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Antimicrobial activity of probiotic bacteria

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ABSTRACT

Probiotic strains were isolated from different cheeses (turkey and domiatii and cottage and kariesh cheese) and fermented products (yoghurt, raib, zeer milk and kishk). The isolates were screened for rate of growth increase in MSR broth at pH 3, pH4, acid production after 48 hours and degree of bile salt (0.3%) tolerance, strength of adhesion and clotting time (3 to 48 hrs) of skim milk. The good fourteen isolates identified as two *Lactobacillus spp* (S4b1 and S2a3), eleven *Bifidobacterium* spp. (RC1 b8, RC2 b1, SC1a4, RC4b2, FC1b1, RC2b4, RC4a3, LZ1a3 and LZa7) and one *Streptoococcus* spp. (RC2b3), were used against several human *(Staphyllococcus aureus*. and *Eschericia coli*) and plant (*Rhizoctonia solani* and *Fusarium oxysporum*) pathogens by examining their in vitro antimicrobial properties.

Antibacterial activity of the good fourteen selected probiotic isolates in this test exhibited varying degrees of inhibitory activity against human pathogenic *Staphyllococcus aureus*. The isolates LZb8, S4b1 and RC2b3 exhibited the superior antibacterial activity with inhibition zones (I.Z.) ranged 8.3 - 8.4 mm followed by the isolates Kb2, LZa7 and Y2a5. The least activity was recorded for the isolates SCa4 and RC4b2 (I.Z.) ranged 2.3-2.5 mm. The antibacterial activity of the same probiotic isolates against human pathogenic *Eschericia coli* was almost similar to that obtained against *S. aureus*, and followed the same pattern. The isolates LZb8, S4b1 and RC2b3 possessed the highest activity, while the isolates SCa4 and RC4b2 were highly significantly the least active. It seems that the inhibitory activity of the isolates against *E. coli* was slightly less as compared to that obtained against *S. aureus.*

The antifungal activity of the same14 probiotic isolates was tested against the plant pathogenic *Rhizoctonia solani* and *Fusarium oxysporum*. All probiotic isolates were highly significantly active on both fungi as compared to the control, showing % growth inhibition (%GI) ranges of 26.7-52.3 %, and 17.1 -51.2 % against the first and the latter fungi, respectively . The most active isolates against *R. solani* were RC4b2 (52.3 % GI), followed by both RC4b3 and RC1b8 (47.6 % GI). Also, the most active isolate against *F. oxysporum* was Y2a5 (51.2 GI).

Keywords: Antibacterial activity, probiotic, antifungal activity, human and plant pathogenic.

INTRODUCTION

During the past two decades probiotic (health promoting) micro-organisms have been increasingly included in various types of food products, especially in fermented milks.

The genus *Lactobacillus* has a long history of safe use, especially in the dairy industry, and plays a major role in the production of fermented milk products. Over the past few decades, an increased drive has existed for the isolation of novel *Lactobacillus* strains that exert a beneficial health effect when ingested by humans. Such strains are termed probiotic. According beneficial effects conferred by lactobacilli include inhibition of pathogenic organisms, such as *Salmonella*, *Shigella* and *Helicobacter* (Bernet-Camard *et al*., 1997; Hudault *et*

al., 1997; Aiba *et al.,* 1998; Hammilton-Miller, 2003; Sgouras *et al.,* 2004).

The technological application of probiotic organisms in fermented dairy products aims to combine the potential health benefits of the bacteria with their ability to grow in milk, resulting in a nutritionally healthy and desirable product for the consumers. Gomez *et al.,* (1997) evidenced a bacteriocin-like substance produced by a new strain of *Streptococcus sp*., inhibitory to Gram-positive food-borne pathogens.

Probiotics may reduce the incidence of disease or lessen the severity of disease outbreaks. Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer a health benefit to the host" (Reid *et al.*, 2003). The mechanisms used include the production of inhibitory substances against pathogens, competition for essential nutrients and adhesion sites, the supply of essential nutrients and enzymes resulting in enhanced nutrition in the host, and the modulation of interactions with the environment and the development of beneficial immune responses [Verschuere *et al*., (2000), Balcázar *et al*., (2006) and Gomez and Balcázar, (2008)]. Harro *et al.,* 2007 found that application of a multispecies probiotic mixture prevented infectious complications in critically ill patients.

Petros *et al*., (2006) reported that although no bacteriocin activity was detected in vitro, strains *L. casei* Shirota ACA-DC 6002, *L. plantarum* ACA-DC 146 and *L. paracasei subsp. tolerans* ACA-DC 4037 were able to inhibit the adhesion of *Escherichia coli* and *Salmonella typhimurium* to Caco-2 cells. They also induced the secretion of pro and anti-inflammatory cytokines by human peripheral blood mononuclear cells. These three strains were therefore found, in vitro, to possess desirable probiotic properties.

