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RESEARCH ARTICLE

EFFECT OF PROBIOTIC BACTERIA ON THE GROWTH AND MYCOTOXIN PRODUCTION OF TWO TOXIGENIC FUNGI

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ABSTRACT

The effect of probiotic bacteria (Lactobacillus delbrueckii and Lactobacillus rhamnosus) on the growth of two toxigenic fungi Aspergillus flavus and Aspergillus parasiticus were investigated in vitro by the well-disc diffusion method. The concentrated and un-concentrated cell-free extracts were examined, and it was found that the effect was non significant on the growth of fungi. The effect of the metabolites produced by the probiotic bacteria affect the growth of, A. flavus on solid medium and exhibited inhibition zone of 16 mm ($p \leq 0.05$) in the presence of L. delbrueckii and L. rhamnosus separately. However, the inhibition zone of A. parasiticus varied from 16-17mm ($p \leq 0.05$) in the presence of L. delbrueckii and L. rhamnosus, respectively. Also, both bacterial strains showed a reduction of pH in the presence of A. flavus after the addition of L. delbrueckii (4.17-4.00) and L. rhamnosus (4.27-4.09), while in the presence of A. parasiticus, the pH decreased in the existence of L. delbrueckii (4.18-4.01) and L. rhamnosus (4.06-3.96). In addition, the lactic acid concentration of L. delbrueckii and L. rhamnosus were measured in the presence of the two tested fungi individually. It was noted that the acid concentration in the existence of A. flavus was 2.4% while in the existence of A. parasiticus was 2.3%. Furthermore, a significant decrease was observed in the counts of fungal spores of A. flavus and A. parasiticus after the addition of L. rhamnosus and L. delbrueckii, separately. Also, the probiotic strains significantly affect the morphological structure and reduced aflatoxin production of the tested fungi. These probiotic bacteria showed excellent properties for the possibility of using it to prevent fungal contamination to reduce economic loss of agriculture products as well as increase the nutritional value and therapeutic properties of food.

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INTRODUCTION

The food industry has been distressed extensive losses due to microbial spoilage. Filamentous fungi are one of the major categories of spoilage microorganisms that participate in considerable economic losses and human health hazards (Maurya *et al*., 2015). According to the survey provided by Rodrigues and Naehrer (2012), more than 33% of the world's agricultural production is contaminated with mycotoxin resulting in economic damages in the grain industry. Mycotoxins are secondary metabolites produced by filamentous fungi that frequently found in products such as nuts, corn, rice, feed and several other cereals, which can be contaminated in the field during harvest or storage (Köppen *et al*., 2010). Studies have demonstrated their toxigenic, nephrotoxic, hepatotoxic, carcinogenic, immunosuppressive and mutagenic characteristics, and most mycotoxins represent a considerable risk to human and animal health (da Rocha *et al*., 2014). Exposure to mycotoxins almost accidental and may take place by inhalation, ingestion, and dermal contact. Most cases of mycotoxicoses result from eating contaminated food. Human exposure might be direct through cereals or indirect by animal products (such as meat, milk and eggs) (CAST, 2003). For example, the aflatoxin B1 found in contaminated feedstuffs that ingested by lactating dairy cows is bio-transformed into AFM1 which then excreted into the milk (Frobish *et al*., 1986). Several types of research have shown the antifungal properties of some LAB (Tropcheva *et al*., 2014, Sevgi and Tsveteslava, 2015, Bayankaram and Sellamuthu, 2016). Lactic acid bacteria is a group of gram-positive, cocci or rods, non-spore forming that produce lactic acid as a primary end-product of fermentation of carbohydrates (Hütt *et al*., 2006). Most bacteria that used as probiotics belong to the LAB (e.g. *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, etc.) and *Bifidobacteria* (Hütt *et al*., 2006, Savadogo *et al*., 2006). In addition, *Bifidobacterium bifidum*, *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* have completely lowering mycotoxin levels in milk samples (Škrinjar *et al*., 1996).

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Moreover, these bacteria have a long history of use in food as bio-preservation and have been appointed or generally considered as safe for human consumption (Schnürer and Magnusson, 2005, Maurya *et al*., 2015). Therefore, chemical preservatives could be avoided and replaced by natural preservatives. Probiotic bacteria can produce several compounds, act as an inhibitory agent to the growth of the pathogen, these include organic acids i.e. lactic acid, acetic acid, citric acid, and hippuric acid (Pundir *et al*., 2013). Lactic acid bacteria also produce diacetyl, hydrogen peroxide, and bacteriocin as antimicrobial substances. These inhibitory molecules create antagonistic environments for food borne pathogens and spoilage organisms (Kachouri *et al*., 2014, Sevgi and Tsveteslava, 2015). Therefore, the present study investigated (*in vitro*) two strains of identified probiotic bacteria (*Lactobacillus delbrueckii* and *Lactobacillus rhamnosus*) for their ability to inhibit the growth of toxin-producing aspergilli (*Aspergillus flavus* and *Aspergillus parasiticus*).

