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ISOLATION CHARACTERIZATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM FRUIT JUICES AND THEIR EFFICACY AGAINST ANTIBIOTICS

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Abstract

The presence of lactic acid bacteria (LAB) in dairy products has long been established but the occurrence of this particular group of bacteria in Pakistani fruits has rarely been focused. Therefore, different lactic acid bacteria generally found in fruits, cultivated in Pakistan were studied using microscopic analysis as well as the technique of ribotyping. Following the culture enrichment method randomly selected fruit samples were analyzed. The initial identification was based on conventional morphological and biochemical analysis while the final confirmation was done by utilizing advanced molecular tools (i.e., 16S rRNA gene amplifications). Prior to all these manipulations the growth conditions were carefully optimized for the respective strains. The surveillance of all strains after experiencing an acid shock mimicking the harsh acidic surroundings of gastrointestinal tracts reflects their probiotic potential. The results of antibiotic sensitivity test demonstrated an unusual high rate of kanamycin and oxacillin resistance among isolated LAB strains. Hence it can be hypothesized that use of these antibiotics against pathogens would not disrupt the natural microbiota of gastrointestinal tract which mainly consist of lactic acid bacteria. The study finally led us to conclude that *Lactobacillus plantarum* was the most abundant type of lactic acid bacteria distributed in most of the fruit samples studied, on the other hand *Leuconostoc mesenteroid* subsp. *mesenteroides* showed sporadic occurrence. The presence of such bacteria in raw fruits satisfies the nutritive and microbial profile of fruits to be a healthy food.

Introduction

Fresh produce like fruits and vegetables, are the normal part of the human diet and are consumed in large quantities in most civilizations. Traditionally, fruits and vegetables have been regarded as microbiologically safer than other unprocessed food items such as meat, milk, eggs, poultry and sea food. These products are rich in carbohydrates and poor in proteins with pH value from 7.0 to slightly acidic and provide a suitable niche to several bacteria, yeasts and moulds (Wiessinger *et al*., 2000; Trias *et al*., 2008).

In contrast to vegetables, fruits have good record from public health standpoint. Many fruits possess a natural defense mechanism. Fruits contain organic acids in quantities adequate enough to contribute a pH value of 4.6 or lower. The pH and the type of the acid itself are the major influence that select for predominant microflora in fruits. Acid tolerant microbes like fungi (Splittstoesser, 1987) and lactic acid bacteria are demonstrated as a part of autochthonous microflora of tomatoes owing to its low pH and organic acids (Brackett, 1988; Sajur *et al*., 2006). Food borne bacteria capable of causing human illness can not grow at a pH less than 4.0, so edible portion of most fruits precludes the involvement as substrate for proliferation of human pathogen.

Rich nutrients, such as carbohydrates, minerals, nitrogen compounds and a low pH environment are necessary for the growth of LAB. So, fruit juices serve as an excellent substrate for proliferation of LAB. Lactic acid bacteria and microorganisms most frequently used as probiotic agents also exist as the part of indigenous microflora of fruits (Alvarez & Oberhelman, 2001). An extensive research was focused on isolation of LAB from a variety of fruits (Bae *et al*., 2006; Chambel *et al*., 2006; Duangjitcharoen *et al*., 2006; Nyanga *et al*., 2007; Trias *et al*., 2008; Yanagida *et al*., 2008; Chen *et al*., 2010). LAB especially genus *Lactobacillus* are one of the most distinguished probiotics and have been reported to be a valuable adjunct to food, ultimately promoting health and vitality to an enormous extent.

Lactic acid bacteria (LAB) have also been described as bioprotective agents (Milani *et al*., 1998). Efficient pathogens' inhibition has been achieved by LAB (Bennik *et al*., 1999). LAB competes with the microbes by modifying the microenvironment with their metabolic end products (Niku-Paavola *et al*., 1999). Moreover, the traditional food preservatives that may lead to the formation of potentially carcinogenic by-products (Nitrosamines from nitrites) have created an interest in natural antimicrobials (Roller, 2003). During last few years there have been great efforts to use living microorganisms as an alternative to chemical pesticide (Janiswicz & Bors, 1995). Strains of the lactic acid bacteria are one of the most relevant biocontrol agents that are categorized as biological control agents (BCA) (Milani *et al*., 1998).

