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Full Paper

Reduction of sterigmatocystin biosynthesis and growth of food-borne fungi by lactic acid

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Food contamination by fungi and mycotoxins presents a problem for food safety even today. Since lactic acid (LA) has Generally Recognized As Safe (GRAS) status, the aim of this research was to determine its potential in protection of food against mycological and mycotoxicological contamination. In this study, LA showed an inhibitory effect on the growth of food-borne fungi (*Penicillium aurantiogriseum* K51, *Aspergillus parasiticus* KB31, *Aspergillus versicolor* S72, and *Aspergillus niger* K95) and on biosynthesis of sterigmatocystin (STE). For the antifungal effect of LA on the growth of food-borne fungi, the disc diffusion and microdilution methods were performed. The effect of LA on the STE biosynthesis by *A. versicolor* was determined using an LC-MS/MS technique. The largest inhibition zone was observed for *A. versicolor* (inhibition zone of 24 ± 0.35 mm), while there were no inhibition zones for *A. niger* and *A. parasiticus* at all tested LA concentrations. The minimal inhibitory concentration (MIC) of LA on fungi ranged from 25.0 mg/mL to 50.0 mg/mL, while the minimum fungicidal concentrations (MFCs) ranged from 50.0 mg/mL to 100.0 mg/mL. Complete inhibition of STE biosynthesis by *A. versicolor* was observed at an LA concentration of 50.0 mg/mL. The obtained results showed that LA could be efficient for protection of food against mycological and STE contamination.

Key words: antifungal activity, fungi, lactic acid, sterigmatocystin

INTRODUCTION

Fungal growth is a major problem for many food products and is responsible for more than 10% of global food spoilage [1]. Moreover, fungal spoilage is the main cause of substantial economic losses in food production and might also be regarded as a source of mycotoxins, which are involved in public health problems [2, 3]. For this reason, any decrease of fungal growth is of great interest.

Sterigmatocystin (STE) is a secondary metabolite a variety of fungal species (*Aspergillus* spp., *Eurotium* spp., *Emericella* spp., *Drechslera* spp., *Bipolaris* spp., *Chaetomium* spp., and *Penicillium* spp.). STE has been isolated from a variety of different foods (cereals, cheese, bread, spices, coffee, soya, beans, pistachios, and other nuts) and feeds [4–6]. This mycotoxin shows a variety of toxicological, mutagenic, and carcinogenic effects in animals (liver, kidney, and renal necrosis in rats) and is thought to be involved in the etiology of chronic liver disease in people living in Africa [4, 7]. Due to these toxic effects, STE was classified as a 2B carcinogen (possible human carcinogen) by the International Agency for Research on Cancer. STE and aflatoxin B_1 (which is a common very toxic contaminant in a variety of foods and animal feeds) have a same precursor in their biosynthesis. [4, 8].

The modern concept of reducing fungal contaminations and their toxic metabolites, mycotoxins, increasingly involves the application of natural food preservation methods, such as the use of natural antimicrobials, without adverse effects on the consumer or the food itself [9]. The use of organic acids is considered to be a good alternative in many food processing industries due to their natural origin and preservative, antioxidant, flavoring, and acidifying properties, as well as their low cost [10]. Lactic acid (LA), as produced by LA starter culture bacteria or as a food additive, functions as a natural antimicrobial agent. LA is generally recognized as safe and has a GRAS status [11]. Among other organic acids, LA is recognized as a biopreservative in

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naturally fermented food [12], and numerous applications of LA for decontamination of meat have been described [13–16]. As reviewed by Doores [17], LA is able to inhibit the growth of many types of food-borne pathogens. In particular, it is considered to be an appropriate agent to suppress fungal growth and mycotoxin biosynthesis [18].

Several studies showed inhibitory activity of LA against bacterial [19–21] and fungal growth [21–24]. However, there are a limited number of studies related to the effect of LA on mycotoxin biosynthesis [25–28].

