PRELIMINARY *IN VITRO* STUDY ON ANTIFUNGAL ACTIVITY OF SOME LOCAL LACTOBACILLI AND LACTIC STREPTOCOCCI ISOLATES

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ABSTRACT:

The agar – well diffusion method was used to assay *in vitro* the antifungal activity (AA) of the metabolites of five lactobacilli (Lb) and five Lactic streptococci (LSt) isolates obtained from samples of Egyptian dairy products towards four indicator phytopathogenic fungi: *Rhizoctonia solani, Sclerotuim cepivorum, Fusarium oxysporum* and *Penicillium* sp.The AA was expressed as percent of fungal growth inhibition (% FGI) .

The results revealed that the crude cell-free culture supernatants (crude CFCS) of both Lb and LSt isolates showed variations in their AA , and the ranges were 48 – 63 % FGI and 38 – 52 % FGI for Lb and LSt isolates , respectively .Also ,the four indicator fungi showed differences in their sensitivity to the CFCS of the tested bacteria . The *Penicillium* sp. was the most sensitive indicator fungi with 60% FGI and 54% FGI for Lb and LSt isolates, respectively.

In general, the AA of Lb isolates was relatively higher (54% FGI is average) than that of LSt isolates (44% FGI in average). Neutralization (pH 6.5) of crude CFCS, elimination of H_2O_2 or both caused a decrease in AA in most cases indicating a role of organic acids and H_2O_2 in AA.

Treatment of the neutralized, H_2O_2 – free CFCS with the proteolytic enzymes , Pronase completely inactivated the AA in most cases and slightly reduced it in some cases, indicating the presence of a proteinaceous antifungal substances in the crude CFCS of both Lb and LSt isolates . This study has proven the *in vitro* potential of some local Lb and LSt isolates to inhibit growth of some plant pathogenic fungi.

Key words: Lactobacilli, lactic streptococci, antifungal activity, Phytopathogenic fungi.

INTRODUCTION

Lactic acid bacteria (LAB) are microorganisms that have been used for centuries to prepare and preserve food and for ensiling different crops for animal feed. During the last twenty years there has been a growing interest in use of microorganisms and / or their metabolites to prevent spoilage and to extend the shelf- life of foods. LAB are of particular interest as biopreservation bacteria. Their preserving effect mainly relates to the formation of lactic acid, acetic acid and hydrogen peroxide; competition for nutrients; and the production of bacteriocin and other antimicrobial compounds **(Stiles, 1996).**

Reviewing the literature reveals that a majority of the publications study antibacterial activity of LAB and that only few present data concerning antifungal activity their **(Magnusson and Schnürer, 2001).** However, most publications on antifungal activity of LAB merely study the antifungal

activity towards food and feed spoilage fungi but rarely investigated the antifungal activity. Moreover, towards plant pathogenic fungi.

The possibility of using metabolites of LAB with GRAS (generally regarded as safe) status as a biotechnological solution to plant fungal diseases could be a promising and safe alternative for the chemical fungicides. Therefore, the objective of this work was to study *in vitro* the ability of metabolites of some local lactobacilli and local streptococci isolates obtained from samples of dairy products to inhibit plant pathogenic fungi.

MATERIALS AND METHODS

1. Materials

Samples of raw milk, fermented milk (Laban rayeb), Domiati cheese, Karech cheese and Yoghurt (Zabadi) were obtained from markets. The screened 32 Lactobacilli isolates and the four fungal indicators obtained from the microbial collection of the Agricultural Microbiology Department, Faculty of Agriculture, Fayoum University. MRS agar **(De man *et al.*, 1960)** was used for isolation and purification of Lactobacilli (Lb) and Lactic Streptococci (LSt) isolates, while MRS broth was used for growing these isolates to obtain culture supernatants. PDA medium was used for growing fungi and for performing the agar - well diffusion assay.

