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Inhibition by Antimicrobial Food Additives of Ochratoxin A Production by *Aspergillus sulphureus* and *Penicillium viridicatum*

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The effects of antimicrobial food additives on growth and ochratoxin A production by *Aspergillus sulphureus* NRRL 4077 and *Penicillum viridicatum* NRRL 3711 were investigated. At pH 4.5, growth and toxin production by both *A. sulphureus* and *P. viridicatum* were completely inhibited by 0.02% potassium sorbate, 0.067% methyl paraben, 0.0667% methyl paraben, and 0.2% sodium propionate. At pH 5.5, 0.134% potassium sorbate and 0.067% methyl paraben completely inhibited growth and ochratoxin A production by both fungi. Sodium bisulfite at 0.1%, the maximum level tested, was found to inhibit growth of *A. sulphureus* and *P. viridicatum* by 45 and 89%, respectively. Toxin production was inhibited by 97 and 99%, respectively. Sodium propionate (0.64%) at pH 5.5 inhibited growth of *A. sulphureus* and *P. viridicatum* by 76 and 90%, respectively. Toxin production was inhibited by >99% for each fungus. Antimicrobial agents were ranked as to effectiveness by comparing the level required for complete inhibition of ochratoxin A production to the highest antimicrobial agent level normally used in food. At pH 4.5, the most effective inhibitor of growth and toxin production was potassium sorbate, followed by sodium propionate, methyl paraben, and sodium bisulfite, respectively, for both fungi. However, at pH 5.5, the most effective antimicrobial agents for inhibiting ochratoxin production were methyl paraben and potassium sorbate, followed by sodium propionate. Sodium bisulfite was not highly inhibitory to these toxigenic fungi at the higher pH value tested.

The ochratoxins constitute a group of structurally related metabolites produced by several species of *Aspergillus* and *Penicillium*. Ochratoxin A (7-carboxyl-5-chloro-8-hydroxyl-3-4-dihydro-3-methyl isocoumarin linked to L-β-phenylalanine) was the first toxin discovered in this group (24). In addition to being associated with nephropathy in chickens (5) and swine (11, 12), ochratoxin A has been found to be toxic to species such as poultry, rats, mice, dogs, cattle, brine shrimp, and bacteria (10, 16). Ochratoxin A is teratogenic in mice (8), rats (2), chick embryos (7), and hamsters (9). In the United States, the ochratoxins have not been studied as intensively as the aflatoxins because most reports of its occurrence have been unsubstantiated. However, ochratoxin has been widely studied in Scandinavian countries because of its occurrence in small grains such as barley and because of its toxicity to swine (10). In a survey of a farm at which pigs are slaughtered, ochratoxin A was found in tissues of 18 of 19 pigs fed ochratoxin-contaminated barley, and residue levels in tissue were as high at 67 μg/kg (10). Residues of ochratoxin A have also been found in kidneys, livers, and muscular tissues of slaughtered poultry at concentrations between 4.3 and 50 μg/kg (5). The only observable lesion in afflicted animals is often evidenced as kidney damage; therefore, the remaining parts of the carcass may pass meat inspection, and the transfer of ochratoxin A from animal feed to human food is possible.

There have been few studies on the chemical control of ochratoxin A production. Vandegraft et al. (22, 23) have investigated the effects of fumigants on ochratoxin production in stored grain and have found that none reduced ochratoxin production. The insecticide dichlorvos, at 30 mg/100 ml of culture medium, inhibited ochratoxin production by 80% (25). In a later study, Vandegraft and co-workers (21) examined grain perservatives such as propionic acid and ammonia in corn to determine their effects on ochratoxin production. Both 1% propionic acid and 2% ammonia significantly reduced mold growth and ochratoxin production.

The present study was designed to evaluate the effectiveness of four antimicrobial food additives, sodium propionate, methyl paraben, sodium bisulfite, and potassium sorbate, at pH 4.5 and 5.5, for inhibition of ochratoxin A production and mycelium formation by *Aspergillus sulphureus* and *Penicillium viridicatum*.

MATERIALS AND METHODS

Test organisms and growth medium. *A. sulphureus* NRRL 4077 and *P. viridicatum* NRRL 3711, known ochratoxin-producing fungi, were used throughout this study. Cultures were inoculated onto 2% yeast extract–4% sucrose (YES) agar slants, incubated for 2 weeks at 28°C to produce spores, and stored at 4°C until needed. Conidial suspensions were prepared in a sterile aqueous solution of Triton X-100 (0.005% [vol/vol]) immediately before inoculation.

