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Lactic Acid Bacteria as Antimycotic and Antimycotoxins Agents against Toxigenic *Fusarium* species Associated to Maize Grains Stored in Egyptian Markets

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Fusarium **species are worldwide causal agents of huge damage of storage cereals. Moreover their toxigenic potential is a health risk for both humans and animals. In the present work infected seed samples of maize were collected from local markets in Egypt. Thirteen isolates of *Fusarium* species were initially identified by phenotype based methods then genotyped using the partial sequence of translation elongation factor-1α (TEF1-α) gene. Furthermore, fumonisin-producing isolates were identified using species-specific primers (PQF5-F/PQF5-R) and (FUM5P2-F/FUM5P2-R) based on partial sequence of FUM1 gene. The results indicated that an amplicon of 60-bp for four isolates identified as *F. proliferatum* and an amplicon of 70-bp amplicon for three isolates identified as *F. moniliforme* were generated. Five strains of lactic acid bacteria (LAB) *viz. Lactobacillus delbrueckii* subsp. *lactis* DSM 20076, *Lactobacillus acidophilus* DSM 20079, *Pediococcus acidilactici* NNRL B-5627, *Lactobacillus. sakei* LB 706 and *Enterococcus faecalis* were screened for their ability to inhibit *Fusarium* isolates growth and/or bind fumonisin B1. *Lb. delbrueckii* subsp. *lactis* DSM 20076 was the most efficient strain at removing fumonisin B1 toxin (76.67 %). However, *Lb. acidophilus* DSM 20079, *Lb. sakei* LB 706 and *P. acidilactici* NNRL B-5627 showed the greatest inhibitory effect against *Fusarium* isolates. Therefore the use of LAB as a source of natural antimycotic and antimycotoxin agents showed promising, economical and successful strategy for preserving food products from *Fusarium* spoilage.**

Key words: Maize, *Fusarium*, Fumonisin, PCR, Lactic acid bacteria, Antifungal, Toxin binding.

Maize (*Zea mays L.*) is an important cereal crop of the world and has economic value in livestock. It is considered as one of the two important cereal crops in Egypt and plays a fundamental role in human and animal feeding. Increasing maize production during the last period became one of the most important goals of the Egyptian government to satisfy human and animal demands[1]. Food and feed spoilage moulds cause great economic losses worldwide. It is estimated that between 5 and 10% of the world's food production is wasted due to fungal deterioration[2]. Of all agricultural commodities, maize is the one showing the greatest contamination with *Fusarium* toxins and therefore, its products are of major concern for animal and human health[3, 4]. *Fusarium* spp. are the most prevalent mycotoxin producing fungi[5]. In addition to damaging plant tissues some

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Fusarium species produce fumonisins (FBs), a group of mycotoxins as secondary metabolites[6]. *Fusarium moniliforme, Fusarium proliferatum* are known to produce a number of mycotoxins, primarily fumonisins[7].

The natural occurrence of fumonisins in maize has become an important concern for human and animal health throughout the world[8], these mycotoxins are common contaminants of corn and maize specially corn based-snacks which are a type of food that very popular among children and the contamination of this type of food may be hazardous to those susceptible young age consumers[9]. Twenty-eight fumonisins have been isolated and they can be divided in four series known as A, B, C and P. Fumonisin B1 (FB1), FB2 and FB3 are the principal fumonisins analyzed as natural contaminants of cereals, of which FB1 is the most common in maize[10,11]. In Egypt, a study with yellow maize, white maize and maize meal showed FB1 contamination of 80%, 33.3% and 53.8% of cases, respectively[12]. Moreover its toxigenic potential is a health risk for both humans and animals, increased levels of fumonisins in moldy maize kernels have been previously linked to the high incidence of human esophageal cancer[13]. Consumption of FB1-contaminated feed causes pulmonary edema in pigs and equine leukoencephalomalacia in horses. To experimental animals (rats and mice) FB1 is nephrotoxic and hepatotoxic[14].

Detection of fumonisin-producing fungal species by morphological characters sometimes is not enough for accurate identification of fungal isolates at the species level. Furthermore, both morphological and mating type characterization are time consuming and require considerable expertise in *Fusarium* taxonomy and physiology[15, 16]. As identification of *Fusarium* species is critical to predict the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive and reliable specific diagnosis of *Fusarium* species.

Various PCR assays have been developed for the identification of toxigenic species of *Fusarium*. Some of them are based on single copy genes directly involved in mycotoxin biosynthesis while others are species-specific[17]. The translation elongation factor 1-α (TEF) gene appears to occur consistently as single-copy in *Fusarium*, and shows a high level of sequence polymorphism among closely related species, even when compared with the intron-rich portions of protein-coding genes such as calmodulin, β-tubulin and histone H3. Thus, TEF has become the marker of choice as a single-locus identification tool in *Fusarium*[18, 19].