2. Methods

2.1. Isolation of lactic streptococci bacteria

Samples of 10 ml or 1g of fresh milk or dairy products were added to 1/4 strength sterile Ringer's solution, and 10-fold serial dilutions were prepared. Hundred µl of dilutions were plated onto MRS agar plates and incubated at 30˚C for 48h. Colonies with *Streptococcus* - like morphology were isolated and purified. Gram- positive, catalase- negative, non motile ovoid cells in pairs or chains, that able to clot milk and grow in 0.1% methylene blue milk and not able to liquefy gelatin were tentatively considered lactic streptococci isolates.

2.2. Preparation of cell - free culture supernatants

Lb and LSt isolates were grown in MRS broth at 32˚C for 18h. The cultures were centrifuged at 10000 xg for 15 min at 4˚C and the resulted supernatant was designated crude cell - free culture supernatant (crude CFCS). To eliminate growth inhibition caused by organic acids or by hydrogen peroxide, the pH of the crude CFCS was adjusted to 6.5 or treated with catalase (1mg ml^{-1}). These supernatants were used immediately or stored at -20˚C until needed.

2.3. Antifungal activity assay

This was assayed using the agar-well diffusion method**,** in which molten PDA dispensed in sterile Petri dishes. After solidification of medium in the plate and dried it for 30 min. under a laminar flow hot, four wells of 7 mm diameter were bored in each plate. Aliquots (100 µl) of crude or treated cell-free culture supernatants (CFCS) were pipetted in the wells. After preincubation of plates at 4˚C for 6h to allow diffusion of the antimicrobial substances, an agar plug (7mm diameter) was removed from culture of indicator fungi and placed in the center of fresh PDA plates (photo 1 & 2). The plates were incubated at 30˚C until the fungal growth in the control plates reach at least the edge of the well, and then the radius (mm) of the inhibition zone around the well was measured. The antifungal activity was expressed as fungal growth inhibition percent (FGI %) which equal to:

100 x (Rc-R_T)/Rc, where **Rc** is the radius (mm) of fungal growth in control plates and **R_T** is the radius (mm) of fungal growth in treated plates.

2.4. Effect of a proteolytic enzyme.

Samples of neutralized, H_2O_2- free CFCS were treated with the proteolytic enzyme Pronase (100µg ml^{-1}) and incubated at 37˚C. After incubation for 1h, the antifungal activity was determined. MRS broth treated with enzyme and untreated CFCS samples served as controls **(Magnusson and Schnürer, 2001).**

RESULTS AND DISCUSSION

Consumers demanded for reduced use of chemical fungicides have stimulated research on microbial antifungal compounds. It has been suggested that some LAB produce a wide spectrum of compounds that might act synergistically towards filamentous fungi **(Magnusson *et al.*, 2003).**

Therefore, continues evaluation of different LAB strains isolated from different sources for antifungal properties could lead to biofungicides which could be used as safe alternatives for the overuse of the harmful chemical fungicides.

In the present study, thirty- two Lactobacilli (Lb) and twenty- six Lactic Streptococci (LSt) isolates obtained from samples of dairy products, were screened for antifungal activity (AA) using agar-well diffusion method with *Rhizoctonia solani* as indicator fungi. Of these isolates, 21 Lb and 15 LSt isolates were found to be able to inhibit growth of *Rhizoctonia solani* (data not shown). Five isolates from each bacteria which showed AA of at least 30% fungal growth inhibition were selected to investigate the AA of their crude cell- free culture supernatants (crude CFCS), neutralized (pH 6.5) crude CFCS, catalase- treated crude CFCS, and neutralized, catalase- treated crude CFCS towards four phytopathogenic fungi, *Rhizoctonia solani, Sclerotuim cepivorum, Fusarium oxysporum* and *Penicillium* sp.,

The results (Table 1&2) revealed that the crude cell free culture supernatants (CFCS) of both Lb and LSt isolates showed variations in there AA, the ranges of activity were 48% - 63% and 38%- 52% for Lb and LSt isolates, respectively.