The aim of this study was to investigate the effect of LA on the growth of fungal species (*Penicillium aurantiogriseum, Aspergillus parasiticus*, *Aspergillus versicolor*, and *Aspergillus niger*) isolated from food and STE biosynthesis by *A. versicolor*.

MATERIAL AND METHODS

Preparation of LA solutions

For the antifungal testing, the LA (L-(+)-lactic acid) (Sigma-Aldrich Co. LLC, Taufkirchen, Germany) was added to distilled water to achieve the following solution concentrations: 5, 10, 20, 50, 75, 102, 209, 321, and 975 mg/mL. Solutions were sterilized by cold sterilization using membrane filters (pore size 0.2 µm; Sartorius AG, Goettingen, Germany). Prepared solutions were stored at 4°C until usage.

Fungi and preparation of fungal spore suspension

The following fungal cultures were chosen for antifungal investigation: *P. aurantiogriseum* K51, *A. parasiticus* KB31, *A. versicolor* S72, and *A. niger* K95. Fungal cultures were isolated from food. *P. aurantiogriseum* and *A. niger* were isolated from cakes. *A. versicolor* was isolated from fresh salads prepared with carrots, lettuce, and red radish. *A. parasiticus* was isolated from corn flour. Isolation and identification of fungal cultures were performed according to Pitt and Hocking; Klich; Samson *et al.*; and Samson and Frisvard. [1, 29–31].

Fungal cultures were kept on Sabouraud Dextrose Agar (SDA; Merck, Darmstadt) at 4°C as a part of the collection of the Laboratory of Food Microbiology at the Faculty of Technology, University of Novi Sad, Serbia.

Seven-day-old fungal cultures grown on SDA were used for preparation of the fungal spore suspension at a final concentration of 10^6 spores/mL. The fungal spore suspension was prepared in a medium (10 mL) which contained 0.5 mL/100 mL of Tween 80. The fungal concentration in the suspension was determined by hemocytometer.

Disc diffusion method

The disc diffusion method was used to estimate the antifungal potential of LA against selected fungal cultures according to the method of Leboffe and Pierce [32], with certain modifications described in our previous research [33]. SDA was used as the basic medium. The following LA quantities were used: 15, 20, and 25 µL for the concentrations of 102, 209, and 321 mg/mL and 1.5, 2.5, 5, and 10 µL for the concentration of 975 mg/mL. Briefly, the entire surfaces of SDA plates were inoculated with fungal spore suspension (10^6 spores/mL). Six-millimeter sterile filter paper discs (blank; HiMedia, Mumbai, India) were placed on the inoculated agar surfaces of the plates. LA solution (in the quantities mentioned above) was added onto the sterile filter paper discs. For control samples, sterile distilled water was used instead of LA solution. Inoculated plates were incubated at 25°C for 72 hr. After incubation, the zones of inhibition were measured, and the results were expressed in mm.

Microdilution method

The microdilution method was performed according to the method described by Marcello *et al.* [34] and Barbour *et al.* [35], with certain modifications described in our previous research [33]. The following media were used in this method: Sabouraud Dextrose Broth (SDB; Merck, Darmstadt) and SDA. Inoculated microtiter plates were incubated at 25°C for 48 hr, and inoculated SDA plates were incubated at 25°C for 72 hr. The lowest LA concentration at which there was no visible fungal growth in SDB was considered to be the minimum inhibitory concentration (MIC). The lowest LA concentration at which there was no growth on the SDA after re-inoculation from the liquid medium (SDB) was considered to be the minimum fungicidal concentration (MFC).

