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Isolation of Acidophilic Lactic Acid Bacteria Antagonistic to Microbial Contaminants

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Abstract.- Four species of lactic acid bacteria viz. *L. bulgaricus*, *L. casei, L. acidophilus* and *L. salivarius* were isolated from a local yogurt sample. The bacterium *L. salivarius* showed maximum growth at 37°C with initial pH 6. *L. bulgaricus* and *L. acidophilus* also grew best at 37°C but with initial pH 4.The bacterium *L. casei* showed maximum growth at 25°C at pH 5. All the four bacteria formed smooth textured and white yogurt at 2% inoculum. *L. acidophilus* tolerated acid upto 1 pH . However, *L. bulgaricus* and *L. salivarius* showed more resistance to acid when cultured in yogurt as compared to their cultivations in the selective medium. These lactic acid bacteria demonstrated antimicrobial activity against different isolates of *E. coli*, *Staphylococcus* sp., *Salmonella* sp. and yeasts. The *L. acidophilus* was found to be the most potent antagonistic microbe against *E. coli*, while the isolate *L. bulgaricus* expressed vivid antifungal activity. Acid tolerance, yogurt fermentation and bacterial and fungal antagonistic characteristics of these bacterial isolates render them good candidates for their consideration as probiotics.

Key words: Acid tolerant bacteria; bacterial antagonism; lactobacilli; probiotics.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram positive, non-respiring, non-spore forming, cocci or rods which produce lactic acid as major end product from fermentation of carbohydrates. They are important in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, milk, cassava and pickled (fermented) vegetables. Moreover, majority of microorganisms used as probiotics belong to the LAB and bifidobacteria. Within the group of LAB, *Lactobacillus* species are most commonly utilized group of microorganisms for their potential beneficiary properties as probiotics. The antagonistic activity of such bacteria is known to inhibit a large number of enteric and urinary pathogenic bacteria (Gilliland, 1990; Battcock and Azam-Ali, 1998; Hutt *et al*., 2006).

Fuller (1992) defined the term probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance''. A number of issues addressing affectability to inhibit the growth of pathogens, functional and technological aspects must be considered while developing a probiotic product (Sareela *et al.*, 2000; Yeung *et al*., 2002; Pascual *et al*., 2008). *Lactobacillus acidophilus* which survives lowest pH ranges and tolerates the bile too has been proved very successful in preparation of yogurt harboring effective probiotic. These characteristics has made it most effective tool against lactose malabsorption and intolerance (Mustapha *et al*., 1997; de-Varese *et al*., 2001). Introduction of *Lactobacillus acidophilus* in milk makes it more effective in lowering the body cholestrol level (Sarkar, 2003).In fermented foods, LAB display numerous antimicrobial activities. This is mainly due to the production of organic acids and other compounds, such as bacteriocins and antifungal peptides (De-Vuyst and Leroy, 2007; Simova *et al*., 2009)

In Pakistan, enteric infectious health hazards are related to the consumption of contaminated food and water. Presence of coliform bacteria in majority of the drinking water supplies and in various foods, especially those served semi or uncooked (Qazi and Qureshi, 2002; Qazi *et al*., 2006a,b) may contributes to a large number of gastrointestinal infections in this country**.** The milk and milk products used by the masses are mostly processed at household and sale points levels in semi-controlled conditions. There is in fact no quality assurance of such products in this country. Thus, need of local bacterial source for yogurt production containing

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probiotic(s) capable of decontaminating the present levels of objectionable microbial contaminations can not be overemphasized. The present investigation reports isolation and characterization of four *Lactobacillus* species having potential application in the local dairy industry such as yogurt fermentation, source of probiotics and microbial contaminants reducing agents of the product.