MATERIALS AND METHODS

Cultures and media

The fungal toxigenic strains, *Aspergillus flavus* (EMCC 274) which produce aflatoxin ($B_1$and B_2) and *Aspergillus parasiticus* which produce (EMCC 886) aflatoxin (B_1, B_2, G_1, and G_2) were purchased from Egypt Microbial Culture Collection (Cairo MIRCEN), Egypt. They were renovated into potato dextrose broth and incubating for 3 days at 28°C. This broth from was used to inoculate potato dextrose agar (PDA) plates which then incubated for 7 days at 28°C. The two probiotic strains, *Lactobacillus rhamnosus* (EMCC 1105) and *Lactobacillus delbrueckii* (EMCC 1145), were refreshed in de Mann Rogosa and Sharpe (MRS) broth via incubating at 37°C overnight before which they were streaked onto MRS agar then incubated at 37°C for 48 h. All the tested bacteria were obtained from Egypt Microbial Culture Collection (Cairo MIRCEN), Egypt.

Antifungal effect of probiotic bacteria

Antifungal effect of the probiotic cell-free extracts on fungal growth by well-disc diffusion method For the cell-free extracts preparation, 200 ml of MRS broth was inoculated with bacterial strains and incubated at 37°C for 5 days. The cultures were then centrifuged to pellet the cells, the supernatants were filtered using sterilized Millipore filter (Muhialdin *et al*., 2013). The cell-free extract concentrated by vacuum rotary evaporator (bath temperature of 60°C, the rotational speed of 90 rpm/min under vacuum) to obtain a 15-fold concentrated supernatant. Potato dextrose agar plate was inoculated with 1 ml of spore suspension (10^5 spores/ml).Wells with a diameter of 8 mm were made and loaded with 200 µL of each concentrated and un-concentrated cell-free probiotic extracts. Plates were incubated in aerobic conditions at 28°C for 48 - 72 hr. The antifungal effect was assayed by measuring the diameter of clear zones around the wells.

Microbial viable cell count

Spread plate technique was used for the total counts of bacteria and fungi. One milliliter of each broth culture sample was diluted with 9 ml sterile peptone water. From the third dilution, 1 ml was spread-plated on duplicate plates of MRS agar and PDA (Shori and Baba, 2012, Chukwuemeka and Ibe, 2013). The MRS plates were incubated at 37°C for 3 days, while PDA plates incubated at 25°C for 5 days.

Determination of pH and titratable acidity

The MRS broth media have been inoculated with each probiotic bacterial and fungal strain in different culture flasks and were measured to determine the changes of pH in the seven days of incubation period by using pH meter (Denver instrument – ultrabasic pH). Also, during incubation period MRS broth cultures were titrated every two days by diluting 1 ml of the broth samples in 9 ml of distilled water and titrated via (0.1N) NaOH with a few drops of phenolphthalein (60% ethanol) indicator (Iland *et al*., 2000). The first persistent pale pink color was determined as an endpoint. Total lactic acid % was calculated by the following formula:

$$Titratable\ acidity\ (\%)_{lactic\ acid} = \frac{d.f \times V_{NaOH} \times 0.009 \times 0.1}{V_{ml}} \times 100$$

Dilution factor (d.f.) = 10

V_{NaOH} = Volume of NaOH used to neutralize the lactic acid
0.009 = conversion factor, 1 ml NaOH (0.01 N) neutralizes 0.009g of lactic acid

