The capacity of silage inoculant bacteria to bind aflatoxin B₁ in vitro and in artificially contaminated corn silage

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ABSTRACT

The objectives were to examine the aflatoxin B₁ (AFB₁)-binding capacity of silage bacteria and factors affecting the responses. Experiments 1 and 2 examined the effects of bacterial strain and population on the AFB₁-binding capacity of 10 bacteria. When applied at 10⁶ cfu/mL to an in vitro medium, only *Lactobacillus plantarum* PT5B bound the AFB₁ and the binding capacity was low (4%). When applied at 10⁹ cfu/mL, all 10 bacteria bound AFB₁, but *L. plantarum* R2014 (*Lp*) and EQ12, *Lactobacillus buchneri* R1102 (*Lb*), and *Pediococcus acidilactici* R2142 and EQ01 (*Pa*) had the greatest capacity (23.9 to 33%). Experiment 3 examined the AFB₁-binding capacity of viable and nonviable (HCl-treated) forms of *Lp*, *Lb*, and *Pa* at different pH. Nonviable *Lb* and *Lp*, but not *Pa*, increased AFB₁ binding. Binding of AFB₁ was greatest at pH 2.5 and least at pH 8. As the nonviable *Lb* and *Lp* that bound AFB₁ in experiment 3 would not be effective silage inoculants, experiment 4 examined effects of benign versus severe treatments (85 vs. 100°C; pH 2.5 vs. <1) on the viability of *Lp*, *Lb*, and *Pa*. The population of bacteria was reduced from 9 to 4 log cfu/mL by treatment with HCl at pH 2.5 and to 2 log cfu/mL by 85 or 100°C, whereas acidification at pH <1 eliminated the bacteria. Experiment 5 determined the effect of the ensiling duration and benign treatment methods [37 (viable cells) or 85°C (heated cells) or acidification with HCl at pH 2.5 (acid-treated cells)] on binding of AFB₁ and silage quality during the fermentation of corn forage. Corn forage was ensiled after treatment with only deionized water (control), AFB₁ (30 µg/kg of fresh forage), or a mixture of AFB₁ and 10⁹ cfu/g of each of the treated bacteria. Adding AFB₁ alone to corn forage reduced the pH decline during the first 3 d

of ensiling and increased or tended to increase butyric acid concentration and final pH after ensiling for 21 d. Bacterial inoculation inhibited these negative effects. The fermentation profile of silage treated with *Lb* and *Pa* did not differ from those of the control silage. In all silages treated with the toxin, the AFB₁ concentration decreased linearly (from 30 to ≤0.35 µg/kg) within 3 d of ensiling. Certain silage bacteria can bind AFB₁ but the efficacy depends on several factors.

Key words: aflatoxin B₁, lactic acid bacteria, silage

INTRODUCTION

Certain strains of selected lactic acid bacteria (LAB) are added to forage to improve fermentation during ensiling. When inoculated homofermentative LAB dominate the epiphytic population of LAB on forages, a rapid drop in pH ensues due to fermentation of water-soluble carbohydrates (WSC) into lactic acid. This process minimizes losses of DM, energy, and nutrients during the fermentation. Various strains of *Lactobacillus plantarum* are used as inoculants, but many are only active after the pH of the ensiled forage falls below 5.0 (McDonald et al., 1991). Therefore, many commercial inoculants contain mixtures of pediococci or enterococci, which are active within the pH range of 5.0 to 6.5 (Henderson, 1993). However, some homofermentative inoculants enhance the spoilage of silage upon exposure to air because they produce insufficient quantities of the antifungal acetic and propionic acids that inhibit the growth of spoilage yeasts and molds (Weinberg et al., 1993; Filya et al., 2000). In fact, the relatively high lactic acid and residual WSC concentrations after homolactic fermentations can enhance the population of spoilage yeasts that use them as growth substrates.

Heterofermentative LAB are typically used as aerobic stability enhancers because they ferment WSC and lactic acid into acetic acid, which is antimycotic. Muck (1996) reported that *Lactobacillus buchneri* treatment of corn forage at ensiling resulted in high concentrations of acetic acid (up to 5.87% of DM) and greater aerobic

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stability of the silage. Similar results were reported by Driehuis et al. (2001), who noted that inoculation of *L. buchneri* alone or in combination with *Pediococcus pentosaceus* and *Lactobacillus plantarum* enhanced the aerobic stability of grass silage.

Aflatoxins (AF) are carcinogenic, mutagenic, teratogenic, and immunosuppressive secondary metabolites of *Aspergillus* fungi (Eaton and Gallagher, 1994). The economic impact of mycotoxin contamination of food and feeds in the United States is estimated at \$1.4 billion per year (CAST, 2003). Aflatoxin B₁ (AFB₁), the most toxic form, can be transformed from dairy cow feeds to aflatoxin M₁ and excreted in milk (Veldman et al., 1992; Xiong et al., 2015). Both forms are carcinogenic and AF cause up to 155,000 cases of liver cancer annually in humans (Liu and Wu, 2010). Consequently, development of additives to sequester AF and other mycotoxins in food and feeds is critical. The potential of LAB to bind AF was summarized by Ahlberg et al. (2015). El-Nezami et al. (1998) reported that *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, and *Lactobacillus casei* can bind AF in aqueous solutions. Binding of AFB₁ in liquid media and of aflatoxin M₁ in milk by LAB have also been reported (Haskard et al., 2001; Pierides et al., 2000). Haskard et al. (2000) reported that nonviable LAB had greater AFB₁-binding capacity than viable cells. This is important because it suggests that AFB₁-binding by LAB can be maintained in distal parts of the gastrointestinal tract despite potential loss of bacterial viability in the acidic abomasum. However, effects of methods of altering the viability of bacteria, such as boiling, autoclaving, irradiating, and acidifying, are not clear and all the studies on AFB₁ binding by LAB have involved probiotic bacteria used in the food industry. To our knowledge, no research has been conducted on the ability of silage bacteria to bind AFB₁. Yet, if effective at binding the toxin, silage bacteria could be used to detoxify forages contaminated with AFB₁ due to stresses such as droughts, lodging, and fungal or disease infestation (Queiroz et al., 2012; Teller et al., 2012) or silages. Inoculation with such bacteria at ensiling could also reduce the need for adding mycotoxin sequestering agents to the diets of dairy cows.

The objectives of the current study were to examine the AFB₁-binding capacity of 10 silage bacteria and to examine effects of the bacterial dose, viability, strain, and prevailing pH on AFB₁ binding (experiments 1–3). We hypothesized that the AFB₁-binding capacity of LAB would be strain- and species-dependent and would be greater in nonviable bacteria and at higher bacterial doses or more alkaline pH values. The objective of experiment 4 was to examine effects of severe or milder bacterial viability-reducing methods on the bacterial

population. Our hypothesis was that reducing the severity of acid and heat treatments would reduce their adverse effects on the bacteria and the bacterial population without curtailing the AFB₁-binding capacity and other beneficial effects of the LAB on the fermentation. The objective of experiment 5 was to determine the effect of the most promising viability-altering methods in experiment 4 and the ensiling duration on binding of AFB₁ by silage inoculant bacteria in ensiled corn forage. We hypothesized that inoculated LAB would bind AFB₁ in silages contaminated with AFB₁ and the bacterial treatments would increase the AFB₁-binding capacity of the LAB without affecting their beneficial effects on the fermentation.