MATERIALS AND METHODS

Isolation and characterization of bacteria

de Man Rogosa Sharpe (MRS) selective growth medium acidified with 1NHCl to pH 5.3 was used for the isolation of lactic acid bacteria (Merck, 1996-1997). Yogurt sample collected from the cafeteria of the University of the Punjab Quaid-e-Azam Campus, Lahore was spread on the MRS medium and the plates incubated anaerobically for 72 h at 37°C. Pure cultures of representative colonies were made and preserved on nutrient agar slants for further experiments. For determining cytochrome status, a given isolate was inoculated on nutrient agar containing 0.1% KCN and the plates were incubated at 37°C for 48 hours. Isolates were characterized for Gram's stain, cell morphology, capsule, endospore, motility, catalase and oxidase activities as described by Benson (1994), while tests of aeration, nitrate reduction, sulfide, indole production, CO_2 from glucose and H_2S production were performed according to Merck (1996-1997).

Growth at 15°C and 45°C in tubes containing MRS broth (Collins, 1995) and arginine hydrolysis were noticed (Schillinger and Luke, 1989). For carbohydrate fermentations glucose, fructose, raffinose, rhamnose, glactose, lactose, mannitol, maltose and sucrose were employed with bromothymol blue (Schillinger and Luke, 1989; Merck, 1996-1997). Growths in the presence of 3% and 10% NaCl in nutrient broths were also determined. Based upon the results of above characteristics the bacterial isolates were identified as described by Holt *et al*. (2000).

Growth optima of the bacterial isolates

Bacterial isolates were inoculated in MRS broth and incubated at 25°C (RT), 37°C and 50°C for 48h. For optimization of pH, they were grown in MRS broths having pH 4, 5 and 6 at their respective optimum growth temperatures. The isolates were then incubated in orbital shaker at 100rpm and without aeration at their corresponding pH and temperature optima. MRS broth tubes were inoculated with 1%, 5% and 10% inocula and incubated at their corresponding predetermined optimum growth conditions. For all the experiments, after 48 h growth was determined at 600nm on a Spectronic 20D spectrophotometer against un-inoculated MRS broth as control.

Experimental procedure

Yogurt formation

Bacterial cells were obtained by centrifugating 72 hrs' incubated MRS broth culture at 5000rpm.Following washing the pellets two times with 0.9% saline solution. The cells were suspended in the saline to achieve the O. D. of 0.635 to 0.978. Skimmed milk, 10ml was then inoculated with 2% to 10% inocula for each *Lactobacillus* species and the culture bottles incubated at 43 °C for two days. Then the yogurt formed was stored in freezer for further use.

Acid tolerance

Sterile waters of pH 1, 1.5, 2.0, 2.5 and 3.0 were introduced with 0.1ml of a given 48 hours incubated bacterial culture in MRS broths. The contents of the test tubes were mixed well on the vortex for half minute and incubated at 37°C. After 40 minutes 0.1 ml from each tube was inoculated into the MRS broth and incubated at 37°C for 48 hours. Growth in these test tubes was observed by taking O.D. at 600nm. The cultivation in yogurt was similarly processed for their acid tolerance, however, growth following the acid exposure was observed by streaking the samples on MRS agar plates.

Antibiosis of the isolates

Antagonistic effects of the *Lactobacillus* bacteria against *Escherichia coli, Pseudomonas* sp. *Salmonella* sp. and some yeast isolates were determined by disc diffusion method .Cultures of test organisms were obtained from stock culture of the Microbiology Laboratory, Department of Zoology, University of the Punjab, Lahore and

revived in nutrient broth at 37°C for 24h. Then 20µl culture of a given organism was spread on surface of nutrient agar.

Forty eight hrs incubated culture of a given *Lactobacillus* species in MRS broth was centrifuged at 5000rpm for 10 minutes. The supernatant was saved in sterile bottles and subsequently filtered through Millipore filters of 0.22µm pore size. Watmann filter paper No.1 discs of 9mm diameter were loaded with 150µl of a given cell free culture fluid and allowed to dry. The loaded paper discs were then placed on the test organism inoculated plates and pressed gently. All the plates were kept in incubator at 37°C for 24 hours. Growth inhibition zones were measured to the nearest fraction of a millimeter. Supernatant of experimental yogurt were also processed similarly. Besides growth inhibition zones, thin growth around the experimental discs was noticed as retardation zone and the given cell free culture fluid was considered to be microbistatic. Antagonistic results of various bacterial and yeast isolates were compared by employing single factor analysis of variance and Student's t-test.