In the last few decades probiotic potential of lactic acid bacteria and their role in inhibition of pathogenic organisms have opened new horizons in the fields of medical sciences and food biotechnology. The use of LAB has been rationalized based on advances in microbiological sciences. The industrial importance of LAB has boosted the scientific advances in research areas such as microbiology, physiology, genetics, and more recently genomics (Mozzi *et al*., 2010). Genes encoding the 16S-rRNA are excellent phylogenetic markers for genus and species level identification and their sequences can generate vast information about the composition of complex bacterial system i.e., human intestinal microbiota. The studies regarding the presence of lactic acid bacteria in dairy products has long been established

but research communities had rarely focused the microflora of fruits for the existence of this particular group of bacteria in Pakistan. However, the microenvironment and nutritive profile of carbohydrate-rich fruits are quite conducive for such bacteria to multiply. Therefore, the probability of finding the specific microbiota of interest is likely to be highly substantial. Lactic acid bacteria are enormously valued not only for their commercial or industrial application but also their significance as a probiotic, antimicrobial agents, bioprotectants and human intestinal commensals is widely acknowledged.

Therefore, in the present investigation the microflora of different fruit samples of Lahore, Pakistan were studied with special focus on lactic acid bacteria. This study provides a prolific scope for commercial preparation of natural probiotic fruit drinks and could serve as an excellent alternative to conventional probiotic dairy products. Such a scenario could be a beneficial story to emerge in the area of food biotechnology.

Materials and Method

Sample collection: Fruit markets in different areas of Lahore, Pakistan were visited and the seasonal fruits of reasonable quality were purchased on random basis between the months of March-2010: July-2010. The samples were collected in sterile plastic seal bags and brought to the laboratory within 24 hours of purchase for routine analysis (Table 1).

Table 1. The list of fruit samples used in the study.

S. No.	Scientific name(s)	Common english name(s)	S. No.	Scientific name(s)	Common english name(s)
1.	*Carica papaya*	Papaya	11.	*Prunus armeniaca*	Apricot
2.	*Citrus paradise*	Grape Fruit	12.	*Prunus domestica*	Plum
3.	*Citrus reticulata*	Orange	13.	*Prunus persica*	Peach
4.	*Cucumis melo*	Melon	14.	*Psidium guajava*	Guava
5.	*Ficus* spp.	Fig	15.	*Punica granatum*	Pomegranate
6.	*Fragaria* spp.	Strawberry	16.	*Saccharum officinarum*	Sugar cane
7.	*Malus* spp.	Apple	17.	*Solanum lycopersicum*	Tomato
8.	*Mangifera indica*	Mango	18.	*Syzygium cuminutesi*	Jamun
9.	*Manilkara zapota*	Cheeko	19.	*Vitis vinifera*	Grape
10.	*Musa* spp.	Banana	20.	*Ziziphus mauritiana*	Ber

Isolation of lactic acid bacteria: To analyze the targeted microflora 'LAB', the fruit samples brought to the laboratory were thoroughly washed with tap water following a final rinse with sterile saline (0.9%) water. Each fruit was processed separately and diced in a sterile Petri plate. Small portion of the solid fruits sample under study was chopped manually with sterile cutter while the citrus fruits (oranges, grapefruits) 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 9 mL of sterile MRS broth (10 g Casein peptone; 10 g Meat extract, 5 g Yeast extract, 20 g Glucose, 1 g Tween-80, 2 g K_2HPO_4, 5 g Na-acetate, 2 g $(NH_4)_2$ citrate, 0.2 g $MgSO_4$-$7H_2O$, 0.05 g; $MnSO_4$-H_2O; pH: 6.2 -6.5) and incubated overnight at 37^{o}C for 48hours with shaking at120 rpm. After incubation samples were appropriately diluted and spread on MRS-agar (MRS broth media with 1.2% agar) plates at a concentration of 50 µL/10mL enriched broth. The plates were incubated an aerobically at 35^{o}C for 24–48 h with upside down until the bacterial growth was evident. Colonies by random selection were picked up and purified by replating on MRS agar plates and further characterized.