MATERIALS AND METHODS

Experiments 1 and 2: Effects of Bacterial Strain on AFB₁ Binding at 2 Doses

Bacteria Preparation. Ten silage inoculant bacterial strains (Table 1) from Lallemand Animal Nutrition (Milwaukee, WI) were separately grown for 24 h at 37°C in de Man-Rogosa-Sharpe (MRS) broth (Becton, Dickinson and Co., Sparks, MD), and bacterial populations were enumerated by serial dilution and plate counting on MRS agar. For each bacterium, a volume of the culture broth containing 10⁶ (experiment 1) or 10⁹ (experiment 2) cfu/mL of bacteria was centrifuged (3,000 × *g* for 10 min at 10°C). The resulting bacterial pellet was washed with 5 mL of Milli-Q (Millipore, Billerica, MA) water (Peltonen et al., 2001).

AFB₁ Working Solution. Solid AFB₁ (≥98% purity; Sigma, St. Louis, MO) was suspended in benzene-acetonitrile solution (97/3, vol/vol) to obtain an AFB₁ solution of approximately 2 mg/mL of AFB₁. The AFB₁ solution was added to PBS (pH 7.3) to prepare a 5 µg/mL of AFB₁ solution, and the benzene acetonitrile solution was evaporated by heating in a water bath (70°C, 5 to 10 min) under a fume hood.

AFB₁-Binding Assay. Bacterial pellets were suspended in 1.5 mL of AFB₁ solution (5 µg/mL of AFB₁ in PBS) for 24 h at 20°C in quadruplicate. A bacteria control (bacteria pellets suspended in PBS) and an AFB₁ control (5 µg/mL of AFB₁ in PBS) were also incubated. Subsequently, the suspension was centrifuged at 3,000 × *g* for 10 min at 10°C. Supernatant samples (200 µL) containing unbound toxin were frozen at –20°C and sent to the Missouri Veterinary Diagnostic Laboratory (Columbia) to quantify the presence of unbound aflatoxin by reverse phase HPLC (Haskard et al., 2001). The bacteria control was used as a blank and the AFB₁ control was used to quantify the total AFB₁ added.

Table 1. Percentage of aflatoxin B₁ (AFB₁) bound in an aqueous solution by applying 10⁶ (experiment 1) or 10⁹ (experiment 2) cfu/mL of silage bacteria¹

Bacteria species	Strain	Bacteria population, cfu/mL	
		10 ⁶	10 ⁹
<i>Lactobacillus plantarum</i>	R2014	0.83 ^{ab}	33.0 ^a
<i>L. plantarum</i>	EQ12	1.08 ^{ab}	28.4 ^{abc}
<i>L. plantarum</i>	PT5B	4.27 ^a	19.3 ^{cd}
<i>Lactobacillus buchneri</i>	R1102	0.04 ^{ab}	30.3 ^{ab}
<i>Pediococcus acidilactici</i>	R2142	0.00 ^b	23.9 ^{abcd}
<i>P. acidilactici</i>	EQ01	0.66 ^{ab}	25.4 ^{abcd}
<i>Pediococcus pentosaceus</i>	EQ44	0.23 ^{ab}	21.6 ^{bed}
<i>P. pentosaceus</i>	IA38	0.96 ^{ab}	18.2 ^d
<i>Propionibacterium jensenii</i>	SE253	0.00 ^b	19.7 ^{cd}
<i>Propionibacterium acidipropionici</i>	EQ42	1.82 ^{ab}	18.9 ^d
SEM		0.88	3.78
<i>P</i> -value		0.04	<0.001

^{a-d}Means within a column with different superscripts differ, $P < 0.05$.

¹Supplied by Lallemand Animal Nutrition, Milwaukee, WI.

Experiment 3: Effects of Bacterial Viability and the Prevailing pH on AFB₁ Binding

Bacteria Preparation. *Lactobacillus plantarum* R2014 (**Lp**), *L. buchneri* R1102 (**Lb**), and *Pediococcus acidilactici* EQ01 (**Pa**) were grown and centrifuged as described in experiment 1 and 2 to obtain bacterial pellets containing 10⁹ cfu/mL. The bacterial pellets were incubated for 1 h at 37°C in either 4 mL of PBS (viable cells) or 2 M HCl (dead cells). Dead cells were then washed twice with 4 mL of PBS. All bacterial samples were centrifuged (3,000 × *g* for 10 min at 10°C) and the supernatant was discarded.

AFB₁-Binding Assay. The AFB₁ working solution was prepared as described in experiment 1, except that 5 µg/mL of AFB₁ solution was prepared with HCl or NaOH instead of PBS to achieve pH values of 6, 2.5, and 8 to simulate the pH in the rumen, abomasum, and small intestine of dairy cows, respectively. The AFB₁-binding assay procedure was similar to that in experiment 1. The concentrations of AFB₁ in bacteria controls (bacteria pellets suspended in HCl or NaOH to achieve pH of 6, 2.5, and 8) and AFB₁ controls (5 µg/mL of AFB₁ in HCl or NaOH to achieve pH of 6, 2.5, and 8) were also measured.

Experiment 4: Effect of Acid and Heat Treatments on the Bacterial Population

The *Lp*, *Lb*, and *Pa* bacteria were prepared as described in experiment 1 and 2. Bacterial pellets were extracted from 1 mL of broth containing 10⁹ cfu/mL by centrifugation (3,000 × *g* for 10 min at 10°C). The pellets were incubated in 4 mL of PBS at 20 (control), 85 (heated cells), or 100°C (boiled cells) in a water bath.

Additional treatments involved incubating bacterial pellets at 20°C for 1 h in 4 mL of either concentrated (2 M HCl; pH <1) or weak (10^{-2.5} M HCl; pH 2.5) acid. Subsequently, the number of resulting bacteria was determined by making serial 10-fold dilutions in peptone water, pour plating aliquots (1 mL) in MRS agar (Becton, Dickinson and Co.), and incubating for 2 d at 30°C.

Experiment 5: Effects of the Ensiling Duration and LAB Treatment on Binding

AFB₁ Working Solution. Solid AFB₁ (≥98% purity; Sigma) was suspended in benzene-acetonitrile solution (97/3, vol/vol) to obtain an AFB₁ solution of approximately 2 mg/mL of AFB₁. The AFB₁ solution was added to PBS (pH 7.3) to prepare a 9 µg/mL of AFB₁ solution, and the benzene acetonitrile solution was evaporated off by heating in a water bath (70°C, 5 to 10 min).

Bacteria. The bacteria *Pa*, *Lb*, and *Lp* containing 3 × 10¹² cfu were incubated in quadruplicate for 1 h in 30 mL of PBS at either 37°C (viable cells) or 85°C (heated cells) or in 30 mL of HCl adjusted to pH 2.5 with HCl at 37°C (acid-treated cells). Subsequently, all bacteria were washed twice with PBS, centrifuged (3,000 × *g* for 10 min at 10°C) and resuspended in 25 mL of Milli-Q water (bacteria solution).

