Sys Rev Pharm 2020;11(12):1699-1703
A multifaceted review journal in the field of pharmacy

EVALUATION OF ANTAGONISTIC POTENTIAL OF LACTOBACILLUS ISOLATES AGAINST PHYTOPATHOGENIC FUNGI AND PATHOGENIC BACTERIA IN VITRO

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Abstract

The present research was conducted to evaluate the antagonistic potential of lactic acid bacteria (LAB) isolates in vitro using agar well diffusion method. In this research, four LAB isolates were selected to investigate their antifungal and antibacterial activity against two phytopathogenic fungi and four pathogenic bacteria. Cell-free supernatants at different fold concentrations (50, 25 and 12.5) ml/l of the four LAB isolates were employed. It was found that all cell-free supernatant folds of the four selected LAB isolates were able to inhibit the growth of all phytopathogenic fungi (Fusarium oxysporum and Alternaria sp) and pathogenic bacteria (E. coli, Klebsiella sp, Salmonella sp, Staphyllococcus aureus). However, the degree of inhibition varied depending upon LAB isolate and microorganism involved. Some LAB isolates revealed effective inhibition activity while others exhibited lower response. Among different LAB isolates, lactobacillus bulgaricus exhibited the strongest antifungal activity against Fusarium oxysporum in all three cell-free supernatant fold concentrates. On the other hand, L.paracasei, L. acidophilus and L. rhamnosus cell-free supernatants showed higher inhibition activity against pathogenic bacteria whereas L. bulgaricus was least active. E. coli was the most inhibited bacterium by L. paracasei and L. acidophilus. LAB cell-free supernatants also showed wide spectrum inhibition activity against both gram positive and gram-negative bacteria. It was also found that the third fold concentrate showed higher inhibition activity than first- and second-fold concentrates against all tested microorganisms, except the second fold concentrate of L. acidophillus which showed higher inhibition effect against both tested fungi. Antibiotics were used to investigate

Keywords: Lactic acid bacteria (LAB), antagonism, well diffusion method, phytopathogenic fungi, pathogenic bacteria, cell free supernatant.

INTRODUCTION

Lactic acid bacteria (LAB) have received attention of several researchers in search for an alternative method to treat pathogenic microorganisms. A number of studies showed that LAB species produce various compounds that exert antagonistic activity and several researchers have used antagonistic microorganisms to control plant and human pathogens and food spoilage microorganisms against pathogenic bacteria (1). LAB species are relatively diverse group of bacteria and can be found in diverse habitats such as plants, meat, gastrointestinal tracts and dairy products and are involved in production of fermented foods (2). Fresh fruits and vegetables harbor various *Lactobacillus* species. LAB species have been widely used as probiotics. Microbial Antagonism is a method of inhibiting pathogenic microorganisms by secreting substances that interfere with the life cycle of the microorganism (3). The mechanisms of antimicrobial activity of LAB species may be through the production of several substances such as organic acid, hydrogen peroxide, antibiotics and bacteriocins (4,5).

Both *Fusarium* and *Alternaria*are known as major plant pathogenic fungi. *Fusarium* species are known to cause several plant disease infections such as wilting and seed and root rot diseases, causing severe infection to crop plants such as tomatoes and chick peas (6,7). In addition, they are known to cause yield and quality reduction of cereals. The diseases caused by *Alternaria* species are very common and are worldwide in their occurrence. Important host plants include tomatoes, cauliflower, carrots, potatoes, citrus and a number of ornamentals and weeds. *Alternaria* generally attacks the aerial parts of its host. Certain fusaria species are able to produce mycotoxins which are toxic secondary metabolites and can be accumulated in the infected plants and stored material. Many *Alterinaia* species also produce toxins that diffuse into host tissues ahead of the fungus.

There is growing interest in search for alternative antimicrobial agents for fighting increasing resistance of pathogenic bacterial strains to available antibiotics. The development of antibiotic resistance in bacteria and other microorganisms are of public concern in that patients could develop antibiotic resistance by contacting a resistant microorganism or the emergence of a microorganism in the patient's body when treated with antibiotic, furthermore fungi have acquired resistance to many of the conventional chemical treatments (8), therefore the search for alternative agents against bacteria and fungi is of priority in fighting the

diseases the cause.