The results in also show that the four indicator fungi exhibited differences in sensitivity towards crude CFCS of the screened Lb and LSt isolates. *Penicillium* sp. was the most sensitive with 60% fungal growth inhibition (FGI %) for Lb isolates and 54% for LSt isolates. Whereas**,** the least sensitive fungi were *R. solani* (42% FGI) incase of the Lb isolates and *F. oxysporum* (34% FGI) in case of LSt isolates.

In general, the overall average FGI % against all the four indicator fungi of Lb isolates (54%) was higher than that of LSt isolates (44%).

In a study by **DeMuyncka *et al.*, (2004),** 17 lactic acid bacterial strains showed fungal growth inhibition zones never exceeded a radius of 3-4 mm. In the present study, the radius of inhibition zones (data not shown) ranged between 9 and 21 mm for crude CFCS of Lb isolates and 8 -17 for crude CFCS of LSt isolates, indicating that the Egyptian LAB isolates could be promising source for antifungal compounds.

The precise mechanism of antifungal action of the antifungal compounds is difficult to elucidate due to complex and commonly synergistic interactions between different compounds **(Corsetti *et al.*, 1998; Niku-Paavola *et al.*, 1999).**

Neutralization (pH 6.5) of CFCS of both Lb and LSt isolates decreased the AA in some cases and increased it anther cases (Table 1&2). The later cases may suggest that some of the antifungal substrates in the crude CFCS may be exhibit their full activity at neutral pH.

The reports regarding the role of organic acids from microbial source in suppressing fungal growth have remained quite insufficient. The inhibitory effect of organic acids are still a complex and unresolved issue, as little purification or characterization of these compounds has been carried out. Propionic acid and its salts have been found to negatively affect fungal growth particularly at low pH **(Woolford, 1984). Magnusson *et al.*, (2003)** recently screened large number of LAB isolates of LAB from different environments for antifungal activity. Several of the strains showed strong inhibitory activity against the moulds *Aspergillus fumigatus, Aspergillus nidulans, Penicillium anomala.* The authors found the degree of fungal inhibition was not only related to production of acetic or lactic acids. In addition, antifungal cyclic dipeptides were identified after HPLC separation and several other active fractions were observed suggesting a highly complex nature of the antifungal compounds.

Elimination of H_2O_2 generally caused a slight decrease in the antifungal activity, suggests the weak antifungal effect or the low concentration of H_2O_2 in the crude CFCS. The observed reduction in AA of the CFCS after neutralization (pH 6.5) or after H_2O_2 elimination indicates a role for both in the AA of the crude CFCS of the tested LAB.

Roy *et al.*, (1996) isolated an antifungal compound from *Lactococcus lactis,* after enzymatic treatment with proteolytic enzymes, the antifungal activity disappeared indicating the proteinaceous nature of the antifungal substance. The substance was not however characterized further.

In the present study, treatment of the neutralized, H_2O_2- free crude CFCS with the proteolytic enzyme pronase (data not shown) completely inactivated AA of crude CFCS of some isolates and slightly reduced it in crude CFCS of another isolates. **Gourama, (1997)** found that the inhibitory effect of a *Lactobacillus casei* strain against two *Penicillium* species was slightly reduced by treatment with trypsin and pepsin. This indicating a pretinacious nature of a part of the antifungal substances. Also, the above mentioned result may indicate the presence of other antifungal substances beside organic acids, H_2O_2 and antifungal peptides contribute to the overall antifungal activities of the crude CFCS of the screened Lb and LSt isolates. It has been suggested that some LAB produce a wide spectrum of compounds that might act synergistically towards filamentous fungi **(Magnusson *et al.*, 2003).**

In Conclusion, the results of this study show clearly that the growth of some plant pathogenic fungi can be restricted by substances produced in cultures of some local Lb and lactic St isolates. These results with other reported results explore the possibility of using metabolites of LAB to control fungal plant diseases.

