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Antagonism of *Lactobacillus* Species against *Xanthomonas Campestris* Isolated from Different Plants

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ABSTRACT

Several members of the lactic acid bacteria are known to produce antibacterial substances. The antibacterial effect has been ascribed to the production of antibiotics or antibiotic-like substances such as *Lactobacillus plantarum.* In the present study, preliminary tests were conducted to investigate possible antagonism between plant-associated Lactic Acid Bacteria (LAB) and some phythogenic bacteria like *Xanthomonas campestris* PTCC 1473. The ultimate aim would be the implementation of LAB for the biological control of bacterial plant disease. LAB were isolated on MRS agar containing 0.1 % (w/v) cycloheximide and were mainted in MRS broth. The colony and cell morphologies of the pure cultures were examined and the following tests were done to identify the isolates. Antibacterial activity performed by well diffusion and disk diffusion methods. Then the diameter zone of inhibition (mm) measured. Any *Lactobacilli* was isolated from products like potato and onion and in contrast, the isolates of these bacteria recovered from tomato, radish, lettuce, carrot and soybean. There were more *Lactobacilli* in surface areas of spoiling plants especially tomato and soybean. The isolated bacteria from radish and tomato were *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively. Relationship between mean diameter of zone of inhibition with type of method (well diffusion and blank disk) and kind of microbial solution (Supernatants and suspensions) were significant. Amongst of isolated *Lactobacilli*, *L. plantarum* against *X. campestris* PTCC 1473 showed good activity. If the results of this method to be similar with in vivo conditions in future, it can be optimized for neutralization of bacteria pests in plants.

KEY WORDS: Lactobacillus species, Xanthomonas campestris, antagonistic effects, phytopathogen

1-INTRODUCTION

Among probiotics, *Lactobacillus* the most consumed bacteria are used in food industry. These bacteria can change Lactose and other similar carbohydrates into Lactic acid. Probiotic bacteria produce different substances that are inhibitors of the Gram-positive and Gram-negative. These compounds can be pointed short-chain organic acids such as acetic acid and lactic acid or hydrogen peroxide and bacteriocin-like peptide compounds. These substances not only reduces the number of viable cells of pathogenic metabolic but may also affect on metabolism of bacteria or production of toxins by them [1,5]. During of production and processing of agricultural products have occurred vast economical losses due to microorganisms that causes rot in kind of plants postharvesting. Small wounds or cuts occure during harvesting and transportation easily by some pathogens such as *Xanthomonas campestris* and *Erwinia carotovora* that as common spoilage bacteria of fresh fruits and vegetables [9, 12]. The interactions of Lactic acid bacteria with other bacteria have been investigated in food products and widely in fermented foods. However, information on the occurrence of *Lactobacilli* on living plants is low, and no information is available on the interactions of plant-associated Lactic acid bacteria with phytopathogenic bacteria [8, 12]. *Lactobacilli* particularly found in damage and wounded plant tissue [3, 12]. In the present study the possible antagonistic effects between *Lactobacilli* isolated from different plants and some phytopathogen evaluated. The necessity of this study is the use of *Lactobacillus* as a biological control of plants bacteria disease. The primary way to control spoilage caused by microorganisms in a variety of plants is the use of chemical materials. Because of possible toxic hazards many of these substances, health concerns about pesticides, microbial resistance and very high price these new chemicals cause the researchers go looking for other solutions [11]. Among the bacteria used as biological agents, strains of the genera *Bacillus, pseudomonas* and *Lactobacillus* can be noted [12].

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2-MATERIALS AND METHODS

In a study over 7 months, antagonistic properties of *Lactobacillus spp.* isolated from different plants (13 *Lactobacillus spp.* isolated from tomato, soybean, radish, lettuce and carrots) were studied.

*2.*1 Selection and preparation of Lactobacillus species

In this method, 1 g of chopped plant sample with 9 ml quarter- strength Ringer solution was poured in test tube and vortexed very well, then one ml of solution was poured into a second tube and up to 10^{-6} dilutions were prepared. Then 50 microliters of the dilutions was poured on MRS agar plates containing 0.1 % (W/V) cycloheximide (to inhibit possible fungal contamination) and then were placed in an anaerobic jar and were incubated for 24 to 48 hours at 37° C. After this period, colonies isolated on MRS agar plate (Merck) and *Lactobacillus* species of these isolates were identified by comparing their sugar fermentation patterns with the scheme described in Bergey's Manual of systematic Bacteriology [4,7].

*2.*2 -Preparation of culture supernatant

Cell-free culture supernatants of the selected *Lactobacillus* isolated grown in MRS broth for 24 h at 30° C were obtained by removing the cells following centrifugation at 6000 rpm for 15min and sterilizing the supernatants by filtration through 0.2 µm pore size filter. The supernatant was stored for further studies in 4°C.

