

## Inhibition of Fungal Growth And Mycotoxin Productions Using Lactic Acid Bacteria Isolated From Milk And Fermented Food

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**Abstract:** Twenty one bacterial isolates were obtained from the normal habitats of lactic acid bacteria (LAB). They were isolated from women milk, sheep milk, yoghurt, pasteurized milk and fermented food "pickles". Screening of all isolates of LAB for antimicrobial activity on MRS medium revealed that the highest antimicrobial activities were against *Aspergillus niger*, *Penicillium* sp. and *Aspergillus flavus*. No activity was observed against *A. ochraceus* and *Fusarium sporotrichoides*. The isolate W9 was the most active isolate and it was characterized and identified through physiological and biochemical tests, in addition to examination with light and scanning electron microscope as *Lactobacillus bulgaricus* W9. The maximum antimicrobial activity of the selected isolate W9 was achieved using MRS medium after 48 hours of incubation in aerobic conditions at 30°C and pH 6 of the culture medium. The antimicrobial agent was extracted and crude extract affected the growth of a number of fungi and aflatoxins production by *A. flavus*. The antimicrobial compound decreased fungal growth and mycotoxins production. Thus, they can be used as food biopreservative.

**Keywords:** Lactic acid bacteria, *Aspergillus flavus*, mycotoxins, antifungal, *Lactobacillus bulgaricus*

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### I. Introduction

Molds and yeasts cause major problems in food and feed as spoilage organisms. They are particularly important because they produce mycotoxins<sup>(1)</sup>. Bio-preservation, the use of microorganisms to preserve food and feed, has been considered as an alternative to the use of chemical preservation in the expectation that they could be safer<sup>(2)</sup>. Lactic acid bacteria (LAB) are a broad group of aerotolerant Gram positive, bacteria, catalase-negative, non-spore forming rods and cocci, usually non-motile and ferment carbohydrates to form lactic acid as the major end product<sup>(3)</sup>. With occasional exception, Lactic acid bacteria are widely distributed in several raw materials (milk, meat, and flour), soil, silage and waste products<sup>(4)</sup>.

Most representatives of LAB do not pose any health risk to man and are designated as "Generally Recognized as Safe" (GRAS). They have commonly been used as a starter culture and play an essential role in manufacturing of a wide variety of fermented food such as curd, cheese, yoghurts, dry sausages, beers and sourdoughs<sup>(5)</sup>.

Lactic acid bacteria (*Lactobacillus fermentum* and *Lactobacillus rhamnosus*) showed growth inhibition of the mycotoxin-producing *Aspergillus*. *L. rhamnosus* O236 from sheep milk showed the highest fungal inhibition<sup>(6)</sup> while LAB isolated from fermented cereal gruels inhibited at least one aflatoxin-producing fungi to varying extents<sup>(7)</sup>. *Lactobacillus coryniformis* subsp. *coryniformis* produced proteinaceous compounds with a broad spectrum inhibitory action against several molds such as *Aspergillus fumigatus*, *A. nidulans*, *Penicillium roqueforti*, *Mucor hiemalis*, *Talaromyces flavus*, *Fusarium poae*, *F. graminearum*, *F. culmorum*, and *F. sporotrichoides*<sup>(8)</sup>. Various mechanisms have been suggested to be responsible for the inhibitory effects of the bacteria on fungal growth, such as nutritional competition, secondary metabolites, pH or combinations of these mechanisms<sup>(9)</sup>. The inhibitory activity towards moulds could be considered a characteristic for the selection of LAB used as starter cultures in grain ensiling of animal food in order to prevent avian fungal infection<sup>(6)</sup>. The aim of this work was to isolate lactic acid bacteria from different habitats and screen these isolates against some mycotoxigenic fungi. The effect of *Lactobacillus bulgaricus* on growth and aflatoxins production of *Aspergillus flavus* was determined.

### II. Materials And Methods

#### Bacterial isolates

Twenty one bacterial isolates were obtained from women milk, sheep milk, yoghurt, pasteurized milk and fermented food. All isolates of lactic acid bacteria were selected and purified on De Mann, Rogosa and Sharpe (MRS) medium after incubation for 48 hr. at 30°C and pH 6.

### Fungal isolates

The toxigenic fungi, *Aspergillus niger*, *Aspergillus flavus* *Penicillium* sp., *Alternaria* sp., *Trichosporon mycotoxinivorans* and *Cladosporium* sp. in addition to the non toxigenic fungi, *Aspergillus ochraceus* and *Fusarium* sp. were obtained from the culture collection of Biology Department, Faculty of Science, KAU.