The Aim of the present research was to evaluate *in vitro* the antagonistic activity of four cell-free supernatants of LAB against two phytopathogenic fungi and four pathogenic bacteria.

MATERIALS AND METHODS

Four lactic acid bacteria (LAB) isolates, *L. bulgaricus, Lactobacilluscasei*, *L. rhamnosus,* and *L. acidophilus*were used as antagonists in this research. Antifungal and antibacterial activities were performed using the cell-free supernatants of these four LABs against the two phytopathogenic fungi and four pathogenic bacteria.

LAB isolates

The Four LAB isolates were isolated from different sources and identified based on phenotypic characteristic and a standard commercial identification kit **(**50 CHL kit (bioMerieux, Lyon, France)) as *Lactobacillus bulgaricus, L. paracasei subsp paracasei, L .rhamnosus, and L. acidophillus*. Three LAB isolates were isolated from fresh vegetables (*L. paracasei*from cabbage and *L. bulgarisu* from vegetables) obtained from local markets in Baghdad-Iraq, (*L. rhammosus*) isolated from canned okra brought from local market and *(L. acidophilus)* isolated from Glovit commercial product (commercial probiotic tablets). The LAB present in these tablets, according to the manufacturer, is *L. acidophillus*. Strains of the so-called *L. casei* group comprising the species *L. casei, L. paracasei* subsp. *paracasei* and subsp. *Tolerans*. All LAB were isolated by culturing on DeMan-Rose-Sharpe (MRS) (Oxoid Ltd, Basingstoke, UK) under anaerobic conditions.

The test microorganisms

The test of potential antagonism was made by using agar well diffusion method**.** The two phytopathogenic fungi used for test were *Fusarium oxysporum* and *Alternaria* sp and the pathogenic bacteria were *E coli* (G-), *Salmonella* sp. (G-), *Klebsellasp* (G-) *and staphylococcus aureus* (G+). The fungus *Fusarium oxysporum* was obtained from the Agriculture Organic Research Center in Abu Graib in Baghdad and *Alternnria* sp. isolated from infected tomato plants and identified according to Bergies (9). The pathogenic bacteria were collected from samples obtained from patients from local hospitals in Baghdad. Pathogenic bacteria were identified using AP 20 kit and on the basis of the colony morphology and Gram-negative (G-) or Gram-positive (G+) staining method. According to Bergey's manual of systematic bacteriology**.** LAB species were cultured in agar or broth MRS medium at 37^{0}C for 24, 48 and 72 hr. under anaerobic conditions. The two phytopathogenic fungi activated, cultured, maintained on Potato Dextrose Agar (PDA) (DifcoLaboratories, Detroit, MI, USA). The incubation period for Phytopathogenic fungi was 4 d at 28-30°C. Whereas Pathogenic bacteria activated, cultured and maintained on Nutrient Agar at 37ºC for 24 hr. Spore suspension method was employed for inoculation of the fungi on the medium**.** Phytopathogenic fungi were tested on (PDA) whereas pathogenic bacteria tested on Muller-Hinton Agar medium (MRS) (10, 11). Tests for pathogenic bacteria were carried out at 37ºCfor 24, 48 and 72 h incubation.

Extraction of LAB Cell-free Supernatants (CFS)

Overnight broth culture of LAB was standardized to 1.5x10^8 cells using 0.5 McFarland turbidity standards**.** Cultures of MRS broth in flask (1000ml) were inoculated with standardized culture of LAB inoculation (0.1mlLAB broth/10mlMRS broth medium) and incubated anaerobically at 37°C for 48-72 h, **since** LAB are anaerobic species to ensure enough bacterial growth was obtained. Cell-free supernatants of *L. bulgaricus, L. parcasei, L. rhamnosus,* and *L acidophilus* were prepared according to method used by (12). To prepare cell-free culture filtrate, LAB bacterial suspension was cold centrifuged at 5000 rpm for 10 min and the supernatant was separated from pellet and centrifuged again. The supernatant was then incubated under anaerobic condition at 37°C for 24 hr to make sure that there was no bacterial growth appeared. This cell-free supernatant was then filtered through Millipore filter 0.22µm pore-size filter (Millex-HA; Millipore, S.A., Molsheim, France). Each of the four LAB cell-free supernatant was concentrated (evaporated) in incubator at 40^{0}C into three folds concentrates; 100, 50 and 25ml, started with 200ml culture. These concentrates were used to test against the phytopathogenic fungi and pathogenic bacteria. The sterile LAB cell-free supernatants were stored in refrigerator at 4^{0}C until used.

