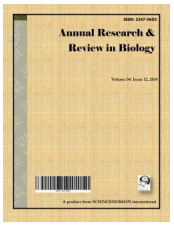

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# Biocontrol Activity of *Bacillus megaterium* against the Fungus *Aspergillus flavus* Isolated from Poultry Feeds in Algeria

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author LL designed the study. Author ND wrote the protocol and the first draft of the manuscript and managed the analyses of the study. Author ND managed the literature searches. Both authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This study aimed to evaluate the antagonist effect of the bacteria *Bacillus megaterium* against the toxigenic fungus *Aspergillus flavus using different methods.*
**Methodology:** According to the method described by Vincent et al. (1991) and Arras (1993) we have determined the effect of *B. megaterium* on the growth of *A. flavus* using fungal disc and fungus spore respectively. In order to see the effect of cell free supernatant of *B. megaterium* on the dry mass of *A. flavus,* culture bacteria of 5 days old was centrifuged; filtered and cell-free supernatant was incubated with 5 mm circular plug of *A. flavus.* After incubation at 28°C for 9 days. The dry mass was determined by weighting every 72 h and compared with the control. Elucidation of antagonistic mechanism of *B. megaterium* was examined using the following tests: Hydrogen Cyanide production, production of Ammonia ($NH_3$) and production of extracellular enzymes such: Protease, Chitinase and Amylase.
**Results:** The results showed that *B. megaterium* is an antagonistic bacterium that has been shown high effectiveness against the fungus *A. flavus* isolated form poultry feeds in Algeria. Results

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indicated an almost entirely decrease (47.56%) of mycelial growth using fungal disc and (40.75%) using fungus spore. In cell-free supernatant *in vitro* assay, *B.megaterium* showed significant inhibitory activity against *A. flavus* when the dry mass of mycelium decrease from 1.25 g to 0.83 g compared with the control.

**Conclusion:** This research shows that *B. megaterium* is quite important and effective as biocontrol agent against the toxigenic mold *A. flavus* in poultry feeds. This inhibition action is probably due to the synergistic effects of the factors such as the production of antibiotics and the extracellular enzymes such as protease, cellulase, chitinase and amylase.

*Keywords: Biocontrol; poultry feeds; Bacillus megaterium; Aspergillus flavus.*

## 1. INTRODUCTION

Poultry feeds are exposed to several phytopathogens contaminants such as fungi. Fungi occurrence and growth on poultry feeds is one of the major threats to poultry economic and health in addition to their negative impacts on nutritional and organoleptic properties. Fungi can also synthesize different mycotoxins which affect the nutritional properties of the poultry feeds and represent a worldwide problem for animal and human health. More than 100.000 fungal species are considered as natural contaminants of agricultural and food products [1].

The majority of the toxic species belong to the genera *Aspergillus, Penicillium, Fusarium* and *Alternaria*, they are widely distributed in soil, air and plant material [2].

There is a serious hygienic risk about fungi contaminants of industrial animal feed endangering safety of food of animal origin especially milk, meat and eggs [3].

Mycotoxins secreted by fungi not only affect the nutritional and organoleptic properties of the feeds, but also represents a worldwide problem for human and animal health include hepatotoxicity, nephrotoxicity, immunotoxicity and genotoxicity [4,5].

However, an increase in the public concerning the negative health and environmental effects had made a compulsory search of alternative control methods [5]. Thus the biocontrol of toxigenic fungi in feeds by antagonistic microorganisms could be an alternative to chemical methods.

Several bacteria reduce the production of mycotoxins, such as *Bacillus megaterium* which has been reported as efficient antagonist bacteria against contaminant fungi in various food products, including dairy products [4,6-8]. However, the potential of *B. megaterium* as antagonist against toxigenic fungi *in* poultry feeds has not been explored. In this study we have evaluate the antagonistic effect of the bacteria *B. megaterium* against the toxigenic fungus *A. flavus.*

## 2. MATERIALS AND METHODS

### 2.1 Fungal material

*A. flavus* was isolated from six samples of poultry feeds collected from factories of Sétif State, Algeria.

Ten grams of each sample was added to 90 ml peptone water 0.1%(w/v) and kept at room temperature for approximately 30 min. This mixture was then shaken and diluted to final concentrations of $10^{-2}$, $10^{-3}$ and $10^{-4}$. Then, 100 µl of each dilution was spread on the surface of solid media PDA (Potato Dextrose Agar); plates were incubated at 28ºC for 7 days.