Phenotypic characterization of selected isolates: The isolates were characterized for gram and catalase reaction and fermentative catabolism of various carbohydrates. The strains were also tested for their ability to grow at lower pH values (2.0, 2.5, 3.0, 3.5, 4.0, 4.5). The fermentative behaviour was determined for various carbohydrates in Bromothymol blue lactose broth (peptone, 0.35 g; Tryptone, 0.35 g; NaCl, 0.5 g; Bromothymol blue, 0.004 g distilled water, up to 100; pH:7.0) supplemented with inverted Durham's tube and 1.5% of carbohydrate to be tested (arabinose; fructose; galactose; glucose; lactose; maltose; mannitol; raffinose; sucrose, xylose). The strains showing positive gram reaction and negative catalase test were selected and the glycerol stocks were stored at -80^{o}C.

Antibiotic sensitivity test: Antibiotic resistance of the isolated bacterial cultures was determined using discs. The susceptibility to antibiotics of LAB strains was tested through Bioanalyse® Antimicrobial susceptibility tests discs. Fifteen (15) LAB strains were tested against 10 available antibiotics with following concentrations. Ampicillin (A^{10}: 10mcg), Azithromycin (At ^{15}: 15mcg), Ceftazidime (CA^{30}: 30 cmg), Clarithromycin (CW^{15}: 15 mcg), Erytromycin (E^{15}: 15 mcg), Gentamicin (GEN^{10}: 10 mcg), Kanamycin (K^{30}: 30 mcg), Nitrofurantoin (NF^{300}: 300 mcg), Oxacillin (OX^1: 1 mcg), Vancomycin (VA^{30}: 30mcg). The freshly grown, well isolated bacterial colonies were closely streaked on pre-dried MRS agar plates (48x48x34 cm) in 2 cm long lines to form a growth lawn. Antibiotic discs were placed on streaked plates at appropriate distances and incubated for 24 h at 37^{o}C. After incubation the zones formed by each antibiotic were measured using a millimeter scale.

Identification of isolates: The sequence analysis of 16S ribosomal RNA (16S rRNA) gene was employed for identification of bacterial strains. Genomic DNA of selected isolates was isolated by the protocol devised by Rodringuez & Tait (1983) with some amendments. ~1.5 Kb long fragment of the 16S rRNA gene was amplified from the extracted DNA using eubacterial universal primers [Forward primers: P8 (5' AGAGTTTGATCCTGGCTCAG

'3) and Reverse primers: P_C1544 (5' AAGG AGGTGATCCAGCCGCA 3')] specific for 16S rRNA gene (Heller *et al.,* 1997). The amplified products were directly cloned in pTZ57R/T vector using InsTAclone Cloning Kit (Fermentas) according to the instructions provided by the manufacturer. The clones were confirmed for the presence of amplicons via double digestion of plasmids using restriction enzymes *Pst*1 and *Eco* R1. The confirmed clones were repurified using QIA spin Miniprep Kit # 27106. The clones were sequenced using universal primers. The partial genome sequence of approximately 1500 bp long 16S rRNA gene was obtained and sequence homologies were analysed by comparative studies using "The National Center for Biotechnology Information (NCBI) using weblink (http://www.ncbi.nlm.nih.gov/) and Basic Alignment Search Tool (BLAST). The sequences were then aligned with two closest sequences via ClustalV-Multiple Sequences Alignment using web links (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequences were then submitted to Genbank databases.

