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Lactic Acid Bacteria from Fresh Fruits and Vegetables as Biocontrol Agent of Foodborne Bacterial Pathogens

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ABSTRACT

The presence of lactic acid bacteria (LAB) in dairy products has long been established but the occurrence of the particular group of bacteria in fruits has rarely been focused. Therefore, different lactic acid bacteria generally found in fruits, cultivated in Pakistan were studied. Following the culture enrichment method randomly selected fruit samples were analyzed. About 12 different isolates of *Lactobacillus spp.* **were obtained from 25 different fruits and vegetables. The identification was based on conventional morphological and biochemical analysis. Prior to all these manipulations the growth conditions were carefully optimized for the respective strains. This study evaluated the efficacy of lactic acid bacteria (LAB) isolated from fresh fruits and vegetables as biocontrol agents against the foodborne human bacterial pathogens (***Escherichia coli, Enterococcus faecalis, Proteus sp.* **and** *Pseudomonas aeruginosa***). The antagonistic activity of LAB strains were tested in vitro and all tested microorganisms except few were inhibited by at least one isolate. Cell-free supernatants of selected antagonistic bacteria were studied to determine the nature of the antimicrobial compounds produced.**

Keywords: Antagonistic activity, Lactic acid bacteria, Mircoorganisms, Probiotics.

INTRODUCTION

Lactobacillus is a genus of gram positive facultative anaerobic or microaerophilic rod-shaped bacteria, occurring in chains. They are non-sporulating, facultatively heterofermentative, catalase negative, cytochrome oxidase negative and non motile. The lactobacilli are strictly fermentative and have complex nutritional requirements (Madigan *et al*., 1997). They are a major part of the lactic acid bacteria (LAB) group, named as such because most of its members convert lactose and other sugars to lactic acid (Makarova *et al*., 2006). In humans they are present in the vagina and the gastrointestinal tract, where they make up a small portion of the gut flora. Lactic acid bacteria may isolate from fermented foods, dairy products, grain products, meat and fish products, pickles, sour dough, fruits & vegetables. In nature, all plant surfaces contain Lactobacilli in low numbers (Mundt and Hammer, 1968) and also grow luxuriantly with other lactic acid bacteria in manure decaying plant material, especially decayed fruits. Hence, Lactobacilli are also important for the production and spoilage of fermented vegetable feed and food (e.g. silage, mixed pickles) and beverages (e.g. Beer, Wine, Fruit Juices). These organisms have been used for many years for production of fermented food. Lactic acid bacteria are chiefly responsible for the raw material through the production of organic acids, mainly lactic acid. In addition, their production of acetic acid, ethanol, aroma compounds, bacteriocins,

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exopolysaccharides, and several enzymes is of importance. Lactic acid bacteria produced different antimicrobial molecules such as lactic acid, acetic acid, hydrogen peroxide, CO_2 and bacteriocin. Bacteriocin is widely known to inhibit spoilage microorganism and food borne pathogens, thereby extending shelf life of food products.

Lactic acid bacteria exert strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens. In addition, some strains may contribute to the preservation of fermented foods by producing bacteriocins (Adeniyi *et al*., 2006). Research on bacteriocins from lactic acid bacteria has expanded during the last decades, to include the use of bactericocin or producer organisms as natural food preservatives. Adeniyi *et al*. (2006) reported varied inhinbitory acitivities of lactic acid bacteria isolated from indigenous fermaented diary foods against commonly encounted bacteria implicated in urinary tract infections (Coconnier *et al*., 1993). The killing activity of anti-*Salmonella enterica* serovar Typhimurium produced by Lactobacillus and Bifidobacterium strains in the persence of Luria broth (LB) has also beed reported (Graver and Wade, 2011). A small number of clinical studies suggest that eating yogurt with *L. acidophilus* cultures may also help. Some people also use *L. acidophilus* to treat or prevent vaginal yeast infections. Some clinical research suggest *Lactobacillus acidophilus* may be effective when used to prevent diarrhea (caused by eating contaminated food). The health benefits derived by the consumption of foods containing probiotic bacteria are well documented and more than 90 probiotic products are available worldwide. To provide health benefits, the suggested concentration for probiotic bacteria is suggested to be 10^6 cfu/g of a product (FAO/WHO, 2010).