RESULTS

Colonies of all the isolates had convex elevations and attained, in general, round configurations on MRS agar. Colonies of the isolates identified as *L. casei* and *L. acidophilus* were nucleated and feathery, respectively. All the species' colonies had opaque and creamy consistency except, those of *L. salivarious* which appeared rubbery. Some other colonial features of the isolated bacteria are shown in Table I. The bacterial isolates expressed Gram +ve streptobacilli cell morphology. All the four isolates were found negative for capsular, catalase, oxidase and cytochrome tests. Results of acid fermentation from different sugars are shown in Table I. Based upon the described colonial and physiochemical characteristics, the bacterial isolates were identified after Holt *et al.* (2000) as *L. bulgaricus, L. casei, L. acidophilus* and *L. salivarious.*

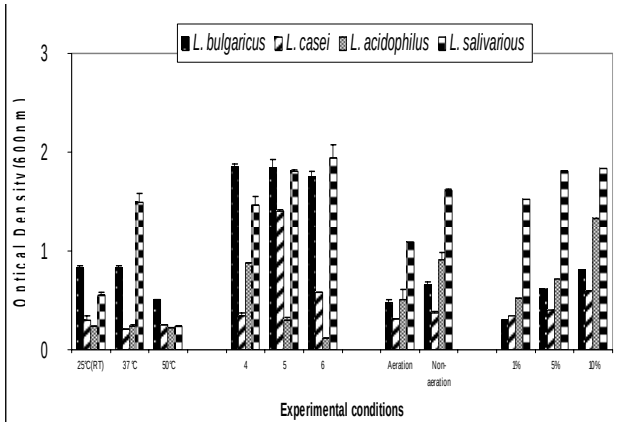
At 48 hours post incubation, majority of the isolates yielded best growth at 37°C under low acidic environment (pH 4 and 5), except the *L. salivarius* which grew best at RT. All the isolates

Table I.- Some colonial and physiobiochemical characteristics and identification of LAB isolated from yogurt sample.

Species.	Colonial characteristics			Acid from sugar fermentation							
	Colour	Margin	Size (mm)	Fructose	Sucrose	Raffinose	Rhamnose	Glactose	Lactose	Mannitol	Maltose
L. bulgaricus	Off-white	Irregular convex	1.3	-	-	+	-	-	-	-	+
L. casei	Off-white	Irregular and raised	4	+	+	-	-	+	+	+	+
L. acidophilus	Off-white	Irregular	1.2	+	-	-	-	-	+	-	-
L. salivarious	Light yellow	Smooth irregular	2	+	+	-	+	-	+	+	+

All the isolates were non-motile facultative anaerobes and found negative for endospore,nitrate reduction, sulfide, Voges Proskaur test, indole, CO_2, H_2S production and arginine hydrolysis. The four species grew at 45ºC, however, no growth was seen at 15ºC and 10% NaCl concentration. *Lactobacillus acidophilus* grew at 3% NaCl. –, negative; +, positive.

revealed highest growth with 10% inocula without aeration (Fig. 1). All the species produced yogurt following inoculations in sterile milk, however, more smooth texture was observed at 2%, 4%, 6% as compared to 8% and 10% inocula. . The bacteria showed, in general, improved acid tolerance in yogurt as compared to their performance in MRS. The bacterium *L. acidophilus* grew well following exposure of pH 1 both in acid water as well in yogurt. The three other species expressed growth following exposure to the pH of 1.5 in yogurt while in acid water treatment they could not grow below pH 2.5.