Antifungal activity was obtained against the vegetative stage and cysts of *Saprolegnia parasitica*, with cysts showing a higher susceptibility., morphological changes observed within hyphae suggested that T1 could be a potential cytoplasmic toxin, Lategan *et al.,* (2006). Schillinger and Jéssica (2010) found that during a screening procedure for lactic acid bacteria exhibiting antifungal activity against an ochratoxin-producing *Penicillium nordicum* (BFE 487), numerous strains were observed to produce zones of inhibition against the mould on MRS agar. The comparison of the antifungal effect of culture supernatants from selected LAB strains with un-inoculated MRS medium acidified to the respective pH by addition of HCl or lactic acid showed that the culture supernatants were more effective in inhibiting *P. nordicum* growth than the acidified MRS medium, indicating that besides acetic and lactic acid other metabolic products of the LAB contribute to the inhibition.

The aim of this study was to apply in vitro tests to evaluate the probiotic potential of *Lactobacillus spp, Bifidobacterium* spp. and *Streptococcus spp* strains isolated from dairy sources, and to select candidate probiotic strains that fulfill the established criteria and could therefore be potentially used as novel probiotic strains in the food industry and against several human (*Staphyllococcus aureus*. and *Eschericia coli*) and plant (*Rhizoctonia solani* and *Fusarium oxysporum*) pathogens by examining their in vitro antimicrobial properties.

MATERIALS AND METHODS

Identification of isolates:

Probiotic strains were isolated from different cheeses (turkey and domiatii and cottage and kariesh cheese) and fermented products (yoghurt, raib, zeer milk and kishk). The isolates were screened for rate of growth increase in MSR broth at pH 3, pH4, acid production after 48 hours and degree of bile salt

(0.3%) tolerance, strength of adhesion and clotting time (3 to 48 hrs) of skim milk (Ali *et al.,* 2010 and 2012).

The good fourteen isolates were enumerated on the MRS agar, after anaerobic incubation at 37 ◌̊C for 48 hrs. The selectivity of the growth conditions was confirmed by microscope appearance of the cells from single colonies, gram stain and catalase test, was done to identifying the isolates, Ali *et al.,* (2012).

Indicator bacteria and fungi for antimicrobial tests:

a- Bacteria: *Escherichia coli* and *Staphylococcus aureus* were obtained from Agric. Microbiology Dept., Fac. Agric., Minia Univ.

b- Fungi: plant pathogenic fungi, i.e. *Rhizoctonia solani* and *Fusarium oxysporum* were obtained from Plant Pathology Dept., Fac. Agric., Minia Univ.

Antimicrobial activity:

a)- Against bacteria:

Antibacterial effect of all selected isolates against indicator bacteria was determined by the agar-well diffusion method according to (Fleming *et al.,* 1985) and (Ashraf *et al.,* 2009). *Escherichia coli* and *Staphylococcus aureus* were used as indicator bacteria. Supernatants of probiotic isolates were monitored for antibacterial activity against indicator bacteria inoculated on nutrient agar. A volume of 100ul of cell-free supernatants was filled in 7-mm diameter wells cut in the nutrient agar. The diameter of the inhibition clear zone was measured after 48 hrs of incubation.

b)- Against plant pathogenic fungi:

This assay was carried out using the agar-well diffusion method according to (Elbadry, 2008) in which molten PDA was dispended in sterile Petri-dishes. After solidification of medium and dryness, 4 wells of 7-mm diameter were bored in each plate. A volume of 100ul of cell-free culture supernatants of the tested isolates was pipetted in the wells, and the plates were kept at 4 ◌̊C for 6 hrs to allow diffusion of the antimicrobial substance. An agar plug (7-mm) was removed from culture of indicator fungi (*Rhizoctonia solani* and *Fusarium oxysporum*) and placed in the center PDA plates, then the plates were incubated at 30 ◌̊C until the fungal growth in the control plates reached at least the edge of the wells. Then, the radius (mm) of the growth in the treatment and the control were measured. (results were recorded after 4 days for *R. solani* and after 8 days for *F. oxysporum*).

The antifungal activity was calculated according to the following formula:

% FI = (Rc – Rt /Rc) x 100

Where:

%FI = % fungal inhibition.

Rc= radius of growth zone in the control.

Rt = radius of growth zone in the treatmen.

Statistical analysis:

All results were statistically analysed using Duncan's Multiple Range test (Duncan, 1955) was applied for meancomparison when one-way analysis of variance (ANOVA) showed significant differences at the 95% confidence level.

RESULTS AND DISCUSSION

The good fourteen isolates, identified as two *Lactobacillus* spp. (S4b1 andS2a3), eleven *Bifidobacterium* spp. (RC1 b8, RC2 b1,SC1a4, RC4b2, FC1b1, RC2b4, RC4a3, LZ1a3 and LZa7) and one *Streptoococcus* spp. (RC2b3), (Fig. 1a and b). These results agree with Durst et al.,(1998)who found that the first studies were mainly carried out with bacteria of the genus *Lactobacillus*, but later experiments included preparations consisting of *Bacillus* spp. (preparations in spore form), *Enterococcus faecium* and *Saccharomyces cerevisiae* .Gomez *et al.,* (1997) evidenced a bacteriocin-like substance produced by a new strain of *Streptococcus* sp., inhibitory to Gram-positive food-borne pathogens.

