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INHIBITION OF MYCOTOXIN- PRODUCING FUNGI ISOLATED FROM KARISH CHEESE BY LACTOBACILLI STRAINS

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ABSTRACT

Food- borne fungi cause serious spoilage and some species can produce toxic metabolites (mycotxins), produce serious health problems. Fungi were isolated from forty samples of fresh Karish cheese, collected from Giza governorate, using malt extract and potato dextrose agar media at 25 ^{O}C. The results indicate that members of *Penicillum spp.* and *Aspergillus spp.* were the mainly contaminated molds of all the tested samples. Members of *Penicillum spp.* (*P. roqueforti*, *P.corylophilum* and *P. chrysogenum*) and *Aspergillus spp.* (*A. flavus* and *A. niger*). Spectrum of antifungal activity of *Lactobacillus casei* CH-1, *L. bulgaricus* CH-2 and *L. plantarum* (ATCC 8014) were investigated. Among tested strains *L.plantarum* (ATCC 8014) strain had a strongest inhibitory activity against all isolated of molds from Karish cheese samples. The disc diffusion method was used to evaluate the zone of fungal growth inhibition at various volume of cell-free supernatant. Minimal inhibition concentration (MIC) and minimal fungicidal concentration (MFC) of cell-free supernatant were determined. Cell-free supernatant of *L.plantarum* (ATCC 8014) culture in Karish cheese was studied. No moldes could be observed till 30 days of storage. Therefore, addition of cell-free supernatant from *L.plantarum* (ATCC 8014) to Karish cheese is a good bio-preservatives, preventing fungal spoilage and consequently mycotoxin formation in Karish cheese and recommended to extent the shelf life.

Keywords: Lactic acid bacteria, Antifungal, molds, Karish cheese. Cell-free supernatant.

INTRODUCTION

Food spoilage fungi are undesirable organisms, which responsible for flavor defects, discoloration and poor appearance of the product (Walker, 1977). Furthermore, some species can produce toxic metabolites (mycotxins), causing serious health problems for human health (lund *et al*., 1995; El-Shrief, 2000). These toxins comprise a group of chemically diverse compounds originating from secondary metabolism by molds and are mainly produced by five genera: *Aspergillus, Penicillillium, Fusarium, Alternaria and Claviceps* (Steyn, 1995). The compounds can be carcinogenic, hepatoxic, teratogenic or immunosuppressing (Speijers and Speijers, 2004).

Cheese is considered as one of the most important foodstuffs consumed by human and it contains a source of high quality animal protein having all the essential amino acids (El-Shrief, 2000). Cheese can easily become moldy by a large number of species of fungi and yeasts during ripening and storage in shops or at home (Carter& Cole, 1990). Karish cheese is a soft cheese locally made and commonly consumed in Egypt. It is

characterized mainly by its low fat content and its high acidity resulting by the action of lactic acid bacteria (Abou-Dawood and Abdou, 1973). Fungi and yeasts are important spoilage organisms for different foods. During the last few years there has been a growing interest in bio-preservation, i.e., the use of microorganisms and /or their metabolites to prevent spoilage and to extend the shelf-life of food (Stiles, 1996; Trais *et al*., 2008).

Reduction of fungi growth during the production and storage of food and feed is of great importance. The primary method of control is the use of chemical fungicides. However more of them are nowadays not authorized due to the toxicological risks (Directive91/414/CEE of the EU). Some microorganisms have traditionally been used as bio-preservatives in food and feed. Bio-preservatives allows prolonged shelf life and enhanced safety of foods through natural or supplementary microflora and their antimicrobial products (Schnurer and and Magnusson, 2005). Among the different potential decontaminating microorganisms lactic acid bacteria (LAB) represented unique groups, which widely used in food fermentation, and are one of a particular interest as bio-preservation organisms (Shetty and Jeseprsen, 2006; Voulgari *et al*., 2010). Their preserving effect mainly relates to the formation of lactic acid, acetic acid, hydrogen peroxide, and the production of bacteriocins (Lindgren & Dobrogosz, 1990; Stiles, 1996). Many studies have assessed those bacteriocins as antibacterial effects (Dodd & Gasson, 1994); but there are very few reports on specific antifungal compounds from lactic acid bacteria (Munoz, *et al*., 2010). Therefore, investigations on antifungal activity of lactic acid bacteria (LAB), that combine LAB species or strains which produce different antifungal compounds could be a useful target as novel biopreservatives (Strom, 2005; Munoz *et al*., 2010). Hence, this study aimed to investigate the antifungal activity of Lactobacilli strains against some mycotoxin-producing fungi isolated from Karish cheese Lactobacilli strains.