2.3-Preparation of phytopathogenic bacteria

Lyophilization powder of *X. campestris* PTCC 1473 from scientific and industrial research organization of Iran was prepared and after activation in MHB medium, cultured in synthetic YDC medium with the following composition:

1-	Yeast extract	10 g
2-	Glucose	20 g
3-	Agar	15 g
4-	$CaCO_3$	20 g

After the appearance of colonies, 4-5 colonies from overnight cultures of each strain was added to 3 ml of MHB medium and incubated at 30° C for 1-2 h until turbidity was equivalent to 0.5 McFarland.

*2.4-*Determination of antimicrobial effects

The antimicrobial activity of the isolated strains on *X. campestris* was determinate by the well diffusion assay and disk methods. For well diffusion assay, ten ml of MRS broth was inoculated with each LAB strains and were incubated at 30° C for 48h. After incubation, a cell-free solution was obtained by centrifugation (6000 rpm for 15 min) the culture, followed by filtration of the supernatant through a 0.2 µm pore size filter. The *X. campestris* was incubated in Brain Heart Infusion (BHI) broth, at appropriate temperature for 24 h. Petri dishes with 20 ml of Muller Hinton agar were prepared, previously inoculated with 0.1 ml of a 24 h broth culture of *X. campestris*. Once solified, the dishes were stored for 2 h in a refrigerator. Wells (6mm) then made filled using 100 µl of suspension and cell-free filtrated and incubated for 24 h at 37° C. The antimicrobial activity was determinate by measuring the clear zone around the colonies. An agar disk technique was used to determine whether the *Lactobacillus spp.* were capable of inhibiting the *X. campestris* in vitro. Pour plates were made of the Lactic acid bacteria by mixing 1 ml of a 36-h broth culture in ca. 15 ml of MRS agar. After incubation at 30° C for 48 h, disks with a diameter of 6mm were stabbed from the agar. The disks were placed on agar covered with supernatants of the *X. campestris* in Ringer solution. Sterile MRS agar disks were used as a control. After an incubation period of 36 h at 25°C, the diameter of clear zones surrounding the disks was measured. The experiment was done in triplicate to ensure repeatability.

Table1. Physiological and biochemical characters of *Lactobacillus* strains having an antimicrobial isolated from different plants [4, 7].

Characteristic	*L. plantarum*	*L. brevis*	*L. casei*
Shape	Rod	Rod	Rod
Gram stain	+	+	+
Catalase	-	-	-
Growth at 15/45˚C	+/-	+/-	+/-
Carbohydrate fermentation			
Arabinose	+	+	-
Esculin	+	-	+
Fructose	+	+	+
Galactose	+	d	+
Glucose	+	+	+
Lactose	+	-	+
Maltose	+	+	-
Mannitol	+	d	+
Mannose	+	+	-
Raffinose	+	d	-
Ramnose	-	-	+
Ribose	+	+	+
Sorbitol	-	-	+
Sucrose	+	d	+
Xylose	+	d	-

Symbols: + = 90% or more strains positive, - = 90% or more strains negative, d = 11-89% strains positive

Table2. Antimicrobial activity of *Lactobacillus spp.* against *X. campestris*

Lactobacillus spp.	Origin	Zone diameter (mm)[a]			
		Disk diffusion		Well diffusion	
		Suspension	Supernatant	Suspension	Supernatant
L. plantarum	radish	5	2	6.5	3.5
L. brevis	tomato	0	0	0	0
L. casei	lettuce	0	0	0	0
Lactobacillus spp.	carrot, soybean, tomato	0	0	0	0

[a] Average of triplicate readings

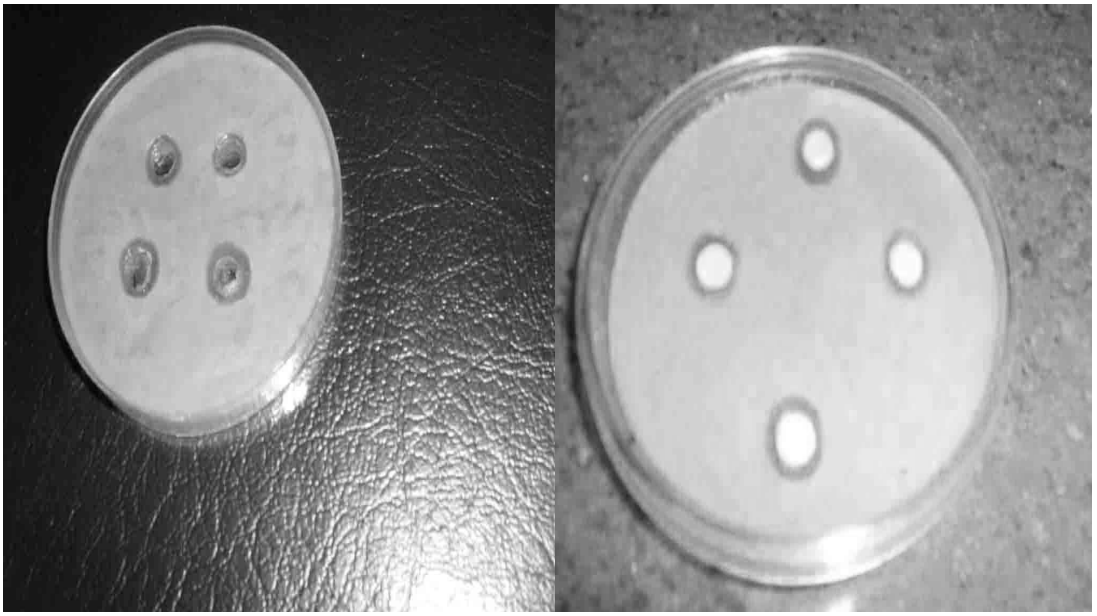

Figure1 .
Inhibition zones surrounding wells of *Lactobacillus Plantarum* cultures placed on a spread plate Of *X. Campestris* PTCC 1473 on MHA