### Selection of the most active bacterial isolate

All isolates of LAB were screened for antimicrobial activity against *Aspergillus niger*, *Penicillium* sp., *Aspergillus flavus*, *Aspergillus ochraceus* and *Fusarium* sp. using modified overlay method that described by Magnusson and Schnurer (2000)<sup>(10)</sup>. The inhibitory effect was determined on plates using an overlay technique; the potentially inhibitory microorganisms were inoculated first on MRS solid agar medium and then the tested fungi were inoculated on a top layer soft PDA media).

### Identification of the most active bacterial isolate

The bacterial isolate that showed maximum antifungal activity in this study was selected and identified to genus level initially by morphological and physiological tests and according to Bergey's Manual of systematic bacteriology<sup>(11, 12)</sup>.

### Effect of the active material on fungal growth, spore production and aflatoxins production

*Lactobacillus* bacterium was grown in De Mann, Rogosa and Sharpe (MRS) broth medium for 48 hours of incubation in aerobic conditions at 30°C and pH 6. The cells were collected and the culture filtrate was precipitated with 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The obtained precipitate was collected in cellophane membrane and dialysis was carried out for several times. The obtained material was lyophilized, weighted and dissolved in 2 ml of dist. water. The antimicrobial activity of the crude extract against different fungi was determined on potato dextrose agar (PDA) using agar well diffusion assay. The antimicrobial activity was measured by the mean diameter of three inhibition zones. Effect of the active material (crude extract) on spore (spore/ml) and aflatoxins production by *Aspergillus flavus* was studied in Czapek-Dox broth medium and compared to control (medium without the addition of the crude material). Czapek-Dox broth medium was prepared, the crude extract (5 mg/ml) was added and the medium was inoculated with *A. flavus*. After growth for 5 days, fungal growth (spore/ml) was determined by counting number of spores/ml using PDA medium. Aflatoxins production by *A. flavus* was determined in the filtrate by thin-layer chromatography (TLC) and Chromato-Vue Cabinet (Model CC- 60, UVP Inc, an Gabriel, California) under visible light or short wavelength UV light (254 nm) and long wavelength (366 nm).

## III. Results

Only twenty one bacterial isolates were obtained from the normal habitats of Lactic acid bacteria. The isolates were from women milk, sheep milk, yoghurt, pasteurizing milk and fermented pickles, respectively. All isolates of LAB were screened for antifungal activity and the results revealed that the highest antimicrobial activities were against *Aspergillus niger*, *Penicillium* sp., *Aspergillus flavus*. No activity was observed against *A. ochraceus* and *Fusarium sporotrichoides* (Table 1, Figure 1, 2). The isolate W9 was the most active isolate and it was characterized and identified through physiological and biochemical tests. The selected isolate was identified as *Lactobacillus bulgaricus* W9. The activity of the crude extract of *Lactobacillus bulgaricus* was tested against different fungi, *Aspergillus niger*, *Aspergillus flavus* *Penicillium* sp., *Alternaria* sp., *Trichosporon mycotoxinivorans* and *Cladosporium* sp. using agar well diffusion method (Table 2, Figure 3). The highest inhibition was against *Aspergillus flavus*, *Alternaria* sp. and *Trichosporon mycotoxinivorans* (diameter of inhibition zones 18-19 mm) and the lowest activity was against *Aspergillus niger* (13.6 mm). *Aspergillus flavus* was selected for the studying of the effect of crude extract on sporulation (spore/ml), spore germination and mycotoxins production. The result in Table (3) shown that the extracted material decreased spore formation to 0.002 x 10<sup>4</sup> spore/ml compared to control (2.0 x 10<sup>4</sup> spore/ml). Moreover, % of spore germination was determined on sterile water agar 1.5 % (W/V). The % of spore germination of *A. flavus* was decreased to 33.7 % compared to control (73%). The detected toxin using TLC was decreased in broth medium containing the crude extract of *Lactobacillus bulgaricus* (Figure 4).

## IV. Discussion

Nearly 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods<sup>(13, 14)</sup>. In addition, poultry for human consumption is generally fed on cereals or their products<sup>(15)</sup>. There was a predominance of *Aspergillus* species during storage period of coffee beans<sup>(16)</sup>. Therefore growth of the fungus in cereal crops could affect humans not only after consumption of infected cereals, but also after chicken consumption. In addition, the presence of toxigenic moulds represents a potential risk of

mycotoxin contamination and considering the worldwide increased use of herbal products as alternative medicine<sup>(17)</sup>.