For evaluation of ochratoxin production and growth, 10^7 fungal spores were added to 100 ml of YES broth in a 500-ml Erlenmeyer flask. The broth cultures were incubated for 14 days at 28°C. Results of preliminary experiments confirmed that optimum ochratoxin production occurred in a 2% (wt/vol) yeast extract–4% (wt/vol) sucrose broth (4) with the fungal strains chosen for experimentation.

Antimicrobial agents. Sodium propionate and sodium bisulfite (Fisher Scientific Co., Atlanta, Ga.), food grade methyl paraben (Ueno Fine Chemical Industries, Inc., Osaka, Japan), and potassium sorbate (Tri-K Industries, Inc., Westwood, N.J.) were evaluated as antimicrobial agents against ochratoxin producers. Methyl paraben was dissolved in 75% ethanol because of its low solubility in water. Other antimicrobial agents were dissolved in water. Solutions of antimicrobial agents were filter sterilized and stored at 4°C until needed.

YES medium was autoclaved at 121°C for 15 min. When

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the culture medium reached ambient temperature, appropriate amounts of the antimicrobial agents were added, and the pH was then aseptically adjusted to pH 4.5 or 5.5 by using varying normalities of sterile HCl and NaOH.

Antimicrobial agents were added to YES broth at the following concentrations: sodium propionate (0%, 0.1%, 0.2%, 0.32%, 0.48%, 0.64%), methyl paraben (0%, 0.011%, 0.022%, 0.033%, 0.067%), sodium bisulfite (0%, 0.0167%, 0.0334%, 0.0500%, 0.0667%, 0.0834%, 0.1000%, 0.1200%), and potassium sorbate (0%, 0.011%, 0.022%, 0.044%, 0.067%, 0.134%). Levels of antimicrobial agents permitted or generally used (6, 14) in foods for human consumption were considered when levels of antimicrobial agents were selected.

Extraction and analysis of ochratoxin. Ochratoxin A was extracted from the broth by the procedures of Scott et al. (17). The chloroform extracts were combined and dried by filtering through anhydrous sodium sulfate to remove water. The chloroform was evaporated to dryness at 60°C with a flash evaporator (Rotovap). The residue was dissolved in chloroform (5 ml). After the samples were filtered through a 0.45-μm Millipore filter, they were sealed in vials and quantitated by high-pressure liquid chromatography (HPLC) for ochratoxin A.

The high-pressure liquid chromatograph (Waters Associates, Milford, Mass.) was equipped with dual model M6000 pumps, a model 440 UV detector (340 nm), a model 420 fluorescence detector (340-nm excitation filter and a 440-nm emission filter), and a U6K septumless injector. A μ-Porasil column (Waters Associates) was used as the stationary phase with a solvent system of benzene-acetic acid-methanol (90:10:5 [vol/vol/vol]). The average retention time for ochratoxin A was 3 min and 40 s at a flow rate of 1 ml/min. An experiment was performed to determine the reproducibility of the HPLC analysis when the injection volume, the sensitivity of the detector, and the concentration were varied. Each of five standard solutions ranging in concentrations from 0.1 to 0.001 mg/ml was injected 10 times into the chromatograph. The standard curve was found to be linear. The coefficient of variation for each series of injections was highly satisfactory (Table 1).

TABLE 1. Reproducibility of the HPLC system[a]

Concn of standard solution (mg/ml)	Mean peak area ± SD[b]	Coefficient of variation (%)
0.1	219.5 ± 6.451	2.94
0.05	178.3 ± 4.234	2.38
0.01	124.2 ± 7.435	5.99
0.005	130.3 ± 6.885	5.28
0.001	124.2 ± 6.888	5.55

[a] Reproducibility was determined with a μ-Porasil column and a solvent system of benzene-acetic acid-methanol at a flow rate of 1 ml per min using the fluorescence detector.

[b] Mean of 10 injections. The peak area is subject to change with a change in the volume of injection and the gain of the fluorescence detector. Peak areas were held between 100 and 250 for optimal visualization.

Samples were spotted on thin-layer chromatographic plates (Redi; Fisher Scientific Co.) which were precoated with silica gel G and developed with benzene-acetic acid (90:10 [vol/vol]. The presence of ochratoxin A was confirmed by boron trifluoride derivatization (1). The recovery of ochratoxin from YES broth was determined by spiking experiments.