Determination of the effect of LA on STE biosynthesis by Aspergillus versicolor

The effect of the LA on *A. versicolor* growth and STE biosynthesis was determined according to the method described in our previous study [36]. Briefly, the effect of LA on STE biosynthesis was determined in a sterile Yeast Extract Sucrose (YES) broth (yeast extract 20 g, sucrose 150 g, $MgSO_4 \times 7H_2O$ 0.5 g, distilled water 885 mL), which was divided into equal volumes (100 mL) and poured into Erlenmeyer flasks (volume 250 mL). LA was aseptically added to the YES broth to obtain the following concentrations: 5, 10, 20, 50, and 102 mg/mL. Each of the YES broths with the different LA concentrations was inoculated with 5 mL of a prepared conidial suspension of *A. versicolor* (10^6 spores/mL). The concentration of the fungal suspension was determined by a hemocytometer. Incubation was performed stationary at 25°C for 7 and 14 days. YES broth inoculated with a conidial suspension of *A. versicolor* without LA addition was used as a control sample. The STE content was determined on the 7th and 14th day of incubation at all applied LA concentrations. After incubation, inoculated YES broth was filtered through Whatman filter No. 1. Then, the obtained *A. versicolor* mycelia were washed with distilled water, placed on pre-weighed Petri plates, and dried (6 hr at 60°C and then overnight at 40°C). The dry weights of the samples and control sample were measured, and the mycelial growth inhibition was calculated as follows: Mycelial growth inhibition (%) = (Control sample weight – Sample weight) × 100/Control sample weight.

The STE content was determined in filtrates using the liquid chromatography tandem mass spectrometry (LC-MS/MS) technique. The inhibition of STE production was calculated as follows:

$$\text{Inhibition of STE production (\%)} = \frac{C1 - C2}{C1} \times 100$$

where C1 was the concentration of STE in the control sample and C2 was the concentration of STE in the treated sample.

Statistical analysis

The tests were performed in with 3 replications. MS Excel was used to calculate means and standard deviations. Significant differences between mean values were determined by one-way ANOVA followed by Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Disc diffusion method results

LA demonstrated an ability to reduce or inhibit the growth of the investigated fungal species. The inhibitory effect depended on the applied concentration of LA, fungal species, and applied method of testing.

The results for the inhibitory activity of LA on the growth of fungal species, obtained by measuring the inhibition zones, at all applied concentrations and amounts of LA are shown in Table 1. LA showed the highest inhibitory effect on *A. versicolor*, while *P. aurantiogriseum* was moderately affected. Most resistant to the LA were *A. niger* and *A. parasiticus* (there was no inhibition zone at all tested LA concentrations). The strongest inhibitory effect of LA on the investigated fungal species was achieved at a concentration of 975 mg/mL (10 µL).

Microdilution method results

The results for the MICs and MFCs of LA against the investigated fungal species are shown in Table 2. The MIC of LA against the investigated fungal species ranged from 25.0 mg/mL (*P. aurantiogriseum* and *A. parasiticus*) to 50.0 mg/mL (*A. niger*). The lowest MFC of LA was 50.0 mg/mL (*P. aurantiogriseum* and *A. parasiticus*), while the highest value was 100.0 mg/mL (*A. niger*; Table 2).

From the results obtained in the microdilution tests (Table 2), it can be seen that low pH in the medium (pH_{MIC} and pH_{MFC} ranged between 2.61 and 2.07) probably contributed to the antifungal effect of LA. Some authors have stated that lower pH and higher MIC are needed to inhibit *Aspergillus* spp., which is in agreement with our study [23, 24]. Moreover, effective use of organic acids depends on the rate of dissociation [11]. At low pH, organic acids are in undissociated forms and thus are probably responsible for the antimicrobial activity [24]. On the other hand, some authors have stated that the precise mechanism of antimicrobials cannot be defined because of complex interaction and synergistic effects between cell-growing compounds [11, 24].

Generally, the research on the antifungal activity of LA against food-borne fungi is limited. Many studies have shown the antifungal activity of an LA bacteria supernatant, in which the predominant antimicrobial agent was LA [37–40]. Some authors have suggested that LA is not sufficiently effective as an antifungal agent [41, 42], while other studies have indicated that LA has potential antifungal activity [23, 43].

