and molds in LB and LBLP than C and LP on d 2 and 4 were attributed to the fungistatic property of AcA produced by the inoculants of *L. buchneri* [2]. The gradual decline of ethanol concentration with the aerobic exposure progress, might be due to its volatilization or metabolized by *Acetobacter* [23].

Effect of inoculant and fungal infection on the bacterial and fungal communities of corn silages

During aerobic exposure, the application of LAB reduced the Shannon and Chao 1 indexes of bacterial and fungal communities in NFI silages, but not in FI silages. This might be due to the proliferation of aerobic bacteria and yeasts, which occupied the predominant roles of the microbial community [20].

In the study, *Acetobacter* became the dominant bacteria after 6 d of aerobic exposure, because it can oxidize ethanol to AcA initially, followed by oxidation of LA and AcA to CO_2 and H_2O [24]. *Acetobacter* is non-fermenting aerobic bacteria and can be found in various environments, it can initiate aerobic deterioration of corn silage with or without the presence of yeasts [25]. Guan, et al. [26] also observed a significant increase in the RA of *Acetobacter* in Napier grass after 2 d of aerobic exposure. *Lactobacillus* is the main bacteria involved in LA fermentation during ensiling and usually dominates the well-fermented silages [27], however, it is rapidly replaced by aerobic bacteria (e.g., *Acetobacter* spp.) once silage is exposed to air [28]. In the present study, the RA of *Lactobacillus* reduced below 1% in silage except for C and LB of NFI silages after 6 d of aerobic exposure. The genus *Klebsiella* and *Enterobacter*, belonging to enterobacteria, are capable to produce ammonia, which can cause animal health issues [29]. In the study, the decline of RA of *Klebsiella* and *Enterobacter* might be attributed to the anaerobic property and intolerance to acid conditions, as reported by McGarvey, et al. [30], who found Enterobacteriaceae could thrive in anaerobic and weak-acidic conditions (pH > 5.4). In the study, the silage pH was not increased up to 5.4 until d 6 of aerobic exposure, which resulted in the substitution of *Klebsiella* and *Enterobacter* by *Acetobacter*. The higher RA of *Acinetobacter* in NFI than in FI silages might be related to the higher AcA concentration, which could be used by *Acinetobacter* as a substrate [31].

Issatchenkia, an acid-tolerant yeast, is the dominant fungal genus in NFI silages regardless of aerobic exposure or inoculation. However, there are marked variations in fungal composition for FI silages during 6 d of aerobic exposure. This discrepancy between NFI and FI silages was attributed to the fungal infection, which resulted in more yeast and molds present and revived in FI silages during aerobic exposure. For FI silages, the marked decline in the RA of *Issatchenkia* was accompanied by the increase of RA of *Kazachstania* and *Zygosaccharomyces* during 6 d of aerobic exposure. Wang, et al. [32] indicated that *Kazachstania* had a strong tolerance to LA and was crucially involved in initiating the aerobic deterioration of corn silage with a relatively low pH and AcA content. Hao, et al. [33] reported that *Zygosaccharomyces bailii* was the sole yeast species isolated from spoilage total mixed ration (TMR) silages and confirmed that the *Z. bailii* could initiate aerobic deterioration of TMR silages. In the study, the RA of *Candida* and *Pichia* decreased below 1% after 6 d of aerobic exposure, this is in contrast to the report by Duniere, et al. [34], who found that *Candida* and *Pichia* were the main spoilage genera after aerobic exposure. Pahlow, et al. [35] indicated that *Issatchenkia*, *Candida*, and *Pichia* are lactate-assimilating yeasts that are the initiators of aerobic degradation of silage. We speculated that *Kazachstania* and *Zygosaccharomyces* underwent more vigorous growth and outcompeted other yeast and molds.

The correlation between microbial community and fermentation profiles was analyzed by RDA. In the present study, bacterial genera such as *Lactobacillus* were found to be positively correlated with LA and AcA, while *Acetobacter* was negatively correlated with LA and AcA regardless of fungal infection. When silage is exposed to air, LA and AA were metabolized by aerobic bacteria,

increasing silage pH, which further inhibited the proliferation of *Lactobacillus* and boosted the dominance of *Acetobacter* [36]. Fungal genera *Kazachstania* and *Zygosaccharomyces* were negatively correlated with LA and were positively correlated with pH. This indicated that *Kazachstania* and *Zygosaccharomyces* were considered dominant fungi during aerobic exposure, which could assimilate lactate and increase pH, initiating the spoilage of silages.