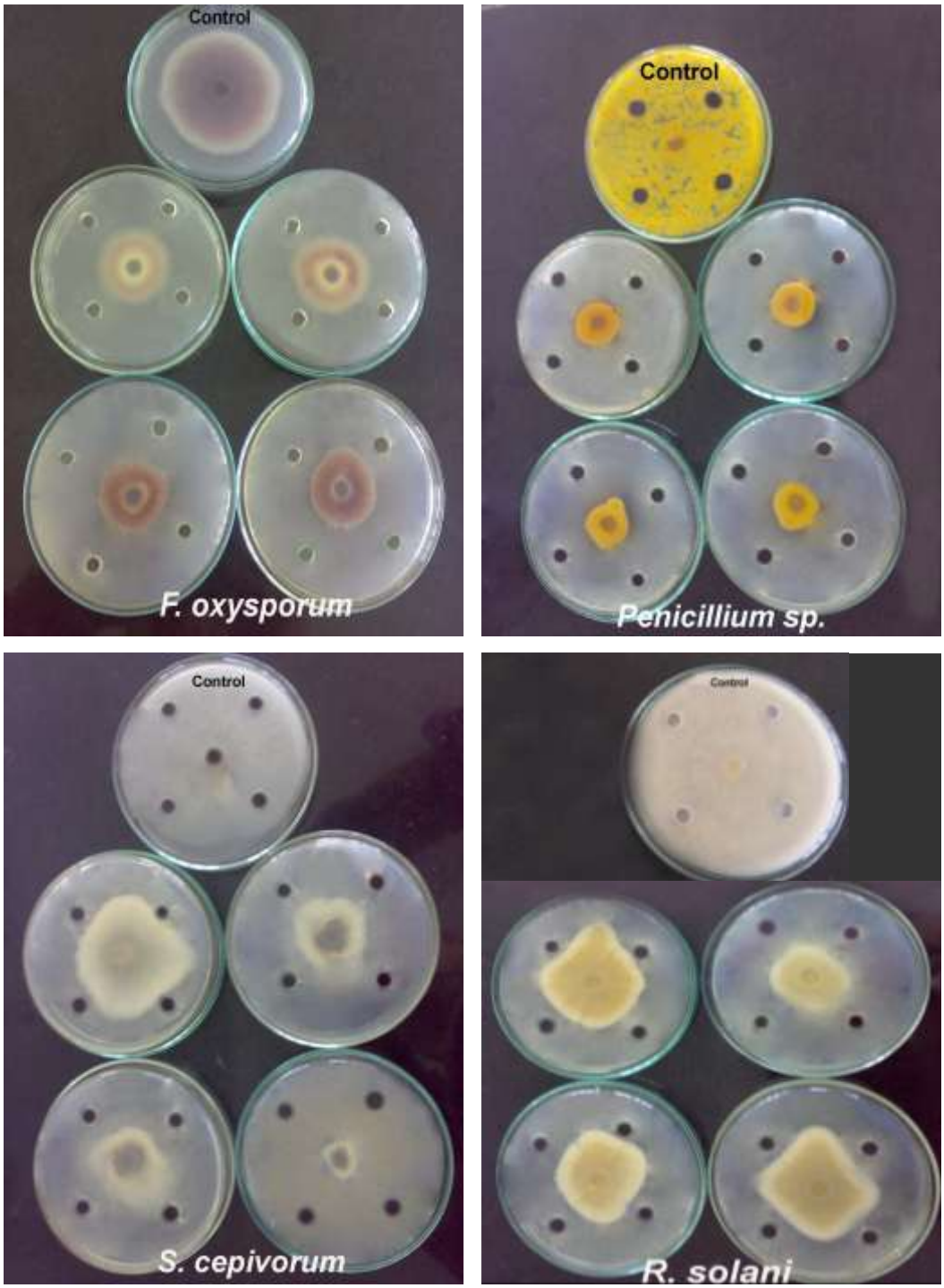

Photo (1): Antifungal activity of CFCS of local Lactobacilli isolates against different plant pathogenic fungi.