In the present investigation the microflora of different fruit samples of Karachi, Pakistan were studied with special focus on lactic acid bacteria. We isolated lactic acid bacteria (LAB) from fresh vegetables and fruit and then tested their potential as bioprotective agents against food-borne human bacterial pathogens. This study was focused in two different areas. First part of the study aimed to isolate Lactobacillus and second part was focused on inhibition properties.

MATERIAL AND METHODS

Sample collection: Fruit markets in different areas of Karachi, Pakistan were visited and the seasonal fruits (apple, banana, cheeko, custard apple, sugarcane, peach, grape, papaya, plum, grape fruit, mango, melon, pomegranate, pear, lettuce, cabbage, tomato and cucumber) of reasonable quality were purchased. The samples were collected in sterile plastic seal bags and brought to the laboratory within 24 hours of purchase for analysis (Table I).

Isolation of Lactic acid bacteria: To isolate LAB, the fruit samples were brought to the laboratory and thoroughly washed with tap water following a final rinse with sterile saline (0.9%). Each fruit was processed separately and diced in a sterile petri plate. Small portion of the solid fruits sample was chopped manually with sterile cutter while the citrus fruits (grapefruit) were squeezed manually till the considerable amount of extract was obtained. Following the culture enrichment method 1 ml of fruit extract so obtained was inoculated into 9ml of sterile MRS broth and incubated overnight at 37ºC for 48 hours in shaking water bath at 120 rpm. After incubation samples were appropriately diluted and spread on MRS-agar plates. The plates were incubated an aerobically at 35ºC for 24-48 hr.

Identification of isolates based on phenotypic characteristics: The isolates were characterized for gram reaction and catalase reaction and fermentative catabolism of various carbohydrates. The strains were also tested for their motility by SIM (sulphide, indole, motility) test. The fermentative behavior for various carbohydrates was determined in Bromothymol blue lactose broth supplemented with 1.5% of carbohydrate to be tested (arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, raffinose, sucrose, xylose).

Assay of Antagonistic activity: For this assay, 48 hrs broth cultures of the LAB were centrifuged at 10,000 rpm for 30 min. The supernatnants were membrane filtered (Millipore, 0.22um) and the pH was measured using pH meter. The pH of sterile MRS broth treated the same way as the LAB culture. The cell free supernantant 100 ul was transferred in to 6mm diameter well bored in Mueller Hinton agar previously seeded with 0.5 MacFarland of clinical isolates of *Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli* and *Proteus sp.*, equivalent to 10^8 CFU/ml. The culture plates were incubated at 37^{o}C for 24hrs and the zones of inhibition measured in millimeter.

RESULTS

A total of 12 lactic acid bacterial strains were isolated from 25 fruit samples collected and analyzed during present course of work. All isolates were identified on the basis of colony/cell morphology and biochemical tests (Figure 1-7). Majority of the colonies on MRS agar plates were whitish to off-white in color, gram reaction showed gram positive rods, catalase test gives negative result and motility was not observed in SIM media (Table I). Small number of the analyzed fruit sample gave colonies of bacillus with positive catalase reaction. All gram positive and catalase negative isolates were selected for further analysis.

The 12 isolated lactic acid bacterial strains were tested for their antagonistic activity against *E. coli, Enterococcus fecalis, Pseudomonas aeruginosa* and *Proteus*. All except few strains show moderate to high inhibition activity against these organisms (Table II).

Table I: Identification test for lactobacilli.