Antimicrobial test

Fungal culture incubated under aerobic conditions at 28-30°C for 3-4 days. Bacterial cultures were incubated at 37 for 24, 48 and 72 hr periods and the diameters of inhibition zones around the wells were measured at each period. Antagonistic activity was evaluated according to the method described by (13). Sterile cork borer size (5) mm was used to bore holes (5) mm. After solidification, agar surface was inoculated with 100 µl (0.1ml) suspension of antagonist bacteria. And 50 µl of each LAB concetrates was added to the each well. Fungal spore suspension of each phytopathogenic fungi was spread on agar surface with 500 µl, and 50 µl of each LAB concentrate was added to each well.

Antifungal and antibacterial activity was evaluated by measuring the diameter of the zone of inhibition against the test microorganisms [14]. The sensitivity of the phytopathogenic fungi and pathogenic bacteria to each LAB supernatant is indicated by clear inhibition zones around the wells and the diameter of clear inhibitory zones were measuredas an index of the degree of sensitivity. Zones of inhibition were measured using calibrated ruler. A control treatment using sterile MRS medium was performed and zone of inhibition was measured which gave negative result, no inhibition effect. Three replicates of each LAB cell-free supernatants were used in this investigation.

Antibiotic test

Eight antibiotic discs were used to determine antibiotic sensitivity of pathogenic bacteria. These antibiotic discs were Rifampicin, Ketoconazole, Novobiocin, Fluconazole, Gentamycin, Amphotericin, Oxacillin and Chloramphenicol. The susceptibility tests for each pathogenic bacterium were made by using disc diffusion method (15). The discs were placed on the solidified agar surface and the formed inhibition zones were recorded. The plates were incubated aerobically at 37^{0}C for 24 hours, pH of supernatants was adjusted to 6.5, the inhibition activity by LAB cell-free supernatants against the phytopathogenic fungi and pathogenic bacteria were exhibited by clear zones of inhibition.

RESULTS AND DISCUSSION

Table 1. Average inhibition activity of four LAB isolates cell-free supernatants against two phytopathogenic fungi

Alternariasp			Fusariumoxysporum			LAB isolate
3	2	1	3	2	1	
11	8	7	25	23	21	L. bulgaricus

15	10	7	16	14	12	L. rhamnosus
12	9	7	13	10	8	L. paracasei
13	15	9	11	12	7	L. acidophillus

Table 2. Average inhibition activity of four LAB isolates cell-free supernatants against four pathogenic bacteria by

Staphyllococcus 2nd			***Salmonella***			***Klebsiella***			***E. coli 1st***			**h**	**LAB isolate**
3	2	1	3	2	1	3	2	1	3	2	1		
14	13	11	12	11	10	14	13	11	15	14	12	24	*L. bulgaricus*
14	13	12	13	12	10	14	13	11	16	14	13	48	
22	21	19	22	21	20	19	18	17	21	20	19	24	*L.rhamnosus 2nd*
23	22	20	23	22	21	20	19	17	22	21	20	48	
23	20	12	24	21	15	23	18	16	24	17	15	24	*L. paracasei 1st*
24	20	14	25	22	16	25	18	17	25	18	16	48	
21	19	17	16	11	8	19	16	14	24	19	17	24	*L. acidophilus 3rd*
22	20	17	17	12	9	20	16	15	25	19	18	48	

The results showed that All LAB isolates of different species used in this investigation showed to reduce mycelia growth of fungi and growth of bacteria. Tables (1,2) showed that LAB cell-free supernatants inhibited the growth of all tested phytopathogenic fungi and pathogenic bacteria by developing zones of inhibition around the well that contain LAB cell-free supernatants. Data in tables (1,2) are the values of an average of the inhibition zone diameters of the three LAB cell-free supernatant fold concentrates against the two tested phytopathogenic fungi and the four pathogenic bacteria. According to (17), who tested LAB antagonistic activity against bacteria, stated that inhibition scored positive if the width of the zone around the colonies of the producer strain was 0.5 mm or larger. Results of our investigation (table 1, 2) showed that all three supernatant fold concentrates (50, 25, 12.5%) exerted inhibition activity though to varying degrees. It was shown that the inhibition activity increased as the LAB cell-free supernatant fold concentrates increased with widest range of inhibition zones revealed by the most fold concentrate (12.5%) in both phytopathogenic fungi and pathogenic bacteria, except with *L. acidophilus* which showed higher inhibition activity in the second fold concentrate (25%) against both