Several strategies (physical and chemical) have been developed to prevent and/or to reduce mycotoxin accumulation in cereals[20]. These are expensive and not practical to implement on farms. An alternative strategy is the use of micro-organisms able to degrade or bind toxins. Recent research revealed that lactic acid bacteria (LAB) are well known for their antifungal activity, which is related to the production of a variety of compounds including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide, phenyllactic acid, bacteriocins and cycle peptides[21, 22]. Several studies dealing with antifungal activity of LAB has highlighted the ability of some strains to repress mycotoxinogenic mould growth through the production of several low molecular weight antifungal metabolites[23]. Gerez *et al.*,[21] screened the ability of LAB strains isolated from different sources for antifungal activity against *Aspergillus niger* CH 101, *Penicillium* sp. CH 102 and *F. graminearum* CH 103, the most common contaminants in bread. They found that from a total of 95 strains homo- and hetero-fermentative LAB tested, the majority of them (63 strains) were able to inhibit the conidial germination, while only four LAB strains inhibited the mycelial growth. Moreover, Sathe *et al.*,[24] have reported the successful use of *Lb. plantarum* to significantly delay the growth of *Aspergillus flavus* and *F. graminearum* in cucumber.

The cell walls of some LAB such as *Leuconostoc* and *Streptococcus* have been reported to be able to bind some mutagenic compounds such as amino acid pyrolysates and heterocyclic amino acids produced during cooking. Many investigations have been conducted to evaluate the ability of LAB to remove other food-contaminating substances including mycotoxins, known for their mutagenic effects[23]. Niderkorn and workers[25] evaluated the ability of 29 LAB and 3 strains of *Propionibacterium* to remove fumonisin B1 and B2 from acidified De Man, Rogosa, Sharpe

(MRS) broth at pH 4.0. Most of the strains were able to remove both toxins, but considerable differences were observed among these strains.

The objectives of this study were to isolate and identify *Fusarium* spp. by partial sequencing of the TEF-1α gene, to analyze the fumonisin-producing ability of identified fungal isolates, to screen the ability of various LAB to inhibit the growth of *Fusarium* isolates and to evaluate the ability of LAB tested to bind FB1 from MRS medium.

MATERIALS AND METHODS

Bacterial strains and culture media

Lb. delbrueckii subsp. *lactis* DSM 20076 and *Lb. acidophilus* DSM 20079 were obtained from German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, *P. acidilactici* NNRL B-5627 was obtained from the Northern Regional Research Laboratory (NRRL, Peoria, IL, USA), *Lb. sakei* Lb 706 was kindly provided by Prof. Ulrich Schillinger (the Institute of Hygiene and Toxicology, BFE Karlsruhe, Germany) and *Enterococcus faecalis* isolate was kindly provided by Dr. Ashraf A. Khalil. Stock cultures of LAB were maintained at -80° C on MRS broth with 25% (v/v) glycerol. To produce fresh cultures, the strains were propagated at 30° C for 14-16 h then at 37 °C for 14-16 h before experimental use.

Mycotoxin preparation

Fumonisin B1 toxin was purchased from Cayman Chemicals Company, USA. Toxin was dissolved in acetonitrile–water in the ratio 1: 1 (v/v) and kept at -20° C until used.

Fusarium isolation from maize samples

Infected maize samples were collected from local markets at Alexandria. A couple methods were followed to isolate *Fusarium* spp. (serial dilution and direct plating) according to Suleiman and Akaajime,[26]. All *Fusarium* isolates were subcultured on Potato Dextrose Agar (PDA) and Spezieller Nahrstoffarmer Agar (SNA) using a single spore technique. Inoculated PDA and SNA media were incubated at 25° C for two to four weeks[15].

Morphological identification of *Fusarium* isolates

Isolates were grown in carnation leaf agar (CLA) according to Fisher *et al*.[27]. Cultural characters were assessed visually and by microscopic examination. Morphological identifications of isolates were made using the criteria of Leslie and Summerell[15]. The morphology of macroconidia, microconidia, conidiogenous cells and the chlamydospores was assessed from cultures grown on SNA and CLA. A total of 13 *Fusarium* isolates were recovered from the maize samples.

Molecular identification of *Fusarium* isolates

DNA extraction

The DNA was extracted from fungal culture using mini preparation method modified according to Edel *et al*.[28]. Isolates were grown on PDA medium at 25° C for 7 days. One mL of extraction buffer (50 mm Tris-HCl pH: 7.5, 50 mm EDTA, 3% SDS) was added in Petri dish, and the mycelium was collected by scraping with a sterile spatula, then placed into ependorf tube. The samples were incubated for 15 min. at 65° C then centrifuged at 12000 *xg* for 15 min. After adding equal volume of phenol–chloroform, the mixture was centrifuged again. The DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 1.5 volume of isopropanol. The pellet was rinsed with ethanol, suspended in Tris-EDTA (TE) buffer, pH 7.4 and stored -20° C until further use.