Fruit Source	Colonial Morphology	Microscopy	Catalase	SIM test	Bromothymolblue test
Grape	Large, gummy colonies	+ve rods	-ve	-ve	+ve
Sugarcane	Flat, gummy colonies	+ve rods in chains	-ve	-ve	+ve
Banana	Small colonies	+ve rods	-ve	-ve	+ve
Apple	Flat, gummy colonies	+ve rods	-ve	-ve	+ve
Peach	Large, gummy colonies	+ve rods	-ve	-ve	+ve
Cucumber	Pin pointed transparent colonies	Gram +ve diplococci	-ve	+ve	-ve
Plum	Large, gummy colonies	Gram-ve rods	-ve	-ve	+ve
Grape fruit	Gummy colonies	+ve cocci	-ve	-ve	+ve
Tomato	Gummy colonies	+ve rods	-ve	-ve	+ve
Papaya	Small colonies	+ve rods	-ve	-ve	-ve
Cheeko	Pin pointed flat colonies	+ve rods in chains	-ve	-ve	-ve
Pomegranate	Spreaded colonies	+ve rods	+ve	----	----
Mango	Pin pointed flat colonies	+ve rods in chains	-ve	-ve	-ve
Melon	Pin pointed flat colonies	+ve rods in chains	-ve	-ve	-ve
Lettuce	Gummy colonies	+ve rods	-ve	-ve	+ve
Cabbage	Gummy colonies	+ve rods	-ve	-ve	+ve

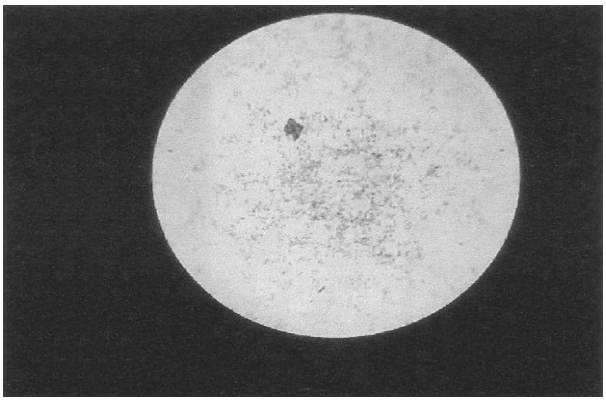

Figure 1: Microscopy of Lactobacillus.

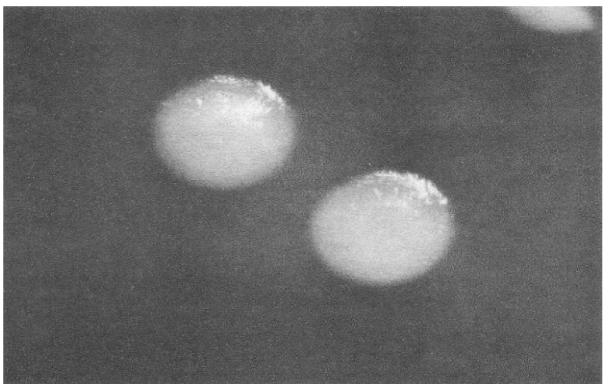

Figure 2: Colonial morphology of Lactobacillus.

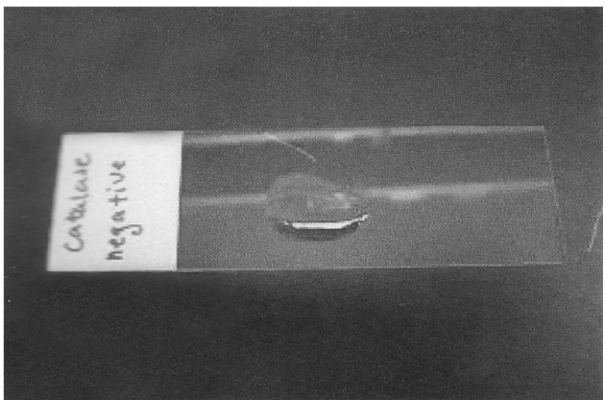

Figure 3: Catalase test.