MATERIALS AND METHODS

Forty samples of fresh Karish cheese were collected randomly from several locations in Giza.

Lactobacillus plantrum (ATCC 8014) strain was obtained from the American Type culture collection (ATCC) (Rockvill, Maryland 20852, USA). Strains of *Lactobacillus casei* CH-1 and *L. bulgaricus* CH-2 strains were obtained from the agent of Chr. Hansens Laboratory (Denmark A/S). They were grown on MRS agar at 30°C in CO2 Oxoid atmosphere generation system (CampyGen Oxoid Ltd., Basingstoke, U.K.). The working cultures were kept on MRS agar at 5°C until use.

Ten grams of each Karish cheese sample were placed in 90 ml of sterile 2% sodium citrate solution and then shaken. One ml of the suspension was spread onto potato dextrose agar plate and another one ml was applied onto malt extract agar plate. Incubation was carried out at 25°C during the period of 5-7 days. Fungal isolates were identified by colony cell morphology and microscopic observation of conidiospore formation according to Alexopoulos *et al*, (1996).

Inocula containing spores were prepared by growing the fungi on malt extract agar slants at 25 ^{O}C for 7 to 10 days (or until sporulation) and then collecting spores were obtained after vigorously shaking the slants with sterile peptone water (0.2%[wt/vol.]). Spores were determined as total number of viable spores per ml. Spores suspension (50µl) was spread on potato dextrose agar plates and then incubated at 30 ^{O}C for 72h and adjusted to 10^5 per ml of sterile peptone water (0.2%) (Magnusson and Schnurer, 2001).

Antifungal activity assays

Two different assays, the overlay method and agar well diffusion method were used to detect antifungal activity. All experiments of assaying the inhibitory activity in the current study were performed in duplicate. The overlay method was performed using MRS agar plates on which lactic acid bacteria were inoculated as two cm long lines and incubated at 30 ^{O}C for 48h in anaerobic jar. The plates were then overlaid with 10ml of malt extract soft agar (2% malt extract, 0.7% agar, Oxoid) containing 10^4 fungal spores (conidia) per ml. The plates were then incubated aerobically at 30 ^{O}C for 5-7 days. The plates were examined for clear zones of inhibition around the bacterial streaks. The area of clear zones was scored as follows: (-) no suppression; (+) no fungal growth on 0.1 to 3% of the plate area per bacterial streak; (++) no fungal growth on 3 to 8% of the plate area per bacterial streak; or (+++) no fungal growth on >8% of the plate area per bacterial streak. The agar well diffusion assay, malt extract agar plates (pH 3.6) containing 104 *A. niger* conidia per ml agar were prepared. Wells with a diameter of 5 mm were cut in the agar using a sterile cork-borer. A droplet of agar was added to each well in order to seal it to avoid leakage, then, 10, 20, 40, or 80µl of MRS broth culture 18h old lactic acid bacteria were added to the wells and allowed to diffuse into the agar during a 3h pre-incubation period at room temperature, followed by aerobic incubation at 30 ^{O}C for 48h. The antifungal activity was scored as follows :(-), no suppression ;(+) weak suppression around the wells; (++) strong suppression with detectable clear zones around the wells; or (+++) very strong suppression with large, clear zones around the wells. (Magnusson and Schnurer, 2001).