PCR amplification

The primers used in this investigation are summarized in Table 1. TEF-1α gene was amplified using the primer pairs EF1T / EF2T to identify *Fusarium* species as described by O'Donnell *et al*.[29]. Based on partial sequence of FUM1 gene, fumonisin producing isolates were identified using primers PQF5-F/PQF5-R developed for *F. moniliforme*[30] and FUM5P2-F/FUM5P2-R developed for *F. proliferatum*[16]. In all cases, amplification reactions were carried out in a volume of 25 µL containing 200 ng of fungal DNA, 25 pM of forward and reveres primers each, 1 U of Taq DNA polymerase (Fermentase, Germany), 10X of Taq DNA polymerase including (20 mM $(NH_4)_2SO_4$; 75 mM Tris–HCl; 50 mM KCl; pH 9) and 50 mM of dNTPs (Fermentase, Germany). PCR was performed in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany). The thermal cycling was carried out through initial denaturation at 95°C for 5 min. followed by 35 cycles of DNA amplification. Each cycle consisted of denaturation at 95°C for 1 min. then annealing at 58°C for 1 min. and extension at 72°C for 1 min.

There was a 10 min. delay at 72 °C at the end of the last cycle. The amplified fragments were separated on 2% agarose gel stained with ethidium bromide, visualized on a UV transilluminator and photographed by Gel Documentation System (Alpha Imager M1220, Canada).

DNA sequencing

Fragments (750 bp) of TEF-1α gene were purified using gel extraction kit (PeqGold). Sequencing of forward strands was performed using 3130xl Genetic Analyzer (Applied Biosystems-Hitachi, Japan) at the Sequencing Service Unit, of the City for Scientific Research and Technology Applications, Borg Elarab, Egypt.

Screening of antifungal activity of viable LAB strains

LAB were screened for antifungal activity using agar well diffusion assay[31]. MRS agar plates containing 10^4 of *F. moniliforme*, *F. proliferatum* and *F. oxysporum* conidia per mL agar were prepared. Wells with a diameter of either 3 or 5 mm were then cut in the agar using a sterile cork-borer. To avoid leakage, a droplet of agar was added to each well. Then, either 40 or 70 µL of viable LAB suspension ($X10^{10}$ CFU mL^{-1}) were added to the wells and allowed to diffuse into the agar during a 5 h pre-incubation period at room temperature, followed by aerobic incubation at 30°C for 48 h. The LAB antifungal effects recorded were graded as follows: (-), no suppression; (+), weak suppression around the wells; (++), strong suppression (with detectable clear zones around the wells); or (+++), very strong suppression, (large, clear zones around the wells).

Preparation of LAB cell-free supernatants

The LAB strains were grown in MRS broth at 30° C or 37° C. After 24 h or longer periods of incubation, cells were centrifuged at 8000 *xg* for 10 min. The supernatants were filtered through a 0.2 µm sterile filter (Rotrand, Schleicher & Schüll, Germany) and frozen at -20° C until use. Tenfold concentrated supernatant of selected strains were prepared by lyophilization.

Screening of antifungal activity of LAB cell free supernatants

Mould agar spot assay was based on the agar spot assay as described by Cabo *et al*.[32]. Two mL of the filter-sterilized culture supernatants were mixed with 8 mL of Malt Extract Agar (MEA), (20 g malt extract, 20 g glucose, 1.0 g peptone, 20 g agar, 1.0 L distilled water) at 55° C and poured into plates. After drying of the plates, 10 µL of *F. moniliforme*, *F. oxysporum* and *F. proliferatum* spore suspension (10^6 CFU / mL) were dropped on the agar, and the plates were incubated for 4–6 days at 25° C. The diameter of the mould colonies was measured then compared with the control in which the supernatant was replaced by MRS medium.

The percentage inhibition of radial mycelial growth of the test fungus was calculated as follows:

$$I = [(C - T) / C] * 100 \quad ...(1)$$

Where, I = Percentage of inhibition, C = Diameter of the fungal colony in control (MEA), T = Diameter of the fungal colony in treatment. All screening tests were done in triplicate.

Screening FB1-binding activity of viable LAB from MRS medium

LAB strains were screened for their ability to bind FB1 according to Niderkorn *et al*.[25]. For each strain, two erlenmeyer flasks containing 200 mL of MRS medium were inoculated with 0.1 mL of an overnight LAB culture, and were incubated at optimal temperature of growth (30 or 37° C) for 24 h. At the end of incubation, cultures were centrifuged (3000 *xg*, 10 min., 5° C), and supernatants were removed. Toxin solutions containing FB1 (10 µg/mL) were prepared by evaporating solvents at 45° C with nitrogen gas, re-dissolving in water (5% v/v in the final solution) and adding MRS broth acidified to pH 4 with lactic acid. For each strain, the bacterial pellet from 200 mL culture was re-suspended in 2 mL of MRS-containing Mycotoxins. Positive controls containing no bacteria and a negative control for each genus of LAB containing no toxin were included. The mixtures were incubated in polypropylene tubes (1 mL per tube) at 30° C for 1 h with shaking (480 rpm) and for 23 h without shaking. At the end of the incubation period, tubes were centrifuged (3000 *xg*, 10 min., 5° C), and the supernatants were directly analyzed for toxins. Assays and positive controls were performed in triplicate.