0.1 = Normality of NaOH

V = Volume of sample for titration

Effect of probiotic bacteria on mycotoxin production

The probiotics inhibitory effect on aflatoxin was performedaccording to the method described by Bayankaram and Sellamuthu (2016).Fungal spore suspensions of *A. flavus* and *A. parasiticus*(1 ml, 10^7spores/ml) were inoculated in 100 ml of the MRS broth in different culture flasks and incubated for 24 h at 28 °C. Then, each culture has been inoculated with 1 ml of each probiotic separately, and all incubated at 28 °C for 7 days. The fungal mycelia were filtered using Whatman No. 1, then aflatoxin was extracted from the filtrate using 25 ml of chloroform and the dissolved aflatoxin was separated in a separator funnel. The separation process was repeated twice for each sample to ensure complete removal of aflatoxin from the filtrate. The chloroform was evaporated in a water bath (60 °C) and the concentrate was re-suspended in 1 ml methanol. Thin layer chromatography (TLC) plates were used for aflatoxin detection by spotting 10 µL aliquots of each extract onto a pre-coated silica gel (DC- Alufolien- Kieselgel, silica gel matrix with fluorescent indicator 254 nm). The plates were developed in the solvent system of toluene-ethyl acetate-formic acid (5:4:1, vol/vol/vol) at the room temperature. The plates were removed from the solvent, and air-dried in a fume cabinet (Samson *et al*., 2000). The presence of aflatoxin was specified by examined the plates under UV light at 365 nm as fluorescent blue spots. Moreover, the aflatoxin content was determined by VICAM fluorometer. The prior technique was applied by Bokhari (2010), Bokhari and Aly (2013) to reveal mycotoxin in various food samples using VICAM fluorometer.

Morphological changes of fungi

The treated growth of fungi was examined under the 100_X objective lens of the light microscope using the immersion oil to detect any apparent changes in fungal structure. Also,

scanning electron microscope (SEM) was used to observe the morphological changes in fungal hyphae and spore formation.

Statistical analysis

Three separate experiments were carried out and triplicate assays from the same experiment were performed. Data were expressed as mean ± SEM. (standard error of the mean). The statistical analysis was performed using one-way analysis of variance (ANOVA, SPSS 16.0), followed by Duncan's post hoc test for mean comparison. The criterion for statistical significance was $p \leq 0.05$.

RESULTS

Antifungal effect of the probiotic cell-free extract on fungal growth. The results in table 1 showed that insignificant differences between the concentrated and un-concentrated cell-free extracts of the probiotics against *A. flavus* and *A. parasiticus*. The concentrated extract of both *L. delbrueckii* and *L. rhamnosus* exhibit a clear inhibition zone (16 mm; $p \leq 0.05$) against *A. flavus* (figure 2) compared to the control plates in figure 1. As shown in figure 3, a zone of inhibition presented after 48 h incubation of *A. parasiticus* by the concentrated cell-free extract of *L. rhamnosus* (17 ± 0.3 mm; $p \leq 0.05$). *L. delbrueckii* extracts showed same results of inhibition against *A. parasiticus* (16 mm; $p \leq 0.05$).

Table 1. The antifungal activity of concentrated and un-concentrated CFE of probiotic strains on the growth of fungi after 48 h at 28 °C

Sample	Mean diameter of inhibition zone (mm) ± SE***			
	L. delbrueckii		*L. rhamnosus*	
	*Un-con.	**Con.	Un-con.	Con.
A. flavus	15 ± 0.5	16 ± 0.5	15 ± 0.3	16 ± 0.8
A. parasiticus	16 ± 0.6	16 ± 0.5	14 ± 0.8	17 ± 0.3

*Un-concentrated cell-free extracts**Concentrated cell-free extracts
***Standard error.

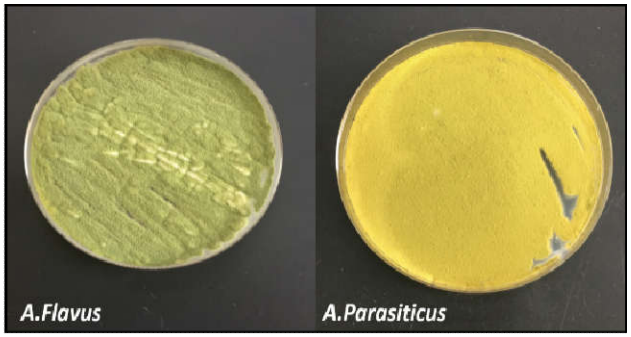

Figure 1.The growth of untreated fungi on PDA plates after 48 h at 28 °C

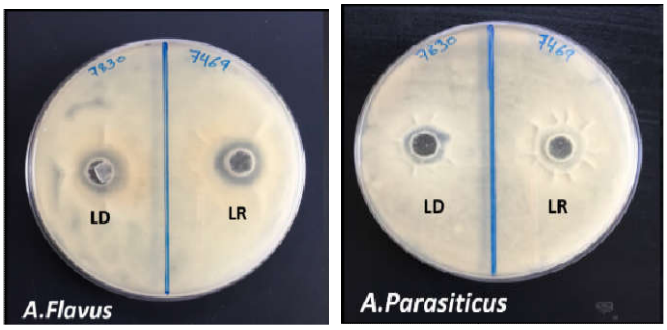

Figure 2. The antifungal activity of un-concentrated CFE of probiotic strains on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* after 48 h at 28 °C. LD = *Lactobacillus delbrueckii*, LR = *Lactobacillus rhamnosus*

