



SHORT COMMUNICATION

In vitro pre-selection criteria for probiotic *Lactobacillus acidophilus* TS1 isolated from fermented milk product, Dahi

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ABSTRACT

Aims: The aim of this research was to evaluate some probiotic traits of *Lactobacillus acidophilus* TS1 strain previously isolated from dahi.

Methodology and results: For this purpose, tested strain was evaluated for their resistance to low pH, tolerance to bile and *in vitro* antibiotics susceptibility. It was observed that the strain TS1 remained viable at pH 3.0 to 6 and bile concentration of 0.1 to 0.3%.

Conclusion, significance and impact of study: *L. acidophilus* TS1 was found resistant to nalidixic acid, ciprofloxacin, gentamycin and vancomycin. The results highlighted the probiotic potential of *L. acidophilus* TS1 which deserves further investigation *in vitro* studies to elucidate its health benefits.

Keywords: probiotics, *L. acidophilus*, bile, antibiotics sensitivity, dahi

INTRODUCTION

In the last two decades, antimicrobial peptides have been gaining attention as antimicrobial alternatives to chemical food preservatives and commonly used antibiotics. Due to emergence of multi-drug resistant bacteria as serious problem over the past decades, major research efforts are aimed at finding effective drug(s). Under such conditions lactic acid bacteria (LAB) and their metabolites are considered to be the best option for antimicrobial agents. Therefore, these LAB are considered to be the useful source of probiotic bacteria, which provide a positive balance for maintaining the normal flora of intestinal tract (Isolauri, 2001). These probiotic bacteria may produce different types of metabolites viz organic acids (lactic and acetic acids), bacteriocins and reuterin which are responsible to inhibit the growth of numbers of pathogens. They also provide an aid in stimulating immune responses, preventing infection by enteropathogenic bacteria, reducing cholesterol, and treating and preventing diarrhea (Reid, 1999). Therefore there is growing interest in the commercial use of lactobacilli isolated from traditional naturally-fermented dairy products such as probiotics. Lactobacilli are known to produce many types of bacteriocins like acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin and

lactobrevin (Alvarez-Olmos and Oberhelman, 2001). In order to provide good health benefits, *Lactobacillus* strains must have to overcome some physical and chemical barriers in the gastrointestinal tract. The most common criteria for probiotics strains selection are acid and bile tolerance as well as resistance to antibiotic (Collins *et al.*, 1998; Petros *et al.*, 2006).

It is well documented that isolation and screening of LAB are mostly carried out from indigenous fermented milk products. Therefore, these products are considered to be the natural source of genetically-stable strains of industrial importance. In this regard the health secret of indigenous dahi is well known due to the presence of probiotic *Lactobacillus* sp. that varies from region to region. Therefore, there is a dire need to access the probiotic potential strain of *L. acidophilus* isolated from arid region of Pakistan. In previous study, a total of fourteen *L. acidophilus* strains have been isolated from dahi. Among these, *L. acidophilus* TS1 strain was considered to be a potential candidate for the broad spectrum antimicrobial activity against Gram positive and Gram negative bacteria (Maqsood *et al.*, 2008). Therefore, the present study was designed to access the some desirable probiotic traits of *L. acidophilus* TS1 such as acid resistance, bile tolerance, and antibiotic resistance *in vitro*.

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MATERIALS AND METHODS

Microorganism isolation and purification

Lactobacillus acidophilus TS1 isolate used in this research was previously isolated from the indigenous dahi (Maqsood *et al.*, 2008). One gram of freeze-dried powder of this isolate was transferred aseptically into 50 mL sterile de Man, Rogosa and Sharpe (MRS) broth supplemented with 0.5% L (+) cysteine-HCl (99.6% purity, Sigma, USA), then incubated at 37 °C for 20 h in an anaerobic jar (Oxoid, UK).

Repeated streaking onto MRS agar plates was used for purification for this isolate. One colony from each plate was picked up and inoculated into 5 mL sterile MRS broth. The isolate was activated by making sub-culturing twice in MRS broth containing 0.5% cysteine-HCl (Sigma), as reducing agent, using 1% inoculum and 18-20 h of incubation at 37 °C in an anaerobic jar (Oxoid).