Antimicrobial Activity of Probiotic Isolates:

a)- Antibacterial activity:

The antibacterial activity of the selected 14 probiotic isolates was tested against both *S. aureus* and *E. coli* using the agar well-diffusion method, and the growth inhibition zones of the indicator bacteria were recorded.

Supernatants obtained from all isolates (14) exhibited varying degrees of inhibitory activity against *S. aureus* Table (1) and Fig. (2). The isolates LZb8, S4b1 and RC2b3 exhibited the superior antibacterial activity with inhibition zones ranged 8.3-8.4 mm, followed by the isolates Kb2, LZa7 and Y2a5 with insignificant deference. The least activity was recorded for the isolates SCa4 and RC4b2 (inhibition zone ranged 2.3-2.5 mm), while the isolates S2a3, RC1b8 and RC4a3 (inhibition zone ranged 3.5-4.8 mm), were moderately active against *S. aureus*.

Table 1: Antibacterial activity of cell-free culture supernatants (CFCS) of probiotic isolates against bacterial pathogens.

Probiotic Isolâtes	Origin Source	Mean values of zone of inhibition (mm) (1) & (2)	
		Staphylococcus aureus	*Escherichia coli*
RC2b4	ras cheese	6.5 b	5.3 c
RC4b2	ras cheese	2.5 e	0.8 f
RC1b8	ras cheese	4.8 cd	2.1 e
RC4a3	ras cheese	8.3 a	3.5 d
RC2b3	ras cheese	4.3 d	6.6 ab
FCb1	domiatii cheese	6.3 bc	4.9 c
SCb2	karish cheese	6.5 b	5.4 bc
SCa4	karish cheese	2.3 e	0.8 f
LZa7	zeer milk	7.3 ab	6.1 abc
LZb8	zeer milk	8.4 a	6.9 a
S4b1	rayeb milk	8.3 a	6.8 a
S2a3	rayeb milk	3.5 de	1.4 ef
Y2a5	yoghurt	7.0 ab	5.6 abc
Kb2	kishk	7.8 ab	6.6 ab

(1) Results were recorded after 24 hrs, and represented means of 8 replicates.

(2) Means followed by the same letter (s) are not significantly at 1% level of probability (Duncan's multible-range test).

The antibacterial activity of the same probiotic isolates against *E. coli* (Table 1 and Fig. 3) was almost similar to that obtained against *S. aureus*, and followed the same pattern. The isolates LZb8, S4b1 and RC2b3 possessed the highest activity, while the isolates SCa4 and RC4b2 were highly significantly the least active. It is worth mentioning that the inhibitory activity of the tested isolates supernatants was slightly less against *E. coli* as compared to that obtained against *S. aureus*, indicating that *E. coli* could be less sensitive.

This agar well-diffusion method, used in this test, proved to be useful for selecting probiotic isolate of *Lactobacillus* spp., that possessing the ability to inhibit or compete with harmful bacteria. DeVuyst and Vandamme (1994) reported that LAB disply a wide range of antimicrobial activities. Among these activities, the production of lactic acid and acetic acid is obviously the most important. However certain strains of LAB are further known to produce bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin and reutericyclin. Many strains also produce bacteriocins and bacteriocin-like molecules that display antibacterial activity. Ashraf *et al.* (2009) revealed that all lactobacilli tested (except *L.*

delbruceki) inhibited the growth of *E. coli* and *S. aureus*. Also, in agreement with the present results, Ronka *et al.* (2003); Erdourul and Erbilir (2006) and Ryan *et al.* (2008) reported that *Lactobacillus* spp., showed a broad inhibitory spectrum against the indicator organisms tested. The inhibitory substance of certain lactobacilli isolates was distinct from bacteriocin, lactic acid, acetic acid produced by those bacteria (Amin *et al.,* 2009). Gomez *et al.,* (1997) evidenced a bacteriocin-like substance produced by a new strain of *Streptococcus* sp., inhibitory to Gram-positive food-borne pathogens.

b)- Antifungal activity:

The antifungal activity of the same14 probiotic isolates was investigated against the plant pathogenic *R. solani* and *F. oxysporum* as indicator fungi. Crude cell-free culture supernatants (CFCS) of all isolates were tested using agar- well diffusion method, and the results are shown in (Table 2) and (Fig. 4). The obtained results showed that the (CFCS) of all probiotic isolates was highly significantly active on both *R. solani* and *F. oxysporum* as compared to the control, showing growth inhibition (GI %) ranges of 26.7-52.3 %, and 17.1 - 51.2 % against the first and the latter fungi, respectively (Table 2). The most active probiotic isolate against *R. solani* was RC4b2, (52.3 % growth inhibition) followed by both RC4b3 and RC1b8 (47.6% growth inhibition), while the least active isolates were RC2b3 (GI: 26.7%), followed by RC2b4, Kb2, S2a3, FCb1 and Y2a5 (GI:33. 7-39.5 GI%).

Table 2: Antifungal activity of cell-free culture supernatants (CFCS) of probiotic isolates against plant pathogenic fungi.