Nucleotide sequence accession numbers: The strain designation and GenBank accession numbers for the sequences derived from 16S rRNA gene analysis are shown in Figs. 1 and 2.

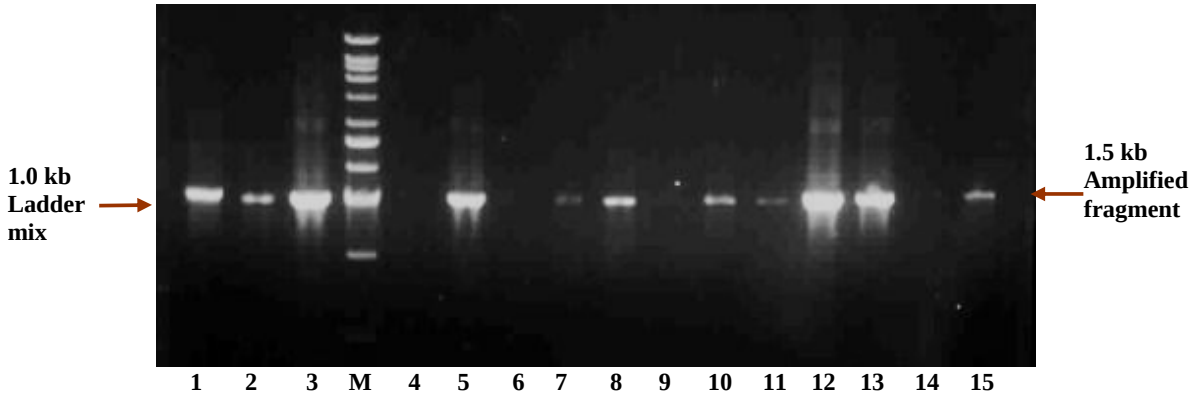

Fig. 1. Amplified PCR product of 16S rRNA gene on 1% agarose gel. PCR was done as describes in materials and methods.

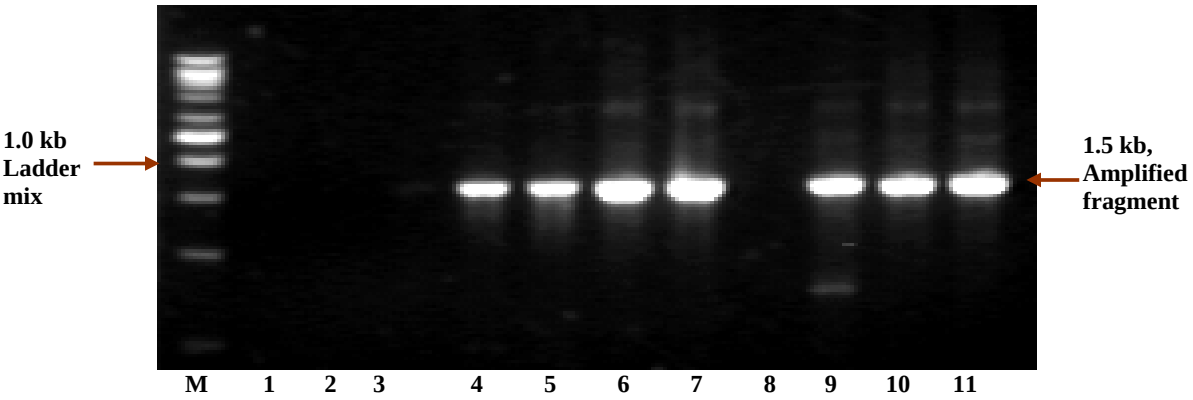

Fig. 2. PCR product of 16S rRNA gene on 1% agarose gel. PCR was done as describes in materials and methods.
The sequences determined in this study have been deposited in the GENbank databases with sequential accession numbers HQ823673, HQ823674, HQ823675 and JF417991.