Lactobacillus plantrium ATCC 8014 strain was inoculated to concentration of 10^5 cells ml-1 in one liter of MRS broth medium under aseptic condition and incubated at 30°C for 18 h. Cell-free supernatant was prepared by centrifugation (5000xg for15 min) and sterile filtration (0.45µm-pore size filter; Millipore). The sterile cell-free supernatant was freeze-dried and resuspended (to 20-fold concentration) in 20 mM citrate-phosphate buffer (pH 3.4). (Strom.*et al*., 2002).

The minimal inhibitory concentration (MIC) and Minimal fungicidal concentration (MFC) were assessed as follow: MFC was determined by a broth dilution method in test tubes, 50µl from each of 1/2, 1/4,1/8,1/16 dilutions of 20 fold- cell-free supernatant were added to 5 ml of malt extract broth tubes containing 10^5 spores /ml. The tubes were then incubated on an incubator shaker. 50µl of MRS broth, were concentrated to 20 fold, used as a control. The highest dilution (lowest concentration), showing no visible

growth, was regarded as MIC. Negative cells (-) from the tubes showing no growth were subcultured on potato dextrose agar plates to determine if the inhibition was reversible or permanent. MFC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates (Rasooli and Abyaneh , 2004).

Fungicidal estimation of the cell-free supernatant:

50µl of 1/4 dilution of aliquots 20 fold-cell-free supernatant was added to 5 ml of malt extract broth tubes containing 10^5 spores /ml and which incubated at 30°C for 15 -120min at increments of 15 min in an incubator shaker. Samples were taken after the time intervals and were cultured on potato dextrose agar for 48h at 30°C. 50µl from MRS broth, were concentrated to 20 fold, used as a control. Microbial colonies were counted after incubation period and the total number of viable spores per ml was calculated. The calculation was converted to percent dead spores using routine mathematical formulae. .(Rasooli and Abyaneh ,2004).

The antifungal activity remaining after exposure to high temperature, different pH values, or proteolytic enzymes were determined using the agar well diffusion assay. The high temperature effect was investigated. 10ml of 20 fold-cell-free supernatant (prepared as described above) were heated to 121°C for 15min.The samples were allowed to cool and then tested for antifungal activity. The pH effect was investigated with another 10ml of 20 fold-cell-free supernatant, adjusted to pH 2.5, 4.0, 5.0, 6.0 and 7.0 with 1M HCl and 2 M NaOH before determining the antifungal activity. MRS broth was concentrated to 20 fold, adjusted to the same pH values and used as a control. The effect of proteolytic enzyme on antifungal activity was investigated with 10ml of 20 fold-cell-free supernatant, were treated with trypsin (Sigma). Samples were adjusted with 1M HCl and 2 M NaOH to the optimum pH value (7.6). For the enzyme, the cell-free supernatant were treated with 100 µg of the enzyme per ml and incubated at 37°C for 1h. Before evaluating the antifungal activity the pH of the cell-free supernatant was readjusted to the initial pH , and the cell-free supernatant adjusted to 20 fold concentrate to serve as control (Magnusson and Schnurer, 2001).

Karish cheese was made as described by Fahmi (1960). Skimmed milk was heated at 65°C for 30min, then cooled to 32°C and inoculated with 3% starter (vol/vol) of *Lactococcus lactis subsp lactis var diacetylactis* then incubated at 32°C till reaching pH 5.5. Cell-free supernatant (20 fold) was added at a rate of 5% before renneting. Curd was hoped to drain at room temperature for 24h. Karish cheese was stored at ~8°C. Samples were taken at time intervals of 0, 7, 14, 21 and 30 days of storage and examined for mycotoxin-producing fungi.

RESULTS AND DISCUSSION

Results in Table (1) indicate that the majority of fungi isolated from 40 samples of Karish cheese collected from local market in Giza belong to *Aspergillus spp. and Penicillium spp Aspergillus spp.* was the dominant