Probiotic Isolâtes	Origin Source	*Rhizoctonia solani*(1)		*Fusarium oxysporum*(2)	
		Mean growth(3) diameter (cm)	Growth inhibition%	Mean growth(3) diameter (cm)	Growth inhibition%
Control	------	8.6 a	------	8.2 a	-----
RC2b4	ras cheese	5.8 c	33.7	5.5 cdef	32.0
RC4b2	ras cheese	4.1 f	52.3	6.8 b	17.1
RC1b8	ras cheese	4.5 ef	47.6	4.7 efg	42.7
RC4a3	ras cheese	6.3 b	26.7	5.4 cdef	34.1
RC2b3	ras cheese	4.5 ef	47.6	4.7 efg	42.7
FCb1	domiatii cheese	5.5 cd	37.2	6.0 c	30.5
SCb2	karish cheese	4.7 e	45.3	4.6 efg	43.9
SCa4	karish cheese	4.6 e	46.5	4.9 defg	40.2
LZa7	zeer milk	4.7 e	46.5	4.5 fg	45.1
LZb8	zeer milk	4.7 e	45.3	4.8 efg	26.8
S4b1	rayeb milk	5.7 cd	33.7	4.6 fg	43.9
S2a3	rayeb milk	5.5 cd	37.2	5.9 cd	28.0
Y2a5	yoghurt	5.3 d	39.5	4.0 g	51.2
Kb2	Kishk	4.8 e	45.3	5.7 cde	41.5

(1) Results were recorded after 4 days *R. solani.*
(2) Results were recorded after 8 days for *F. oxysporum.*
(3) Means followed by the same letter(s) are not significantly different at level 1% of probability (Duncan's multible-range test).

In case of the inhibitory activity against *F. oxysporum* (Table 2) and (Fig. 5), the most active probiotic isolate was Y2a5 (51.2 GI%), which differed highly significantly from the least active isolate (17.1 GI %), and both were highly significantly different from the control.

In an earlier study, Lavermicocca *et al.* (2000) reported that strains of *Lactobacillus plantarum*, isolated from sourdough and grass silage, display antifungal activity, due to the production of organic acids, other low-molecular-mass metabolites, and /or cyclic dipeptides.Magnusson *et al.* (2003) tested the antifungal activity of a large number of *Lactobacillus* isolates from different environments. Several of those isolates exhibited strong inhibitory activity against the moulds *Aspergillus fumigatus*, *A. nidulans* and *Penicillium anomala.* Also, DeMuyncka *et al.* (2004) mentioned that 17 lactic acid bacterial strains showed fungal growth inhibition zones never exceeded 3-4 mm. In addition, Elbadry (2008) tested the antifungal activity of five lactobacilli against four pathogenic fungi (*Rhizoctonia, Sclerotuim, Fuzarium* and *Penicillium*). He found that the crude cell free culture supernatants (CFCS) showed variation in their antifungal activity ranged 48-63 % fungal inhibition zone (F.I. %), and *Penicillium* sp., was the most sensitive indicator fungi. Antifungal activity was obtained against the vegetative stage and cysts of *Saprolegnia parasitica,* with cysts showing a higher susceptibility., morphological changes observed within hyphae suggested that T1 could be a potential cytoplasmic toxin, Lategan *et al.,* (2006).

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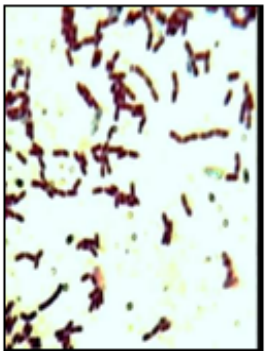
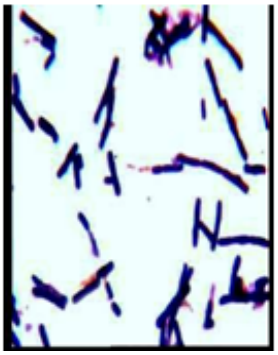
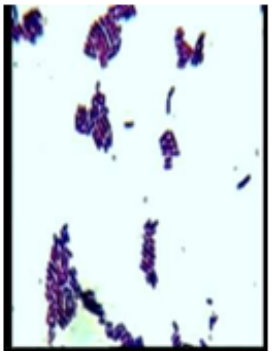


RC2b3 (***Streptococcus*** spp.)



S2a3 (*Lactobacillus* spp.)



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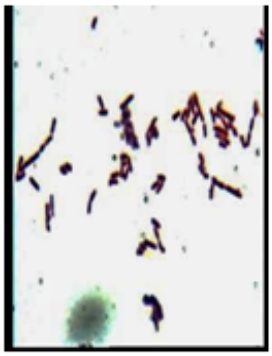
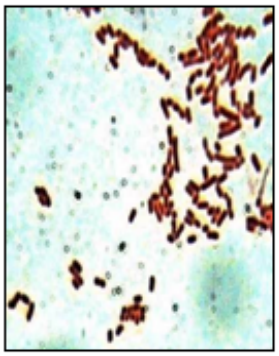
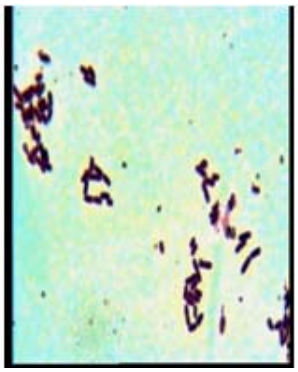


Y2a5 (*Bifidobacterium* spp.)