Results

A total of 15 lactic acid bacterila strains were isolated from 20 fruit samples collected and analysed during present course of work. All isolates were primarily identified on the basis of colony/cell morphology and biochemical tests. Majority of the colonies on MRS agar plates were small whitish to off-white in color; gram positive catalase negative, and were able to grow under anaerobic conditions suggested the related- ness of these species with lactic acid bacteria. Small number of the analyzed fruit samples gave colonies of yeasts with positive catalase reaction. All gram positive and catalase negative isolates were selected for further analysis. All of the selected isolates were found to be heterofermentative and were gas producers. The results of carbohydrate fermentation behavior were found comparable to those stated in Bergyes Manual of Bacteriology, Vol 2. The selected strains were then carefully optimized for growth conditions. Almost all strains were viable at the lower temperature of 28 ^oC but growth retardation occurred at an elevated temperature of 45^oC. The ability of selected strains to survive in low pH conditions were analyzed after subjecting the strains to a low pH shock. Most of the strains were found to survive between pH range of 2.0-4.5. But some of the strains could survive at pH range of 2.0- 2.5.

Concerning antibiotic resistance all 15 isolates bacterial strains were tested for their susceptibility and resistance against 10 available antibiotics. Almost all strains were sensitive to 50% of the 10 antibiotic used in the test but maximum sensitivity was observed for oxacillin and kanamycin.

For strains identification up to specie level 16S rRNA sequence analysis was carried out. The results identified 4 of the 15 isolated bacterial strains as *Lactobacillus plantarum;* one strain as uncultured bacterium and one as *Leuconostoc mesenteroides* ssp. *mesenteroides.*

S. No.	Strain code	Fruit source	Specie	% Homology
1.	MNFS: 1	Orange	-	-
2.	MNFS:3	Grapes	*Lactobacillus plantarum strain* AF1	100%
3.	MNFS:7	Strawberry	*Lactobacillus plantarum strain* AF1	99%
4.	MNFS:8	Grapes	*Lactobacillus plantarum strain* STIII	99%
5.	MNFS:9	Grape fruit	*Uncultured bacterium*	99%
6.	MNFS:10	Ber	*Leuconostoc mesenteroides*	99%
7.	MNFS:11	Plump	*Lactobacillus plantarum strain* ST III	99%
8.	MNFS:14	Jamen	-	-

Discussions

Lactic acid bacteria are generally considered as microorganisms with no pathogenic activities (Sexline *et al.,* 1996). Several clinical applications of LAB against human diseases have attracted the attention of many research groups. Therefore, the bacterial strains reported in the present study appear valuable because these strains also belong to the group lactic acid bacteria. In the course of present work a collection of about 15 LAB was achieved from about 20 different seasonal fruits between the months of March-2010: July-2010.

In the present study the approaches in preliminary identification of isolated bacterial strains of various bacterial species were morphological characterization like positive growth on MRS agar medium, negative catalase activity, colony color, positive gram tests, 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 almost each and every study 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. Instead a predominance of yeasts by this method were found. This correlated the different yeasts species as widespread pollutants isolated from plant leaves, marula fruits, flowers, pulp and juices and occurrence of yeasts in association with LAB on fermentable substrates (Sanni, 1993; Caplice & Fitzgerald, 1999; Fleet, 1999; Jespersen, 2003; Nout, 2003; Nyanga *et al.,* 2007). Consequently, the present investigation focused on enrichment culture as an approach to determine the diversity of lactic acid bacteria associated with fruit samples and used microaerophilic conditions that would prevent the growth of other microbes and their potential to interfere with the recovery of lactic acid bacteria (Bae *et al.,* 2004). 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. The finding of Sieiro *et al*., (1990), determined that only a minority of LAB could be isolated from grape surfaces in one vineyard, and attempts to isolate these bacteria from grape surfaces failed in another vineyard. However, contrary results were reported by Renouf *et al*., (2007; 2008). In the light of contrary results reported by these authors, the possible explanations for failure to isolate lactic acid bacteria from some specimens under consideration are: (i) lactic acid bacteria may not be present in samples (ii) isolation method could be inadequate; and (iii) may be other reasons.

