African Journal of Microbiology Research Vol. 5(12), pp. 1428-1436, 18 June, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR10.737
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Screening and characterization of new potentially probiotic lactobacilli from breast-fed healthy babies in Pakistan

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Accepted 15 June, 2011

Probiotic bacteria are used for the protection and improvement of human intestinal microflora with added health effects. In search of new probiotics, 61 lactobacilli isolates were screened for survival in acidic conditions and simulated gastrointestinal juices, tolerance to ox-gall and antimicrobial activity. Selected strains (NWS09, NWS11, NWS14, NWS19 and NWS29) were identified by sequencing their 16S rRNA gene and 16S-23S rRNA gene spacer region, and further characterized by the absence of transferable antibiotic resistance, adhesion to Caco-2 cells and survival in gastrointestinal tract of BALB/c gnotobiotic. NWS09 had transferable resistance to erythromycin conferred by *erm*(B) gene, while NWS14 was resistant to tetracycline. NWS29, identified as *Lactobacillus fermentum* showed remarkable tolerance to simulated gastrointestinal juices and bile and highest antimicrobial activity against 5 food borne pathogens by producing heat (100°C) and pH resistant bacteriocins. *L. fermentum* NWS29 was also found to be highly adhesive to human caco-2 cells *in vitro* as compared to NWS11 and NWS19 and was isolated in highest numbers from BALB/c gnotobiotic after 24, 48 and 72 h of orogastric inoculation. *L. fermentum* NWS29 was identified as a probiotic strain that can be incorporated in functional foods for human use.

Key words: Probiotic, *Lactobacillus*, acid and bile resistance, antimicrobial activity, Caco-2 cells.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). The major organisms used as probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, but also other strains have been used. Probiotics can prevent or reduce the gastrointestinal disorders (Lenoir-Wijnkoop et al., 2007), risk of allergy (Boyle and Tang, 2006; Ouwehand, 2007). Although, there has been a growing interest in using lactobacilli isolated both from naturally fermented products and humans for health benefits (Lim and Im, 2009). However, it has been preferred and proposed to use probiotics from human origin. Probiotic effects are species and strain specific which require careful screening for the selection of a true probiotic lactobacilli (Collins et al., 1998; Morelli, 2000; Vinderola et al., 2008). Probiotic bacteria must have the ability to survive passage through the gastrointestinal tract by overcoming physical and chemical barriers, especially acid and bile stresses (Del Piano et al., 2006; Mattila-Sandholm et al., 2002), adherence to intestinal epithelial cells (Schillinger

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et al., 2005) and protective effect against pathogens (Saarela et al., 2002).

Attachment and colonization of gut epithelium is very important to exert beneficial effects as it prolongs the time for microorganism, to influence the immune system and microbiota of the host (Forestier et al., 2001; Kirjavainen et al., 1998). Although, lactobacilli generally show a long history of safe use and have acquired the "Generally recognized as safe" (GRAS) status, their safety must be carefully assessed, with particular attention to transferable antibiotic resistance (Mathur and Singh, 2005; Saarela et al., 2000; Salminen et al., 1998). It is not fully clear how probiotic microbes exert their beneficial effect on health, but the most documented and well accepted mechanisms are the production of antimicrobial substances, the competition for nutrients, the competitive exclusion of pathogen binding, removal of toxins and immunomodulation (Isolauri et al., 2001; Parvez et al., 2006).

Exploration of new environments and populations for distinct strains of a probiotic species provide an opportunity for better and safest probiotic strains. There have been a number of reports for isolating the probiotic lactobacilli from human gastrointestinal tract (Dunne et al., 2001; Koll et al., 2010; Morelli, 2000); none is from healthy babies in Pakistan. With different environments, social set up and immunity in Pakistani babies, we aimed to isolate, identify and characterize probiotic lactobacilli. The isolates were characterized by (i) tolerance to low pH, (ii) resistance to bile, (iii) survival in simulated gastrointestinal juices, (iv) antimicrobial activity, (v) antibiotic resistance profile (vi) adhesion to intestinal cells and (vii) survival in BALB/c mice. The competitiveness of selected isolates with reference to a probiotic strain *Lactobacillus rhamnosus* GG was also evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Wild type lactobacilli (n = 61) were isolated (NWS01-NWS61) from stools of breast-fed healthy babies (n = 19) from Pakistan. All samples were blended and diluted in phosphate buffer saline and 10 fold dilutions were plated onto MRS agar plates (Oxoid) and incubated at 37°C for 48 h in anaerobic environment (Anaerogen, Oxoid). Distinguished colonies were selected, purified and stored in MRS broth with 15% (w/v) glycerol at -20°C. *L. rhamnosus* GG ATCC 53103 was used as control strain for probiotic evaluation. *Escherichia coli* ATCC25922, *Salmonella typhi* CMCC50013, *Shigella dysenteriae* CMCC51383, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC15313 were used as indicator strains for antimicrobial activity. *Enterococcus faecalis* ATCC 29212 and *E. faecium* ATCC 35667 were used as control strains in antibiotic sensitivity testing. Prior to all experiment bacterial isolates were subcultured at least twice.