After extraction, mycelia were separated from the broth culture by filtering with a Buchner funnel with a vacuum and Whatman no. 1 filter paper. The mycelial mat was transferred to preweighed filter paper, dried at 60°C for 24 h, and weighed on a Mettler analytical balance to determine the amount of growth (dry weight).

Analysis and experimental design. The entire experiment was replicated twice (three separate runs). Three flasks were prepared for each treatment for each replicate. An analysis of variance was performed to analyze the data. When significant differences were found, the Duncan multiple range test was used to determine the significant differences among mean values.

RESULTS AND DISCUSSION

Mycelium inhibitors. Figure 1 shows the effects of potassium sorbate, methyl paraben, sodium bisulfite, and sodium propionate on growth of *A. sulphureus* NRRL 4077 and *P. viridicatum* NRRL 3711 in YES broth at pH values of 4.5 and 5.5. The ability of the food additives to inhibit mycelium production varied widely. However, a significant decrease ($P < 0.05$) in mycelial weight was obtained with the addition of each of the four antimicrobial agents. Potassium sorbate was highly effective for reducing fungal growth at pH 4.5. Although potassium sorbate is listed as a generally recognized as safe (GRAS) substance, its level of use in food does not generally exceed 0.2% (6, 14). Potassium sorbate was found to completely inhibit fungal growth at 0.134%, two-thirds of the normal use level, when the pH of the broth was 5.5. Originally, potassium sorbate levels of 0, 0.022%, 0.044%, 0.067%, and 0.134% were studied. However, mycelium production was not detected even at 0.022% at pH 4.5. The effectiveness of potassium sorbate at pH 4.5 was approximately seven times higher than that at pH 5.5 based on the amount used and the percent inhibition.

Methyl paraben was 100% effective in the inhibition of mycelial growth at low levels. The growth inhibition patterns were similar with methyl paraben, regardless of the pH values (Fig. 1). A significant decrease in weight of mycelial mat occurred with 0.033% methyl paraben.

Sodium bisulfite is not generally used in food at levels in excess of 500 ppm because of a noticeable taste above this level (6). Because inhibition at 500 ppm was very poor at pH 5.5, higher levels were tested. At 1,000 ppm, sodium bisulfite inhibited growth of *A. sulphureus* and *P. viridicatum* by 85 and 91%, respectively. At 500 ppm, sodium bisulfite inhibited growth of *A. sulphureus* and *P. viridicatum* by 9 and 11%, respectively, at pH 5.5 (Fig. 1). To obtain 100% inhibition of growth for both fungi, 667 ppm sodium bisulfite was required at pH 4.5. Significant decreases in mycelial weight occurred from 334 to 500 ppm at pH 4.5 and 834 to 1,000 ppm at pH 5.5, respectively (Fig. 1). The reduced antifungal activity of sodium bisulfite at pH 5.5 versus 4.5 is typical of antimicrobial agents that are most active in the undissociated form.

The practical use of sodium propionate as a fungal inhibitor was significantly related to pH. With 0.20% sodium propionate, growth of *A. sulphureus* was inhibited by 13% at pH 5.5 compared with 100% at pH 4.5 and, similarly, 16% compared with 100% for *P. viridicatum* NRRL 3711. At 0.64% sodium propionate, 80 and 88% inhibition of growth occurred at pH 5.5 for *A. sulphureus* and *P. viridicatum*, respectively.

P. viridicatum was found to produce more mycelium than *A. sulphureus*. It was found that both strains produced more mycelial mat at pH 4.5 than at pH 5.5. Similar results have