Higgins and Brinkhaus [22] investigated the inhibitory activity of organic acids on fungal growth. The results of their investigations showed that even at an LA concentration as high as 0.5%, the growth of *Aspergillus* spp. and *Penicillium* spp. was inhibited by 60%. These authors suggested that effective inhibition can be achieved at a higher LA concentration (4.53 mg/mL). El-Gazzar *et al.* [44] showed that LA at a concentration of 2% (200 mM) inhibited the growth of *A. parasiticus*. Škrinjar *et*

Table 1. Lactic acid antimicrobial activity against fungal species by the disc diffusion method

Lactic acid concentration (mg/mL)	Diameter of inhibition zone (mm)				
	Amount (µL)	*Penicillium aurantiogriseum*	*Aspergillus versicolor*	*Aspergillus parasiticus*	*Aspergillus niger*
102	15	-	-	-	-
	20	-	-	-	-
	25	-	-	-	-
209	15	-	-	-	-
	20	-	-	-	-
	25	8.0 ± 1.15*	10.0 ± 0.00	-	-
321	15	-	7.6 ± 0.57	-	-
	20	7.0 ± 0.57	11.0 ± 1.00	-	-
	25	10.0 ± 0.00	12.6 ± 0.57	-	-
975	1.5	-	17.0 ± 0.77	-	-
	2.5	-	19.0 ± 0.00	-	-
	5	-	21.0 ± 0.56	-	-
	10	11.0 ± 0.00	24.0 ± 0.35	-	-

*Values represent means ± standard deviation calculated from three parallel tests. A hyphen (-) indicates that there was no inhibition zone.

Table 2. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of lactic acid for fungal species

Fungi	pH_{MIC}	Minimal inhibitory concentration (MIC) (mg/mL)	pH_{MFC}	Minimal fungicidal concentration (MFC) (mg/mL)
Penicillium aurantiogriseum	2.61	25.0	2.33	50.0
Aspergillus parasiticus	2.61	25.0	2.33	50.0
Aspergillus versicolor	2.44	37.5	2.17	75.0
Aspergillus niger	2.33	50.0	2.07	100.0

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al. [27] investigated the effect of LA on the growth of *Penicillium* spp. isolated from dairy products. The results of their study showed only reduced growth of *P. aurantiogriseum* in a medium containing 2.75% of LA. At higher concentrations, the inhibitory activity of LA against *Penicillium* spp. was increased by lowering the pH of the substrate [27].

Some authors have suggested that *Penicillium* species are more resistant to fungicides than *Aspergillus* species [45, 46]. Pundir and Jain [21] showed that LA had an inhibitory effect against *Aspergillus* spp. (50%), while it was ineffective for inhibiting *Penicillium* spp.

The inhibitory effect of lactic and acetic acids on the growth of toxigenic and non-toxigenic *Aspergillus flavus* species was studied by León Peláez *et al.* [47]. The results of their study suggested that the inhibition of some toxigenic *A. flavus* species required a higher concentration of LA compared with the non-toxigenic species. The MIC of the non-toxigenic *A. flavus* species was 357.7 mM, whereas those of the toxigenic species ranged from 274 to 405.4 mM.

Several authors have confirmed the synergistic antifungal effect of LA and phenyllactic acid [37, 38, 48]. Lavermicocca *et al.* [48] found that the synergistic action of phenyllactic and LA on *A. niger* increased inhibition by 30% compared with the inhibition obtained with only phenyllactic acid.