Forage Harvesting, Inoculating, and Ensiling. A corn hybrid was grown at the Dairy Research Unit, University of Florida, and harvested at 35% DM with a Claas Jaguar 900 (Claas of America LLC, Columbus, IN) forage harvester on December 5, 2013. The forage was chopped to achieve a theoretical length of 1.9 cm, thoroughly mixed, and subsampled (3 kg) into 14

$\times 21$ cm, thick-walled polyethylene bags. The forages were treated with 10 mL of deionized water (control) or 10 mL of the AFB₁ solution (containing 30 μ g/kg of the toxin by adding 10 mL of the 9 μ g/mL AFB₁ solution to 3 kg of corn forage), or with the AFB₁ solution and 1×10^9 cfu/g of fresh forage of each of the *Lp*, *Lb*, and *Pa* preparations that had been treated with or without the less-severe acid and heat treatments (37 (viable cells) or 85°C (heated cells) and acidification with HCl at pH 2.5 (acid-treated cells). The 1×10^9 cfu/g of fresh forage bacterial population was achieved by adding 3×10^{12} cfu of the bacteria to 3 kg of forage. The appropriate treatment cocktail was sprayed on each of 4 replicate samples of the forage within individual bags and mixed thoroughly, after which the bags were sealed and stored at ambient temperature (20°C) for 0, 1, 2, 3, 7, 14, and 21 d. At each of these opening times, a 100-g sample of the silage was taken for AFB₁ and chemical analysis and the silo bags were resealed after the air in the bags was evacuated with a vacuum pump (Zing, Bissell, Grand Rapids, MI). Samples for d 0 were taken 2 h after applying inoculants and aflatoxin and before sealing.

Laboratory Analyses. Silage fluid was extracted by blending 15 g of silage and 30 mL of distilled water in a stomacher (UL Lab-Blender 400, Seward Laboratory, London, UK) for 2 min. The fluid was filtered through 2 layers of cheesecloth, frozen at -20°C , and sent to the Missouri Veterinary Diagnostic Laboratory to quantify the presence of unbound aflatoxin by HPLC (Haskard et al., 2001). Concentrations of AFB₁ were only measured on samples taken after 0, 1, 2, and 3 d of ensiling because by 3 d, the concentrations were below the Food and Drug Administration Action Level for AFB₁ in dairy cattle feeds (20 μ g/kg; FDA, 2011).

Silage extracts for analysis of VFA, pH, and ammonia were prepared by blending 15 g of silage and 135 mL of distilled water for 2 min in the stomacher. Lactic acid and VFA concentrations were measured by HPLC (Muck and Dickerson, 1988). The pH was determined with a digital pH meter (Accumet AB15, Fisher Scientific, Hampton, NH). Ammonia-N was analyzed with a Technicon Auto Analyzer (Technicon, Tarrytown, NY) and an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric N quantification. Ammonia-N and VFA concentrations were analyzed only on samples ensiled for 21 d.

Subsamples of each replicate were dried in a forced-air oven at 60°C for 48 h and ground to pass the 1-mm screen of a Wiley mill (A. H. Thomas, Philadelphia, PA). The chemical composition of each replicate was measured in duplicate. Ash (600°C for 8 h), NDF, ADF, and CP were analyzed on samples taken at 0 and 21 d of ensiling. Concentrations of NDF and ADF were

determined using the method described by Van Soest et al. (1991) and AOAC International (2000), respectively, in an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat-stable α -amylase was used in the NDF assay but sodium sulfite was not, and the results are expressed exclusive of residual ash. Crude protein was measured by Dairy One Forage Laboratory (Ithaca, NY) using a Leco FP-528 Nitrogen/Protein Analyzer (Leco Corporation, St. Joseph, MI).

Statistical Analysis

Experiments 1, 2 and 3 were analyzed as completely randomized designs with 4 replicates per treatment. The statistical model for experiments 1 and 2 included the effect of the bacterial strain. Experiment 3 had a 3 (bacteria) $\times$ 2 (viabilities) $\times$ 3 (pH values) factorial treatment structure and the data were analyzed with a statistical model that included bacterial strain, viability, pH, and all interactions of these terms. Means within the same bacterial species were separated with the Tukey test. Polynomial contrasts were used to determine effects of increasing the pH after the IML procedure of SAS (ver. 9.1, SAS Institute Inc., Cary, NC) was used to generate coefficients for unequally spaced bacterial dose means.

Experiment 4 had a completely randomized design, a 3 (bacteria) $\times$ 5 (viability-reducing treatments) factorial treatment structure, and each treatment had 2 replicates. Data were analyzed with a statistical model that included bacterial strain, treatment and their interactions.

Experiment 5 had a completely randomized design with a 3 (bacteria) $\times$ 3 (processing methods) $\times$ 4 (silage sampling times for AFB₁) or 7 (silage sampling times for DM and pH) factorial treatment structure with positive (toxin) and negative (control) control treatments. Fermentation parameters were analyzed with a 3 $\times$ 3 factorial treatment arrangement because fermentation data were separately analyzed for samples taken on d 0 and 21. Data for AFB₁, pH, and DM were analyzed with a statistical model that included bacteria, processing method, silage sampling time, and all interactions of these terms. The model included a repeated statement and the TOEP covariance structure was used to account for repeated sampling over time because it generated lower Akaike information criteria than others. Data for fermentation parameters were analyzed with a similar model that excluded the sampling time effect. The PDIF statement of SAS was used for pairwise comparison of means from the same ensiling duration. The REGRESSION procedure of SAS was used to examine the relationship between ensiling duration and toxin concentration in silage extract. Treat-

ment effects on rates and intercepts generated from the regression, as well as those on the chemical composition and fermentation measures of the d-21 silage, were analyzed using the model mentioned above excluding the effect of and interactions with time. Polynomial contrasts were used to examine effects of ensiling duration. Coefficients for unequal spacing treatments were generated using the IML procedure of SAS. The following contrasts were used to examine the following treatment effects: control versus other treatments, control versus toxin, control versus *Lp* or *Lb* or *Pa*, and viable versus processed forms of each bacterium. Other than those for the regression in experiment 5, data were analyzed using the GLIMMIX procedure of SAS and means were separated with the Tukey test. Normality of the data was confirmed by examining the distribution of residuals using the normal probability, quantile-quantile, and predicted mean plots. Differences were declared at $P < 0.05$ and tendencies at $0.5 \geq P < 0.1$ in all experiments.

RESULTS AND DISCUSSION

Experiments 1 and 2

Binding of AFB₁ by 10^6 cfu/mL of silage bacteria in experiment 1 was generally low (0 to 4.27%) compared with studies conducted with other gram-positive bacteria (Table 1; El-Nezami et al., 1998; Hernandez-Mendoza et al., 2009a). Only *L. plantarum* PT5B bound ($P < 0.05$; 4.27%) AFB₁ significantly. The percentage of AFB₁ bound by all other silage bacteria did not differ ($P > 0.05$) from 0%. The generally low AFB₁ binding at 10^6 cfu/mL may be due to the low population of silage bacteria used for the test. El-Nezami et al. (1998) reported that a *L. rhamnosus* population of 2×10^9 cfu/mL or greater was necessary for binding ($P < 0.05$) of AFB₁.