FB1 concentration was determined using reserved-phase HPLC technique according to Horwitz and Albert, [33] using column C18 (Kinetex 2.6u C18 100A 150X4.6mm, Phenomenex) as follows: The mobile phase consisted of methanol and emission wavelength for all fumonisins were 335

nm and 440 nm, respectively (florences detector). The percentage of toxin removed was calculated by using the following equation:

Toxin removed (%) = [(1 - Peak area of toxin in the supernatant) / Peak area of toxin in the positive control] x 100 ...(2)

RESULTS

Morphological identification

Stored maize grains are prone to fungal attack, especially at a moderate temperature and high humidity. A total of 30 fungal isolates were isolated from twenty maize samples obtained from five different local markets and assessed visually by microscopic examination. Based on morphological and cultural characteristics, 13 isolates belonged to *Fusarium* spp. These isolates were identified as *F. moniliforme*, *F. oxysporum*, *F. proliferatum* and *F. culmorum*. *Fusarium* isolates were recovered from five maize samples out of twenty (Table 2).

Molecular characterization

The species-specific PCR assays confirmed the identification of *Fusarium* spp. associated with maize. PCR amplifications gave an amplification band of the expected size for each species tested (Table 1), where, the primer set EF1T-F/EF1T-2 amplified the expected 750 bp DNA fragment from all *Fusarium* isolates (Fig. 1). Specific primer (PQF5-F/PQF5-R) was used to analyze for fumonisin producing isolates. Three isolates (*F. moniliforme*) showed the expected 70-bp amplicon (Fig. 2), while an expected 60-bp fragment (Fig. 3) for four isolates (*F. proliferatum*) was generated using species-specific PCR primers (FUM5P2-F/FUM5P2-R).

Strains identification by TEF-1α partial gene sequencing

PCR products (750 bp) using specific primer (EF1T-F/EFT-R) of five isolates (EMF1, EMF4, EMF6, EMF9 and EMF10) were subjected to DNA sequence analysis. The obtained sequences were analyzed using BLAST DNA (http://www.ncbi.nlm.nih.gov/). Isolates EMF4 and EMF6 showed similarity 95% and 84%, respectively with *F. oxysporum*. However, isolates EMF9 and EMF10 showed similarity 99% and 98%, respectively with *F. proliferatum*. Isolate EMF1 showed 99% similarity with *F. moniliforme*.

Table 1. Sequence of primers used in the experiments

PCR Assay	Primer name	Primer sequence (5′/3′)	PCR conditions*	Species-Specific	Amplicon size (bp)	Reference
TEF	EF1T-F	ATGGGTAAGGAGGACAAGAC	58°C, 1 min., 35 cycle	All *Fusarium* species	750	O'Donnell *et al.* [29]
	EF1T-R	GGAAGTACCAGTGATCATGTT				
FUM1 (Fv)	PQF5-F	GAGCCGAGTCAGCAAGGATT	60°C, 1 min., 35 cycle	FUM1 (*F. verticillioides*)	70	Lopez-Errasqu1n *et al.*[30]
	PQF5-R	AGGGTTCGTGAGCCAAGGA				
FUM1 (Fp)	FUM5P2-F	CCCCCATCATCCCGAGTAT	60°C, 1 min., 35 cycle	FUM1 (*F. proliferatum*)	60	Jurado *et al.* [16]
	FUM5P2-R	TGGGTCCGATAGTGATTTGTCA				

* PCR conditions provided in the column are annealing temperature, extension time and number of extension cycles

Table 2. Potential mycotoxigenic and fumonisin production of *Fussarium* isolates

Isolate[1]	Identity assigned	Fumonisin production[1]	FUM1 (Fv)[2]	FUM1 (Fp)[3]
EMF1	*F. moniliforme*	+	+	-
EMF2	*F. moniliforme*	+	+	-
EMF3	*F. moniliforme*	+	+	-
EMF4	*F. oxysporium*	-	-	-
EMF5	*F. oxysporium*	-	-	-
EMF6	*F. oxysporium*	-	-	-
EMF7	*F. proliferatum*	+	-	+
EMF8	*F. proliferatum*	+	-	+
EMF9	*F. proliferatum*	+	-	+
EMF10	*F. proliferatum*	+	-	+
EMF11	*F. Culumorum*	-	-	-
EMF12	*F. Culumorum*	-	-	-
EMF13	*F. Culumorum*	-	-	-

[1]Isolate codes were managed according to sample location (Egypt), origin of the isolates (Maize) and fungal genus (*Fusarium*). [2]Positive (+) and negative (-) represent detected and nondetected fumonisin production. [3]Positive (+) and negative (-) represent detected and nondetected amplicon for FUM1 gene assays.