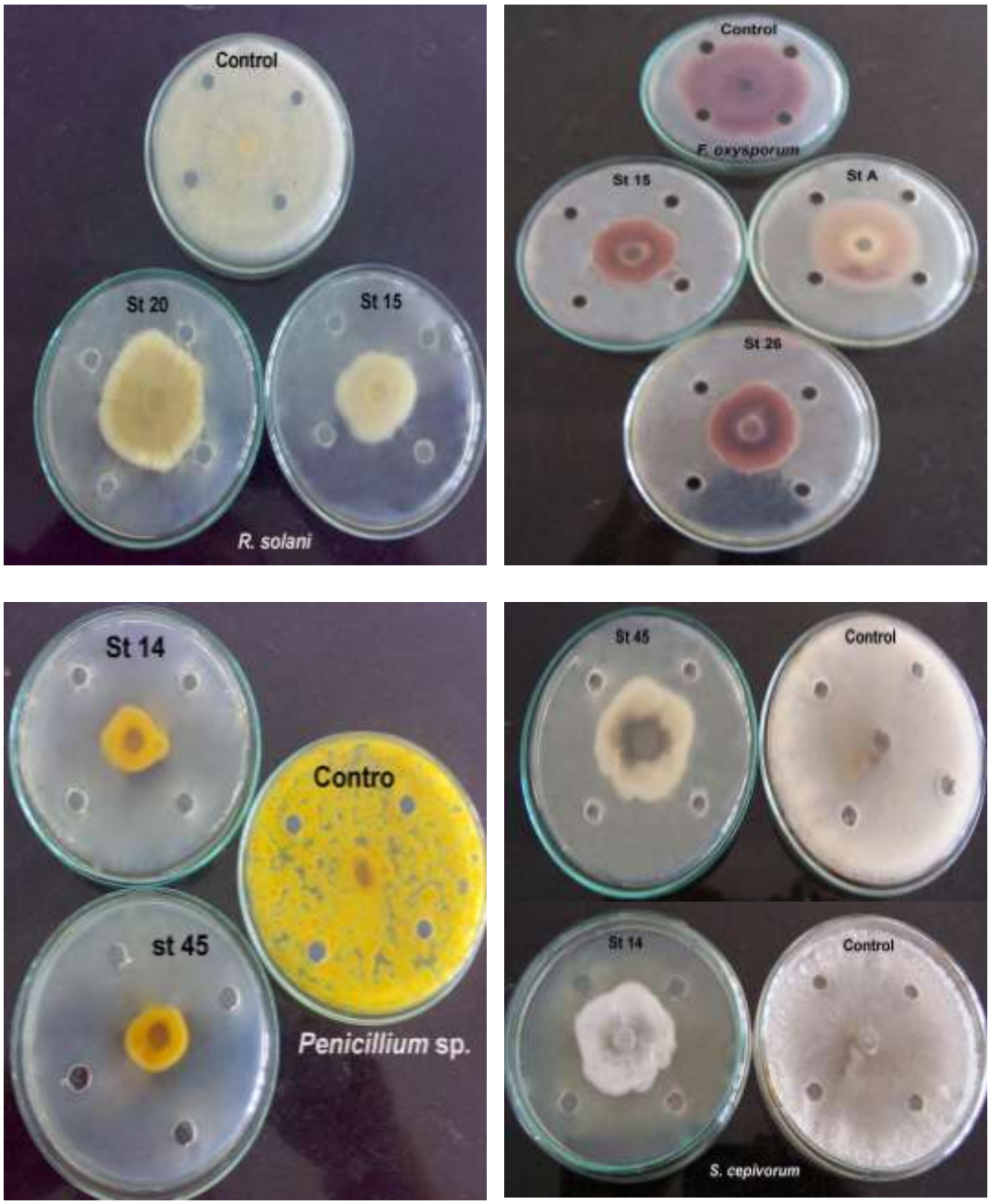

Photo (2): Antifungal activity of CFCS of local lactic Streptococci isolates against different plant pathogenic fungi.