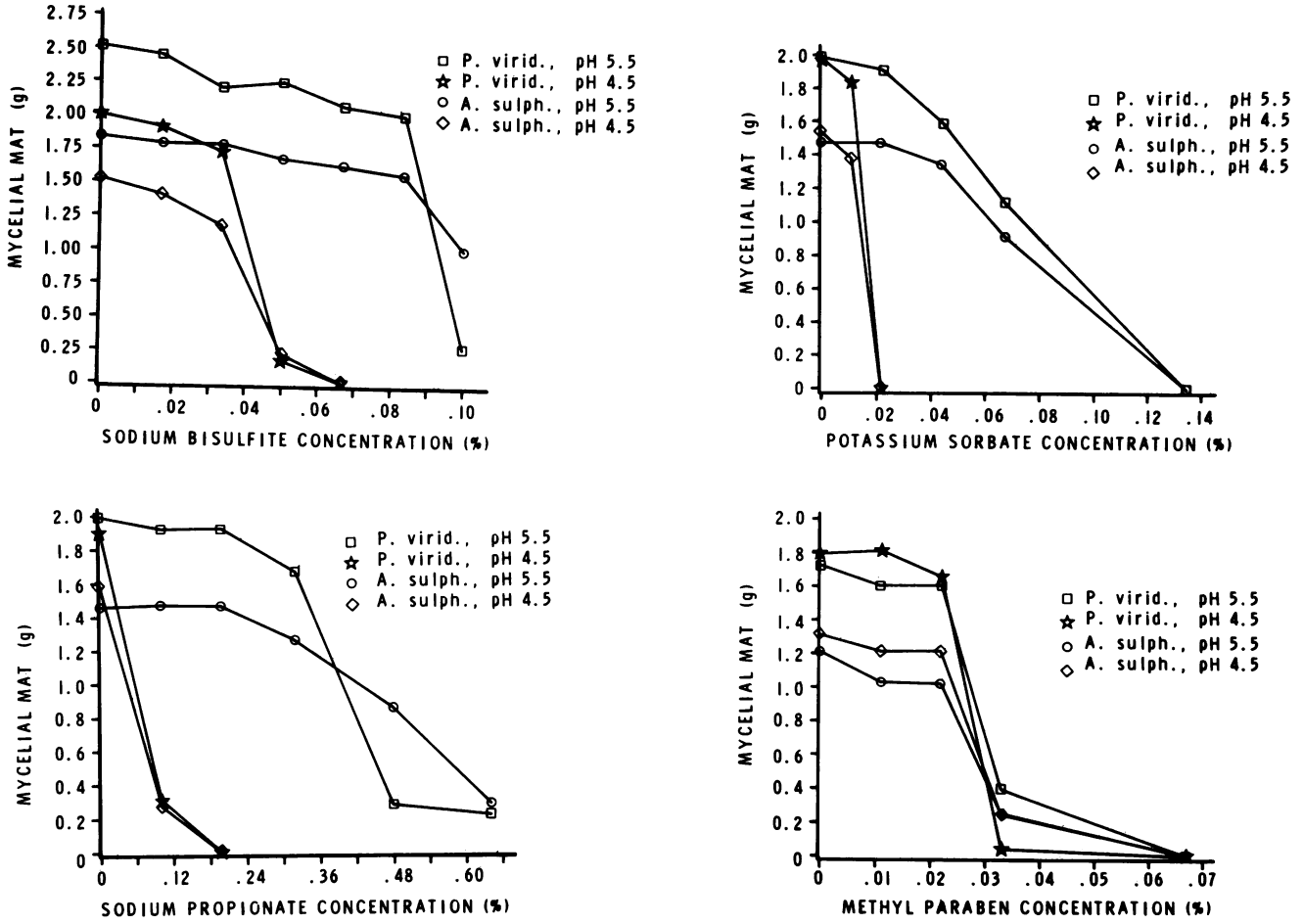

FIG. 1. Effect of antimicrobial agents on the growth of *A. sulphureus* and *P. viridicatum* when incubated at 28°C for 2 weeks.

been reported by Lai et al. (13). Generally, the inhibition patterns for antimicrobial food additives were almost identical for *A. sulphureus* NRRL 4077 and *P. viridicatum* NRRl 3711 at pH 4.5. However, at pH 5.5 and at higher concentrations, the former was generally found to be more resistant to the antimicrobial agents than was the latter.

Toxin inhibition. Tables 2 and 3 show the effects of antimicrobial food additives on ochratoxin A production by *A. sulphureus* NRRL 4077 and *P. viridicatum* NRRL 3711 in YES broth at two pHs. The addition of the food additives resulted in a significant decrease in ochratoxin A production. Potassium sorbate (0.134%) and methyl paraben (0.067%) completely inhibited toxin production by both fungi at the two pH levels tested without exceeding levels normally used in food for human consumption. However, two times the amount of sodium bisulfite and three times the amount of sodium propionate normally used did not completely inhibit toxin production at pH 5.5. By increasing the concentrations of sodium bisulfite and sodium propionate to 0.1 and 0.64%, more than 90% inhibition in ochratoxin A production was achieved for both strains at both pH values (Tables 2 and 3).

