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Growth and Aflatoxin Production by *Aspergillus parasiticus* NRRL 2999 in the Presence of Lactic Acid and at Different Initial pH values

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ABSTRACT

Twenty-five milliliters of glucose-yeast-salts medium containing 0, 0.5, 0.75, 1.0, 1.5 and 2.0% lactic acid with an initial pH of 3.5 or 4.5 were inoculated with 1 ml of a spore suspension containing 10^6 conidia of *Aspergillus parasiticus* NRRL 2999 and incubated at 28°C for 10 d. The pH of the medium, weight of mycelium and aflatoxin production were determined after 3, 7, and 10 d of incubation. Amounts of aflatoxin produced were determined using reversed-phase high-performance liquid chromatography. Cultures grown in the presence of 0.5 and 0.75% lactic acid at an initial pH of 4.5 produced more aflatoxin B_1 than did the other cultures at the end of 3 d of incubation. This was not true for aflatoxin G_1; with increasing concentrations of lactic acid, cultures produced decreasing amounts of aflatoxin G_1. Also, cultures growing in the medium with an initial pH of 3.5 produced more aflatoxin B_1 in the presence of lactic acid at the end of 3 d of incubation than did control cultures. Cultures growing in the presence of 0.5 and 0.75% lactic acid produced the most aflatoxin. Maximum amounts of aflatoxin G_1 were produced after 7 d of incubation, with cultures growing in the presence of 0.5 and 0.75% lactic acid producing the most. Lactic acid did not inhibit growth (mycelium weight) of cultures in the medium with initial pH values of 3.5 or 4.5 except there was a slight decrease in mycelial weight when the medium contained 0.5% lactic acid and had an initial pH value of 3.5.

Certain strains of *Aspergillus parasiticus* and *Aspergillus flavus* produce a group of toxic metabolites collectively known as aflatoxins. Aflatoxins B_1, B_2, G_1 and G_2 are the most commonly encountered forms, with the former being the most potent and exhibiting LD_{50} values in the range of 0.5-1.0 mg of toxin/kg of body weight for such animals as the duckling, guinea pig, rabbit and dog *(23)*. Aflatoxin-producing molds can grow under various environmental conditions *(18)*, and on a numerous agricultural and food commodities *(6,17)*.

Lactic acid is one of the primary acids formed during some natural fermentation processes and is found in sauerkraut, pickles, green olives, fermented milks, cheese, certain sausages, and in other fermented foods of plant origin. Reports indicate that *A. flavus* and *A. parasiticus* can grow and produce toxin on cheese *(5,22)*, and on meats such as sausages, bacon and salami *(7,8,30,32)* which may contain lactic acid.

Subramanian and Marth *(31)* reported that lactic acid was more detrimental to growth of *Salmonella typhimurium* in sterile skim milk than HCl but was less detrimental than citric acid. Lactic acid is an excellent inhibitor of sporeforming bacteria at pH 5.0 but totally ineffective against yeasts and molds *(33)*, whereas it is approximately four times more effective than malic, citric, propionic and acetic acid in limiting growth of *Bacillus coagulans* in tomato juice *(27)*. Combinations of lactic and citric acids, and potassium sorbate synergistically delayed initiation of growth or restricted total growth of *Salmonella, Yersinia enterocolitica, Pseudomonas fluorescens,* two strains of lactic acid bacteria and four strains of osmophilic yeast *(25,26)*.

The antimicrobial action of organic acids is well known. These acids exert their effect through their undissociated molecules *(2,10,20,21)*. If this is true, activity of the acids is dependent on pH, which determines the degree of dissociation. At a low pH, the proportion of undissociated molecules is greater than at pH values approaching neutrality.

This study was conducted to determine the antimicrobial effect of various concentrations of lactic acid on growth and aflatoxin production by *A. parasiticus* in a glucose-yeast extract-salts medium at pH 3.5 and 4.5; selected pH values normally occur in some fermented foods and are close to the pK_a of lactic acid (3.08).

MATERIALS AND METHODS

A glucose-yeast-salts medium described by Yousef and Marth *(34)* was used. *A. parasiticus* NRRL 2999 was the test culture and was obtained from the Northern Regional Research Center, U.S.D.A, Peoria, IL. The mold was grown on slants of Mycological agar incubated at 28°C. After 7 d, spores were harvested by adding sterile distilled water and glass beads to cultures; glass beads helped to dislodge spores from the mycelium when shaken. The spore suspension was pooled in a sterile 125-ml Erlenmeyer flask. The number of spores/ml was determined with the plate count method using Mycological agar and incubation at 28°C.