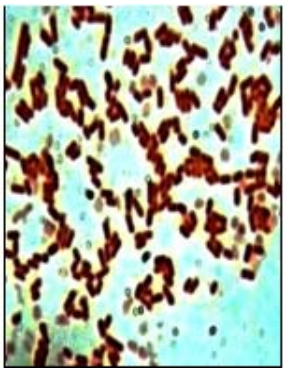


Kb2 (***Bifidobacterium*** spp.)

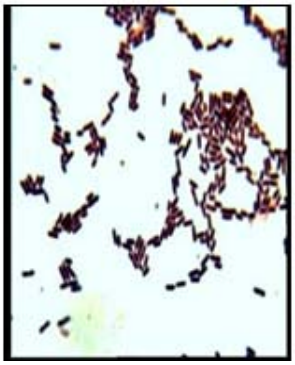
Fig. 1a: Finally selected probiotic lactic acid bacteria from dairy products and Kishk.



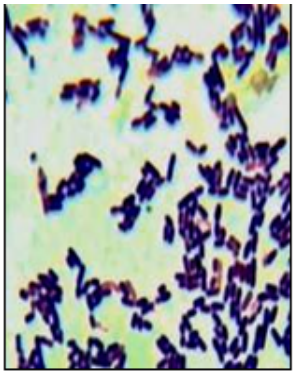
RC1b8 (***Bifidobacterium*** **spp.)**



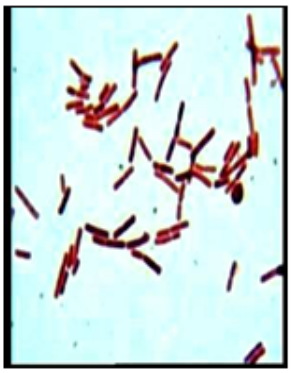
RC4b2 (***Bifidobacterium*** **spp.)**



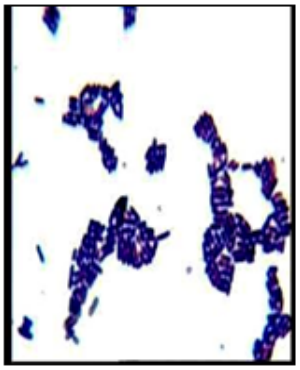
FC1b1(***Bifidobacterium*** **spp.)**



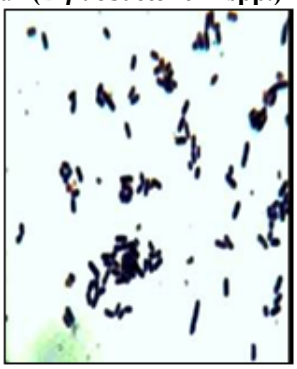
SC1a4 (***Bifidobacterium*** **spp.)**



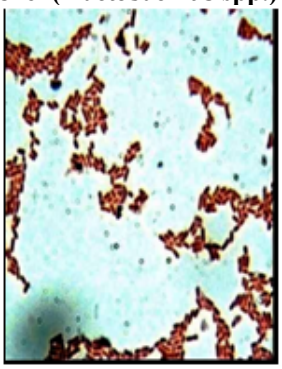
S4b1(***Lactobacillus*** **spp.)**



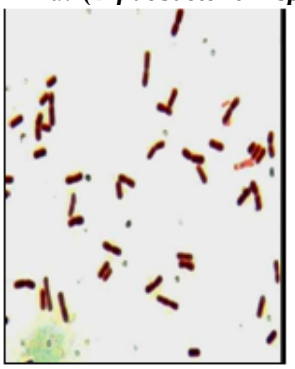
LZ1a7 (***Bifidobacterium spp.***)



LZ1b8 (***Bifidobacterium*** **spp.)**



RC2b4 (***Bifidobacterium*** **spp.)**



RC4a3 (***Bifidobacterium*** **spp.)**

Fig. 1 b: Finally selected probiotic lactic acid bacteria from dairy products.