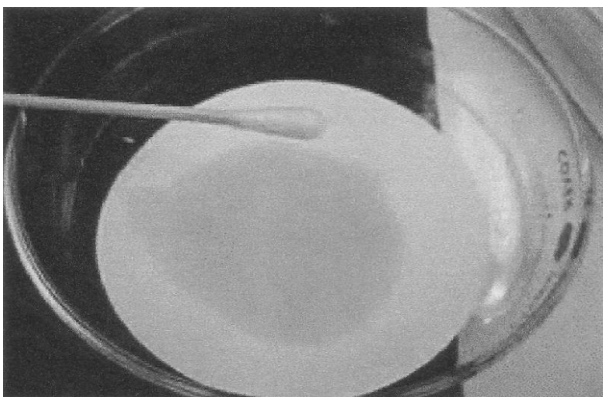

Figure 4: Oxidase test.

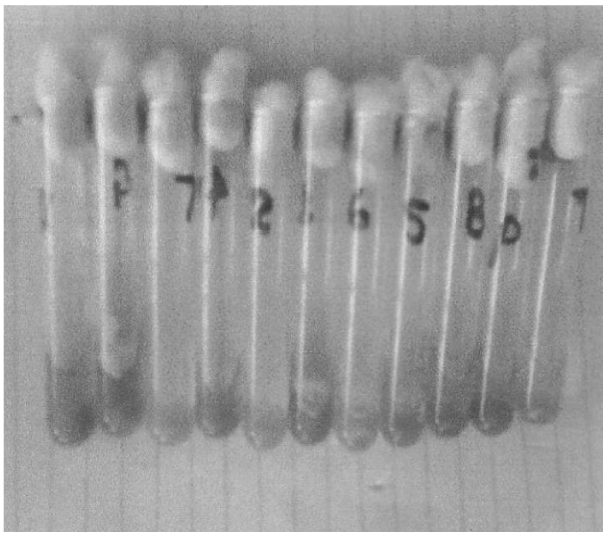

Figure 5: Bromothymol blue Lactose broth.

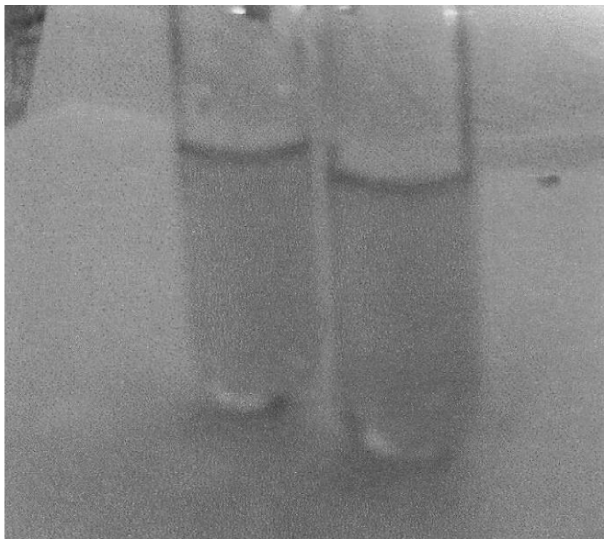

Figure 6: SIM Test.

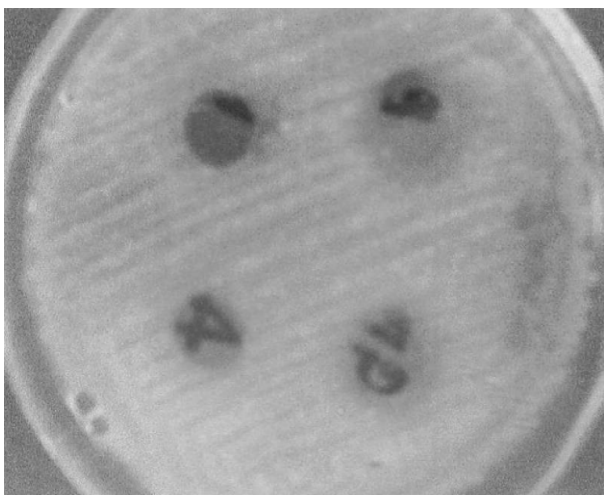

Figure 7: Zone of Inhibition of LAB against bacterial pathogens.