Inhibition of mycotoxigenic fungi is necessary in order to avoid toxin formation in food and feed. According to our results, natural control of the microflora could be realized by beneficial microorganisms. The number of publications on antifungal agents from LAB is still low<sup>(18)</sup>. LAB produced a variety of antimicrobial compounds. Various mechanisms have been suggested to be responsible for the inhibitory effects of the bacteria on fungal growth, such as nutritional competition, secondary metabolites, pH or combinations of these mechanisms<sup>(9)</sup>. *Lactobacillus acidophilus* and *Bifidobacterium animalis* strains are able to detoxify the mycotoxins. Both species can be used for the production of probiotic fermented foods, therefore our findings may contribute to the development of strategies for the detoxification of contaminated plant derived products with these toxins by use of LAB<sup>(19)</sup>. Other authors have suggested that aflatoxin biosynthesis was inhibited by LAB but that the bacteria were not efficient enough to remove aflatoxin from the medium<sup>(20)</sup>. Our studies confirm previous studies demonstrating the inhibitory activity by LAB against a mycotoxin-producing fungus. *Lactobacillus bulgaricus* W9 isolated from sheep milk and selected for its technological properties showed highest fungal inhibition of the microorganisms assayed. The inhibitory activity of lactobacilli against moulds could be due to different factors. It is worthy to mention that the obtained 21 bacterial isolates, which were screened for their antimicrobial activity against some mycotoxigenic fungi using modified overlay method described by Magnusson and Schnurer, (2000)<sup>(10)</sup>. Onilude et al., (2005)<sup>(7)</sup> and Munoz et al., (2010)<sup>(6)</sup> used the modified overlay method to detect the antimicrobial activity of lactic acid against aflatoxin-producing fungi.

Many authors demonstrated the antifungal activity of lactic acid bacteria<sup>(21,22)</sup>. Batish et al.<sup>(23)</sup>, observed that the antifungal activity of a *L. acidophilus* strain was maximum at 30°C after 48 h incubation, whereas increasing the incubation period resulted in a lower antifungal activity. These anti-mycotoxinogenic metabolites could also be produced during LAB growth<sup>(24)</sup>. Growth, cell numbers, morphological characters and toxin production of *Aspergillus flavus*, treated with the crude extract of crude extract filtrate were determined and compared to that obtained for untreated *Aspergillus* (control). The growth and the spores number were decreased by the crude antimicrobial agent. Our results are in agreement with those previously observed by Onilude et al., (2005)<sup>(7)</sup> that showed that the lactic acid bacterial isolates affect different *Aspergillus* species prior to the sporulation of the latter. *Lactobacillus fermentum* RS2 was observed to exhibit maximum inhibition on mycelial development for most of the *Aspergillus* species while *Lactobacillus* spp. had the lowest. Thyagaraja and Hosono, (1994)<sup>(25)</sup> suggested that aflatoxin biosynthesis was inhibited by LAB but the bacteria were not efficient enough to remove aflatoxin from the medium. The interaction between mycotoxin producing fungi and other microorganisms is a common phenomenon in nature that can affect fungal growth and/or production of mycotoxins<sup>(26)</sup>.

The use of antifungal LAB instead of chemical preservatives would enable the food industry to produce organic food without addition of chemical substances. In addition to the already known excellent properties of LAB they could enhance the nutritional value and prolong conservation of food<sup>(6)</sup>.