When the silage bacteria were applied at 10^9 cfu/mL in experiment 2, each bacterium exhibited some AFB₁ binding (18 to 33%), but the results were strain-specific ($P < 0.001$; Table 1). This result agrees with previous research, in which the AFB₁ bound by 10^9 cfu/mL of 8 strains of *L. casei* varied from 14 to 49% (Hernandez-Mendoza et al., 2009a). Reasons for the strain-specificity of AFB₁ binding are unknown but differences in cell wall components, particularly peptidoglycan, may be implicated (Lahtinen et al., 2004).

Lactobacillus plantarum R2014 and EQ12, *L. buchneri* R1102, and *Pr. acidilactici* R2142 and EQ01 achieved among the greatest AFB₁-binding capacities (33.0, 28.4, 30.3, 23.9, and 25.4%, respectively), whereas *Pr. pentosaceus* IA38 and *Propionibacterium acidipropionici* EQ42 had among the lowest (18.2 and 18.9%, respectively). Of the former, *L. plantarum* R2014, *L. buchneri*

R1102, and *Pr. acidilactici* EQ01 were examined further because of their high binding capacities and wide use and efficacy as silage inoculants.

Experiment 3

Binding of AFB₁ depended on the bacterial species and viability and the prevailing pH (pH $\times$ viability $\times$ species, $P < 0.001$; Table 2). The greatest binding of AFB₁ within a bacterium was achieved by dead *Lp* and *Lb* and viable *Pa* at pH 2.5 (60.5, 66.5, and 56.9%, respectively), whereas dead *Pa* at pH 8 bound no AFB₁ (0%). More AFB₁ was bound across pH and viability treatments by *Lp* and *Lb* compared with *Pa* (28.7 and 30.5 vs. 12.2%, respectively; $P < 0.001$). Binding efficacy generally decreased in a quadratic manner as the acidity of the culture media decreased. Binding of AFB₁ was greater by dead versus viable *Lp* and *Lb* (32.6 vs. 24.8% and 43.3 vs. 17.8%, respectively), but viable *Pa* was more effective than dead *Pa* (21.92 vs. 2.44%).

The basis of AF binding by bacteria is not well understood. El-Nezami et al. (1998) suggested that the AFB₁-binding capacity of bacteria depends on bacterial cell wall structure because gram-positive bacteria were more effective at binding aflatoxin than *Escherichia coli*, which is gram-negative. Polysaccharides and peptidoglycan are 2 of the main cell wall components considered responsible for binding mutagens by LAB (Zhang and Ohta, 1991). Haskard et al. (2001) examined the AFB₁ binding ability of 12 LAB strains and noted that nonviable bacteria (heat- and acid-treated) bound and retained greater amounts of AFB₁ than viable bacteria. Haskard et al. (2001) suggested that peptidoglycan and polysaccharides were likely greatly affected by heat and acid treatments because heat may denature protein or induce formation of Maillard reaction products between polysaccharides and peptides or proteins, and acids may break glycosidic linkages in polysaccharides or the amide linkages in peptides or proteins. Therefore, they concluded that these treatments likely perturbed the cell wall and reduced its integrity, thus facilitating binding of the toxin to cell wall peptidoglycan and polysaccharides. The *Lb* and *Pa* in our study have the same type of peptidoglycan (L-lysine-D-aspartic acid), whereas that of *Lp* is the meso-diaminopimelic acid-direct type (Schleifer and Kandler, 1972). However, dead *Lb* and *Lp* and viable *Pa* were the most promising treatments in our study. This suggests that type of cell wall peptidoglycan did not fully explain their different effects on binding of AFB₁. Hernandez-Mendoza et al. (2009b) suggested that teichoic acid in the bacterial cell wall may be necessary for binding AFB₁, as its absence reduced the binding capacity. In contrast, Lahtinen et al. (2004) reported that teichoic acid does not partici-

Table 2. Effects of species (B), viability (V) and the prevailing pH on the percentage of aflatoxin B₁ (AFB₁) bound by 3 silage bacteria (experiment 3)

pH	Bacterial species, ¹ AFB ₁ bound, %									
	<i>Lp</i>			<i>Lb</i>			<i>Pa</i>			<i>P</i> -value
	Viable	Dead	SEM	Viable	Dead	SEM	Viable	Dead	SEM	
2.5	56.2 ^b	60.5 ^a	51.5 ^b	66.5 ^a	2.91 ^{bc}	0.57	56.9 ^a	2.91 ^{bc}	0.57	0.001
6.0	10.1 ^e	15.8 ^d	1.47 ^d	34.1 ^c	4.40 ^{bc}	0.57	6.76 ^b	4.40 ^{bc}	0.57	
8.0	8.05 ^e	21.6 ^c	0.32 ^d	29.2 ^c	0.00 ^c	0.58	2.05 ^c	0.00 ^c	0.58	
SEM	0.80	0.80	0.80	0.85	0.80	0.80	0.80	0.80	0.80	
Contrast ²	L, Q	L, Q	L, Q	L, Q	L, Q†	L, Q	L, Q	L, Q	L, Q	

^{a-e}Means within a bacterium with different superscripts differ, $P < 0.05$.

¹*Lp* = *Lactobacillus plantarum* R2014, *Lb* = *Lactobacillus buchneri* R1102, and *Pa* = *Pediococcus acidilactici* EQ01. Each bacterium was supplied by Lallemand Animal Nutrition, Milwaukee, Wisconsin, and applied at 10^9 cfu/mL.

²Linear (L), quadratic (Q) effects ($P < 0.05$); Qt = quadratic trend ($P < 0.10$).

pate in the binding process. More research is needed to identify and elucidate the cell wall components that enable binding of AFB₁ by bacteria.

It is noteworthy that dead *Lb* and *Lp* bacteria were more effective at binding AFB₁ than the viable forms at each pH. Acid treatment likely damaged the cell wall integrity of bacteria, thus allowing binding or degradation of AFB₁ by intracellular components.

That increasing acidification of the culture medium increased binding of AFB₁ may be attributable to additional transformation of AFB₁ to aflatoxin B_{2a} by LAB in the increasingly acidic environment (Megalla and Hafez, 1982). The results of our study contrast with a report (Haskard et al., 2001) that AFB₁ binding by *L. rhamnosus* did not differ as pH increased from 2 to 10. This discrepancy may be due to the differing effects of acidification on cell wall components of different bacterial species.

Experiment 4

In experiment 3, killing the cells with 2 M HCl enhanced the AFB₁ binding ability of *Lp* and *Lb*; however, dead forms of these bacteria cannot ferment substrates and improve forage preservation during ensiling. Therefore, this experiment was designed to examine effects of severe or benign methods of altering the viability of the bacteria on their population with the assumption the benign methods will increase bacterial binding of AFB₁ without killing them or excessively curtailing their population. The viability of the bacteria depended on processing treatment, species, and their interaction (Table 3; $P < 0.001$). All treatments reduced the bacteria population relative to the control treatment considerably. The 2 M HCl treatment killed all the bacteria, as in experiment 3. Heating bacteria at 85°C decreased the population from 9 to <5 log cfu/mL, representing up to a 45% reduction. Boiling the cells at 100°C prevented the growth of *Lp* and reduced the population of *Lb* and *Pa* by approximately 6 log cfu/mL, which amounts to a reduction of over 70%. Ding and Shah (2007) also reported that incubation of *L. plantarum* at 65°C instead of room temperature (22°C) for 1 h reduced the population by 6 log cfu/mL. Charalampopoulos et al. (2003) reported that reducing the culture pH of a phosphate-buffered medium from 7 to 2.5 dramatically reduced the cell density of *L. plantarum* from 10^{10} to less than 10^5 cfu/mL.