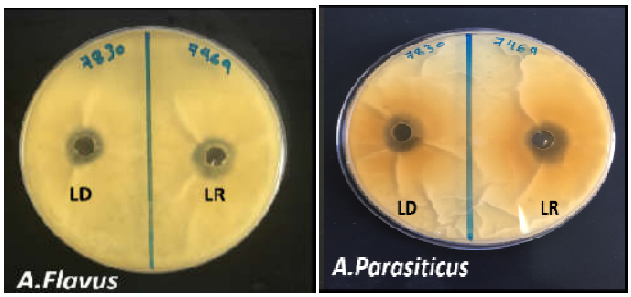

Figure 3. The antifungal activity of concentrated CFE of probiotic strains on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* after 48 h at 28 °C

Effect of probiotic bacteria on fungal spore counts

Table 2 showed a significant decreased of *A .flavus* counts with addition of *L. rhamnosus* compared to the control sample during 3rd,5th and 7th day of incubation ($1.8\times10^2 \pm 0.04$, $7\times10^1 \pm 0.08$ and $2\times10^1 \pm 0.03$ cfu/mL, respectively) also similar effects of *L.delbrueckii* on *A. flavus* counts to $1.9\times10^2 \pm 0.1$, $1.1\times10^2 \pm 0.05$ and $2\times10^1 \pm 0.07$ cfu/mL ($p \leq 0.05$). Therefore, a pronounced reduction in *A. parasiticus* counts occurred on the 7-day incubation in the existence of *L. rhamnosus* and *L. delbrueckii*was $1 \times 10^1 \pm 0.03$ and $1 \times 10^1 \pm 0.06$ cfu/mL, respectively ($p \leq 0.05$). In contrast, bacteria counts were increased for both *L. rhamnosus* and *L.delbrueckii* in the presence of *A. flavus* and *A. parasiticus* individually as shown in table 3. Changes of pH and titratable acidity (% TTA) during incubation of fungi with different probiotic bacterial strains In the present study, *L. rhamnosus* and *L.delbrueckii* showed the lower pH values in the presence of *A. parasiticus* (3.96 ± 0.008, 4.01 ± 0.04; $p \leq 0.05$). Moreover, *L. delbrueckii* with the presence of *A. flavus* showed a reduction in pH during 7 days of the incubation period (4.27 ± 0.008, 4.09 ± 0.04; $p \leq 0.05$). In contrast, lactic acid concentration increased during the incubation period of the same strains (1.8 ± 0.05 - 2.4 ± 0.06%; $p > 0.05$). Also, *L. rhamnosus* showed high lactic acid concentration in the existence of *A. flavus* (1.65 ± 0.03 – 2.43 ± 0.05 %; $p \leq 0.05$). Therefore, the lactic acid concentration of *L. rhamnosus* and *L.delbrueckii* increased during the incubation period from 1.5 ± 0.06 to 2.3 ± 0.05% ($p \leq 0.05$) in the presence of *A. parasiticus*.

Table 2. Effect of the probiotic bacterial growth on the number of fungal spores during seven days of incubation at 28 °C

The tested fungi	Counts (spore/ml ± SE* $\times10^2$)		
	3 Days	5 Days	7 Days
A. flavus	9.2± 0.03	28.5 ± 0.05	45.1 ± 0.05
A. parasiticus	8.4 ± 0.06	27.9 ± 0.02	42.7 ± 0.03
L. delbrueckii + A. flavus	1.9 ± 0.1	1.1 ± 0.05	0.2 ± 0.07
L. rhamnosus + A. flavus	1.8 ± 0.04	0.7 ± 0.08	0.2 ± 0.03
L. delbrueckii + A. parasiticus	2.2 ± 0.08	0.6 ± 0.04	0.1 ± 0.06
L. rhamnosus + A. parasiticus	1.3 ± 0.06	0.5 ± 0.05	0.1 ± 0.03

*standard error

Table 3. Changes in probiotic bacterial counts during seven days of incubation at 28 °C