Table 3. Antifungal activity of viable LAB on toxigenic *Fusarium* isolates

LAB strains	Inhibition zone*				
	F. moniliforme EMF1	*F. moniliforme* EMF2	*F. proliferatum* EMF9	*F. proliferatum* EMF10	*F. oxysporium* EMF4
Lb. delbrueckii subsp. *lactis* DSM 20076	-	-	+	++	+
Lb. acidophilus DSM 20079	++	++	++	++	+++
P. acidilactici NNRL B-5627	++	++	++	++	++
Lb. sakei LB 706	+++	+++	+++	+++	+++
Enterococcus faecalis	+	+	-	-	++

*Inhibition zone: (-), no suppression (+), no fungal growth on 1~30% of plate area/bacterial streak; (++), no fungal growth 30~60% of plate area/bacterial streak; (+++), no fungal growth on > 60% of plate area/bacterial streak. Experiment was conducted in 3 replicates for each test.

Table 4. Antifungal activity of LAB cell-free supernatants on toxigenic *Fusarium* isolates

LAB strains	Percentage inhibition of fungal mycelial growth (I)*				
	F. moniliforme EMF1	*F. moniliforme* EMF2	*F. proliferatum* EMF9	*F. proliferatum* EMF10	*F. oxysporium* EMF4
Lb. delbrueckii subsp. *lactis* DSM 20076	50	56.25	100	97.5	100
Lb. acidophilus DSM 20079	75	75	100	100	93.75
P. acidilactici NNRL B-5627	81.25	81.25	100	100	100
Lb. sakei LB 706	100	100	100	99	100
Enterococcus faecalis	62.5	68.75	87.5	93.75	93.75

Experiment was conducted in 3 replicates for each test. Radial growth measured in cm.
*I = [(C - T) / C] x 100.

Table 5. Detoxification of fumonisin B1 from culture medium (MRS) by viable LAB strains

LAB species	Bacterial concentration ($X10^{10}$ CFU mL^{-1})	Fraction FB 1 removal (%)*
Lb. delbrueckii subsp. *lactis* DSM 20076	2.18	76.67
Lb. acidophilus DSM 20079	2.36	47.88
P. acidilactici NNRL B-5627	2.26	72.65
Lb. sakei LB 706	2.17	46.26
Enterococcus faecalis	2.34	41.46

Bacteria from 100% concentrated cultures were incubated in MRS broth acidified at pH 4 at 30° C for 1 h with shaking (480 rpm) and for 23 h without shaking. Final toxin concentration in MRS broth was 10 µg mL^{-1}.
*Toxin removed (%) = Peak area of toxin in supernatant/peak area of toxin in the positive control X 100

Screening of antifungal activity of viable and cell free supernatant LAB strains

F. proliferatum isolates which were reported as two ubiquitous species frequently found to be contaminants of food, reduce cereal yields and are major sources of fumonisins and trichothecenes which are mycotoxins noxious for human and animal health. Five strains of LAB were screened for inhibitory activity towards five fungal isolates. Varying degrees of inhibition were detected against the moulds (Table 3). *F. oxysporum* isolate, which was the most sensitive indicator strain, was highly inhibited by all the bacterial strains. Three strains of LAB *viz*. *Lb. acidophilus* DSM 20079, *Lb. sakei* LB 706 and *P. acidilactici* NNRL B-5627 showed the inhibitoriest effect against all fungal isolates. No zones of inhibition were observed with *L. delbrueckii subsp. lactis* DSM 20076 against *F. moniliforme* isolates (Figure 4). Also, no zones of inhibition were observed with *Enterococcus faecalis* strain against *F. proliferatum* isolates.

''Mould agar spot assay" used to assess the antifungal activities for cell free culture supernatant. All LAB strains showed a spectrum of activity against the majority of the fungal isolates (Table 4). *P. acidilactici* NNRL B-5627 showed strong antifungal activity against all *Fusarium* isolates especially against *F. moniliforme* (Figure 5). *Lb. sakei* LB 706 strain showed the most antagonistic activity against all fungal isolates followed by *Lb. acidophilus* DSM 20079 and *P. acidilactici* NNRL B-5627 strains. *Lb. delbrueckii* subsp. *lactis* DSM 20076 and *Enterococcus faecalis* strains showed antifungal activities less than the other three strains.