The differences in sensitivity observed among the fungal species may be related to their capacity to alter cell metabolism in response to an acidic environment. The antifungal activity of organic acids depends on the pH value. At lower pH values of a medium, LA will exist substantially in its non-dissociated form, in which molecules easily pass through the cell membrane and cause loss of viability and cell destruction [49]. Alakomi *et al.* [11] reported that the antimicrobial activity of LA is largely, but not totally, ascribed to the non-dissociated LA form. Lind *et al.* [23] showed that *Penicillium roqueforti* was most sensitive to the effect of LA at pH 3 (no growth), while at pH values of 5 and 7, the MIC value was 70 mM. The MIC of LA for *Aspergillus* spp. ranged from 200–250 mM (pH 3) to >500 mM (for pH 5 and 7). Gerez *et al.* [24] found that the MIC_{50} of LA for *Penicillium* sp. depended on the pH and ranged from 80 mM (pH 3.5) to 160 mM (pH 6), while that for *A. niger* ranged from 180 mM (pH 3.5) to >300 mM (pH 6).

The inhibitory activity of LA against fungi may depend on the applied method of determination. The results obtained in our study showed that the microdilution method required a lower concentration of LA to inhibit the tested fungi (Tables 1 and 2). Reasonable disagreement of the results between the disc diffusion and microdilution methods is probably due to the fact that the LA solutions had closer contact with fungi when used in the latter method [50]. Lind *et al.* [23] explained that the inhibitory activity of LA determined by the microdilution method might be higher than that compared to the activity determined by the disc diffusion method due to direct contact of fungal conidia with the acid. Unlike the microdilution method, testing with the disc-diffusion method was dependent on diffusion of antimicrobial agents through agar media [50, 51].

Jiang (Master's Thesis 2009) observed that the diffusion of LA in a medium, and the nutrients requirements and growth rates of individual species, can influence the results. Some studies indicate that *A. niger* and several *Penicillium* species have the ability to reduce organic acids concentration (it they are present at lower concentrations in the medium) by decarboxylation. Also, fungi have the ability to use organic acids, including LA, as a relatively good source of carbon, with the number of carboxylic groups being an important parameter that stimulates (polycarboxylic organic acids) or inhibits (mono- and unsaturated organic acids) fungal growth [52].

Effect of LA on STE biosynthesis by Aspergillus versicolor

The results for the inhibitory effect of LA on STE biosynthesis by *A. versicolor* in YES broth with 7 and 14 days of incubation are shown in Table 3. The results shown in Table 4 present the effect of LA on *A. versicolor* growth inhibition in YES broth after 7 and 14 days of incubation. LA demonstrated the ability to reduce or inhibit STE biosynthesis and mycelial growth. The inhibitory effect of LA proportionally increased with the increase in LA concentration and lowering of the pH of YES broth. The period of exposure to LA also affected fungal growth and STE biosynthesis. LA concentrations in the range of 5 to 20 mg/mL demonstrated a slight to moderate ability to inhibit fungal growth and consequently STE biosynthesis. At concentrations above 50 mg/mL, there was no fungal growth and thus no STE biosynthesis.

As can be seen in Table 4, *A. versicolor* growth was inhibited by LA, and consequently, STE biosynthesis was reduced. An LA concentration of 5 mg/mL (pH 4.04) partially inhibits STE biosynthesis and mycelial growth. The highest inhibitory activity (for a concentration of 5 mg/mL) was detected after 14 days of incubation, and it amounted to 20% for STE biosynthesis and 13.7% for *A. versicolor* growth. Increase of the LA concentration to 20 mg/mL (pH 3.19) substantially hindered STE biosynthesis

Table 3. Effect of lactic acid on sterigmatocystin (STE) biosynthesis by *Aspergillus versicolor* at 25°C in YES broth

Lactic acid concentration (mg/mL)	Initial pH of YES broth	Sterigmatocystin (ng/mL) (mean ± SD[b])		Inhibition (%)	
		Days			
		7	14	7	14
Control	6.36	40.0 ± 4.75^{b*}	80.0 ± 7.35^{d}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
5	4.04	38.4 ± 5.00^{b}	64.0 ± 6.18^{c}	4.0 ± 1.11^{a}	20.0 ± 0.4^{b}
10	3.69	36.0 ± 6.00^{b}	52.0 ± 4.45^{b}	10.0 ± 4.4^{b}	35.0 ± 0.4^{c}
20	3.19	32.0 ± 5.57^{b}	44.0 ± 3.25^{b}	20.4 ± 4.5^{c}	45.0 ± 1.0^{d}
50	2.78	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	100.0 ± 0.0^{d}	100.0 ± 0.0^{e}
102	2.47	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	100.0 ± 0.0^{d}	100.0 ± 0.0^{e}

*Values represent means ± standard deviation calculated from three parallel tests. Values with different superscript letters with in a column are significantly different ($p<0.05$). Superscript letters indicate statistically significant differences.