Probiotic potential of *L. acidophilus* TS1

In order to study the probiotic properties, bacteriocin producing strain of *L. acidophilus* TS1 was further investigated for its ability to tolerate acid and bile along with resistance to certain commonly used antibiotics.

Acid tolerance

Activated culture of TS1 strain was examined for acid tolerance from pH 2 to 6 in MRS medium incubated at 37 °C at the rate of 1% according to the procedure of Charteris *et al.* (1998). Aliquots of 0.1 mL were removed after 3 h for determination of viable counts. Total viable counts were determined by the pour plate method in MRS agar after serial dilutions in buffered peptone-water (0.1% bacteriological peptone, 0.85% NaCl). Plates were incubated anaerobically at 37 °C for 48 h.

Bile tolerance

Bile tolerance was checked in MRS broth containing 0.1-0.5% bile salt (Oxoid). Before testing for bile tolerance, *L. acidophilus* TS1 was incubated for 18 h at 37 °C in MRS broth without bile. After centrifugation (7000 rpm for 10 min, 4 °C), the collected cells were re-suspended in 0.5 mL MRS broth was used as inoculum. A volume of 5 mL MRS broth containing 0.1-0.5% bile salts was inoculated with 50 µL of the inoculums and incubated for 3 h at 37 °C. The bacteria were plated and enumerated after 24 h of incubation.

Antibiotic susceptibility test

The disc diffusion method was used to check the antibacterial susceptibility of the *L. acidophilus* TS1 on MRS agar plates (Klare *et al.*, 2005). The assay was performed as follows: MRS broth was inoculated by *L.*

acidophilus TS1 at 37 °C. Turbidity of bacterial cultures was adjusted to 0.5 McFarland and with the help of sterile cotton swab a lawn was made of this culture on MRS agar plate by streaking the entire surface of the MRS agar plates three times and turning the plate 60 degree between streaking to obtain uniform inoculation. Later, ten antibiotic disc (6 mm) of size:, nalidixic acid (30 mg), ampicillin (30 mg), penicillin (10 AU), amoxicillin 30 mg, ciprofloxacin (5 mg), tetracycline (30 mg), erythromycin (15 mg), vancomycin (30 mg), gentamicin (10 mg), sulphamethoxazol (25 mg) were placed aseptically on the inoculated plates and incubated at 37 °C for 24 h. The diameter of the zones of inhibition were measured and results were expressed as sensitive (S; 26-33 mm), intermediate (I; 12-16 mm) and resistant (R; 0-12 mm).

RESULTS

Probiotic potential for *L. acidophilus* TS1

Acid and bile tolerance of *L. acidophilus* TS1

The effect of acid and bile tolerance of selected strain was determined by growing the bacteria in MRS broth containing Oxgall of different concentration of 0.1 – 0.5% (w/v) and pH (2, 3, 4, 5 and 6) at 37 °C respectively. Results in Table 1 revealed that *L. acidophilus* TS1 remained their survival in the presence of Oxgall % concentration ranging from 0.1 to 0.3 % and pH at 3.0 to 6.0.

Antibiotic susceptibility test

L. acidophilus TS1 was also tested for its susceptibility against different antibiotics commonly used against different infectious bacterial strains. It was found that *L. acidophilus* TS1 was sensitive to various chemotherapeutic agents viz amoxicillin ampicillin, erythromycin, tetracycline and penicillin, resistant to nalidixic acid, ciprofloxacin, gentamicin and vancomycin (Table 2), and was intermediately susceptible to sulphamethoxazol.