Generally, toxin production decreased as mycelium formation decreased. However, the antimicrobial agent inhibition of toxin was more significant than mycelial inhibition. Although the reason for this has not been shown experimentally, it is a logical finding because in toxin biosynthesis the excess acetate and malonate formed by primary biosynthesis are used. Thus, even small changes in primary biosynthesis can have major effects on secondary biosynthesis.

In the controls, *A. sulphureus* produced about 100 times more ochratoxin A than did *P. viridicatum*. Penicillian species frequently produce maximum toxin at lower temperatures than do aspergilli. This is true of *P. viridicatum*, which has optimal ochratoxin production at 20°C (18, 19). However, *P. viridicatum* produced more mycelial weight than did *A. sulphureus* in YES broth at 28°C. Lai et al. (13) have reported that pH 5.5 is better for ochratoxin A production than pH 4.5. Nevertheless, in this study, no significant difference in ochratoxin A formation was observed as a result of pH differences in the control flasks. It was also found that the standard deviations of mean values for toxin were much higher than the deviations of mean values for mycelium. The large variation for toxin could be due to the fluctuation of incubation temperature, extraction procedures, degradation during heating and evaporation, HPLC quantitation, and biological differences. Results of our experiments showed that only minor variations were due to the HPLC analysis. Recoveries of ochratoxin in spiking experiments ranged from 83 to 95%. Despite a report by Chu and Butz (3) that ochratoxin A is very stable when stored under refrigeration, the degradation of standard solution for HPLC determinations could also be a factor that contributes to the variation.

Trenk et al. (20) have found that ochratoxin A can persist

in foods even after 3 h of autoclaving. The removal of ochratoxin A would be difficult once foods are contaminated, and the best protection would be to prevent toxin formation through proper drying and the addition of antimicrobial agents. The effectiveness of the four antimicrobial food additives and the effects of pH values were evaluated in YES broth to provide information for future applications in grains.

It is well known that the activity of antimicrobial food additives, except for the parabens, is dependent on pH. Usually, the lower the pH, the higher the effectiveness. The optimum pH range for sodium propionate and sodium bisulfite is between 2.5 and 5.0 and between 3.0 and 6.5 for potassium sorbate. The parabens are effective at above pH 7 (6). In this study, potassium sorbate and methyl paraben were effective for inhibiting fungal growth at low concentrations at both pH 4.5 and 5.5. Although the level required to completely inhibit fungal growth and ochratoxin A production was the same for methyl paraben at pH 4.5 and 5.5, the percentages of inhibition were still affected by pH. Vandergraft et al. (21) have reported that no ochratoxin A was detected in corn when the corn was treated with 1% propionic acid. The pH of corn is about pH 6.0 (15). Because the addition of 0.64% sodium propionate resulted in 99% inhibition in toxin production, a level between 0.64% and 1% is expected to inhibit ochratoxin A production by 100%. Because of its poor inhibition of the fungi at pH 5.5, sodium bisulfite may not feasibly be added into most foods because its strong odor is detectable when the level exceeds 500 ppm.

TABLE 2. Effect of antimicrobial agents on ochratoxin A production by *A. sulphureus* NRRL 4077 in YES broth incubated at 28°C for 2 weeks

Antimicrobial agent	Level (%)	Mean toxin level ± SD (μg/100 ml of broth) at the following pHs[a]:	
		4.5	5.5
Potassium sorbate	0	2,297 ± 44^{b}	1,914 ± 37^{b}
	0.011	696 ± 8^{c} (70)	1,621 ± 30^{b} (15)
	0.022	0^{d} (100)	472 ± 13^{c} (75)
	0.044	0 (100)	196 ± 4^{d} (90)
	0.067	0 (100)	4 ± 0^{d} (99)
	0.134	0 (100)	0^{d} (100)
Methyl paraben	0	2,733 ± 45^{b}	1,591 ± 33^{b}
	0.011	111 ± 4^{c} (96)	407 ± 22^{c} (74)
	0.022	4 ± 0^{c} (99)	49 ± 2^{d} (97)
	0.033	3 ± 0^{c} (99)	22 ± 0^{d} (99)
	0.067	0^{c} (100)	0^{d} (100)
Sodium propionate	0	1,611 ± 31^{b}	1,770 ± 46^{b}
	0.10	17 ± 0^{c} (99)	1,430 ± 33^{c} (19)
	0.20	0^{d} (100)	635 ± 26^{d} (64)
	0.32	0 (100)	227 ± 9^{e} (87)
	0.48	0 (100)	201 ± 24^{e} (89)
	0.64	0 (100)	10 ± 1^{e} (99)
Sodium bisulfite	0	2,188 ± 27^{b}	2,516 ± 29^{b}
	0.0167	416 ± 21^{c} (81)	2,129 ± 24^{c} (15)
	0.0334	264 ± 14^{d} (88)	1,950 ± 16^{c} (22)
	0.0500	4 ± 5^{e} (99)	997 ± 17^{d} (60)
	0.0667	0^{e} (100)	460 ± 13^{e} (82)
	0.0834	0 (100)	186 ± 9^{f} (93)
	0.1000	0 (100)	71 ± 4^{f} (97)