Acid tolerance varies between bacteria species; for instance, some species of *Lactobacillus* can grow at pH 4.4, but others cannot (Axelsson, 2004). In our study, *Lp* was more acid tolerant when treated with pH 2.5 HCl than *Lb* and *Pa* (4.36 vs. 2.95 and 2.36 log cfu/mL, respectively; $P < 0.05$). Differences in acid tolerance by

Table 3. Effects of viability-altering method on the population of bacteria in experiment 4

Bacteria, ¹ log cfu/mL	Viability-altering method					SEM	P-value		
	Control	pH 2.5 ²	pH <1 ³	85°C	100°C		Bacteria	Method	Bacteria × method
<i>Lp</i>	9.05 ^a	4.36 ^{b,x}	<1 ^d	2.48 ^{c,y}	<1 ^{d,y}	0.08	<0.001	<0.001	<0.001
<i>Lb</i>	9.12 ^a	2.95 ^{c,y}	<1 ^d	4.87 ^{b,x}	2.34 ^{c,x}	0.05			
<i>Pa</i>	9.05 ^a	2.36 ^{c,y}	<1 ^d	4.73 ^{b,x}	2.60 ^{c,x}	0.10			
SEM	0.05	0.14	0.0	0.05	0.07				

^{a-d}Means within a row with different superscripts differ, $P < 0.05$.

^{x,y}Means within a column with different superscripts differ, $P < 0.05$.

¹Bacteria (10⁹ cfu/mL): *Lp* = *Lactobacillus plantarum* R2014, *Lb* = *Lactobacillus buchneri* R1102, and *Pa* = *Pediococcus acidilactici* EQ01.

²pH 2.5 was achieved using 10^{-2.5} M HCl.

³pH <1 was achieved using 2 M HCl.

bacteria have been attributed to the variation among bacteria in the synthesis of proteins and mechanisms involved in acid response (van de Guchte et al., 2002).

Based on the resulting bacterial populations, the most promising viability altering treatments that are likely to maintain the beneficial effects of bacterial inoculation on silage preservation are acid treatment at pH 2.5 for *Lp* and heating to 85°C for *Lb* and *Pa*. Both of these treatments warrant further examination because they maintained populations of at least 2.4 log cfu/mL across species. The results of experiment 3 were that dead cells of *Lp* and *Lb* and viable cells of *Pa* were more effective at binding AFB₁ at low pH. In this experiment, heating the bacteria at 85°C killed about 6.5 log cfu/mL of *Lp* and maintained populations of approximately 5 log cfu/mL of *Lb* and *Pa*. Therefore, this treatment may have been the best at maintaining the viability of *Pa* and killing sufficient *Lp* and *Lb* to increase AFB₁ binding and maintaining sufficient bacterial populations to elicit their desired effects on silage preservation. The effects on silage fermentation and AFB₁ sequestration of treating the bacteria with heat at 85°C or acid at pH 2.5 were examined in experiment 5.

Experiment 5

To evaluate if the LAB could sequester AF in the real world, we tested the binding of AFB₁ by LAB in artificially contaminated corn silage. To our knowledge, this is the first study that used bacteria to reduce AF concentration in animal feed. Also, silage bacteria were used in our study, which could potentially benefit animals by both decreasing AF in feed and improving silage nutritional quality and preservation.

Chemical Composition. The chemical composition of the chopped corn forage did not differ among treatments (Table 4). The summer-grown corn forage had relatively higher NDF and ADF and lower CP concentrations compared with spring-grown hybrids in

Florida (Kang et al., 2009; Arriola et al., 2011), partly reflecting typical effects of high temperatures on forage chemical composition (Romero, et al., 2013) as well as an atypical 4-wk period of low precipitation before the corn was harvested.

Changes in pH and DM During Ensiling. During ensiling, the DM concentration (Table 5) of the control silage decreased in a quadratic manner, whereas that of the toxin and *Lp* silages decreased linearly ($P < 0.10$) and those of *Lb* and *Pa* silages did not differ. Concentrations of DM did not differ among forages ensiled for the same duration except on d 2, when values for control and toxin silages tended to be the least ($P = 0.06$), and on d 7, 14, and 21, when the control silages had or tended to have greater values than most other ensiled forages ($P < 0.10$). The DM concentration of the toxin silage decreased at a faster rate than that of control.

All pH values declined quadratically during ensiling (Table 5). By d 3 after ensiling, the pH of silages inoculated with bacteria had decreased to approximately 4.5 or less, but it took 7 and 14 d for the toxin and control silages to achieve these values, respectively. Therefore, bacterial inoculation increased the rate of acidification of the silages. This response differed with the bacteria species and processing treatment. The viable *Lp* silage consistently had lower pH values than the control silage through d 3, and it tended to have the least ($P = 0.06$) pH after 21 d of ensiling, whereas other inoculated bacteria only had lower pH values than control silages through d 1. *Lactobacillus plantarum* is perhaps the most widely used bacteria in silage inoculants because it meets virtually all of the criteria defined by Whittenbury (1961) for ideal silage inoculants. These include growing vigorously, competing with and dominating other organisms, using a homofermentative pathway, acid tolerance, fermenting hexose and pentose sugars, and having no action on organic acids (McDonald et al., 1991). In our study, homofermentative *Lp* seems to have dominated the fermentation, leading to the rapid

pH decrease within 3 d of ensiling and maintenance of this low pH for 21 d. This likely reflects efficient homofermentation of sugars to lactic acid by *Lp*.

AFB₁ Reduction. Certain LAB strains have reduced the AFB₁ concentration of aqueous solutions (El-Nezami et al., 1998; Fazeli et al., 2009). However, to our knowledge, the ability of either such bacteria or silage bacteria to bind aflatoxin in silage never has been examined. In our study, no treatment $\times$ time interaction ($P = 0.22$) was detected for the concentration of AFB₁ in silages (Table 6). The concentrations of AFB₁ in d 0 silages were less than 2 $\mu\text{g/kg}$ despite addition of a PBS solution containing 30 $\mu\text{g/kg}$ of AFB₁ 2 h earlier. This rapid reduction of the toxin concentration may have been due to immediate binding of the toxin by bacteria, but the causes and mode of action are unclear. More than 70% of AFB₁ was bound immediately when LAB were added to a solution containing 5 $\mu\text{g/mL}$ of AFB₁ (El-Nezami et al., 1998). Binding of AFB₁ by bacteria has been attributed to cell wall polysaccharides and peptidoglycans, particularly their AA (Haskard et al., 2001; Niderkorn et al., 2007).