Fig, 1. Optimization of different growth conditions for LAB.

Cell free culture supernatants of LAB exhibited varying degrees of inhibitory activity against *E. coli*. *L. acidophilus* showed highest inhibitory activity against *E. coli* isolates. A significant inhibition was also observed against a strain of *Staphylococcus* sp. *Lactobacillus salivarious* showed highest antibacterial activity against an isolate of *Salmonella* sp. (Sa-1) and highest antifungal activity against the yeast-5Y with inhibition zone of 15mm. *L. bulgaricus* and *L. salivarius* were found potent inhibitors of *Salmonella* and *Staphylococcus* sp., respectively (Table II).

DISCUSSION

The *Lactobacillus* species reported here produced yogurt and tolerated highly acidic conditions *i.e.*, upto pH 1. These characteristics together with their antagonistic effects against frequently food contaminating microorganisms render them good probiotics candidates. Yogurt has long been supplemented with *Lactobacillus acidophilus* to enhance mucosal and systemic immune responses to cholera (Tejada-Simon *et al*., 1999). *Lactobacillus casei* has been reported to be involved in complex as well as simple yogurt productions (Zanini *et al*., 2007). Isolates reported in this study appear valuable as a single *Lactobacillus* species can be selected for desirable features to obtain yogurt and other fermented milk products fortified with probiotic's advantages.

Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant to survive in the human gastrointestinal tract. The time from entrance to pass through the stomach has been estimated to be approximately 90 minutes (Berada *et al.,* 1991). Stresses to organisms begin in the stomach, with pH between 1.5 and 3.0, and in the upper intestine that contains bile (Lankaputhra and Shah, 1995; Corzo and Gilliland, 1999). Survival at pH 3.0 for 2 h is considered optimal acid tolerance for probiotic strains (Usman, 1999). Strains of lactobacilli reported in this study showed varying levels of growth at pH 3.0, 2.5, 2.0, 1.5 and 1.0 after a 40 minutes incubation period. All the species, in general, survived best under the acidic conditions, while *L. acidophilus* tolerated well upto pH 1. Comparable results have been reported by Liong and Shah (2005) who indicated that *L. acidophilus* and *L. casei* survived best under the acidic conditions.

Commercial lactic acid bacterial products have been one of the major health related foods in the world. When a LAB is used as probiotic, it should be safe and possess some basic characteristics, such as the tolerance to acid, ability to adhere to human intestinal epithelium and having antagonistic activity against bacterial pathogens (Johnson and Case, 1995). Bacteriocins derived from lactic acid bacteria are usually small, heterogeneous, cationic proteins consisting of 30 to 60 amino acid residues (Lima and Andreatti, 2005). However, owing to their protein nature these antagonistic substances are inactivated by the proteolytic enzymes (Mathot *et al*., 2003; Pascual *et*

Table II.- Antibacterial and antifungal activities of cell free MRS culture fluids of the LAB.

Test organism code	*L. bulgaricus*	*L. casei*	*L. acidophilus*	*L. salivarious*
E. coli- E4	12± 0.28and 17R±0.01	11.5±0.00	11±0.57	11±0.28
E. coli-E5	$12^{c,}$±1.15 and 14R±0.03	11.5^{c}±0.28	$25^{a,b,10}$±0.57	11^{c}±0.57
E. coli-E6	14^{b}±0.57	$11.5^{a,c}$±0.28	$15^{b,d}$±0.28	12.5R±0.28
E. coli-E7	13^{c}±0.28	13^{c}±0.17	$26^{a,b,10}$±0.57	14^{c}±0.86
E. coli-E8	$13.5^{b,c,d}$±0.86	11^{a}±0.17	11^{a}±0.57	11.5^{a}±0.57
Salmonella sp-Sa-1	12^{b}±0.28	11^{d}±0.57	N	14^{a}±0.17
Salmonella sp-Sa-2	$15^{b,d}$±0.17	$11^{a,d}$±0.28	N	$13^{a,b}$±0.17
Staphylococcus sp-St-1	16R±0.02	N	12^{d}± 0.28and 20R±0.06	12^{c}±0.28
Staphylococcus sp-St-2	14.2^{d}±0.28	N	N	13^{a}±0.57
Yeast sp-1Y	13*±0.50	N	N	12*±0.11
Yeast sp-4Y	14±0.10	EGD	N	N
Yeast sp-5Y	14*±0.11	EGD	16R±0.10	15*±0.05
Yeast sp-6Y	12*±0.15	N	N	13* ±0.05 EGZ