Figure 2.
Inhibition zones surrounding disks of *Lactobacillus plantarum* cultures placed on a spread plate of *X. campestris* PTCC 1473 on MHA

3-CONCLUSION AND DISCUSSION

A total of 13 *Lactobacillus spp.* isolated from different plants were tested for antimicrobial activity. *Lactobacillus* isolated from radish was *L. Plantarum* and effected on phytopathogenic bacteria like *X. campestris* very well. *Lactobacillus spp.* was isolated from 100 samples of vegetables, randomly obtained from the field and market. The measured densities of *Lactobacillus spp*. differed significantly among product types and sources. Samples obtained directly from field always showed higher densities than vegetables obtained in the markets. No *Lactobacillus* could be detected on the surface of tubers and bulbs such as potatoes and onions. In contrast, higher densities were found in tomato, soybeans, radish, lettuces and carrots. The major antimicrobial effect produced by *Lactobacillus* strains was acidification of the medium. In fact, many pH-neutralized supernatant lost their inhibition ability. *L. brevis* and *L. plantarum* grew well at 15°C but could not grow at 45°C. None of *Lactobacilli* isolated were able to produce gas from glucose. All of them were glucose, maltose and fructose positive but *L. brevis* unlike *L. plantarum* was not able to use Esculin. According to the data from the analysis of variance test, relationship between mean diameter of zone of inhibition with type of method (well diffusion and blank disk) and kind of microbial solution (suspensions and supernatants) were significant (Table2). Suspension of *L. plantarum* in well diffusion method was the highest average zone of inhibition of growth and *L. brevis* didn't have a significant impact on *X. campestris.* In compared with the suspension, the supernatant of *L. plantarum* was produced less zone of inhibition. Well diffusion agar method was better than blank disk assay(Table2).In recent years, the antagonistic effects of *Lactobacillus* spp. isolated from dairy products has been studied very much but on *Lactobacilli* isolated from plants and their effect on phytopathogenic bacteria are available a few resources. *Lactobacillus spp.* was founded particularly in damaged and wounded plant tissues [12]. In this paper, we report studies of the distribution and identification of *Lactobacilli* occurring on plants. The aim of this study was the necessity of the using of *Lactobacillus* as biocontrol agents of bacterial plant disease. Visser et al. (1986) were isolated a variety of Lactic acid bacteria, from plant surfaces and plant-associated products and were found to be antagonistic to test strains of the phytopathogenes *Xanthomonas campestris* and *Erwinia carotovora* and *Pseudomonas syringae.* According to their research all the pathogens were inhibited by *L. plantarum.* Trias et al. (2008) evaluated the efficacy of lactic acid bacteria isolated from fresh fruits and vegetables as biocontrol agents against the phytopathogenic and spoilage bacteria and fungi, *Xanthomonas campestris* and *Erwinia carotovora* and *Penicillium expansum, Monilinia laxa,* and *Botrytis cinerea.* The survival of *Lactobacillus* in postharvest conditions makes these bacteria most adequate to prevent postharvest spoilage [10]. According to Trias et al. (2008) research *L. plantarum* affected on *X. campestris* that was consistent with our results but in our study *Lactobacillus* spp. isolated from surface of spoilage plant tissue. In this study, dilutions of samples were prepared for isolation of *Lactobacillus* and pour plate method on agar medium were cultured in anaerobic conditions. Many of researchers have been studied the antimicrobial effects of *Lactobacillus* over the years [1, 5, 10]. These studies have shown that *Lactobacilli* were cultured on selective or specific medium and under anaerobic conditions are able to produce antimicrobial compounds. Lactic acid bacteria are believed to be safe because they have been long established as the normal flora in fermented food. Thus, they have great potential for use in biopreservation [10]. The preserving effects of Lactic Acid Bacteria (LAB) are due to the production of antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocin or related substances [5]. Bacteriocins are proteinaceous compounds that mainly inhibit closely related species [6]. MRS agar medium used for the growth of mesophilic *Lactobacillus.* This medium have high amount of Acetate with low pH is a good medium for the growth of mesophilic *Lactobacillus* [2]. In the present study isolated *Lactobacilli* didn't grow in 4°C and optimum temperature was 30-37° C. in 15 and 45° C had a weak growth so these bacteria were mesophile. In vitro assays showed that LAB could inhibit phytopathogens and encourage the development of biocontrol agent from this bacterial group. Moreover, some LAB strains are able to inhibit more than one phytopathogen, which must be taken into account in considering a wide range of plant protection [10].

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