Table (1): Antifungal activity of cell-free culture supernatants (CFCS) of local *Lactobacilli* isolates.

Isolate	Origin	Treatment	Fungal Growth Inhibition (FGI %)				Average*
			Rhizoctonia solani	*Sclerotuim cepivorum*	*Fusarium oxysporum*	*Penicillium* sp.	
Lb1	Domiati cheese	Crude CFCS	32	38	58	63	48
		Neutralized CFCS	38	33	47	70	
		H_2O_2- free CFCS	25	32	55	53	
		Neutralized, H_2O_2-free CFCS	30	25	38	63	
Lb2	Fermented milk	Crude CFCS	42	50	57	68	54
		Neutralized CFCS	32	50	58	62	
		H_2O_2- free CFCS	40	43	52	62	
		Neutralized, H_2O_2-free CFCS	33	50	48	62	
Lb3	Fermented milk	Crude CFCS	63	60	62	68	63
		Neutralized CFCS	51	52	58	73	
		H_2O_2- free CFCS	59	58	60	63	
		Neutralized, H_2O_2-free CFCS	45	55	48	57	
Lb4	Yoghurt	Crude CFCS	35	63	58	48	51
		Neutralized CFCS	28	55	58	50	
		H_2O_2- free CFCS	30	55	50	60	
		Neutralized, H_2O_2-free CFCS	35	53	52	52	
Lb5	Raw milk	Crude CFCS	37	65	62	52	54
		Neutralized CFCS	30	57	58	55	
		H_2O_2- free CFCS	25	60	52	57	
		Neutralized, H_2O_2-free CFCS	37	58	55	58	
		Average*	42	55	59	60	54

Table (2): Antifungal activity of cell-free culture supernatants (CFCS) of local lactic *Streptococci* isolates.

Isolate	Origin	Treatment	Fungal Growth Inhibition (FGI %)				Average*
			Rhizoctonia solani	*Sclerotuim cepivorum*	*Fusarium oxysporum*	*Penicillium* sp.	
St1	Fermented milk	Crude CFCS	31	35	20	65	38
		Neutralized CFCS	30	28	31	58	
		H_2O_2- free CFCS	31	33	18	62	
		Neutralized, H_2O_2-free CFCS	20	25	33	49	
St2	Fermented milk	Crude CFCS	33	43	58	49	46
		Neutralized CFCS	25	33	48	60	
		H_2O_2- free CFCS	30	45	50	41	
		Neutralized, H_2O_2-free CFCS	22	35	41	52	
St3	Karech cheese	Crude CFCS	39	58	32	38	42
		Neutralized CFCS	30	50	25	28	
		H_2O_2- free CFCS	26	55	29	32	
		Neutralized, H_2O_2-free CFCS	19	48	27	24	
St4	Yoghurt	Crude CFCS	40	65	35	68	52
		Neutralized CFCS	35	42	28	73	
		H_2O_2- free CFCS	37	53	30	63	
		Neutralized, H_2O_2-free CFCS	30	48	23	57	
St5	Fermented milk	Crude CFCS	47	42	25	50	41
		Neutralized CFCS	40	33	19	63	
		H_2O_2- free CFCS	42	40	22	57	
		Neutralized, H_2O_2-free CFCS	35	35	20	58	
		Average*	38	49	34	54	44

*Average of antifungal activity of crude CFCS which represents the overall activity.

REFERENCES

Corsetti, A.; Gobbetti, M.; Rossi, J. & Damiani, P. (1998). Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. Appl. Microbiol. Biotechnol. 50, 253-256.

De Man, J. C.;Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130-135.