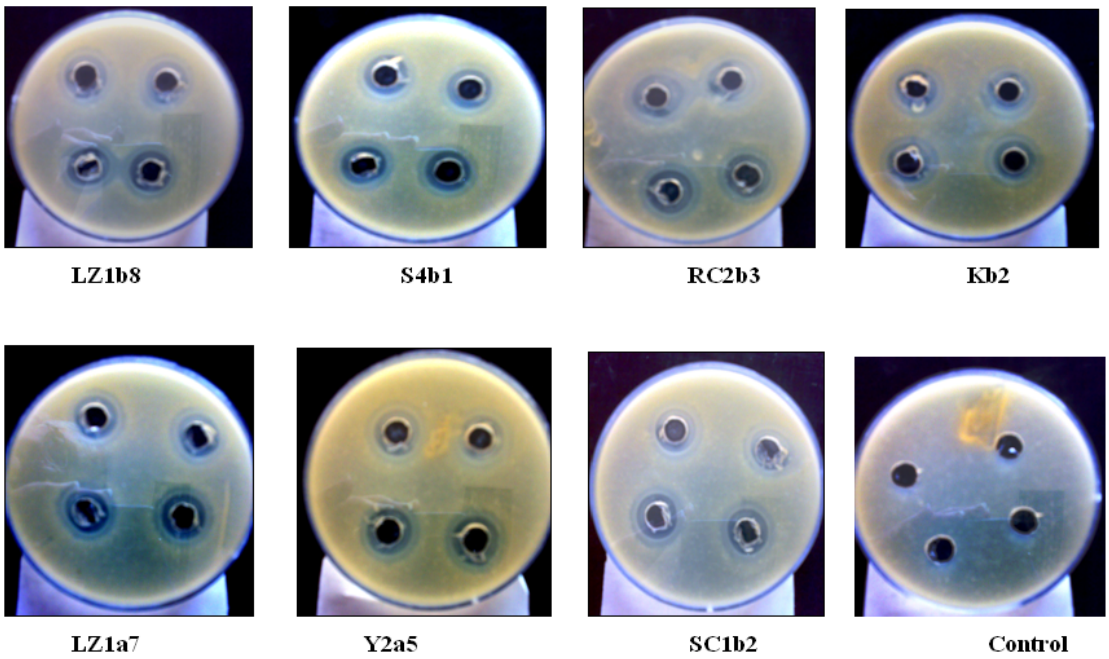


Fig. 2: Examples of antibacterial activity of selected probiotic isolates against *Staplyllococcus aureus.*

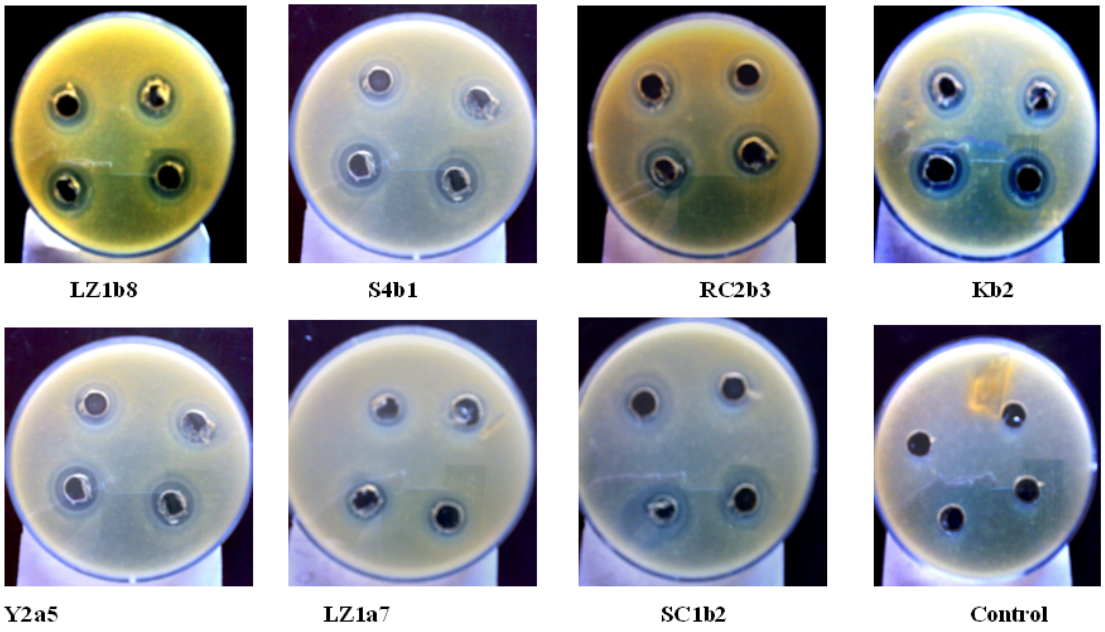


Fig. 3: Examples of antibacterial activity of selected probiotic isolates against *Escherichia coli*