DISCUSSION

Ingested probiotics are exposed during their transit through the GIT to successive stress factors that influence their survivability. Passing through the stomach, which has a pH as low as 1.5, is one hurdle that faces probiotics on their way to the intestines (Marteau *et al.*, 1997). Probiotic bacteria must first survive transit through the stomach, before reaching the intestinal tract (Dunne *et al.*, 2001). The food transit time through the human stomach is about 90 min. Accordingly, probiotic bacteria should be able to tolerate acid for at least 90 min (Chou and Weimer, 1999). The effect of acidity on the viability of the *L. acidophilus* TS1 is presented in Table 1. There was a

Table 1: Effect of acid and bile tolerance on survival percentage of *L. acidophilus* TS1 after 3 h of incubation at 37 °C.

pH					Oxgall%				
2.0	3.0	4.0	5.0	6.0	0.1	0.2	0.3	0.4	0.5
7±0.3	61±0.2	72±0.2	84±0.4	98±0.3	98.7±0.3	96.6±0.2	94.7±0.3	–	–

Each value in the table represents the mean value ± standard deviation from triplicates.
 –: No growth

Table 2: Antibiotic susceptibility test of *L. acidophilus* TS1 against different antibiotics.

Antibiotics	Zone size (mm)	Susceptibility Pattern
Nalidixic acid (30 µg)	0	R
Ampicillin (30 µg)	26	S
Ciprofloxacin (5 µg)	10	R
Tetracycline (30 µg)	30	S
Penicillin (10 U)	32	S
Sulphamethoxazol (25 µg)	14	I
Erythromycin (15 µg)	16	S
Gentamicin (10 µg)	12	R
Amoxicillin (30 µg)	33	S
Vancomycin (30 µg)	0	R

R: resistant; I: intermediate sensitivity; S: susceptible

marked variation in counts after 3 h of exposing to different level of pH.

It has been reported that approximately 2.5 L gastric juice (pH ± 2.0) is secreted daily in the stomach is variable and strain-dependent (Clark *et al.*, 1993; Charteris *et al.*, 1998; Zavaglia *et al.*, 1998; Charteris *et al.*, 1998), forming an environment which inhibits the growth of most of the microorganisms (Kimoto *et al.*, 1999). Growth of strain *L. acidophilus* TS1 was repressed at pH 2.0, but not completely inhibited, suggesting that they may have some intrinsic resistance to acid and may survive conditions in the small intestine. Similar results have been reported for *L. plantarum* 423, *L. salivarius* 241, *L. curvatus* DF38 and *Lactococcus lactis* subsp. *Lactis* HV219 (Brink *et al.*, 2006; Todorov *et al.*, 2007). The ability of probiotic bacteria to survive passage through the stomach). Loss of viability has also been reported for *Lactococci* in stimulated gastric juice (Kimoto *et al.*, 2000).

Another factor that should be considered in selecting probiotic culture is bile resistance, which enables a selected strain to survive, grow and perform therapeutic benefits in the intestinal tract (Usman and Hosono, 1999). Bile acids are synthesized from cholesterol and conjugated to either glycine or taurine in the liver, then stored in the gall bladder. They are secreted from the gall bladder into the duodenum in the conjugated form (500-700 mL/d). These acids then undergo extensive chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) in the intestine as a result of microbial activity (Dunne *et al.*, 2001).

The bile stress for ingested microorganisms in the gastrointestinal tract (GIT) is complex because bile concentrations and residence times vary in each compartment of the GIT (Marteau *et al.*, 1997). Gilliland *et al.* (1984) and Gilliland and Walker (1990) pointed out the