Appropriate amounts of glucose-yeast-salts medium were dispensed into each of a series of 500-ml Erlenmeyer flasks and autoclaved at 121°C for 15 min. Following autoclaving, suitable amounts of 100% (w/v) lactic acid (Baker) were added to obtain the desired concentrations (0.0, 0.5, 0.75, 1.0, 1.5 and 2.0%). Twelve milliliters of a spore suspension containing ca. 10^6 conidia/ml was added to each flask and the pH of the medium was adjusted to 3.5 or 4.5 by aseptically adding 10 *N* HCl or 40% NaOH. The medium was then dispensed into sterile 125-ml Erlenmeyer flasks. Each flask contained 25 ml of medium and ca. 10^6 conidia.

Determinations of pH, mycelium dry weight and aflatoxins B_1 and G_1 were made after 3, 7 and 10 d; methods were previously described *(28)*. Statistical analysis was done using the Minitab statistical package on an IBM personal computer.

RESULTS AND DISCUSSION

pH of medium containing various concentrations of lactic acid and with an initial pH of 3.5 and 4.5

Results (Table 1) indicate that lactic acid had a buffering effect because after 3 d of incubation, the rate of decrease in pH decreased with an increasing concentration of lactic acid. This was also true for cultures growing in the presence of 1.5 and 2% lactic acid, where there was an increase in pH, even though there was no significant difference ($p>0.05$) in mycelium dry weight between these cultures and those with 0 and 0.5% lactic acid. No change in pH was observed for cultures with 1% lactic acid. Similar trends in changes in pH were observed for cultures growing in the medium with an initial pH of 3.5 (Table 2); a decrease in pH occurred in all cultures except when the medium contained 2% lactic acid where there was no change in pH.

TABLE 1. *The pH values of cultures of* A. parasiticus *containing various concentrations of lactic acid and incubated at 28°C; initial pH of medium was 4.5.*

Conc. of lactic acid (%)	Incubation (d)		
	3	7	10
0	2.53	4.60	6.10
0.5	4.04	6.38	6.74
0.75	4.26	6.78	6.95
1.0	4.45	6.45	6.71
1.5	4.61	6.91	6.78
2.0	4.73	7.11	7.04

TABLE 2. *The pH values of cultures of* A. parasiticus *containing various concentrations of lactic acid and incubated at 28°; initial pH of medium was 3.5.*

Conc. of lactic acid (%)	Incubation (d)		
	3	7	10
0	2.66	3.15	3.81
0.5	2.78	6.43	6.74
0.75	2.92	6.38	6.84
1.0	3.15	5.99	6.61
1.5	3.36	5.96	6.57
2.0	3.50	6.11	6.40

After 7 d of incubation, there was an increase in pH of cultures growing in the presence of lactic acid. When cultures grew in the absence of lactic acid, the increase in pH was not substantial compared to cultures with lactic acid. This was also true for cultures growing in the medium with an initial pH of 3.5. After 10 d of incubation, a net increase in pH was observed for all concentrations of lactic acid at both initial pH values of 3.5 and 4.5. These results agree with those of Buchanan and Ayres *(4)* who observed an increase in pH of mold cultures containing lactic acid and incubated 7 d. The decrease in pH after 3 d of incubation is associated with growth and the increase in pH after 7 d of incubation may result from elevated levels of nitrogen in the medium *(9)* and/or autolysis of fungal cells *(34)*.

Mycelium dry weight

Data in Table 3 indicate that at pH 4.5 lactic acid, regardless of concentration, had no inhibitory effect on growth of the mold. There was no significant difference ($p>0.05$) between the mycelium dry weight of cultures growing in the absence or presence of lactic acid. Also, there was no significant difference ($p>0.05$) in mycelium dry weight among cultures growing in the presence of different amounts lactic acid. Data in Table 4, suggest that lactic acid at concentrations of 0.75, 1.0, and 1.5% initially may have slightly stimulated growth of the mold since more mycelia were produced after 3 d at these concentrations than at 0, 0.50 and 2%, but the differences were not significant ($p>0.05$). After 7 d of incubation, there was no difference ($p>0.05$) in mycelium dry weight among cultures growing in the medium with an initial pH of 4.5, but there appeared to be a general decrease in mycelium weight, a trend we saw in previous work *(16,28)*, and that also has been reported by others *(29,34)*. This was not true for cultures growing in the medium with an initial pH of 3.5, as a significant ($p<0.05$) increase in mycelium dry weight was observed when the medium contained 0, 0.5, 1.5 and 2% lactic acid. After 7 d, all cultures had sporulated except the culture growing in the absence of lactic acid in the medium with an initial pH of 3.5. Pohlmeier and Buller-