Taxonomic identification of *A. flavus* was made according to microscopic criteria in accordance with appropriate keys.

Fungal culture was maintained on slants of PDA at 4ºC. Spore suspension of *A. flavus* was prepared by collecting spores from 5 days-old colonies (growth on PDA at 28ºC) in peptone water (2 g/l) with 0.015% tween 80 to assist the dispersal of conidia. The spore concentration was enumerated by a hemocytometer.

### 2.2 The Antagonistic Bacteria

The antagonistic bacteria used in this work was isolated from apple in a previous study in the Laboratory of applied microbiology at the University of Farhat Abbas Sétif 1(Algeria).The methodology and techniques used for the identification (morphological, physiological and biochemical tests) are described in Bergey s Manual of Systematic Bacteriology. The isolate was maintained on nutrient agar slants at 4ºC.

#### 2.2.1 Antagonistic activity of *B. megaterium*

*2.2.1.1 Using fungus spore*

The test was carried out to see the effect of *B. megaterium* on the spore *in vitro*, according to the method described by Arras [9].The bacterium isolate of *B. megaterium* was grown on NB (Nutrient broth) and tested *in vitro* on PDA to evaluate its inhibitory potential against the spore of *A. flavus* . Each test was carried out by streaking in the form of a cross on Petri dishes 20 µl of twenty four hour old culture of bacteria. 20 µl of suspension of spores with density of $10^4$ spore/ml of *A. flavus* was placed in each plate at 3 cm from bacteria stripe. The plates with only suspension of spores but without bacterial streaks were used as the control. All plates were incubated at 28+2ºC for 7 days and inhibition of the radial growth of the *A. flavus* was measured. Each treatment was replicated three times. Colony diameter of the *A. flavus* was measured and compared with the control. Percentage inhibition of the fungus by the *B. megaterium* was calculated by using the formula as follows:

I=(C-I)*100/C

Where: I: Percent inhibition of mycelium.
C: Growth of mycelium in the control
T: Growth of mycelium in the treatment.

*2.2.1.2. Using fungal disc*

The test was carried out to see the effect of *B.megaterium* on the growth of mycelium in vitro, according to the method described by Vincent et al. [10]. *B.megaterium* culture was grown overnight (approximatly$10^9$ cfu/ml) and 3 µl of culture was spotted on plates of PDA and incubated for 48h at 28ºC. 5.0 mm circular plug of *A. flavus* from the leading edge of 7 days old culture on PDA was placed on the opposite side of each well. The plates with only *A. flavus* without bacterial streaks severed as the control. The inoculated plates were incubated at 28ºC for 7 days and the inhibition of radial growth of the *A. flavus* was measured. Each treatment was replicated three times. Colony diameter of *A. flavus* was measured and compared with the control. Percentage inhibition of the fungus was calculated by the formula described above.

### 2.3 Effect of *B. megaterium* Cell-free Supernatant on the Dry Mass of *A. flavus*

According to the method of Krebs et *al* [11] with some modifications *B. megaterium* was grown in nutrient broth for 5 days at 28ºC. After incubation, cultures were centrifuged and filtrated to separate bacterial cells. The cell-free supernatant was loaded devised in 100 ml Erlenmeyer flasks containing 25 ml of filtrate which had been incubated with a 5 mm circular plug of *A. flavus* from the leading edge of 7 days old culture. The Erlenmeyer flasks with only *A. flavus* in Potato Dextrose Broth (PDB) served as the control. All flasks were incubated at 28ºC for 9 days and the dry mass was determined by weighting every 72 h and compared with the control. The experiment was conducted by triplicate.

### 2.4 Elucidation of Antagonistic Mechanism

Bacteria isolate was examined for elucidation of the possible mechanism underlying their antagonistic behavior, using the following tests:

#### 2.4.1 Hydrogen cyanide (HCN) production

HCN production by *B. megaterium* was tested according to the method described by Prashar *et al [7].* Plates with whatman No.1 filter paper pads inside their lids were powered with glycine supplemented (4.4 g/l) trypricase soy agar (TSA) medium and streak inoculated with 24 h old bacteria isolate. The filter paper padding was soaked with sterile picrid acid solution and the lid was closed. Inoculated plates were sealed properly and incubated for 5 days at 28ºC and observed for color change of the filter paper padding. Degree of HCN production was evaluated according to the color change ranging from yellow to dark brown.