[a] Values within treatments followed by the same letter (*b* through *f*) are not significantly different ($P < 0.05$) by the Duncan multiple range test. Values in parentheses are precent inhibition.

TABLE 3. Effect of antimicrobial agents on ochratoxin A production by *P. viridicatum* NRRL 3771 in YES broth incubated at 28°C for 2 weeks

Antimicrobial agent	Level (%)	Mean toxin level ± SD (μg/100 ml of broth) at the following pHs[a]:	
		pH 4.5	pH 5.5
Potassium sorbate	0	21 ± 4.0^{b}	18 ± 3.0^{b}
	0.011	4 ± 1.0^{c} (81)	17 ± 3.0^{b} (6)
	0.022	0^{d} (100)	14 ± 1.0^{c} (22)
	0.044	0 (100)	12 ± 1.0^{c} (33)
	0.067	0 (100)	7^{d} (61)
	0.134	0 (100)	0^{e} (100)
Methyl paraben	0	19 ± 6.0^{b}	22 ± 8.0^{b}
	0.011	21 ± 5.0^{b}	13 ± 4.0^{c} (41)
	0.022	2 ± 2.0^{c} (89)	7 ± 3.0^{d} (69)
	0.033	2 ± 0.1^{c} (89)	1 ± 0.1^{e} (95)
	0.067	0^{c} (100)	0^{e} (100)
Sodium propionate	0	19 ± 3.0^{b}	21 ± 6.0^{b,c}
	0.10	5 ± 2.0^{c} (69)	34 ± 24.0^{b}
	0.20	0^{d} (100)	12 ± 4.0^{b,c} (43)
	0.32	0 (100)	1 ± 0.1^{c} (95)
	0.48	0 (100)	2 ± 0.1^{c} (99)
	0.64	0 (100)	0^{c} (100)
Sodium bisulfite	0	21 ± 3.0^{b}	22 ± 7.0^{b}
	0.0167	4 ± 1.0^{c} (81)	19 ± 2.0^{b} (13)
	0.0334	2 ± 1.0^{d} (90)	12 ± 2.0^{c} (46)
	0.0500	2 ± 0.1^{e} (99)	9 ± 1.0^{c} (58)
	0.0667	0^{e} (100)	4 ± 2.0^{d} (80)
	0.0834	0 (100)	0.8 ± 0.3^{e} (96)
	0.1000	0 (100)	0.1 ± 0.1^{e} (99)

[a] Values within treatments by the same letter (*b* through *e*) are not significantly different ($P < 0.05$) by the Duncan multiple range test. Values in parentheses are percent inhibition.

Antimicrobial agents were ranked as to their effectiveness by comparing the level required for complete inhibition of ochratoxin A production with the highest level of antimicrobial agents normally used in food. At pH 4.5, the most effective inhibitor of growth and toxin production was potassium sorbate. In descending order, it was followed by sodium propionate, methyl paraben, and sodium bisulfite. However, at pH 5.5, the most inhibitory antimicrobial agents were potassium sorbate and methyl paraben, followed by sodium propionate and sodium bisulfite. Both *A. sulphureus* and *P. viridicatum* followed similar patterns of inhibition when exposed to antimicrobial agents at pH 4.5 and 5.5.

For foods with pHs between 5 and 6, such as rye and sorghum, the antimicrobial agent of choice for future studies would be methyl paraben or potassium sorbate. The application of these antimicrobial agents is feasible, although there are problems to be overcome in the application and distribution of the antimicrobial agents in grain. Corn silage and similar products with pHs of about 4.5 should be tested with methyl paraben, potassium sorbate, or sodium propionate.

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