Table II: Antagonistic activity of Lactic acid bacteria.

Organism code	*E. coli*	*Enterococcus*	*Proteus*	*Pseudomonas*
1	0 mm	0 mm	11mm	2mm
2	0 mm	0 mm	10mm	0 mm
3	3mm	0 mm	9mm	8mm
4	0 mm	4mm	7mm	0 mm
5	5mm	6mm	0 mm	0 mm
6	6mm	0 mm	0 mm	3mm
7	7mm	4mm	11mm	0 mm
8	0 mm	0 mm	0 mm	0 mm
9	0 mm	0 mm	0 mm	0 mm
10	5mm	3mm	0 mm	2mm
11	0 mm	0 mm	0 mm	0 mm
12	0 mm	0 mm	7mm	0 mm

DISCUSSION

This study evaluated the efficacy of LAB isolated from fresh fruits and vegetables as biocontrol agents against the food borne pathogens. The densities of LAB in fruit and vegetable products usually range from 102 to 106 CFU/gm (Ongeng *et al*., 2006). Lactic acid bacteria are generally considered as microorganisms with no pathogenic activities (Sexline *et al*., 1996).

In the present study, the approaches in preliminary identification of isolated bacterial strains were morphological and biochemical characterization like positive growth on MRS agar medium, negative catalase activity, colony color, gram positive staining reaction, outcomes of microscopic analysis and carbohydrate fermentation pattern. All were to suggest the presence of lactic acid bacteria in the samples under consideration. These identification approaches were in agreement with the previous studies on lactic acid bacteria (Sudi *et al*., 2008).

The present study had little success in isolating lactic acid bacteria from fruit samples by agar plate culture method. Instead a predominance of Bacillus by this method was found. This correlated the different bacillus species as widespread pollutants in the environment. Consequently, the present investigation focused on enrichment culture as an approach to determine the diversity of lactic acid bacteria associated with fruit samples. Enrichment substantially increased the isolation frequency of lactic acid bacteria from the above stated samples. Nevertheless many of the fruit samples in present study did not give detectable lactic acid bacteria. Present investigation deals not only with the distribution of lactic acid bacteria in fruits, but also with antagonistic activity.

Several clinical applications of LAB against human diseases have attracted the attention of many research groups. Therefore, in vitro antagonistic assays performed revealed that LAB strains isolated from fresh fruit and vegetable products had antagonistic

avtivity against common foodborne pathogens like *E. coli, Proteus sp., Enterococcus sp.* and *Pseudomonas sp.* A previous work using LAB showed that most of the selected strains had good antagonistic activity against food borne pathogens, including *Listeria monocytogenes, Salmonella typhimurium,* and *Escherichia coli* (Trias *et al*., 2008). A major advantage of using LAB as biocontrol agents is that they are considered as GRAS (generally recognized as safe) and usually comply with all recommendations for food products. Moreover, LAB is natural colonizer of fresh fruit and has been previously described ad good antagonists of several bacteria and fungi in different food products (Stiles and Holzapfel, 1997). Studies on the antifungal properties of LAB are relatively scare, when this effect was reported; it was attributed to the production of proteinaceous compounds (Rouse *et al*., 2008) or organic acids (Goueama, 1998). The preferred antimicrobial substances produced by the strains described in this study are organic acids, this was the case for all the bacteria and fungi tested. The combination of different organic acids, such as lactic and propionic, has been reported to have a synergistic fungistatic effect (Batish *et al*., 1997).

CONCLUSION

In conclusion, fruits and vegetables have been found in this study to be carriers of some LAB and present potential sources of the organisms. Since LAB strains have inhibitory effect against number of bacterial pathogens, they can be used in food industry to ensure the safety of different food products.

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