FB1 binding from MRS medium

The results indicated that *Lactobacillus, Enterococcus* and *Pediococcus* strains at bacterial concentrations ranged from 1×10^9 to 1×10^{11} CFU/mL were able to remove up to 75 % of FB1 from liquid media. At similar bacterial concentrations, *Lb. delbrueckii* subsp. *lactis* DSM 20076 removed FB1 toxin with an efficiency of 76.67%, while

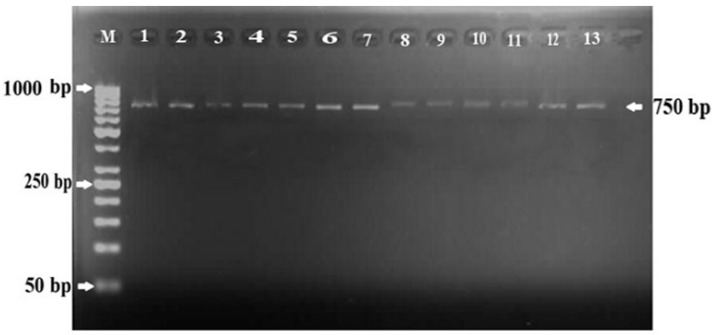

Fig. 1. Species-specific PCR detection of different *Fusarium* spp. generated by *Fusarium*-specific primers sets EF1T-F / EF1T-R. Lane (M): 50-bp DNA ladder (MBI Fermantase, Leon-Rot, Germany), Lanes 1 to 13: *Fusarium* isolates

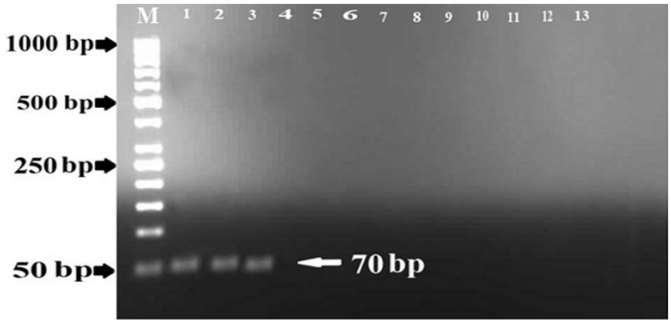

Fig. 2. Species-specific PCR detection of Fumonisin-producing isolates using *Fusarium*-specific primers (PQF5-F/PQF5-R), developed for *F. moniliforme*. Lane (M): 50-bp DNA ladder (MBI Fermantase, Leon-Rot, Germany), Lanes 1 to 3: *F. moniliforme* isolates; Lanes 4-13: other *Fusarium* isolates

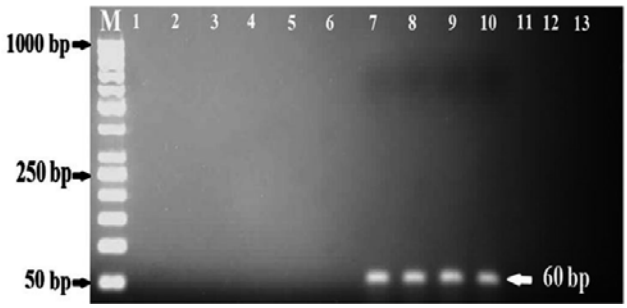

Fig. 3. Species-specific PCR detection of Fumonisin-producing isolates using *Fusarium*-specific primers (FUM5P2-F/FUM5P2-R), developed for *F. proliferatum*. M: 50-bp DNA ladder (MBI Fermantase, Leon-Rot, Germany); Lanes 7 to 10, *F. proliferatum* isolates; Lanes 1-6 and 11-13, various *Fusarium* isolates