Effect of inoculant and fungal infection on mycotoxins of corn silages during aerobic exposure

In the study, the concentrations of most mycotoxins increased over 6 d of aerobic exposure, indicating that toxigenic fungi revived during aerobic exposure [37]. The larger increases of AFs, ZEN, and DON concentrations in FI than in NFI silages confirmed that artificially infected *A. flavus* and *F. graminearum* might revive and produce mycotoxins during aerobic exposure. Ferrero, et al. [38] indicated that the aerobic environment allowed the proliferation of *A. flavus* and enhanced the production of AFB_1. Vandicke, et al. [39] also reported that some *Fusarium* species were even able to survive at low oxygen levels (< 0.5%). The fungal spores that survived in the silage may reactivate and produce *Fusarium* toxins due to exposure to oxygen during feed-out.

The more effective of inoculants on reducing AFB_1 contamination in NFI than FI silages might be attributed to the revival of toxigenic fungi and higher mycotoxins in FI silages, which attenuated the effects of inoculants on reducing AFB_1 contamination. Ma, et al. [9] reported that *L. plantarum*, *L. buchneri*, and *Pediococcus acidilactici* could bind to AFB_1 in an in vitro medium. Dogi, et al. [40] suggested that inoculation with *Lactobacillus rhamnosus* strongly inhibited the fungal growth (*F. graminearum*, *Aspergillus parasiticus*, etc.) and mycotoxin production (AFs, ZEN, DON, etc.). However, it is unexplainable that the AFB_2 in LB of FI silage was decreased during aerobic exposure in the study.

Effect of inoculants and fungal infection on aerobic stability of corn silages

The fungal infection shortened the aerobic stability of silages regardless of inoculation. This was attributed to the artificial fungal infection, which resulted in more yeast and molds surviving in FI than in NFI silages. Keshri, et al. [41] reported that the poor aerobic stability of corn silages was due to the high number of lactate-assimilating yeasts. In the study, the aerobic stability of NFI silages was improved by the application of LB and LBLP as compared with C of NFI silages. Kung, et al. [17] also found that inoculant containing *L. buchneri* could extend the aerobic stability of silages challenged with air stress, which was attributed to the antifungal property of AcA produced by *L. buchneri*. Romero, et al. [42] reported that applying a combination inoculant of *L. buchneri* and *Pediococcus pentosaceus* increased the AcA concentration and decreased yeasts and molds in corn silages, which in turn indirectly extended the aerobic stability.

Conclusion

The fungal infection disturbs the microbial community and silage fermentation, weakening the effect of LB on AcA production. *Kazachstania* and *Zygosaccharomyces* were dominant fungi and contributed to the aerobic spoilage of FI silages. There were larger increases of AFs, ZEN, and DON concentrations in FI than in NFI, and the inoculants showed more effect on reducing AFB_1 contamination in NFI than in FI silages. The application of LB and LBLP inoculants improved the aerobic stability of corn silage, delayed the onset of aerobic deterioration, and reduced the risk of mycotoxins production after silo opening.

Supplementary Information

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Additional file 1: Fig. S1. The bacterial diversity indexes in non-fungal infection (left) and fungal infection (right) of corn silage during aerobic exposure. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW. 0 d, after silo opening; 6 d, after 6 days of aerobic exposure; FW, fresh weight. **Fig. S2.** The fungal diversity indexes in non-fungal infection (left) and fungal infection (right) of corn silage during aerobic exposure. C, control; LB, *L. buchneri* applied at 1×10^6 cfu/g FW; LP, *L. plantarum* applied at 1×10^6 cfu/g FW; LBLP, *L. buchneri* and *L. plantarum* applied at 0.5×10^6 cfu/g FW. 0 d, after silo opening; 6 d, after 6 days of aerobic exposure; FW, fresh weight. **Fig. S3.** Principal coordinate analysis (PCoA) plot based on Unweighted-UniFrac dissimilarity distance of the bacterial (A) and fungal (B) community between samples. NFI, Non-fungal infection; FI, Fungal infection. C, Control of non-fungal infestation silages; LB, *L. buchneri* treatment of non-fungal infestation silages; LP, *L. plantarum* treatment of non-fungal infestation silages; LBLP, *L. buchneri* and *L. plantarum* treatment of non-fungal infestation silages. **Fig. S4.** Correlation analysis of the mycotoxins and fungal (10 most abundant genera) community compositions. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. **Table S1.** The mycotoxin concentrations (μg/kg DM) of corn silages during aerobic exposure.

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WbW, XC, WkW, and PM: performed the experiment, analysis, and writing. JL, JZ, and AG: performed the editing and revision. XY and TS: designed the experiment. All authors read and approved the final manuscript.

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The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors listed have read the complete manuscript and have approved the submission of the paper.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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