During ensiling, the AFB₁ concentration decreased linearly with time across treatments, though a quadratic trend also was evident in the heated *Lp* silage (Table 6). The relationship between ensiling duration and AFB₁ concentration ranged from being weak for the acid-treated *Lp* silage ($R^2 = 0.31$) to being moderately strong for the heated *Lp* silage ($R^2 = 0.72$; $P < 0.01$); however, neither intercepts nor rates of the rela-

tionships differed among treatments, which indicates that LAB application did not increase the rate of AFB₁ removal from the silage. This is probably because epiphytic bacteria were as effective as inoculated bacteria at binding the 30 $\mu\text{g/kg}$ of AFB₁ added to the forage at ensiling.

In our study, a considerable amount of AFB₁ may have been degraded by the acidity of the silage fluid. Lindenfelser and Ciegler (1970) reported that 4 $\mu\text{g/mL}$ of AFB₁ was converted to aflatoxin B_{2a} in 7 d when mixed in a 1 *M* lactic acid solution. Therefore, such acid-induced degradation may have occurred during ensiling in our study. Further studies are needed to identify the dominant method of AFB₁ removal and or degradation in silage.

Across processing methods, the *Lp* silage had similar AFB₁ concentration to the toxin silage (0.34 ppb vs. 0.39 ng/mL; Table 6), whereas surprisingly, and for unknown reasons, *Lb* and *Pa* silages across processing methods had greater AFB₁ concentration than the toxin silage (0.48 and 0.52 vs. 0.34 ng/mL, respectively). This could be due to instability of the bacterial-AFB₁ complex formed early in the ensiling process, assuming that similar amounts of the toxin were bound by the different bacteria earlier. Haskard et al. (2001) reported that washing bacterial-AFB₁ complexes with Milli-Q water released AFB₁ and the stability of the complexes varied with the bacterial strain.

Bacterial viability had no effect on the AFB₁ concentration of *Lb* and *Lp* silages ($P > 0.10$), but the forage

Table 4. Chemical composition of chopped whole corn plants treated with or without aflatoxin B₁ and bacteria subjected to various viability-altering processes (experiment 5; % of DM or as stated)

Additive ¹	Processing method ²	DM, %	Ash	CP	ADF	NDF
Control		36.3	5.44	7.45	33.5	61.7
Toxin		36.4	4.46	7.18	32.1	60.3
Bacteria species ³						
<i>Lp</i>	Viable	37.3	5.13	7.63	32.2	60.4
	Heat	35.8	4.91	7.78	34.5	54.0
	Acid	37.1	4.82	7.93	29.6	55.2
<i>Lb</i>	Viable	36.4	5.15	7.63	38.9	57.1
	Heat	36.4	4.71	7.3	36.8	55.8
	Acid	33.6	4.87	7.18	28.8	61.3
<i>Pa</i>	Viable	35.1	4.81	7.43	40.6	61.9
	Heat	34.8	4.54	7.18	32.3	53.8
	Acid	34.0	4.81	7.53	32.1	64.8
SEM		1.01	0.22	0.20	2.71	2.83
<i>P</i> -value		0.20	0.13	0.13	0.13	0.12

¹Exactly 30 μg of toxin/kg of fresh forage was prepared in PBS 12 h earlier and applied with or without 10^9 cfu/mL of the relevant bacteria at ensiling (0 h); control = silage treated with only milli-Q water; toxin = silage treated with water and toxin solution.

²Viable = viable cells obtained by incubating in PBS at 37°C; heat = heated cells obtained by incubating in PBS at 85°C; acid = acid-treated cells obtained by incubating in pH 2.5 HCl at 37°C.

³*Lp* = *Lactobacillus plantarum*; *Lb* = *Lactobacillus buchneri*; *Pa* = *Pediococcus acidilactici*. The toxin solution was added to all bacterial treatments.

Table 5. Changes in the DM concentration and pH during ensiling of corn forage treated with or without aflatoxin B₁ and bacteria subjected to various viability-altering processes (experiment 5)

Additive ¹	Processing method ²	Ensiling duration, d							SEM	<i>P</i> -value contrast ³
		0	1	2	3	7	14	21		
DM, %										
Control		36.3	34.7	34.2	37.2	38.1	38.7	36.0 ^{ab}	1.26	Q
Toxin		36.4	34.8	34.2	35.6	34.8	34.7	32.7 ^b	0.75	L
Bacteria species ⁴										
<i>Lp</i>	Viable	37.3	37.8	39.2	35.3	36.7	35.4	34.6 ^{ab}	1.02	L
	Heat	35.8	37.2	38.2	35.6	37.0	34.4	35.5 ^{ab}	0.86	Lt
	Acid	37.1	35.7	37.4	36.3	37.0	33.8	35.5 ^{ab}	1.07	Lt
<i>Lb</i>	Viable	36.4	35.8	37.5	38.7	37.5	35.6	34.6 ^{ab}	1.25	NS
	Heat	36.4	37.1	35.4	36.0	37.2	36.1	35.6 ^{ab}	1.07	NS
	Acid	33.6	35.4	35.0	35.3	34.5	34.4	33.9 ^{ab}	1.07	NS
<i>Pa</i>	Viable	35.1	36.5	34.8	37.0	34.3	35.5	34.8 ^{ab}	0.87	NS
	Heat	34.8	37.0	36.4	36.1	34.2	36.5	37.2 ^a	0.78	NS
	Acid	34.0	37.1	34.9	36.3	33.8	36.3	34.7 ^{ab}	0.67	NS
SEM		1.01	0.87	1.21	0.91	1.08	1.00	0.78		
<i>P</i> -value		0.20	0.20	0.06	0.32	0.05	0.09	0.04		
pH										
Control		6.98 ^a	5.16 ^a	4.71 ^{ab}	4.62 ^{ab}	4.59 ^a	4.19	4.39	0.14	L, Q
Toxin		6.50 ^{abc}	5.23 ^a	4.82 ^a	4.78 ^a	4.45 ^{ab}	4.21	4.23	0.12	L, Q
Bacteria species ⁴										
<i>Lp</i>	Viable	6.00 ^c	4.39 ^c	4.30 ^c	4.31 ^c	4.26 ^{ab}	4.14	3.98	0.07	L, Q
	Heat	6.68 ^{ab}	4.88 ^{ab}	4.48 ^{bc}	4.33 ^{bc}	4.20 ^{ab}	4.04	4.13	0.08	L, Q
	Acid	6.20 ^{bc}	4.53 ^{bc}	4.48 ^{bc}	4.46 ^{abc}	4.40 ^{ab}	4.25	4.19	0.10	L, Q
<i>Lb</i>	Viable	6.20 ^{bc}	4.66 ^{bc}	4.41 ^{bc}	4.37 ^{bc}	4.32 ^{ab}	4.13	4.17	0.06	L, Q
	Heat	6.44 ^{abc}	4.85 ^{ab}	4.57 ^{abc}	4.47 ^{abc}	4.29 ^{ab}	4.10	4.18	0.10	L, Q
	Acid	6.18 ^{bc}	4.68 ^{bc}	4.49 ^{abc}	4.42 ^{abc}	4.27 ^{ab}	4.15	4.12	0.07	L, Q
<i>Pa</i>	Viable	6.21 ^{bc}	4.51 ^{bc}	4.56 ^{abc}	4.39 ^{bc}	4.28 ^{ab}	4.15	4.14	0.06	L, Q
	Heat	6.33 ^{bc}	4.81 ^{ab}	4.43 ^{bc}	4.33 ^{bc}	4.13 ^b	4.06	4.17	0.09	L, Q
	Acid	5.99 ^c	4.60 ^{bc}	4.49 ^{abc}	4.49 ^{abc}	4.42 ^{ab}	4.35	4.57	0.09	L, Q
SEM		0.13	0.08	0.07	0.06	0.09	0.06	0.11		
<i>P</i> -value		0.01	0.001	0.01	0.01	0.04	0.05	0.06		

^{a-c}Means in the same column with different superscripts differed ($P < 0.05$).