genera in Karish cheese samples (55% of total fungi). Two species were identified within this genus *A. flavus* (25%) and *A. niger* (30%). The results agree with Hassanin (1993) who isolated *A. flavus* and *A. niger* from 16 samples of cheese collected randomly from several locations in Cairo and Giza . *A. flavus*, *A. flavipes*, *A niger* and *A. terreus* were also isolated from the Egyptian cheese by El-Shrief, (2000). Penicillium spp was the second dominant genus isolated from tested samples. It appeared in 45% of the total fungi isolates, within this genus, 3 species were identified as *P. roqueforti*, *P.corylophilum* and *P. chrysogenum*, and they were present in 10%, 10% and 25% of the samples, respectively. Many other *Penicillium spp* were also isolated with variable incidences from different cheese samples in Egypt (El-Sawi *et al*., 1994; Abdel-Satar *et al*., 1995 and El-Shrief, 2000). Mycotoxins are mainly produced by five genera: *Aspergillus, Penicillium, Fusarium, alternaria and Claviceps Aspergilli* are the most common fungal species that can produce mycotoxins in food and food stuffs causing serious health-hazardous (Steyn, 1995; Probest *et al*., 2007).

Table (1): Frequency of fungi isolates from Karish cheese*

Fungi strains	Frequency of fungi isolates from Karish cheese	% Frequency of fungi isolates from Karish cheese
P.roqueforti	**4**	**10**
P.corylophilum	**4**	**10**
P.chrysogenum	**10**	**25**
A.flavus	**10**	**25**
A.niger	**15**	**30**

***Total Karish cheese samples were 40**

Results in Table (2) illustrated that among three lactobacilli strains, *Lactobacillus casei* CH-1, *L. bulgaricus* CH-2 and *L. plantarum* (ATCC 8014) only *L.plantarum* (ATCC 8014) strain had strong inhibitory activity against all of the isolated fungi isolated from Karish cheese samples. It was also observed that antifungal activity of *L. plantarum* (ATCC 8014) using well diffusion agar was higher than in overlay system,which may be explained by the better the growth of *L.plantarum* being grown in broth media which produce more antifungal substances as well as other metabolites. Also results in Table (2) revealed that *A.niger* was the highest and more sensitive to antifungal substance produced by *L.plantarum* (ATCC 8014) than *P.chrysogenum*. It was also found that activity of LAB against fungi varied greatly between different fungi species. Pitt and Hocky (1999) reported that *P. roquenforti* and *Pichia anomala*, were considered as “preservative resistant”, and hardly affected by the LAB. On the other hand, Strom *et al.* (2002) isolated a *L. plantarum* strain (MiLAB 393) from grass silage, which had activity against several fungi species in an agar overlay method. It showed no inhibitory activity against *P.roqueforti*. Munoz *et al*., (2010) found that LAB strains *L.fermentation* and *L.rhamnosus* (isolated from sheep milk) showed growth inhibition of mycotoxin-producing *Aspergilus* strain.

Table (2): The inhibitory activity of *L.plantarum* (ATCC 8014) against isolates fungi.

Fungi strains	Activity[a] with overlay system	Activity[b] with agar well diffusion method
P.roqueforti	++	+++
P.corylophilum	++	+++
P.chrysogenum	+	++
A.flavus	++	+++
A.niger	+++	+++

[a] Activity was scored as follows-, no suppression ; + no fungal growth on 0.1 to 3% of the plate area per bacterial streak; ++ no fungal growth on 3 to 8% of the plate area per bacterial streak ; or +++ no fungal growth on >8% of the plate area per bacterial streak.
[b] Activity was scored as follows-, no suppression;+ weak suppression around the wells ; ++ strong suppression with detectable clear zones around the wells; or +++ very strong suppression with large, clear zones around the wells.

The minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) techniques were employed to assess fungistatic and fungicidal effect of the antifungal substance. It was found that a broad spectrum of activity by inhibiting all-fungal isolated strains, with MIC ranging from dilution concentrations 1 to 1/16. Moreover, antifungal substance acted with fungicidal mechanism since no conidial germination was observed after 3 days for most strains at concentrations ranging from 1/2 to 1/8 dilution concentrations according to the fungal strain (Table 3).