Table 4. Effect of lactic acid on *Aspergillus versicolor* growth at 25°C in YES broth

Lactic acid concentration (mg/mL)	Mycelial dry weight (g)		Inhibition (%)	
	Days			
	7	14	7	14
0 (control)[a]	0.94 ± 0.02[e]*	2.03 ± 0.15[e]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
5	0.85 ± 0.02[d]	1.75 ± 0.05[d]	9.2 ± 0.4[b]	13.7 ± 4.0[b]
10	0.75 ± 0.03[c]	1.32 ± 0.07[c]	19.9 ± 0.9[c]	34.8 ± 1.6[c]
20	0.63 ± 0.03[b]	1.17 ± 0.08[b]	32.4 ± 1.5[d]	42.6 ± 0.5[d]
50	0.00 ± 0.00[a]	0.00 ± 0.00[a]	100.0 ± 0.0[e]	100.0 ± 0.0[e]
102	0.00 ± 0.00[a]	0.00 ± 0.00[a]	100.0 ± 0.0[e]	100.0 ± 0.0[e]

*Values represent means ± standard deviation calculated from three parallel tests. Values with different superscript letters with in a column are significantly different ($p<0.05$). Superscript letters indicate statistically significant differences.

and *A. versicolor* growth. This LA concentration also showed the maximal inhibition activity after 14 days of incubation (decreases in STE concentration and mycelial growth of 45% and 34.8%, respectively). Growth and STE biosynthesis by *A. versicolor* at 7 and 14 days of incubation were completely inhibited by the LA concentrations of 50 mg/mL (pH 2.78) and 102 mg/mL (pH 2.47) (Tables 3 and 4).

Generally, research on the influence of LA on STE biosynthesis is limited in comparison with other mycotoxins. Reiss [25] investigated the effects of LA on STE biosynthesis by *A. versicolor* in wheat bread. The results of that study showed that complete inhibition of STE biosynthesis can be achieved at 50 mg/mL of LA, which is in agreement with the results presented in this study. Rasić *et al.* [26] found that the addition of lactic, citric, and acetic acid to yogurt and milk can significantly decrease aflatoxin B_1 (AFB_1) content. The AFB_1 content (1,000 mg/kg) in milk acidified with LA was decreased by 84%.

Reduction of the AFB_1 concentration is associated with its conversion to a less toxic metabolite, B_{2a}. Méndez-Alborez *et al.* [28] investigated the stability and effect of organic acids on the biosynthesis of aflatoxins. They found that LA inhibited AFB_1 biosynthesis by more than 67% (the inhibition was proportional to the LA concentration). Guimarães *et al.* [53] observed that LA is less effective in inhibiting fungal growth and biosynthesis of ochratoxin A by *Penicillium nordicum* MUM08.16 (CBS 112573). On the other hand, Škrinjar *et al.* [27] found that LA at a concentration of 1.5% completely inhibited the ochratoxin A biosynthesis in YES broth by *Penicillium* spp.

CONCLUSION

Applying the disc diffusion method, LA exhibited the strongest effect against *A. versicolor*, while *A. niger* and *A. parasiticus* were not sensitive to LA at the applied concentrations. Moreover, STE biosynthesis and mycelial growth of *A. versicolor* in YES broth during 14 days of incubation were completely inhibited at a concentration of 50.0 mg/mL. The obtained results suggest that LA is a suitable antifungal and antimycotoxigenic agent in food.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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