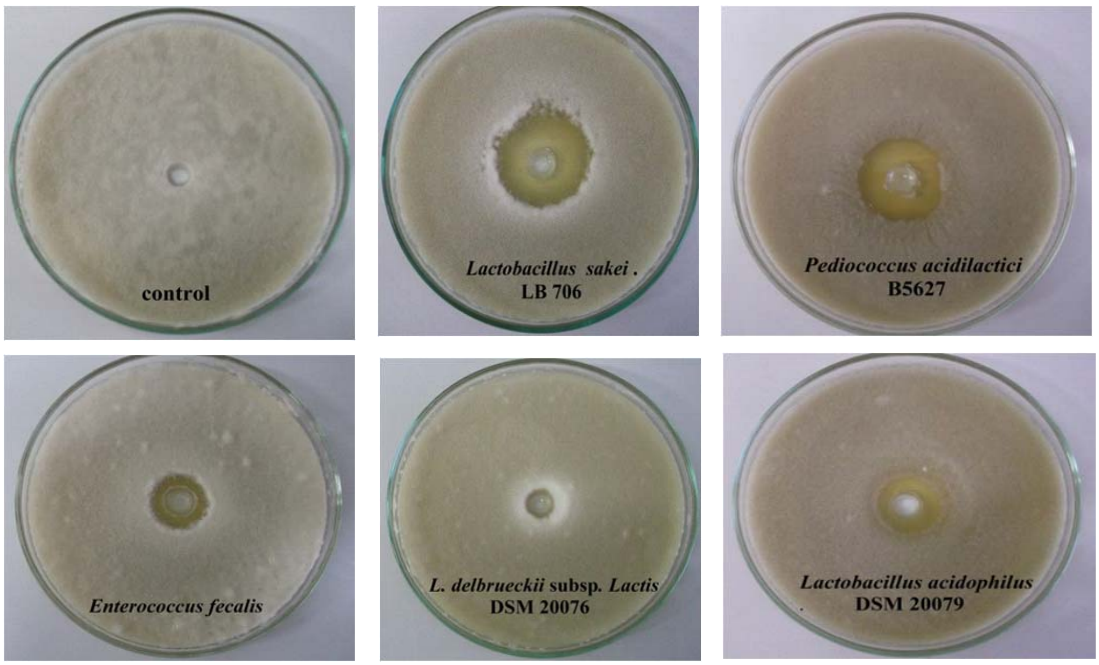

Fig. 4. Antifungal activity of viable LAB strains *viz. Lb. sakei* LB 706, *P. acidilactici* NNRL B-5627, *Lb. acidophilus* DSM 20079, *Enterococcus faecalis* and *Lb. delbrueckii subsp. Lactis* DSM 20076 against the indicator *F. moniliforme* (EMF1). All strains showed inhibitory effects except for *Lb. delbrueckii* subsp. *lactis* DSM 20076

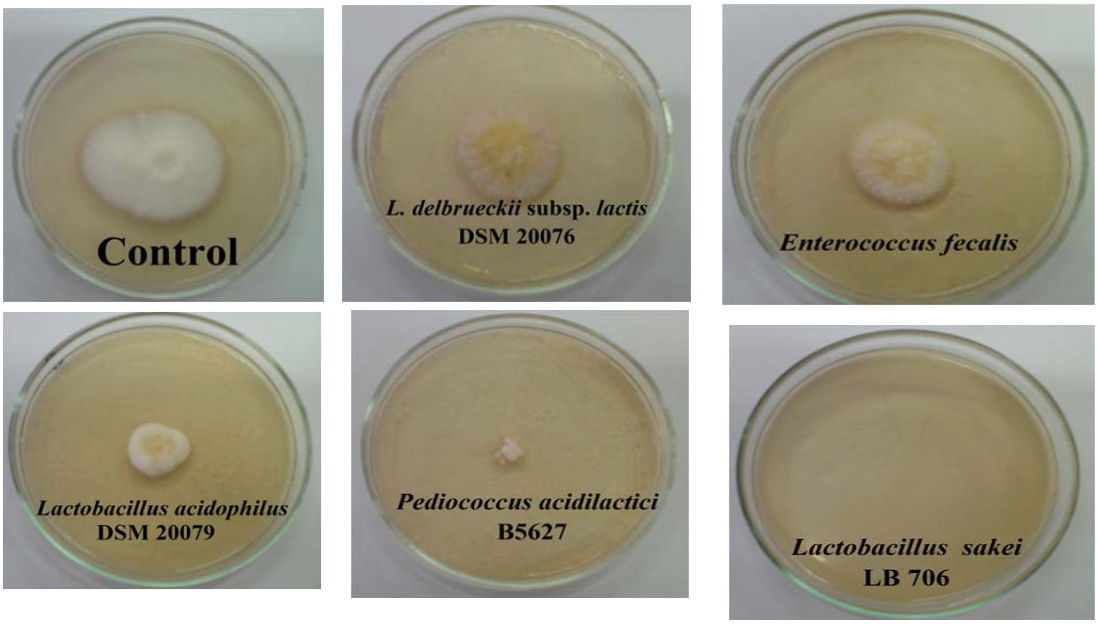

Fig. 5. Antifungal activity of LAB strains cell-free supernatants of *Lb. sakei* LB 706, *P. acidilactici* NNRL B-5627, *Lb. acidophilus* DSM 20079, *Enterococcus faecalis* and *Lb. delbrueckii* subsp. *Lactis* DSM 20076 against *F. moniliforme* (EMF1)

Enterococcus faecalis was the less efficient at removing FB1 toxin from the medium, 41.46% (Table 5).

DISCUSSION

It has been estimated that 25% of the world's crop production is contaminated with mycotoxins. Fumonisins produced by *F. moniliforme* and *F. verticillioides* are the important toxins which have become a major constraint food crops during the last two decades. So, the aim of this investigation concerned *Fusarium* contamination in maize as well as biocontrol of *Fusarium* species associated with stored cereals especially maize. *Fusarium* isolates were recovered from five maize samples out of twenty (25%) which in agreement with the findings of Abo-El Yazeed *et al*. [17] who detect *Fusarium* from 23% of animal feeds. On the other hand Buckley *et al.* [34] could detected *Fusarium* only in 2.6% of equine concentrated feed. Moreover, Khosravi *et al.* [35] isolated *Fusarium* in 6% of animal feed mainly corn seed, barley and corn silage samples collected from Iran.