During the present study, conditions were optimized for best growth of bacterial isolates temperature and pH. Following the incubations at optimal temperatures and pH of specimens, acid tolerance profile and antibiotic sensitivities were determined. All the strains in present investigation showed adequate growth in a pH range of 2.5–4.5 most showed growth at pH 2.5. The results were in agreement with those obtained from previous studies where lactic acid bacterial strains were able to retain their viability when exposed to pH of 2.5-4.0, but were unable to grow at lower pH (Conway *et al.,* 1987; Du Toit *et al.,* 1998; Jacobsen *et al.,* 1999).

The pH in the human stomach ranges from 1-4.5 after a meal (Aiba *et al.,* 1998; Bernet *et al.,* 1997). The survivals of specimens studied in present work ascertain their probiotic potential as they could exhibit substantial viability in highly acidic GI tract suppositories. Present investigation deals not only with the distribution of lactic acid bacteria in fruits, but also with antibiotic resistance conferred by these strains against various antibiotics. Many lactic acid bacteria are resistant to antibiotics. The resistance attributes are often intrinsic and non transmissible (Curragh & Collins, 1992). On the other hand antibiotic resistant probiotics can benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in number due to administration of various antimicrobial agents (Salminen *et al*., 1998). In preceding considerations all isolated strains showed resistivity to 50 to 100% antibiotics. Klare *et al*., (2007) and Aquilanti *et al*., (2007) have reported the antibiotic resistance in lactic acid bacteria from animal and human isolates. Among the antibiotic responses, vancomycin resistance is of aforementioned concern because vancomycin is one of the last antibiotics broadly efficacious against clinical infections caused by multi drug resistant pathogens (Johnson *et al.,* 1990). In present study vancomycin reisistance was conferred by the six among 15 strains while all other strains were susceptible

to vancomycin. The resistance to antibiotics conferred by the lactic acid bacteria may be advantageous, as a growing concern in the selection of probiotic strain is the transfer of resistance genes from microbes to animals through food chain. Resistance benefits are greatly associated with their transferability, therefore further studies should be addressed to analyze the resistance in clinical treatments (Ayeni *et al*., 2009).

As stated by Dellaglio & Felis (2005), phenotypic methods alone are inadequate for identification of lactic acid bacteria and should be confirmed by molecular identification methods to achieve a reliable identification. 16S rRNA gene is found to be a powerful tool for appreciating genetic variability among different species (La Scola *et al.,* 2003). Therefore, during present investigations the genetic discrimination of the related species belonging to Lactic acid bacteria group were obtained via PCR amplifications by using 16S rRNA primers, as demonstrated by Heller *et al.,* (1997). The homology analysis inferred from the 16S rRNA sequence comparison clearly verified that most of the strains clustered with *Lactobacillus plantarum* and one with *Leuconostoc* species. *Lact. planatarum*, was the most frequently isolated species in present study. The molecular confirmations of present work were in agreement with many of the previous reports. The predominance of *Leuconostoc mesenteroides* on tomato surfaces; and from tomato fruit puree had been frequently reported by Sajur *et al*., (2007). The microflora of masau fruit were studied in detail by Nyanga *et al*., (2007). Species such as *L. plantarum; L. lactis* have been frequently found in environments associated with plants (Kostinek *et al.,* 2007; Escalante-Minakata *et al*., 2008; Trias *et al*., 2008). Bacteriocins from *Lact. plantarum* have been frequently studied (Rattanachaikunsopon & Phumkhachorn, 2006; Todorov and Dicks, 2006). Certain strains of *Lactobacillus plantarum* and *L. agilis* have been reported to have probiotic effects (Lee and Salminen, 1995). Therefore, the results obtained in this study are encouraging for the consumption of fruits and fruit juices as a probiotic food.

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