importance of bile tolerance of probiotic strains used as dietary adjunct. The effects of bile salt resistance are shown in Table 1. Bile concentrations in the normal healthy individuals range from 0.3 to 0.5% (Zavaglia *et al.*, 1998; Dunne *et al.*, 1999). *L. acidophilus* TS1 displayed good intrinsic resistance to bile concentrations of 0.1 to 0.3%. Similar levels of bile resistance have been reported by El-Naggar (2004) and Tambekar and Bhutada (2010). The relative good intrinsic resistance of strain *L. acidophilus* TS1 to bile may promote survival in the gastrointestinal tract. However, according to Marteau *et al.* (1997) sensitivity to bile is not necessarily a disadvantage as lysis of cells during passage through the gastrointestinal tract may result in the release of β-galactosidase and improve the digestion of lactose in the small intestine. The mechanism of bile acids resistance is not well-understood yet. Although, many reports indicated that the presence of bile salt hydrolase (BSH) enzyme is responsible for bacterial resistance to bile acids (Gilliland and Walker, 1990; Tanaka *et al.*, 1999). This enzyme has been detected in the gut microflora genera such as *Lactobacillus* and *Bifidobacterium* (Tanaka *et al.*, 1999). Thus, for more accurate assessment of the ability of *L. acidophilus* TS1 strain to survive the stress conditions in the upper GI tract, *in vivo* assessments in further studies are required. Still based on the *in vitro* criteria used in this study, the strain of *L. acidophilus* TS1 can be considered as potential probiotic strain. Although *L. acidophilus* considered among the non-starter LAB flora in indigenous fermented dairy products, selected strain from such source may be used as adjunct culture in mixed starter preparations to deliver possible valuable probiotic activities.

Knowledge related to susceptibility of LAB to antimicrobial agents is limited due to a large number of

genera and species that are present in this particular group of bacteria along with their difference in their resistance pattern (Ocana *et al.*, 2006). Moreover, this resistance pattern and their survival are mostly associated with their adaptability to the environmental conditions (Herrerros *et al.*, 2007). Therefore, in view of the above, before a strain of LAB can be used as a food additive it must undergo antibiotic resistance screening to ensure its safe application. Antibiotics are a major tool utilized by the medical and pharmacological industries to fight pathogenic bacteria; however, antibiotic resistance can cause significant danger and suffering of a number of people with common pathogenic infections, and is a growing problem that complicates the treatment of important nosocomial and community-acquired infections (Mathur and Singh, 2005; Moellering *et al.*, 2007). In order to be used as a probiotic, LAB that exhibit profitable effects on the health of the host must show an ability to resist various antibiotics. Therefore, the antibiotic resistance of acid- and bile-resistant *L. acidophilus* TS1 was also assessed. It was found to be highly resistant to vancomycin, gentamicin, and nalidixic acid and was susceptible to amoxicillin, penicillin, tetracycline, ampicillin and erythromycin. The antibacterial susceptibility tests indicated that *L. acidophilus* TS1 were resistant towards many of the antibiotics tested. The results were as expected as Lactobacilli are known to be naturally resistant against several antibiotics (Charteris *et al.*, 1998; Liasi *et al.*, 2009). In particular, the antibiotic resistance and susceptibility of LAB in the present study, was found to be similar to the results of Zhou *et al.* (2008), who reported that *Lactobacillus* sp. and *Bifidobacterium* sp. were sensitive to erythromycin, tetracycline, and ampicillin, whereas, resistant to that of streptomycin. Many research works on the antibiotic sensitivity and resistance of LAB, which were isolated from various products and human or animal gastrointestinal tracts, have been reported; the Lactobacilli strains isolated from infant faeces were resistant to kanamycin and streptomycin, but affected by amoxicillin, chloramphenicol, erythromycin, penicillin G, and tetracycline (Arici *et al.*, 2004); all *Oenococcus oeni* isolated from wine showed susceptibility to tetracycline, chloramphenicol, rifampicin and erythromycin whereas, they showed resistance to vancomycin, aminoglycosides, trimethoprim and sulfamethoxazole which could represent intrinsic resistance (Rojo-Bezares *et al.*, 2007).

CONCLUSION

It was concluded that the susceptibility and resistance of various LAB to many antibiotics were variable, depending on the species but it is generally considered as an intrinsic property. Amongst the tested antibiotics, vancomycin, an inhibitor of cell wall synthesis is of major concern, as it is one of the last antibiotics broadly effective against clinical infections caused by multi-drug resistance pathogens. *L. acidophilus* TS1 was also found resistant towards vancomycin. Resistance of these antibiotics is usually intrinsic, as it does not transfer the genes to pathogenic or

bacterial flora. Still, there is a need of systematic research to access their resistances against antibiotics in strains intended to use in food and feed in order to avoid their inclusion as starter and probiotics.

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