TABLE 3. *Dry weight of mycelium (g/25 ml of medium) produced by* A. parasiticus *in cultures containing various concentrations of lactic acid and incubated at 28°C; initial pH of medium was 4.5.*

Conc. of lactic acid (%)	Incubation (d)		
	3	7	10
0	0.79	0.83	0.79
0.5	0.75	0.65	0.53
0.75	0.70	0.61	0.53
1.0	0.80	0.67	0.57
1.5	0.82	0.75	0.61
2.0	0.83	0.72	0.63

TABLE 4. *Dry weight of mycelium (g/25 ml of medium) produced by* A. parasiticus *in cultures containing various concentrations of lactic acid and incubated at 28°C; initial pH of medium was 3.5.*

Conc. of lactic acid (%)	Incubation (d)		
	3	7	10
0	0.55	0.69	0.85
0.5	0.58	0.72	0.61
0.75	0.68	0.73	0.58
1.0	0.76	0.84	0.62
1.5	0.71	0.92	0.68
2.0	0.63	0.93	0.77

man *(24)* reported that a substrate (basal salts medium) containing lactate greatly stimulated mold growth but did not increase ochratoxin production. They also observed that a medium devoid of carbohydrates, but containing washed casein or washed casein plus calcium lactate, supported extensive growth of an ochratoxin-producing species of *Penicillium;* however, little or no ochratoxin was produced at 5, 12 or 25°C.

After 10 d of incubation a decrease in mycelium dry weight with a concomitant net increase in pH were observed for all cultures growing in the presence of lactic acid at both initial pH values. No decrease was observed for controls at both pH values. The decrease in mycelial dry weight may have resulted from autolysis of the mycelium with loss of intracellular solutes during filtration; onset of autolysis is usually associated with cessation of growth and initiation of sporulation *(34)*.

Production of aflatoxin

Data in Tables 5 and 6 indicate that biosynthesis and accumulation of aflatoxin were influenced most by concentration of lactic acid, initial pH of the medium and extent of mycelial growth. Generally, after 3 d of incubation, an increase in concentration of lactic acid was associated with a decreased amount of aflatoxin G_1 produced by cultures growing in the medium with an initial pH of 4.5. This was not true for cultures with an initial pH of 3.5, as the cultures growing in the presence of 0.75% lactic acid produced more aflatoxin G_1 than did the others. There was no significant difference ($p>0.05$) in amount of aflatoxin G_1 produced by cultures growing in the presence of 0, 0.5 and 1.0% or between cultures growing in the presence of 1.5 and 2% lactic acid in the medium at an initial pH of 3.5. Results in Table 5 indicate that 0.5 and 0.75% lactic acid had a significant ($p< 0.05$) stimulatory effect of production of aflatoxin B_1 when compared to the control. There was no significant difference ($p>0.05$) in the amount of aflatoxin B_1 produced by cultures growing in the presence of 0, 1.0 or 1.5% lactic acid, whereas cultures growing in the presence of 2% lactic acid produced the least amount of aflatoxin B_1 in the medium with an initial pH of 4.5. Data in Table 6 also show that after 3 d of incubation, cultures growing in the presence of 0.75% lactic acid produced the most aflatoxin B_1, and there was no significant difference ($p>0.05$) in amount of aflatoxin B_1 produced by cultures growing in the presence of 0.5, 1.0, 1.5 or 2% lactic acid. This suggests that at pH 3.5, lactic acid had a stimulatory effect on production of aflatoxin B_1. Yousef and Marth *(34)* reported the ability of mycelia to produce aflatoxin in cultures was greatest after 2 d of incubation and then decreased. Using different molds and cultural conditions, other investigators *(11,15,19)* found that mycelia obtained from 2-, 3- or 4-d-old cultures had greater aflatoxin producting capacity than did younger or older cultures.