Probiotic strain	Counts (cfu/mL ± SE* $\times10^4$)		
	Day 3	Day 5	Day 7
L. delbrueckii	1.95 ± 0.06	3.06 ± 0.05	4.93 ± 0.06
L. rhamnosus	1.92 ± 0.05	3.07 ± 0.07	5.10 ± 0.05
L. delbrueckii + A. flavus	1.93± 0.03	2.78 ± 0.05	4.03 ± 0.08
L. rhamnosus + A. flavus	1.90 ± 0.07	3.03 ± 0.05	4.14 ± 0.03
L. delbrueckii + A. parasiticus	1.98 ± 0.05	2.46 ± 0.02	4.11 ± 0.05
L. rhamnosus + A. parasiticus	1.88 ± 0.05	2.94 ± 0.03	4.17 ± 0.05

*Standard error

Effect of probiotic bacteria on mycotoxin production

The presence of aflatoxin in the control and test samples were detected by TLC plate under UV light as fluorescent blue spots as shown in figure 6. The outcomes given from the analytical procedure, presented in table 4, showed that there was a significant reduction of aflatoxin content in the samples having the bacterial strains, compared to the control. Through affinity column chromatography it was reported that *L. delbrueckii* showed the higher reduction percentage of 97.25 % in the case of *A. flavus*, while 86.25 % in the case of *A. parasiticus* ($p \leq 0.05$). However, *L. rhamnosus* showed the lowest percentage reduction of aflatoxin secretion by *A. parasiticus* of 62.56 %, but in the state of *A. flavus*, the percentage reduction was 78.73 % ($p \leq 0.05$).

Table 4. Effect of probiotic bacteria on the aflatoxin concentration after 7 days

The tested fungi	AFB_1 contentµg/mL ± SE*	Percentage reduction (%)
A. flavus un-treated (control)	64.33 ± 7.45	0.0
L. delbrueckii + *A. flavus*	1.77 ± 0.21	97.25
L. rhamnosus + *A. flavus*	13.44 ± 0.83	78.73
A. parasiticus un-treated (control)	152 ± 15.31	0.0
L. delbrueckii + *A. parasiticus*	20.29 ± 0.77	86.25
L. rhamnosus + *A. parasiticus*	55.97 ± 1.52	62.56

Morphological changes in fungi

The apparent changes of treated fungi were examined under the100_X objective lens of the light microscope. Also, scanning electron microscope (SEM) was used to observe the morphological changes in fungal hyphae and spore formation. The light microscope observation showed that the sporulation of treated *A. flavus*and*A. parasiticus* was completely absent with a swelling hypha as cleared in figure (8)compared to anormal apparent structure in figure (7). The morphological changes in *A. flavus* exposed to the cell-free extract from *Lactobacilli* strains were observed by SEM. The scanning electron microscope observation showed that untreated *A. flavus* hyphae retained complete tubular shapes with the normal appearance of a barrel-like formation, smooth cell walls and spherical-shaped head (figure9). After being treated with the cell-free extracts, the distorted and aberrant morphologies in fungal hyphae were observed. Markedly shrank, and crinkled cell wall and flattened hyphae were obtained from the SEM figure 10. Also, a delay of sporulation process occurred resulted in agradual change in conidia pigmentation from green to yellow was observed in *A. parasiticus*at the periphery of the colonies.

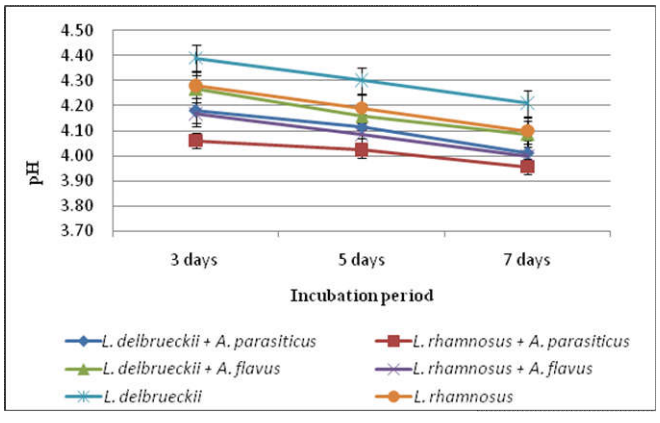

Figure 4. pH changes in growth media during incubation of fungi with different probiotic bacterial strains