¹Exactly 30 µg of toxin/kg fresh forage was prepared in PBS 12 h earlier and applied with or without 10⁹ cfu/mL of the relevant bacteria at ensiling (0 h); control = silage applied with milli-Q water only; toxin = silage applied with water and toxin solution.

²Viable = viable cells obtained by incubating in PBS at 37°C; heat = heated cells obtained by incubating in PBS at 85°C; acid = acid-treated cells obtained by incubating in pH 2.5 HCl at 37°C.

³Polynomial contrasts: L and Q = linear and quadratic effects, respectively ($P < 0.05$); Lt = linear trend ($P < 0.10$); NS ($P > 0.10$).

⁴*Lp* = *Lactobacillus plantarum*; *Lb* = *Lactobacillus buchneri*; *Pa* = *Pediococcus acidilactici*. The toxin solution was added to all bacterial treatments.

treated with viable *Pa* organisms had a greater AFB₁ concentration than heated or acid-treated *Pa* silages ($P = 0.01$). Others also have reported that effects of bacterial viability on binding of AFB₁ are species- and strain-dependent (Haskard et al., 2001), but the underlying reasons for these differences are unknown. Further studies are required to examine binding of AFB₁ in contaminated silage by silage bacteria and to elucidate the mechanisms involved in AFB₁ binding to optimize the response.

Silage Chemical Composition. The toxin-treated silage had less DM than control, *Lp*, *Lb*, and *Pa* silages ($P \leq 0.04$; Table 7), less CP than the control silage ($P = 0.03$), and more NDF than inoculated silages ($P = 0.01$). This indicates that the toxin reduced the concentration of a desirable nutrient and made the silage more

fibrous and hence more prone to limit DMI in cattle. Bacterial viability did not affect any of the chemical component concentrations.

Silage Fermentation Parameters. The *Lp* silage had greater lactic acid concentration than the toxin silage ($P = 0.01$; Table 8). Viable *Lp* and *Pa* silages had greater lactic acid concentrations compared with nonviable *Lp* ($P = 0.01$) and *Pa* ($P = 0.03$) silages, respectively, showing that treating the homofermentative bacterial cells with heat and acid decreased the production of lactic acid. Acetic and propionic acids and ammonia concentrations did not differ among treatments ($P > 0.1$), except that the viable *Pa* silage had greater ammonia ($P = 0.02$) and total VFA ($P = 0.06$) concentrations than heated or acid-treated *Pa* silages. Toxin silages had the greatest butyric acid concentration, and

Table 6. Effect of the ensiling duration on concentrations of the aflatoxin B₁ (µg/kg of fresh forage) in corn silage treated at ensiling with or without 30 µg/kg of the toxin and bacteria that had been subjected to different viability-altering processes (experiment 5)

Additive ¹	Process method ²	Ensiling duration, h					SEM	Polynomial contrast ³	Intercept ⁴	Intercept P-value	Rate ⁴	Rate P-value	R ²	Contrast	Contrast P-value ⁵
		0	24	48	72	ND									
Control (Con)		ND ⁶	ND	ND	ND	ND									
Toxin (Tox)		0.58	0.41	0.15	0.22	0.22	0.07	L	0.54	0.01	-0.13	0.01	0.62	Con vs. Tox	<0.001
Bacteria species ⁷														Con vs. others	<0.001
<i>Lp</i>	Viable	0.61	0.68	0.27	0.26	0.26	0.08	L	0.65	0.001	-0.15	0.001	0.48	Tox vs. <i>Lp</i>	0.43
	Heat	0.50	0.35	0.14	0.17	0.17	0.03	L, Q	0.45	0.01	-0.12	0.01	0.72	Tox vs. <i>Lb</i>	0.05
	Acid	0.65	0.53	0.32	0.26	0.26	0.14	L	0.65	0.001	-0.13	0.01	0.31	Tox vs. <i>Pa</i>	0.01
<i>Lb</i>	Viable	0.80	0.37	0.44	0.30	0.30	0.11	L	0.77	0.001	-0.18	0.01	0.46	<i>Lb</i> viability ⁸	0.98
	Heat	1.05	0.47	0.39	0.24	0.18	0.18	L	0.87	0.001	-0.23	0.001	0.45	<i>Lp</i> viability	0.19
	Acid	0.69	0.46	0.31	0.20	0.13	0.13	L	0.71	0.001	-0.19	0.01	0.40	<i>Pa</i> viability	0.01
<i>Pa</i>	Viable	1.15	0.59	0.46	0.35	0.35	0.12	L	0.95	0.001	-0.22	0.01	0.62		
	Heat	0.81	0.52	0.35	0.09	0.18	0.18	L	0.79	0.001	-0.23	0.001	0.44		
	Acid	0.73	0.61	0.38	0.15	0.10	0.10	L	0.82	0.001	-0.22	0.001	0.66		

¹Exactly 30 µg of toxin/kg fresh forage was prepared in PBS 12 h earlier and applied with or without 10⁹ cfu/mL of the relevant bacteria at ensiling (0 h); control = silage applied with milli-Q water only; toxin = silage applied with water and toxin solution.

²Viable = viable cells obtained by incubating in PBS at 37°C; heat = heated cells obtained by incubating in PBS at 85°C; acid = acid-treated cells obtained by incubating in pH 2.5 HCl at 37°C.

³Linear (L) and quadratic (Q) effects ($P < 0.05$).

⁴No treatment effects on the intercept and rate were detected ($P > 0.1$). Rate and intercept SEM values were 0.06 and 0.14, respectively.

⁵Treatment × time interaction, $P = 0.22$.

⁶ND = not detected.

⁷*Lp* = *Lactobacillus plantarum*; *Lb* = *Lactobacillus buchneri*; *Pa* = *Pediococcus acidilactici*. The toxin solution was added to all bacterial treatments.

⁸Viability = viable vs. nonviable forms.