Table (3): Minimal inhibition concentrations (MIC) and minimal fungicidal concentration (MFC) for the antifungal substance produced by *L.plantarum* (ATCC 8014)

Fungi strains	(MIC)	(MFC)
P.roqueforti	1/4	1/2
P.corylophilum	1/8	1/4
P.chrysogenum	1/2	1
A.flavus	1/8	1/4
A.niger	1/16	1/8

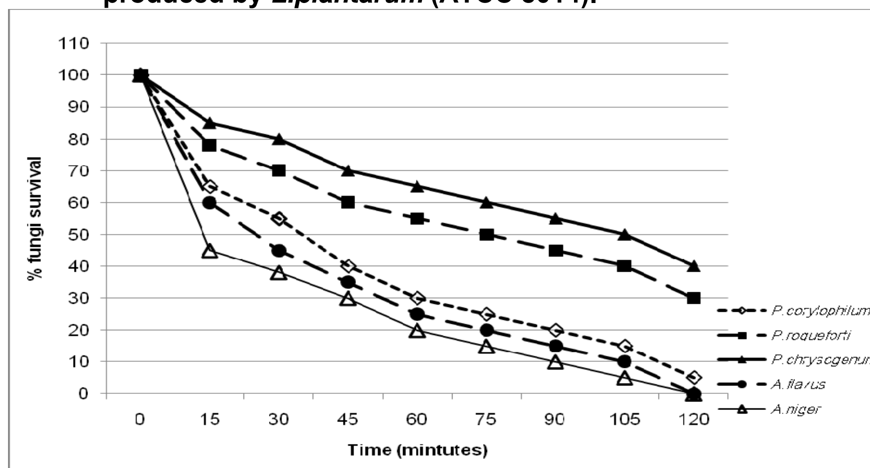
(MIC)= Minimal inhibition concentrations.
(MFC)= minimal fungicidal concentration

Fungicidal estimation of antifungal substance produced by L.plantarum (ATCC 8014)

Results in Fig.(1) illustrated that fungicidal estimation of antifungal substance produced by *L.plantarum* (ATCC 8014) were more than 50% death for *A.flavus* and *A.niger* after 30 min. but the fungicidal was 45% death for *P.corylophilum* strain. *P. chrysogenum* and *P.roqueforti* strains showed some resistant at this time, but 50% death after 75min exposure for *P.roqueforti* after 105 min for *P. chrysogenum* . A 90-100% lethal effect was observed within 2h exposure of the antifungal substance for *A.flavus* , *A.niger* and *P.corylophilum* fungi strains, but 70% for *P.roqueforti* and 60% for *P. chrysogenum* . The cyclic-dipeptides, phenyllactic acid has been found by Lavermicocca *et al*., (2000) in cultures of *L. plantarum* that showed antifungal

activity in bread sour dough. Also Strom (2005) reported that the cyclic-dipeptides phenyllactic acid is only active at high concentrations against fungi.

Fig (1): **Fungicidal estimation at 1/4 dilution of aliquots substance produced by *L.plantarum* (ATCC 8014).**



Effect of temperature, pH and proteolytic enzymes on the antifungal activity.

The antifungal activity was found to be heat stable. (50ml) of 20 fold-cell-free supernatant were heated to 121°C for 15min retained full inhibitory activity against fungi growth. The activity was also stable at low pH values that were between 3.0 and 4.5, but rapidly decreased between pH 4.5 and 6.0. Inhibitory activity was not detected at pH above 6.0. The activity was fully regained after readjustment of the pH to the starting value. The inhibitory activity of cell-free supernatant was totally lost after treatment with proteinase K, and was radically decreased after treatment with pepsin. The observed reduction in antifungal activity of the cell-free supernatant at pH values exceeding 4.5 indicates synergistic effect between lactic acid and other antifungal compounds (Magnusson and Schnurer, 2001).