#### 2.4.2 Production of Ammonia ($NH_3$)

Ammonia production was tested in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water and incubated for 48-72 h at 30ºC. 0.5 ml Nessler's reagent was added to each tube. Development of brown to yellow color was taken as a positive reaction for ammonia production [12].

#### 2.4.3 Protease production

Proteolytic activity was determined using skimmed milk agar. Overnight activated culture was spot inoculated on skimmed milk agar plates and incubated for two days at 30ºC.Plates were observed for bacterial growth, which indicated a positive proteolytic activity [7].

#### 2.4.4 Chitinase production

Chitinolytic activity by *B .megaterium* was evaluated according to the method described by Renwick *et al* [13]. Bacterial culture was grown for 24h on 37ºC. 10 µl of culture was spotted on plates of medium (chitine 4 g, $KHPO_4$ 0.7 g, $KH2PO_4$ 0.3 g, $MgSo_4H_2O$ 0.5 g, $FeSO_4$ 0.01 g, ZnSo4.7H2O 0.01 g, Mncl2 0.001 g, Nacl 0.3 g, yeast extract 0.2 g, Agar 20 g, distilled water 1l), and incubated for 5 days at 30ºC. After incubation the cultures were covered with Lugol solution. Chitinolytic activity was estimated by a clear zone around the bacteria.

#### 2.4.5 Amylase production

Amylase production by *B. megaterium* was tested according to the method described by Amoozegar *et al* [14] using basic culture of gelose Agar with 1% (p/v) of starch.10 µl of freshly grown culture was spotted on plates and incubated at 30ºC for 5 days. After incubation the cultures were covered with Lugol solution. Hydrolyses of starch by amylase was determined by a clear zone around the bacterial colonies.

## 3. RESULTS AND DISCUSSION

### 3.1 Antagonistic Activity of *Bacillus megaterium*

The results of radial inhibition assay against *A. flavus* showed that *B. megaterium* decrease almost entirely the mycelial growth of *A. flavus* (22 mm of colony diameter) compared with control (41 mm; Fig. 1B).

The antagonistic activity of *B. megaterium* against the spores of *A. flavus* showed percentage inhibition of 40.75% compared to the control (Fig. 1A).

Results of the effect of cell-free supernatant of *B. megaterium* on the growth of mycelium of *A. flavus* (Fig. 2) indicated a significant antagonistic effect for antagonistic bacteria after 9 days of incubation. The suppressive effect was best demonstrated in the first 72 h, when the dry mass decreased for 1.25 g to 0.83 g compared to the control.

#### 3.1.1 Elucidation of antagonistic mechanism

The production of HCN and $NH_3$ by *B. megaterium* was carried out using the picric acid and peptone water assay respectively. None of this isolate produced HCN and $NH_3$ (Table 1), but the production of lytic enzymes protease, chitinase and amylase was observed (Table 1) and Fig. 3.

**Table 1. Antagonistic mechanism of *B. megaterium***

| Antifungal metabolite production | |
|---|---|
| HCN | - |
| $NH_3$ | - |
| Protease | + |
| Chitinase | + |
| Amylase | + |

*(-) means no production, (+) means production*

### 3.2 Discussion

Several microorganisms are being used in the control of plant pathogenic microbes. Bacillus-based biocontrol agents are quite important in the management of pests and plant diseases [4]. Varieties of *Bacillus* help to promote the health of crops and control diseases by different mechanism including antibiotic metabolites such as: iturin, bacillomycin, surfactin [15] and competition for nutriments like iron and phosphate [16,17].

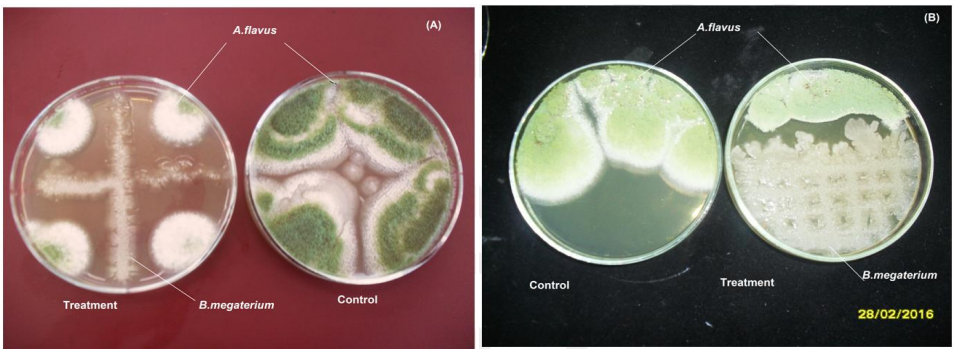


**Fig. 1. Inhibitory effects of *B. megaterium* against A. flavus *in vitro***
*(A): using fungus spore, (B): using Fungal disc*