Although, some species of *Fusarium* were difficult to identify morphologically, identification based on mor-phological characteristics is necessary, as it allows the sorting of *Fusarium* isolates before applying other meth-ods of identification and characterization [15]. In our work 13 out of 30 isolates, were identified as *F. moniliforme* (three isolates), and *F. proliferatum* (four isolates), *F. oxysporum* (three isolates), *F. culumorum* (three isolates) based on morphological characteristics. In recent years, interest has been rekindled among plant pathologists in the use of the PCR-based markers, especially the species-specific PCR assays. It is likely to become a common approach in molecular identification strategies, an effective and fast way to detect fumonisin-producing isolates and no cross-reactions among the fungal isolates tested. However, the molecular identification assay using specific primer (EF1T/EF2T) confirmed the mor-phological results. Accordingly, St'pieD *et al.*[36] have previously reported that the species-specific PCR assays produced an amplicon of a size expected for *F. proliferatum*, confirming the morphological identification of all isolates. In addition, Bayraktar and Dolar[37] found that the species-specific PCR assays confirmed the identification of *Fusarium* spp. associated with onion. In contrast to these findings, Rahjoo *et al.*[19]

and Ghazvini *et al*.[38] reported that identification of *Fusarium* isolates which were identified based on morphologic and cultural characterizations were different from molecular characterization.

F. momiliforme and *F. proliferatum* strains have been tested to their ability to produce FB1 using the PQF5 and FUM5P2 primers respectively. These strains produced FB1, which was previously reported by Abo El-Yazeed *et al*.[6]. In an Argentinean study, only 78 %, of the *F. moniliforme* and *F. proliferatum* produced FB1[39].

LAB have a long history of use in a diversity of food fermentations, where they contribute to the organoleptic properties and safety of the final product[40]. The development of fungicidal resistance together with consumer trends toward safer methods of food preservation has fueled interest in the use of antifungal LAB as biopreservatives[21]. Members of the *Lactobacillus* genus, in particular strains of *Lb. plantarum*, have become prominent players in the field of antifungal research[41, 42]. In a previous study of Magnusson *et al*.,[43] reported that only 4~5% of the isolated microorganism (*Lb. coryniformis. Lb. plantarum* and *P. pentosaceus* showed antagonistic activity against *Aspergillus fumigatus, Aspergillus nidulans, Penicillium commune* and *F. sporotrichioide*. The results of these studies stated that the investigated strains showed varying degrees of inhibition against the *F. moniliforme, F. proliferatum*, and *F. oxysporium.* *Lb. acidophilus* DSM 20079, *P. acidilactici* NNRL B-5627 and *Lb. sakei* LB 706 showed strong activity (+++) against all fungal isolates. In agreement with our results, Kim[44] reported that about 4.16% of the total number of the isolated bacteria had strong activity (+++) against *A. flavus, F. moniliforme, Penicillium commune,* and *Rhizopus oryzae.* In addition, *Lb. plantarum* strain 16 (deposited as NCIMB41875) was determined to possess broad-spectrum antifungal activities and able to prevent the growth of certain fungal spoilers in several food models including fruits, yogurt, and orange juice[45]. Also, El-Mabrok *et al*.,[46] reported that from 324 LAB isolated from different sources of fruits and vegetables, milk, beef, fermented fish and soil, only 30 isolates (9.25%) showed good antifungal activity against *Colletotrichum gloeosporioides.* Optimization and improvement of the antifungal potential of LAB for its use in food and feed preservation holds the key to meeting the general demand by consumers to reduce the use of chemical preservatives and additives in food and feed and the production of high quality, preservative free, safe but mildly processed food with extended shelf life.

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[47]. The ability of LAB to bind mycotoxins, particularly FB1, has been reported in several studies[25, 48]. Where Niderkorn *et al*.,[25] found significant differences among the three strains tested *Lb. rhamnosus* strain GG ATCC 53103, *Lb. delbrueckii* ssp. *bulgaricus* R0149 & *Leuconostoc mesenteroides* R1107, *Leuc. Mesenteroides* used to determine the type of detoxification and the effects of pH and bacterial concentration on the removal of *Fusarium* toxins, the three strains tested, removed FB2 up to 82%, more efficiently than FB1 by 31% only.

Binding property displayed by some selected LAB, resulting in a decrease of mycotoxin bioavailability[23]. The results indicated that *Lactobacillus, Enterococcus* and *Pediococcus* strains were able to remove up to 75%, of FB1 from liquid media; which in agreement with Dalié *et al.*[20] who investigated the effect of *P. pentosaceus* L006 on growth and mycotoxins production by *F. vertciloides*. Lahtinen *et al.*[49] reported that a remarkable effect of *Lactobacillus rhamnosus* strain GG on growth and Aflatoxin B1 production by *Aspergillus* species. Niderkorn *et al*.[50] demonstrated that peptidoglycan (PG) of LAB and more generally PG of Gram-positive bacteria, are the most likely site of FB binding. This result helps to explain the widespread binding of fumonisins by LAB. Existing differences in binding capacity of different bacterial species can be rationally explained by the variation in PG structure.

The current study shows that different species of LAB exhibited antifungal and antimycotoxin activity against a number of common spoilage moulds and mycotoxin. The inhibitory activity is caused by several compounds. In conclusion *Lb. acidophilus* DSM 20079, *P. acidilactici* NNRL B-5627 strains could be promising agents for reducing the *Fusarium* spp. growth and fumonisin B1 contamination of food and feed.

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