Values represent diameters of growth inhibition and growth retardation (R) zones in mm and are mean ± S.E.M of three replicates
Values sharing common superscripts are not significantly different from each other within a rows at $p \leq 0.05$; single factor analysis of variance
Values with asterisk(s) are significantly different from each other within a row at $p = \leq 0.05$ Student's t-test
N, No inhibition zone; EGZ, excessive growth around inhibition zone; EGD, excessive growth around disc
Disc diameter: 9mm

al., 2008; Simova *et al*., 2009). Due to enhancement of immune response and increase of indigenous lactobacilli counts by administration of probiotic strains, suppression of pathogens could be modulated in the host (Michalkiewicz *et al.,* 2003; Monack *et al.* 2004; Songisepp *et al.,* 2005).

Antimicrobial effect exerted by the LAB is mainly due to acid production, hydrogen peroxide, fatty acids, aldehydes and other compounds (Daeschel, 1989). Xanthopoulos *et al*. (2000) indicated that *L. paracasei subsp.* isolated from infant feces had weak antibacterial activity against *Escherichia coli* and *Yersinia enterolitica*. Hutt *et al*. (2006) had also reported that the *Lactobacillus* strains possess intermediate potency for out-competing cystitis-causing *E. coli* from the large intestine. *L. bulgaricus, L. casei, L. salivarious* of this study expressed but weak antibacterial activity against *E. coli.* However, *Lactobacillus acidophilus* showed a strong (26mm zone of inhibition) antibacterial activity against *E. coli*-E6, and expressed a zone of growth inhibition of 25mm against *E. coli* -E5. Goderska and Czarnecki (2007) reported that *L. acidophilus* gave a growth inhibition zone of 26mm against *H. pylori* and *E. coli*. No inhibitory effect against *Salmonella* sp. was observed at all. Millette *et al*. (2007) reported no inhibition against gram negative bacteria after 8 h of fermentation by a mixture of *Lactobacillus acidophilus* and *Lactobacillus casei.* The varying results from different locations may be due to large number of differences inherent to the microbes evaluated as probiotics, test organisms and the physiochemical environments of assays' procedures.

Molds and yeasts are important spoilage organisms in different food and feed systems including yogurt and other fermented dairy products (Pitt and Hocking, 1997). Regarding availability of efficient and safe procedures to prevent fungal growths in raw and prepared foods, LAB producing antibacterial substances have long been reported (El-Gendy and Marth, 1980; Batish *et al*., 1990; Suzuki *et al*., 1991). The species *L. bulgaricus* and *L. salivarious* showed antifungal activities. These acidophilic LAB showing inhibitory activities against the harmful/opportunistic pathogenic bacteria and yeast together with their acid tolerance range and yogurt formation potential may find

useful applications in dairy, food, poultry and pharmaceutical industries. Logically their incorporation in animal feeds and evaluation in terms of growth promotion and enteric infections controlling roles under controlled experimental conditions would be the first step to look forward for their further usefulness.

It is concluded from the present study that the *Lactobacillus* species isolated from the yogurt appeared strong antagonistic against *E. coli* isolates while inhibiting, *Salmonella and Staphylococcus* sp. too. They survived in the pH range of 1-3 and produced yogurt. Fermentation of animal feeds employing some of these microbes and subsequent trial experiments may discern growth and health promoting roles of these "probiotics".

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