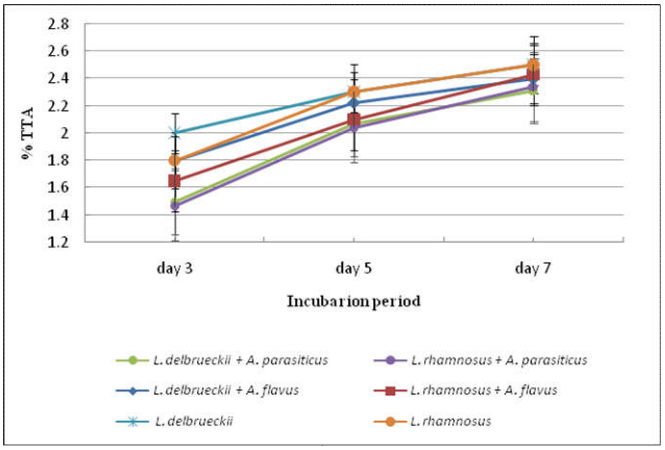

Figure 5. Changes of TT Ain growth media during incubation of fungi with different probiotic bacterial strains

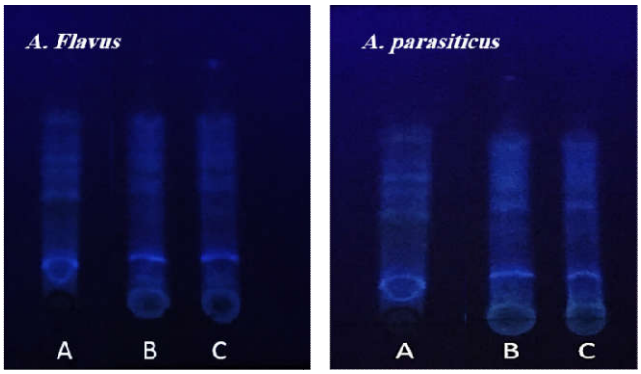

Figure 6. Thin layer chromatographic Separation of aflatoxin secreted by *Aspergillus flavus* and *Aspergillus parasiticus* in the presence of probiotic bacteria under UV light

- Standard aflatoxin B_1
- Treated with *L. delbrueckii*
- C.Treated with *L. rhamnosus*

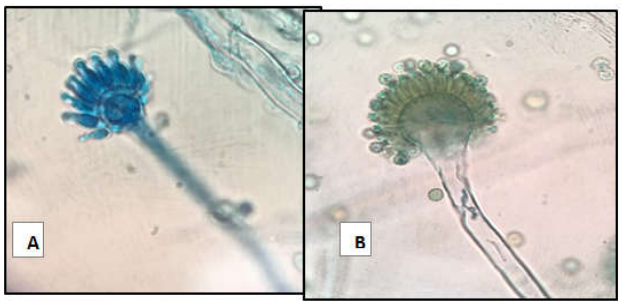

Figure 7. *Aspergillus flavus* (A) and *Aspergillus parasiticus* (B) under light microscope (100 X) after 48 h at 28 °C (untreated)

DISCUSSION

Probiotic bacteria are well recognized for their antagonistic effects against other bacteria. *Lactobacillus* was one of the LAB that has been found to have the most antifungal activity. The effects of probiotic strains against *A. flavus* and *A. parasiticus* using well-disc diffusion method as described by Muhialdin *et al.* (2013). Notable inhibition zones were observed after 48 h of incubation that referred to the secondary metabolites which are responsible for the inhibitory effects of *L.delbrueckii* and *L. rhamnosus*.