TABLE 5. *Accumulation of aflatoxin B_1 and G_1 (μg/25 ml of medium) at 28°C in a medium containing various concentrations of lactic acid; initial pH of medium was 4.5.*

Lactic acid (%)	3 d		7 d		10 d	
	B_1	G_1	B_1	G_1	B_1	G_1
0	746.6	6642.0	1022.0	8691.9	684.5	6923.1
0.5	990.1	5127.8	1065.3	7145.6	750.8	5335.0
0.75	843.2	4498.9	847.9	6526.9	701.9	4982.5
1.0	677.0	3793.8	750.9	5124.6	600.6	5120.0
1.5	689.1	3572.7	632.1	4976.3	594.0	4567.1
2.0	459.4	2839.6	617.8	5067.5	486.0	3389.6

TABLE 6. *Accumulation of aflatoxin B_1 and G_1 (μg/25 ml of medium) at 28°C in a medium containing various concentrations of lactic acid; initial pH of medium was 3.5.*

Lactic acid (%)	3 d		7 d		10 d	
	B_1	G_1	B_1	G_1	B_1	G_1
0	516.2	4534.1	681.8	7280.4	694.0	8442.3
0.5	892.3	4688.5	1022.0	9791.3	759.4	9092.2
0.75	1038.9	6008.2	1011.7	10207.9	717.2	8790.5
1.0	858.3	4724.8	860.4	6309.0	742.4	4723.4
1.5	822.0	3665.8	926.7	6904.8	931.4	5839.6
2.0	773.7	3502.9	831.7	6233.0	1085.3	6512.5

Data in Tables 5 and 6 show that during the 3-to-7-d interval of incubation, the amount of aflatoxin G_1 produced by cultures growing in the absence or presence of lactic acid increased in the medium with an initial pH of 3.5 or 4.5. The increase in aflatoxin G_1 production decreased with increasing concentrations of lactic acid for cultures at an initial pH of 4.5. As for cultures with an initial pH of 3.5, those growing in the presence of 0.5 and 0.75% lactic acid produced more aflatoxin G_1 than did the others. Generally, cultures with an initial pH of 3.5, produced more aflatoxin G_1 in the presence of lactic acid than did cultures with an initial pH of 4.5. This may have resulted because at pH 3.5 more lactic acid was present in the undissociated form than at pH 4.5. More lactic acid is transported across the cell membrane and then converted to lactate as the pH within the hyphae is close to neutral, thereby providing a readily available source of energy or precursors for intermediate compounds in aflatoxin biosynthesis. There was no change in amount of aflatoxin B_1 produced during the 3-to-7-d interval of incubation compared to the 0-to-3-d interval by cultures growing in the presence of lactic acid except for those growing in the absence of lactic acid in the medium with an initial pH of 4.5. As for cultures with an initial pH of 3.5, there was no increase in aflatoxin B_1 production except when the medium contained 0, 1.5 and 2% lactic acid and production of aflatoxin B_1 was enhanced. Abdollahi and Buchanan *(1)* and Buchanan and Stahl *(3)* reported that lactic acid did not induce biosynthesis of aflatoxin when present as a sole carbon source, whereas Buchanan and Ayres *(4)* noted that lactic acid in the presence of glucose stimulated biosynthesis of aflatoxins.

During the 7-to-10-d interval of incubation, there were decreases in amounts of aflatoxin G_1 and aflatoxin B_1 in the medium at both initial pH values. The amount of aflatoxin G_1 increased in cultures at pH 3.5 and in the absence of lactic acid. A similar observation on onset of degradation was made by others *(16,28,29,34,35)*. Data in Tables 5 and 6 show that there was no linear relationship between the amount of aflatoxin present and the rate of degradation. Yousef and Marth *(34)* speculated that aflatoxins are degraded by intracellular fungal enzymes that are liberated following autolysis of the mycelium. Doyle and Marth *(12,13,14)*, after extensive studies, reported that 9-d-old mycelia degraded the maximum amount of aflatoxin at pH values in the range of 5 to 6.5 and at 28°C. The amount of aflatoxin degraded was dependent upon the initial concentration, and aflatoxin G_1 was degraded more rapidly than was B_1. They further concluded that more than one mechanism was involved in degradation of aflatoxins, which explains some observed discrepancies on loss of aflatoxins from cultures of aspergilli.

Results from this study indicate that up to 2% lactic acid in the medium not only supported growth of *A. parasiticus* but at certain concentrations stimulated aflatoxin biosynthesis.

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