Tested phytopathogenic fungi. The results also revealed that different LAB isolates exhibited different antagonistic effect, of which *L bulgaricus* was highly inhibitory to the fungus *Fusarium oxysporum,* while exhibited less inhibition effect against pathogenic bacteria. The results also demonstrated that LAB antagonistic activity against pathogenic bacteria displayed larger inhibition zones than phytppathogenic fungi.

Most past studies focused on the effect of antifungal activity of LAB in food, meat and milk products as biopresevative agents (18), whereas very few *in vitro* studies carried out on the effect of LAB against phytopathogenic fungi (19), those studies investigating the antifungal potential of LAB species *in vitro* revealed the effectiveness of some LAB isolates against phytopathogenic fungi (20) demonstrated that *Lactobacillus rhamnosus, L. plantarum, L. acidophilus, L. casei, L. bulgaricus,* showed high inhibition effect against *Fusarium graminearum.* Another study found that *Fusarium graminearum* was sensitive to 25 different strains of tested LAB and investigation carried out by (21) showed that *L. rhamnosus* was able to inhibit the growth of many spoilage and toxigenic fungi including species in the genera *Fusarium, Aspergillus* and *Penicillium.*The results of our investigation showed that *Fusarium oxysporum* was more sensitive to *L bulgaricus* which exerted inhibition effect ranged between (21-25) mm in all three fold concentrates. *L bulgaricus* exhibited lowest inhibition effect against *Alternaria* sp. (7-11) mm, *L. rhamnosus* came second (12-16) mm and *L. paracasei* came third (8-13) mm. However, *L. acidophilus* showed higher inhibition activity in the second fold concentrate against both *Fusarium oxysporum* (12) and *Alternaria* sp (15) mm.

In contrast more studies carried out on pathogenic bacteria (22). Result obtained by E.C. Emerenini (2014), showed that cell-free culture of LAB isolated from fresh vegetables including *Lactobacillus pentosus,* and *L. plantarum in vitro* inhibited the bacteria that infect tomato (*Xanthomonas campestries, Erwinia caratovora, and Pseudomonas syringae*) by creating clear zones of inhibition around the wells, and the highest zone of inhibition recorded was (15) mm radius and the least (3.50) mm radius. Previous research carried out by (23) using LAB isolates showed that most of the selected strains had good antagonistic activity against the pathogens including *Listeria monocytogenes*, *salmonella thyphimurium* and *E coli* and some were less active (7-9) mm. Results showed that *L. paracasei* was the most effective LAB isolate against all pathogenic bacteria (23-24) mm followed by *L. rhamnosus* (19-22) mm and the least showed by *L. bulgaricus* (12-15) mm**.** Our result is in agreement with that as *L bulgaricus* showed the least inhibition effect.

The results we obtained showed that *L. paracasei, L. rhamnosusand L. acidophilus* exhibited wide range of inhibition zones (23-24) mm, (19-22) mm and (16-24) mm respectively, while *L. bulgaricus* resulted in average (12-15) mm. The results obtained showed that antibacterial potential of cell-free supernatant of *L. paracasei* exerted the most inhibitory activity against the growth of all pathogenic bacteria; *E.coli*(24)mm, *Salmonills* (24)mm*, Staphillococus* (23)mm *and Klebsella (23)mm* in third fold concentrates.*E.coli* was the most inhibited pathogenic bacteria by all four LAB supernatants with inhibition zones ranged between (15-24) mm, followed by *salmonella* (12-24) mm and *Staphyllococcus aureus* and *Klebsiella* which both recorded equal effect (14-23) mm in third fold concentrates. The susceptibilities of various bacteria viz., *E. coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* by the bacteriocin of *Lactobacillus* strains isolated from soil showed inhibitory activity against *E. coli, P. aeruginosa* and *S. aureus* and maximum zone of inhibition was observed against *E. coli* (15mm) and minimum against *P. aeruginosa.*