Table 7. Effect of applying bacteria subjected to different viability-altering processes to corn forage treated with aflatoxin B₁ on the silage chemical composition after 21 d of ensiling (experiment 5; % of DM or as stated)

Additive ¹	Processing method ²	DM, %	Ash	CP	ADF	NDF
Control		36.0	5.33	8.83	34.5	55.2
Toxin		32.7	5.12	8.25	34.9	58.9
Bacteria species ³						
<i>Lp</i>	Viable	34.6	4.80	8.48	33.0	50.4
	Heat	35.5	5.48	8.43	28.5	50.4
	Acid	35.5	4.62	8.30	29.3	46.7
<i>Lb</i>	Viable	34.6	5.08	8.20	33.5	50.7
	Heat	35.6	4.94	8.30	33.9	54.8
	Acid	33.9	4.69	8.43	34.1	50.9
<i>Pa</i>	Viable	34.8	5.26	8.28	33.7	53.5
	Heat	37.2	5.21	8.78	33.8	52.5
	Acid	34.7	4.75	8.03	36.0	50.2
SEM		0.78	0.20	0.18	2.76	1.92
<i>P</i> -value		0.04	0.02	0.12	0.72	0.01
Contrast <i>P</i> -values						
Control vs. others		0.19	0.08	0.02	0.63	0.11
Control vs. toxin		0.01	0.39	0.03	0.91	0.18
Toxin vs. <i>Lb</i>		0.04	0.31	0.78	0.74	0.01
Toxin vs. <i>Lp</i>		0.01	0.47	0.48	0.15	0.01
Toxin vs. <i>Pa</i>		0.00	0.84	0.61	0.89	0.01
<i>Lb</i> viability ⁴		0.91	0.25	0.47	0.88	0.38
<i>Lp</i> viability		0.36	0.24	0.62	0.23	0.44
<i>Pa</i> viability		0.26	0.20	0.58	0.71	0.36

¹Exactly 30 µg of toxin/kg fresh forage was prepared in PBS 12 h earlier and applied with or without 10⁹ cfu/mL of the relevant bacteria at ensiling (0 h); control = silage applied with milli-Q water only; toxin = silage applied with water and toxin solution.

²Viable = viable cells obtained by incubating in PBS at 37°C; heat = heated cells obtained by incubating in PBS at 85°C; acid = acid-treated cells obtained by incubating in pH 2.5 HCl at 37°C.

³*Lp* = *Lactobacillus plantarum*; *Lb* = *Lactobacillus buchneri*; *Pa* =, *Pediococcus acidilactici*. The toxin solution was added to all bacterial treatments.

⁴Viability = viable vs. nonviable forms.

based on the standard error of the means all inoculated silages except the acid-treated *Pa* silage had lower concentrations than the control silage. Toxin silages also had a more rapid reduction in DM concentration and a slower decrease in pH within the first 3 d of ensiling than most other silages. These responses may be attributable to AFB₁ binding by considerable numbers of the epiphytic LAB, such that few of them were available for or effective at fermenting the silage properly.

It is interesting to note that the acetic acid concentrations of the *Lb* silage were not greater than those of the control silage. Being heterofermentative, *L. buchneri* typically ferments sugars to acetic acid, ethanol, and carbon dioxide and ferments lactic to acetic acid (McDonald et al., 1991); however, such fermentations usually require much longer ensiling durations (>50 d; Kleinschmit and Kung, 2006). Thus, it is possible that the 21 d fermentation in our study was too short for *Lb* to affect acetic acid concentration. Also, inoculation with *Pa* typically increases the silage acidification rate (Fitzsimons et al., 1992), but that was not evident in our study.

CONCLUSIONS

This study showed that high concentrations of silage bacteria can bind AFB₁ in vitro, but the binding varies with the population, strain, viability, and medium acidity. Application of AFB₁ to ensiled corn forage reduced the rate of acidification in the first 3 d of ensiling, increased the rate of DM reduction, reduced the CP concentration of the silage, and increased its butyric acid and NDF concentration relative to the control treatment. Inoculation with bacteria prevented adverse effects of the toxin on silage butyric acid and NDF concentration. The AFB₁ concentration of corn forage spiked with the toxin decreased linearly during ensiling across treatments and was reduced to a safe level for feeding within 72 h. Inoculation with bacteria did not affect the rate of AFB₁ removal from the silage. Future work should examine if bacterial inoculants are effective at binding the toxin when silage is contaminated with greater (>30 µg/kg) concentrations of AFB₁ and whether silage bacteria physically sequester or chemically degrade AFB₁.

Table 8. Effect of applying bacteria subjected to different viability-altering processes to corn forage spiked with aflatoxin B₁ on the silage fermentation parameters after 21 d of ensiling (experiment 5; % of DM or as stated)

Additive ¹	Processing method ²	pH	Ammonia, % of TN ³	Lactic	Acetic	Propionic	Butyric	Total VFA
Control		4.39	2.18	1.52	2.97	0.52	0.37	6.52
Toxin		4.23	2.83	1.17	2.97	0.47	0.62	8.44
Bacteria species ⁴								
<i>Lp</i>	Viable	3.98	2.65	3.97	3.50	0.33	0.09	11.7
	Heat	4.13	2.5	2.77	3.34	0.36	0.09	10.3
	Acid	4.19	2.66	2.55	4.11	0.53	0.10	9.77
<i>Lb</i>	Viable	4.17	2.27	0.8	2.90	0.48	0.12	7.08
	Heat	4.18	2.09	2.38	2.38	0.42	0.14	7.81
	Acid	4.12	2.57	0.78	3.04	0.39	0.09	7.87
<i>Pa</i>	Viable	4.14	3.20	2.37	3.34	0.35	0.13	8.51
	Heat	4.17	2.11	2.09	2.28	0.32	0.08	6.92
	Acid	4.57	2.38	0.52	2.47	0.50	0.18	4.49
SEM		0.11	0.31	0.38	0.44	0.11	0.05	1.29
<i>P</i> -value		0.06	0.33	0.001	0.15	0.87	0.001	0.02
Contrast <i>P</i> -value								
Control vs. others		0.08	0.29	0.31	0.89	0.39	0.001	0.20
Control vs. toxin		0.30	0.15	0.56	0.99	0.78	0.001	0.27
Toxin vs. <i>Lb</i>		0.54	0.16	0.77	0.68	0.74	0.001	0.52
Toxin vs. <i>Lp</i>		0.29	0.54	0.01	0.14	0.61	0.001	0.12
Toxin vs. <i>Pa</i>		0.63	0.46	0.34	0.55	0.53	0.001	0.18
<i>Lb</i> viability ⁵		0.92	0.88	0.10	0.73	0.56	0.89	0.62
<i>Lp</i> viability		0.17	0.86	0.01	0.67	0.42	0.90	0.26
<i>Pa</i> viability		0.10	0.02	0.03	0.05	0.65	0.94	0.06

¹Exactly 30 µg of toxin/kg fresh forage was prepared in PBS 12 h earlier and applied with or without 10⁹ cfu/mL of the relevant bacteria at ensiling (0 h); control = silage applied with milli-Q water only; toxin = silage applied with water and toxin solution.

²Viable = viable cells obtained by incubating in PBS at 37°C; heat = heated cells obtained by incubating in PBS at 85°C; acid = acid-treated cells obtained by incubating in pH 2.5 HCl at 37°C.

³TN = total nitrogen.

⁴*Lp* = *Lactobacillus plantarum*; *Lb* = *Lactobacillus buchneri*; *Pa* = *Pediococcus acidilactici*. The toxin solution was added to all bacterial treatments.

⁵Viability = viable vs. nonviable forms.

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