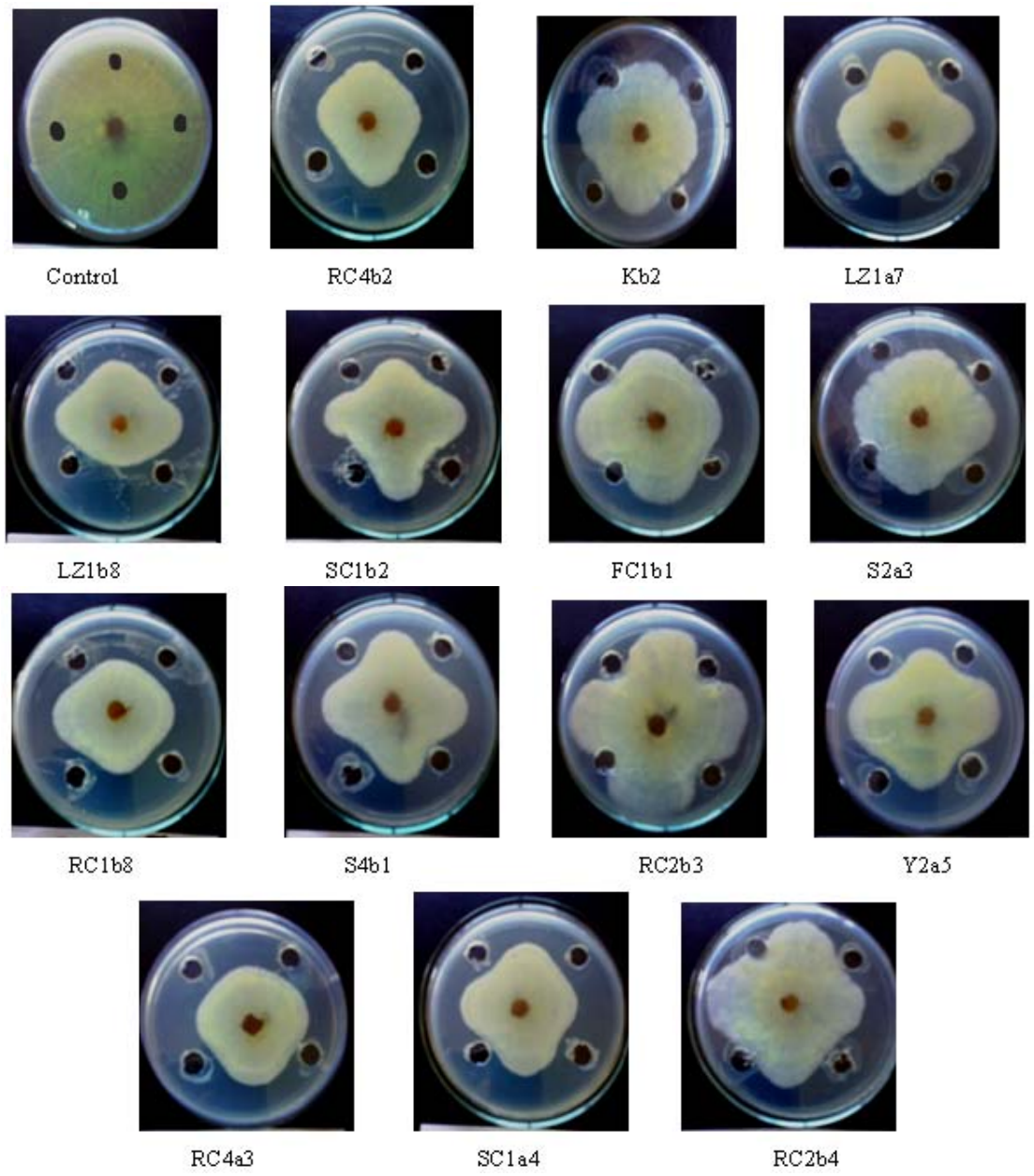


Fig. 4: Antifungal activity of selected probiotic isolates against the plant pathogenic fungus *Rhizoctonia solani*.