Our results agreed with (24) who tested selected LAB isolates and found that most of the isolates exhibited good antagonistic activity against the pathogenic bacteria including *E. coli* and revealed that all *lactobacilli* tested (except *L. delbruceki*) inhibited the growth of *E. coli* and *S. aureus*. The results in table (2) revealed that all four LAB supernatants maintained good inhibition effect against the growth of all four pathogenic bacteria after 24, 48 and 72 hr of incubation. Results revealed that inhibition activity slightly increased after 48 hr, and showed equal inhibition effect after 72 hr similar to results obtained after 48 hr. The

present investigation also showed that LAB cell-free supernatants to have broad spectrum inhibitory effect against both Gram-positive and Gram-negative pathogenic bacteria. The results obtained showed that *Staphylococcus* sp, as a gram-positive bacterium, was found to be inhibited by all LAB supernatants and the range of inhibition zones were between (14-24) mm. Several studies revealed that more than one compound is responsible for the antimicrobial activity, the specific compound or combination of compounds may be work together (in effect) for their potential as antimicrobial. LAB species are known to produce antimicrobial compounds important in the bio preservation of food. Several reports revealed that LAB species produce various substances known to exhibit antagonistic activity against other bacteria. These antimicrobial agents produced by LAB species showed to have good broad-spectrum antimicrobial properties. This activity may be due to the presence of inhibitory substances produced by LAB in supernatants. They have an inhibiting effect on the growth of both gram-negative and gram-positive bacteria, mainly through metabolites agents such as lactic acid, bacteriocins, and hydrogen peroxide. LAB synthesis bactericidal agents that vary in their spectrum of activity. Such compounds consist of hydrogen peroxide, organic acids, lytic agents, bacteriocins or antimicrobial peptides, diacetyl, defective phages and enzymes.

Our results revealed that *L. paracasei, L. rhamnosus and L. acidophillus* found to exert high antimicrobial activity against bacteria and fungi, this may be due to the production and secretion of (effective) antibacterial compounds that was able to reduce the growth of bacteria, (25) found that the cell free supernatants of LAB isolated from salad vegetables were inhibitory to some pathogenic bacteria in an agar well diffusion assay and the result indicated organic acid being responsible.

Table 3. Results of antibiotic tests After 24 h of incubation

Bacteria	Antibiotics effect							
	OX	CIP	TE	AMX	SXT	PRL	C	GIV
E. coli	11	34	11	12	16	32	13	20
Staphyllococus	11	35	9	12	14	16	8	15
Salmonella	11	42	18	18	15	33	20	18
Klebsella	10	33	20	13	13	22	21	15

Antibiotics sensitivity was carried out to test and compare with antagonistic activity of LAB cell-free supernatants against pathogenic bacteria. The results in table (3) showed that the pathogenic bacteria varied in their sensitivity to different test antibiotics which recorded between (8) mm in C and (42) mm in CIP. All test pathogenic bacteria showed resistant to OX with zone of inhibition effect recoded between (10-11) mm and were highly sensitive to CIP (33-42) mm. *Salmonella* was the most sensitive pathogenic bacteria to all tested antibiotics (11-42) mm, in comparison with LAB supernatants. All showed resistance to all the antibiotic discs test except showed sensitivity to this was susceptible.

The present study indicated that the cell-free supernatants of *P. aeruginosa*, a non-acid producing bacterium, had no inhibition effect against the growth of both tested phytopathogenic fungi and pathogenic bacteria. (16). Results of *P. aeruginosa*, showed no inhibition activity was recorded on both testes fungi, as well as bacteria, suggesting that the fungal inhibition seen with *Lactobacillus* cell-free culture was not simply due to nutrient exhaustion.

CONCLUSION

Fresh vegetables may be used as a source of antimicrobial lactic acid bacteria (LAB). *L. paracasei*, *L. rhumnoses, and L bulgaricus* showed to have antifungal and antibacterial effect. The results obtained, showed that LAB isolates exhibited good antifungal activity. The four *Lactobacillus* species investigated inhibited the growth of all fungi which indicate the possibility of using LAB isolates as biocontrol agent. This research revealed the effectiveness of using Lactic Acid Bacteria (LAB) cell-free supernatant as antifungal agents against wilt disease *Fusarium oxysporum* that infects chick peas and *Alternaria sp* that infects tomatoes.

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