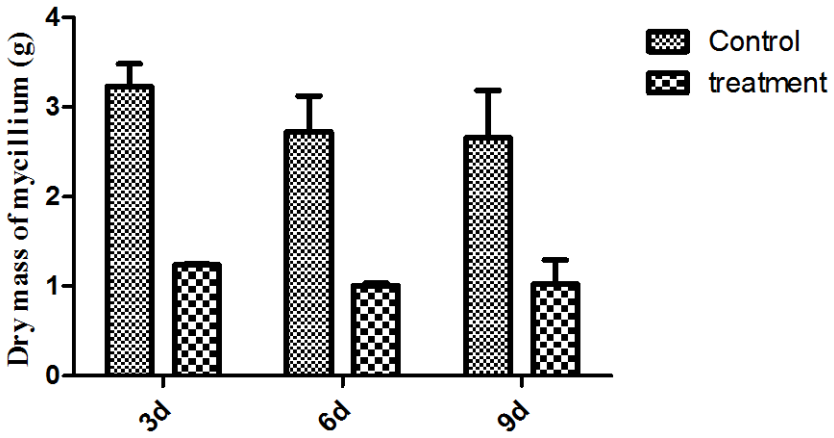


**Fig. 2. Effect of *B .megaterium* cell -free supernatant on the dry mass of *A.flavus.***
*3d……9d: number of day's incubation*

**(A)** **(B)** **(C)**

**Fig. 3. Production of lytic enzymes by *B .megateri***
*(A): Chitinase, (B): Amylase, (C): Protease*

According to the observation in the present study, *B. megaterium* inhibited the growth of *A. flavus* in varying degree ranging from 40.75 to 47.56%. This inhibitory activity of *B. megaterium* was reported to be resulted to antifungal compounds metabolites released into the PDA medium [18], also it has been reported that *B. megaterium* strains were able to inhibit the growth of fungal pathogens due to their ability to produce a wide array of antibiotics such as bacimithrine [19]. In this study the production of secondary metabolites by *B. megaterium* isolate with antifungal properties was demonstrated by cell-free supernatant assay, a significant inhibition of mycelium growth (dry mass) was observed.

Swaddling and Jefferies [20] observed that cell-free supernatant from *B. pumilus* strains inhibited the mycelia growth of *Botrytis cinerea*, also Bertagnolli *et al* [21] demonstrated the efficacy of *B. megaterium* B 153-2-2 contrary *Rhizoctonia solani* with 93.3% of inhibition of mycelium growth.

Moreover, previous studies indicated that mycelial growth inhibited by antifungal bioactive compounds from several *Bacillus* strains such as: Endoproteinase, Endochitinase and phospholipase, also production of extracellular enzymes such as: alfa-galactosidase , amylase and cellulase [21-23,17,24], all of these may be used for degradation of cell walls of the target fungus *A. flavus.*

This research shows that *B. megaterium* is quite important and effective as biocontrol agent .Recherch is continuing to be able to formulate them into microbial agent that will be health and environmentally friendly [4]. Improvement of their

fitness and enhancement of poultry feeds might be a future study.

## 4. CONCLUSION

Poultry feeds may serve as a carrier for a wide variety of microorganisms including pathogenic fungi species like *Aspergillus , Penicillium* and *Fusarium* . Various bacteria such as *B. megaterium* have used as biocontrol agents against this pathogenic fungi .Our results showed that *B. megaterium* is a potential bacterial biocontrol agent against *A. flavus* wish use several mechanisms for biological control.

For further research on the mode of action of biocontrol bacteria to control mycoflora of poultry feed is necessary, particularly to elucidate the specific antifungal mechanisms and their antagonist substances that inhibe growth of fungi.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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