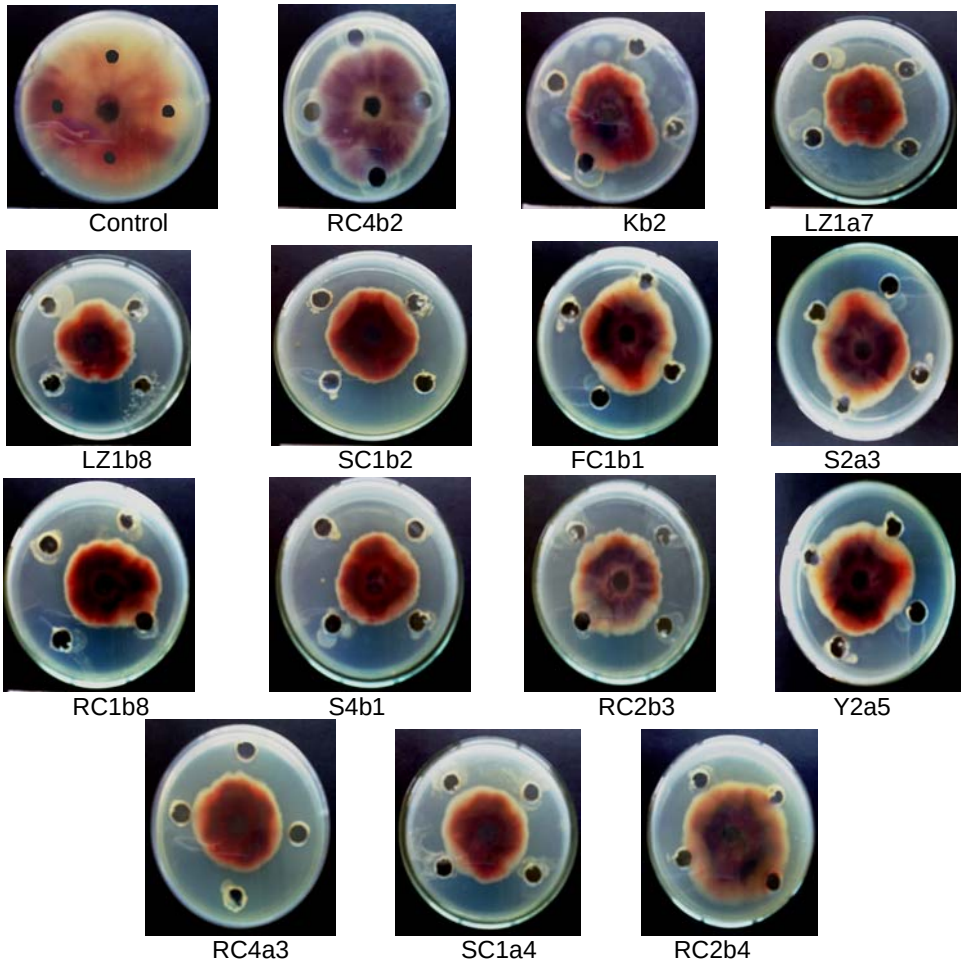


Fig.(5). Antifungal activity of selected probiotic isolates against the plant pathogenic fungus *Fusarium oxysporum.*

ARABIC SUMMARY

نشاط التضاد الميكروبى لبكتريا البروبيوتيك

فاروق شحاتة على - عمر عبد اللطيف عمر سعد- سلوى عادل حسين غريب
قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة المنيا – المنيا مصر

تم حصر سلالات البروبيوتيك المعزولة من الجبن الرومى والدمياطى والقريش والمنتجات اللبنية المتخمرة كالرايب ولبن الزير والكشك من حيث معدل النمو على بيئة MSR السائلة ذات pH 3, pH4 وشدة انتاج الحموضة بعد 48 ساعة ودرجة تحمل النمو فى وجود ملح الصفراء (0,3%) وشدة الالتصاق بجدار الامعاء والمقدرة على تخثير لبن الفرز من 48:3 ساعة.تم اختيار أقوى أربعة عشر سلالة وتم تعريفها (2 عزلة *Lactobacillus spp* (S4b1 and S2a3 و1 عزلة *Streptoococcus* spp. (RC2b3), و11 عزلة *Bifidobacterium spp* (RC1 b8, RC2 b1, SC1 a4,RC4b2,FC1b1,RC2b4,RC4a3,LZ1a3 and LZa7) واستخدمت لدراسة التضاد الميكروبى على عدة ممرضات للانسان (*Staphyllococcus aureus*.and *Eschericia coli*) والنبات (*Rhizoctonia solani* and *Fusarium oxysporum*) .

لتقدير نشاط التضاد ضد البكتريا الممرضة تم إختيارأقوى 14 عزلة بروبيوتيك لاختبارها من حيث قدرتها على تثبيط نمو البكتريا الممرضة للانسان *Staphylococcus aureus*. وقد أعطت العزلات LZ1b8 – S4b1 – RC2b3 نطاق تثبيط للنمو تراوح بين 8.3 – 8.4 مم ، تلاها العزلات Kb2 – LZ1a7 – Y2a5، بينما سجلت العزلات SC1a4 – RC4b2 أقل قدرة على تثبيط نمو *S. aureus* خلال فترة 24 ساعة.

وبالنسبة لإختبار نفس العزلات ضد نمو البكتريا الممرضة للانسان *Eschericia coli* فقد سلكت نفس السلوك فى القدرة على تثبيط النمو، وكانت العزلات LZ1b8 – S4b1 – RC2b3 هى الأعلى فى القدرة على إحداث تثبيط نمو *E. coli* ، بينما كانت العزلات SC1a4 – RC4b2 هى الأقل قدرة. ويبدو من النتائج أن *E. coli* كانت أقل حساسية للتثبيط عن *S. aureus* بواسطة العزلات المختبرة.

ولتقدير نشاط التضاد ضد الفطريات الممرضة للنبات أختبرت نفس ال 14 عزلة بروبيوتيك من حيث قدرتها على تثبيط نمو الفطريات الممرضة للنبات - *Rhizoctonia solani* - *Fusarium oxysporum* وقد أظهرت النتائج أن كل عزلات البكتريا المختبرة ذات قدرة عالية المعنوية فى تثبيط نمو كلا من نوعى الفطر بالمقارنة بالكنترول.

وقد تراوحت نسبة تثبيط النمو 26.7- 52.3 % ، 17.1- 51.2 % ضد الفطريات الأول والثانى على التتابع. وكانت أعلى العزلات البروبيوتيك تأثيرا ضد هذه الفطريات RC4b2 (52.3 % تثبيط للنمو) ثم RC1b8 و RC4b3 (47.6 % تثبيط للنمو) وايضا كانت العزلة Y2a5 (51.2%) الأعلى فاعلية ضد *F. oxysporum* .