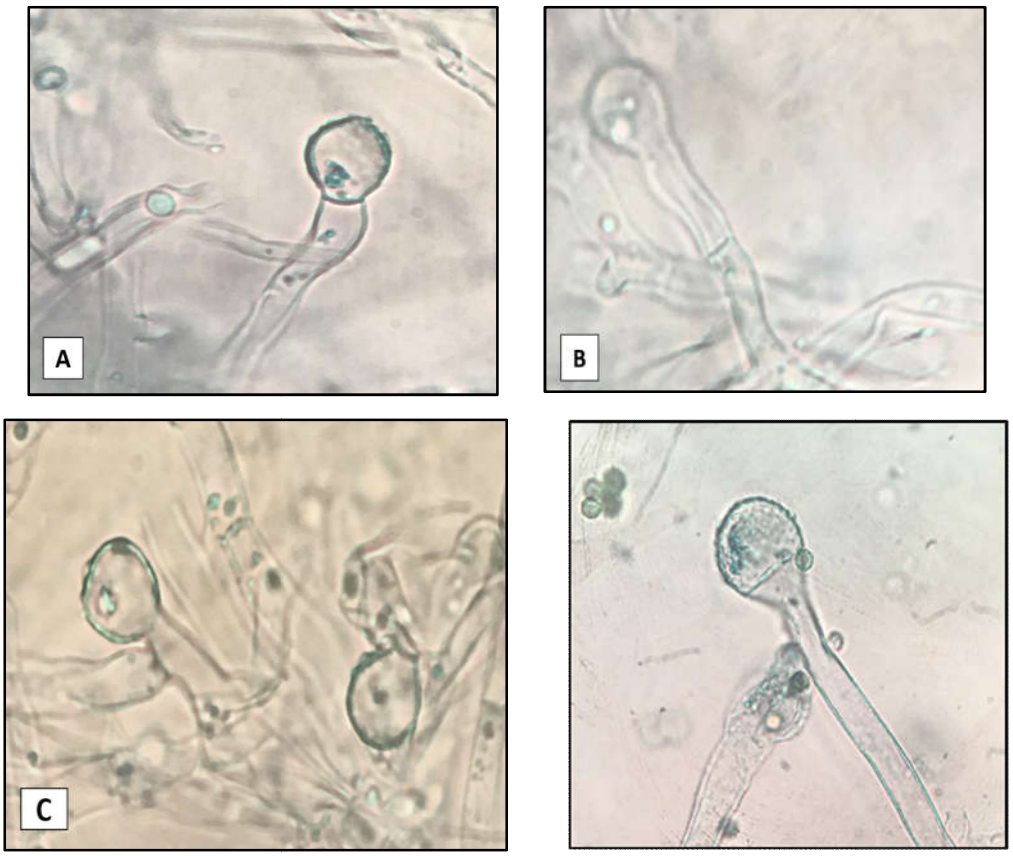

Figure 8. The treated *Aspergillus flavus* (A, B, C) and *Aspergillus parasiticus* (D) under light microscope (100X) after 48 h at 28 °C

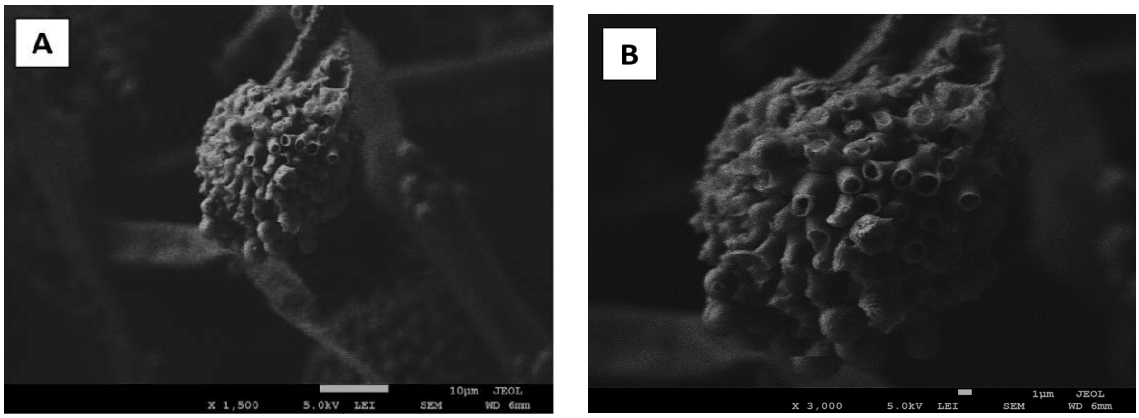

Figure 9.*Aspergillus flavus* (A, B) under scanning electron microscope with different magnification after 48 h at 28 °C(untreated)

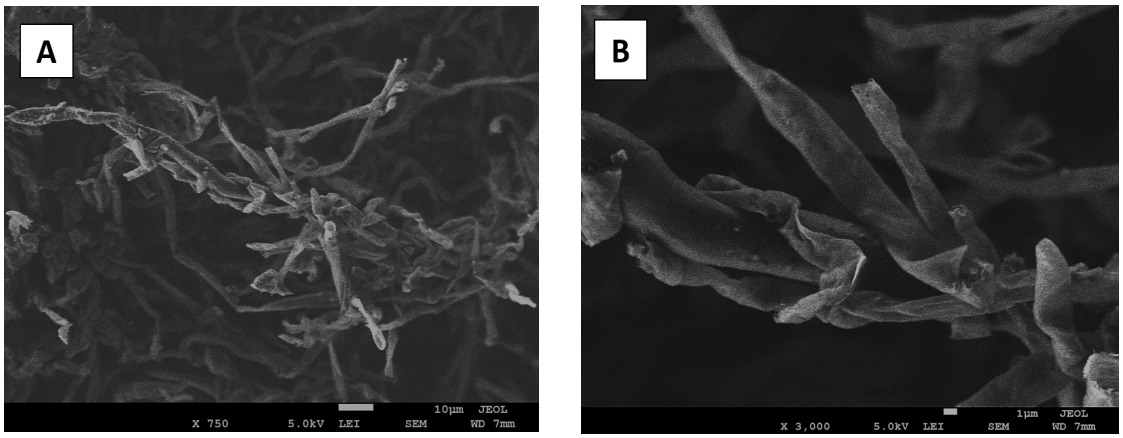

Figure 10.The treated *Aspergillus flavus* (A, B) under scanning electron microscope with different magnification after 48 h at 28 °C