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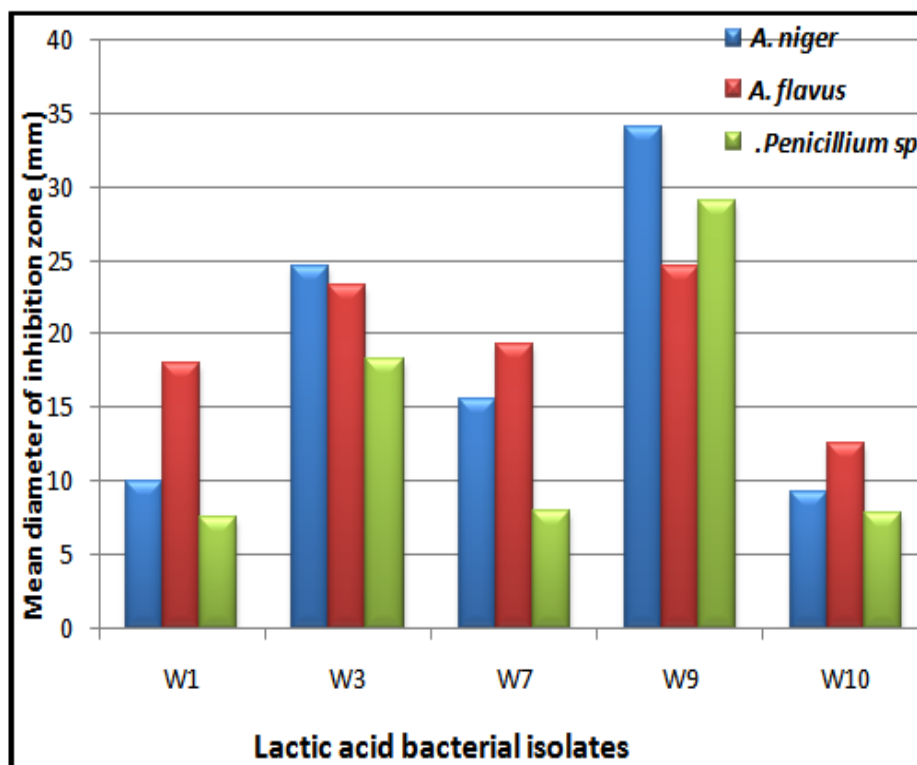
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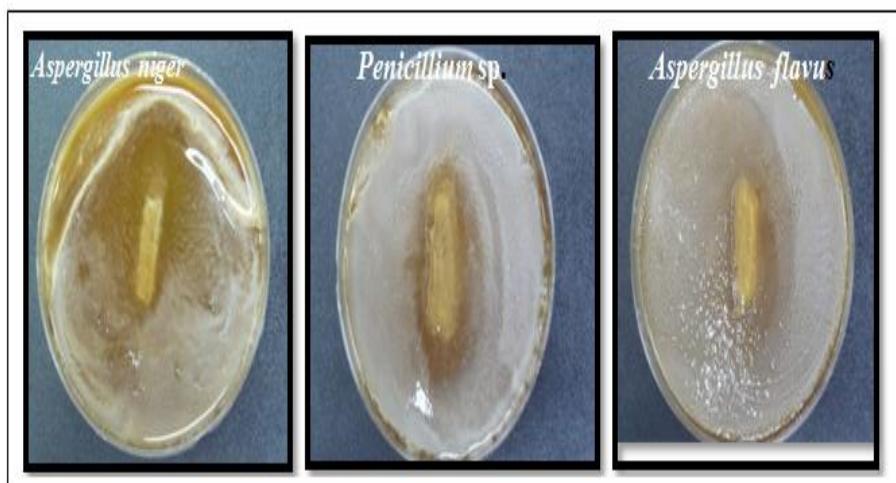
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**Table 1** Screening of the recovered bacterial isolates for antimicrobial activity against some tested fungi using modified overlay method

Isolate code	Mean diameter of inhibition zone (mm) ± SD				
	<i>A. niger</i>	<i>A. ochraceus</i>	<i>A. flavus</i>	<i>Penicillium</i> sp.	<i>Fusarium sporotrichoides</i>
W <sub>1</sub>	10.0±2.0	0.0±0.0	18.0±2.6	7.6±1.5	0.0±0.0
W <sub>3</sub>	24.6±6.4	0.0±0.0	23.3±3.5	18.3±2.0	0.0±0.0
W <sub>7</sub>	15.6±4.0	0.0±0.0	19.3±5.1	8.0±2.6	0.0±0.0
W <sub>9</sub>	34.0±4.5	0.0±0.0	24.6±0.5	29.0±1.0	0.0±0.0
W <sub>10</sub>	9.3±0.5	0.0±0.0	12.6±2.0	7.9±1.7	0.0±0.0