Addition supernatant from *L.plantarum* (ATCC 8014) to Karish cheese,which contained 5% cell-free supernatant (20 fold) was studied. Fungi could not be detected until 30 days of storage at 4°C. Early research suggested antifungal activities of a *Lactobacillus casei* strain which inhibited both the growth and the aflatoxin production of *Aspergillus parasiticus* (El-Gendy&Marth., 1981). Production of fungal inhibitory compounds from *L casei* subsp. *rhamnosus*, all with molecular masses of <1.000 Da was described elsewhere (Vandenbergh, 1993). The antifungal activity of a *Leuconostoc mesenteroides* strain from cheese has been reported, (Suzuki *et al*., 1991). A mixture of *Lactobacillus spp.* isolates from silage was found to reduce fungi growth and spore germination, as well as aflatoxin production of *Aspergillus flavus* subsp. *Parasiticus* (ermaG0urama &Bulln, 1995)

against both *Fusarium avenacum* and the *Gram-negative* bacterium *Pantoea agglomerans*. An antifungal activity of *Lactobacillus sanfrancisco* CBI isolated, from sour dough, was found against bread spoilage fungi of the genera *Fusarium, Penicillium, Aspergillus, and Monilla*. The antifungal activity was caused by formation of several short-chained fatty acids, among which caproic acid was the most important one (Corsetti *et al*., 1998 and Niku-Paavola *et al*, 1999). Okkers *et al*., (1999) found that phenyl- lactic acid and 4-hydroxy- phenyl-lactic acid from a sour dough isolate of *L plantarum* had broad-spectrum fungicidal activity. Characterized the peptide pentocin TV35b from *Lactobacillus pentosus*, to have fungistatic effect on *Candida albicans* and bacteriostatic effect against numbers of gram-positive bacteria. Also, the production of antimicrobial low-molecular-weight compounds other than organic acids, such as benzoic acid, methylhydantion, mevalonolactone, and cyclo- (glycyl-L-leucyl) were reported by (Lavermicocca *et al*., 2000). They were acting synergistically with lactic acid.

It could be concluded that supernatant from *L. plantarum* (ATCC 8014) is useful bioperservative, preventing fungal spoilage, and consequently mycotoxin formation in Karish cheese and recommended to extent the shelf life.

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تثبيط الفطريات المنتجة للسموم المعزولة من جبن القريش بواسطة سلالات من بكتيريا اللاكتيك العصوية

أبوبكر سالم عبدالفتاح * و يحي عبدالمنعم عبدالهادى **
*** قسم السميات الغذائية والملوثات – المركز القومى للبحوث**
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يعد التلوث الفطرى للأغذية من المصادر الخطيرة للفساد ، نظرا لأن بعض أنواع الفطريات يمكن أن تنتج سموم فطرية ينشأ عنها مشاكل صحية خطيرة . وقد أمكن عزل الفطريات من ٤٠ عينة من الجبن القريش الطازج التى تم جمعها من محافظة الجيزة بإستخدام بيئة مستخلص الشعير وبيئة آجار البطاطس والديكسترول عند درجة ٢٥ ºم . وقد إتضح سيادة التلوث بالأفراد التابعة لثلاثة أنواع للجنس *Pinicillium* ، ونوعين تابعين للجنس *Aspergillus* وذلك بالنسبة لكل عينات الجبن القريش التى تم إختبارها . كما أمكن دراسة مجال التثبيط الفطرى بواسطة البكتيريا *L.casei Ch-1* , *L.bulgaricus CH2* and *L.plantarum* (ATCC8014) ، ولوحظ أن إحدى سلالات(ATCC 8041 *L.plantarum* كانت الأقوى فى تأثيرها المثبط لكل الفطريات التى أمكن عزلها من جبن القريش . وقد أستخدمت طريقة قرص الإنتشار لتقدير نطاق تثبيط النمو الفطرى بإستخدام أحجام مختلفة من المحلول الرائق الخالى من النمو البكتيرى، كما أمكن تقدير كل من التركيز المثبط الأدنى والتركيز المثبط للفطر الأدنى فى المحلول الخالى من النمو للبكتيريا (ATCC 8041 *L.plantarum* بالجبن القريش ، ولوحظ خلو الجبن من النموات الفطرية خلال ٣٠ يوم من التخزين . وعلى ذلك، فإن إضافة المحلول الخالى من النمو لتلك البكتيريا إلى جبن القريش يعد كمادة حفظ حيوية جيدة ، بحيث نتج عنها منع التلف الفطرى، وبالتالى منع إنتاج السموم الفطرية بالجبن ، وإطالة فترة الحفظ للجبن.

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