DeMuyncha, C.; Leroy, A.I.J. and De Maeseneire, E.J. (2004). Potential of selected lactic acid bacteria to produce food compatible antifungal metabolilites. Microbiol. Res. 159, 339 – 346.

Gourama, H. (1997). Inhibition of Growth and mycotoxin production of *Penicillium* by *Lactobacillus* species. Lebensmittel -W.und Technologie. 30, 279-283.

Magnusson, J., & Schnürer, J. (2001). *Lactobacillus coryniformis* sub sp. *oryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. 67, 1-5.

Magnusson, J.; Ström, K. Roos, S.; Sjögren, J.& Schnürer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol. Lett. 219, 129-135.

Niku-Paavola, M.-L.; Laitila, A.; Mattila-Sandholm, T. & Haikara, A. (1999). New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. Appl. Microbiol. 86, 29-35.

Roy, U.; Batish, V.K.; Grover, S. & Neelakantan, S. (1996). Production of antifungal substance by *Lactococcus lactis* subsp. *Lactis* CHD- 28.3. Inter. J. food microbiol. 35, 27- 34.

Stiles, M.E. (1996). Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70, 331-345.

Woolford, M.K. (1984). Antimicrobial spectra of organic compounds with respect to their potential as hay preservatives. Grass and Forage Science 39, 75-79.

دراسة معملية اولية على النشاط المضاد للفطريات لبعض العزلات المحلية من بكتريا اللاكتوبسيلس وبكتريا الاستربتوكوكس المنتجة لحامض اللاكتيك

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الهدف من البحث كان دراسة النشاط المضاد للفطريات للمواد التي توجد في راشح المزارع السائلة الخالية من الخلايا (CFCS) لخمس عزلات من كلا من بكتريا الاكتوبسيلس (Lb) وبكتريا الاستربتوكوكس المنتجة لحامض اللاكتيك (LSt) المعزولة من منتجات لبنية محلية، و قد استخدمت أربعة فطريات ممرضة للنبات كدلائل لقياس النشاط المضاد للفطريات التالية:

Rhizoctonia solani, Sclerotium cepivorum, Fusarium oxysporum and *Penicillium* sp.

وقد اوضحت النتائج ان العزلات المختلفة اظهرت اختلافا في قدرتها على تثبيط نمو فطريات الاختبار وكان اكبر نشاط مضاد للفطريات مقدارة 63% في حالة عزلات Lb و 52% في حالة عزلاتLSt ، كما ان الدلائل الفطرية اظهرت فروقا في حساسيتها للبكتريا المختبره و كان الفطر *Penicillium* sp. هو الاكثر حساسية لكل من عزلات الـ Lb و عزلات الـ LSt بينما كان الفطر *R. solani* والفطر *F. oxysporum* هما الاقل حساسية لعزلات الـ Lb و الـ LSt على التوالي.

وبوجة عام كان متوسط نشاط عزلات الـ Lb (54%) تثبيط للنمو الفطري (اكبر من متوسط عزلات الـ LSt (44%) تثبيط للنمو الفطري (و عندما اختبر الـ CFCS بعد معادلته للـ pH 6.5 أو أزالة تأثير فوق اكسيد الهيدروجين او كليهما كان الاتجاة العام هو انخفاض النشاط نتيجة تلك المعاملات وان ظهرت بعض حالات تخالف هذا الاتجاه العام، كما ان المعاملة بأنزيم محلل للبروتين اظهرت ان بعض الـ CFCS لبعض العزلات فقد نشاطه المضاد للفطريات تماما بينما في حالة عزلات اخرى انخفض النشاط قليلا مما يدل على وجود مواد بروتينية مضادة للفطريات.

نتائج البحث تعطي مؤشر جيد على أمكانية أستخدام مواد تنتجها بكتريا حامض اللاكتيك وهى البكتريا الامنه و الغير ضارة بالانسان أو البيئة كبدائل للمبيدات الكيميائية المستخدمة في مقاومة أمراض النبات