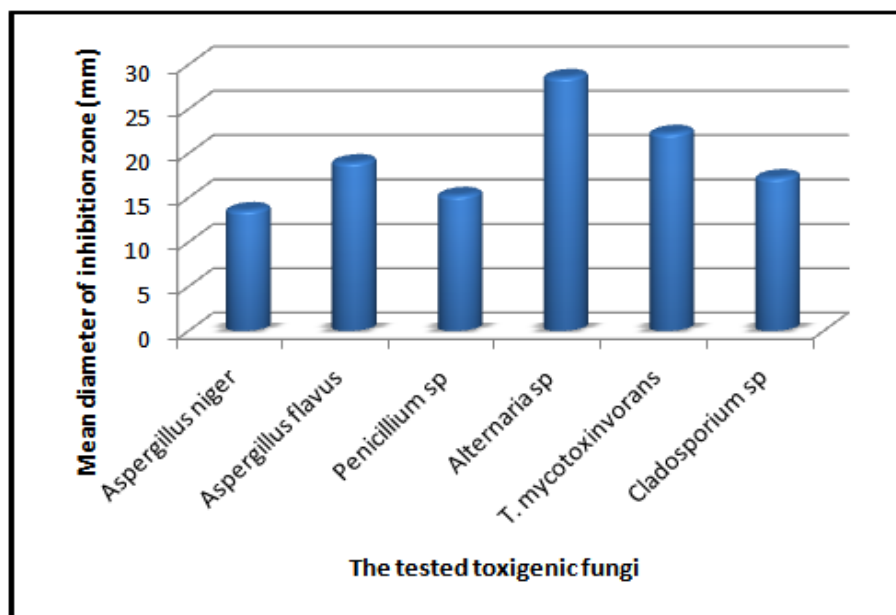
**Figure 1** The antimicrobial activity (inhibition zone mm) of some LAB against some fungi



**Figure 2** Antimicrobial activity of the isolated bacterium W9 against different fungi using modified overlay method

**Table 2 :** The effect of the crude extract of the selected bacterial isolate W9 against some toxigenic fungi using well-diffusion method

The tested toxigenic fungi	Mean diameter of inhibition zone (mm) ± SD
<i>Aspergillus niger</i>	13.6 ±1.5
<i>Aspergillus flavus</i>	19.0 ±1.0
<i>Penicillium sp.</i>	15.3 ±4.5
<i>Alternaria sp.</i>	18.6 ±1.1
<i>Trichosporon mycotoxinivorans</i>	18.3 ±2.0
<i>Cladosporium sp.</i>	17.3 ±1.1



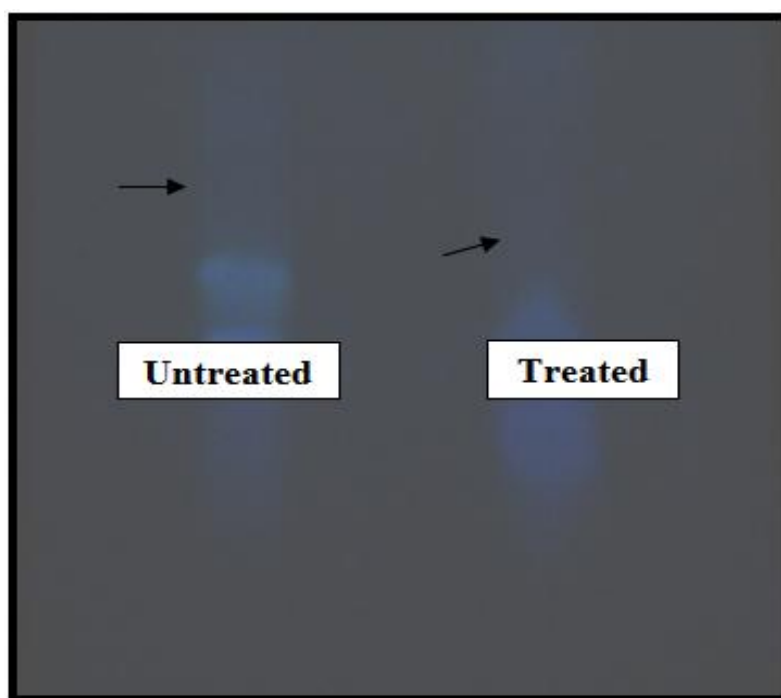
T. mycotoxinivorans : *Trichosporon mycotoxinivorans*

**Figure 3** Antimicrobial activities (inhibition zone mm) of the selected bacterial isolate W9 against some toxigenic fungi.

**Table 3** Effect of the crude extract of lactic acid bacteria on spore numbers of *Aspergillus flavus* and % of germination

	Normal	Treated
Spore number/ml	$2.0 \times 10^4 \pm 3.6$	$0.002 \times 10^4 \pm 1.0^*$
% of germination	$73.01 \pm 4.4$	$33.7 \pm 1.5^*$

\*: Significant at p-value  $\leq 0.05$



**Figure 4** Aflatoxins detection At  $UV_{254}$  in treated and untreated *Aspergillus* extract using UV Light

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