Sevgi and Tsveteslava (2015) found that *L. plantarum* and *L. helveticus* retarded and weakenthe growth of *A. niger* and *P. claviforme*. Moreover, Gerbaldo *et al*. (2012) found that *L. rhamnosus* inhibited the growth of 10 different strains of *A. flavus* at various levels. Also, two strains of *L. plantarum* were studied by Onilude *et al*. (2005) showed antagonistic activity against several strains of *A. flavus* and *A. parasiticus*. As previously mentioned, these metabolites products have been described as varied organic acids (lactic acids, acetic acid, etc.), cyclic dipeptides, hydrogen peroxide phenolic, hydroxyl fatty acids, and proteinaceous compounds. Different combinations of these molecules produced by different strains of probiotic bacteria and suppress fungal growth (Mandal *et al*., 2007, Rouse *et al*., 2008). Also, the high level of organic acids produced by the probiotic bacteria lowering the pH resulting in decline the counts of fungi. Tambekar and Bhutada (2010) and Ammor *et al*. (2006) reported that the production of organic acids lowered the pH, resulting in unfavorable conditions for the growth of toxigenic microorganisms. These acids which have low molecular weight able to pass the membrane and break up into the cell bring about an intracellular acidic exertion. Also, the inhibitory effect might be from the disturbance of membrane, suppression of primary metabolic reactions, and accumulation of poisonous anions (Brul and Coote, 1999). In addition, the concentrated extract of the metabolites product inhibited the sporulation of *A. flavus* and this is a very promising result since *A. flavus* is considered as the second major cause of aspergillosis in human and one of the most poisonous and carcinogenic agents of the micromycetes. In case of *A. parasiticus*, the concentrated extract causesa delay of sporogenesis. The prevention of sporogenesis and conidia germination is a significant outcome due to allergens from spores (Payne and Yu, 2010). Furthermore, the growth and cell numbers of *A. flavus* and *A. parasiticus* treated with *Lactobacilli* were determined and compared to the untreated fungi. A notable decreased growth and cell numbers were observed. Aryantha and Lunggani (2007) reported that the antifungal activity of *Lactobacilli* was due to the competition with fungal strain for available nutrients, no substrate added during the incubation period, where *Lactobacillus sp.* was dominant since having simple living structure and faster growth compared with fungi. Also, bacteria can use the substrate earlier to increase cell biomass, however fungi extend later after restricted of nutrients.

On the other hand, all the examined *lactobacilli* strains in this study exhibited a reduction in aflatoxin-B concentration compared to untreated *A. flavus* and *A. parasiticus* (control). The similar results obtained by Tropcheva *et al*. (2014)which reported a considerable inhibition of aflatoxin production by CFE of *Lactobacillus*. The differences between the bacterial strains in aflatoxin reduction could be due to the variance of the binding sites in each strain. several studies have been reported that some components in the cell wall of lactic acid bacteria are responsible for the aflatoxin binding and removal from solutions (Aryantha and Lunggani, 2007, Elsanhoty *et al*., 2013). According to Haskard *et al*. (2001),the change in the pH of the medium due to the produced metabolites of the bacterial strain plays a major role to the number of binding sites for aflatoxin B_1 on the bacterial cell wall. Also, it was concluded that best adhesion of AFB1 occurred in Low pH media.

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Conclusion

In this research, tow strains *L. rhamnosus* and *L. delbrueckii* have been studied *in vitro* as cultures with promising antifungal activity. These strains have considerable attention for the possibility of using it in preventing fungal contamination due to their low cost and safe for human consumption. Since the cell-free extract of these bacterial strains inhibited the growth of toxigenic fungi, it can be used in the preservation of food and feed as an alternative to synthetic additives. Also, it is possible to use bacterial cells as a biological agent in keeping food from spoilage as well as increasing nutritional value and therapeutic properties of food. Furthermore, the using of bacteria cells in the detoxification of aflatoxin produced in contaminated food and feed to reduce the economic damages resulting from the destruction of contaminated food. However, more *in vivo* studies needed to clarify the mechanisms of the reported antifungal activity to bring close the practical application of the strains.

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