Identification of *Lactobacillus*

All isolates were identified to be *Lactobacillus* by polymerase chain reaction (PCR) using genus specific primers XB5 and LbLMA1-R. For species identification, 16S rRNA gene and 16S-23S rRNA gene spacer regions were amplified and sequenced using primers given in Table 1. DNA sequences were submitted to GenBank.

Resistance to low pH and survival in gastrointestinal juices

Fresh cultures (1 ml) of lactobacilli were centrifuged at 3500 g resuspended in phosphate buffer saline (PBS) and 100 µl was inoculated in 10 mL of MRS broth at pH 7 and pH 2.5. Optical density was recorded after 48 h of incubation in anaerobic environment at 37°C and well grown isolates were selected for future evaluation. Survival of lactobacilli isolates in simulated gastrointestinal juices was determined according to the method described by Bao et al. (2010). Simulated gastric juice (pH 2 and pH 2.5) was inoculated with 1% fresh culture of test isolate and incubated anaerobically at 37°C. Total viable counts were determined at 0, 1, 2 and 3 h of incubation. After 3 h incubation in gastric juice, 10% of culture was inoculated in simulated intestinal juice pH (8.0) and incubated anaerobically. Total viable counts were determined at 0, 3, 6, 12, 24 h of incubation to evaluate percentage survival in intestinal juice. Total viable counts were determined by plating the serial dilutions on MRS agar and incubating in anaerobic environment at 37°C for 48 h.

Tolerance to bile

1% fresh culture of test isolate was inoculated in MRS broth with 0.3% ox-gall and MRS without ox-gall for 24 h, followed by monitoring the absorbance at 600 nm for 9 h or until a 0.3 unit change in O.D. Lag time of an isolate was calculated as difference in time needed for 0.3 unit change in O.D with or without 0.3% ox-gall. To determine the highest level of ox-gall tolerated, isolates showing a lag time of less than 2 h, were selected and inoculated at an amount of 1% in MRS broth with serially higher concentrations of ox-gall as 0, 0.3, 0.4, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0% and incubated in an anaerobic environment at 37°C. Absorbance was recorded at 600 nm at 0, 12 and 24 h.

Antimicrobial activity against pathogens

The well diffusion assay as described by Vinderola et al. (2008) was used to determine the ability of lactobacilli isolates to inhibit intestinal pathogens.

Antibiotic resistance

Antibiotic resistance to penicillin, erythromycin, clindamycin, gentamicin, tetracycline chloramphenicol, streptomycin, kanamycin and vancomycin was determined by E-test on lactic acid bacteria susceptibility test medium (LSM) plates containg 10% MRS medium and 90% iso-sensitest medium (Oxoid, US) and E-test strips (AB Biodisk, Sweden) were applied. Plates were incubated in an anaerobic environment at 37°C and minimum inhibitory concentrations (MICs) were read directly according to the manufacturer's instructions. Breakpoints were adopted from guidelines prepared by European food safety authority (2008). Antibiotic resistance genes including *erm*(B), *tet*(M), *tet*(K), and *tet*(O) were amplified as described previously (Nawaz et al., 2011).

Adhesion to caco-2 cells

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium

Table 1. Nucleotide sequences of primer sets used for PCR in this study.

Target gene	Primer sequence (5'→ 3')	T_a℃	Size (bp)	Reference
16S rRNA	8FLP-F: AGT TTG ATC CTG GCT CAG XB4-R: GTG TGT ACA AGG CCC GGG AAC	55	1380	(Relman et al., 1990) (Xu et al., 2004)
16S-16S/23S spacer	XB5-F :GCC TTG TAC ACA CCG CCC GT LbLMA1-R:CTC AAA ACT AAA CAA AGT TTC	55	250	(Nawaz et al., 2011) Dubernet et al., 2002)
16S-23S rRNA	XB5-F : GCC TTG TAC ACA CCG CCC GT R: TGC CAA GGC ATC CAC CGT	55	420	(Nawaz et al., 2011) (Oliveira and Ramos, 2002)
erm(B)	F:GAA AAG RTA CTC AAC CAA ATA R: AGT AAC GGT ACT TAA ATT GTT TAC	52	642	(Sutcliffe et al., 1996)

(DMEM) (Sigma, USA) with 10% v/v fetal calf serum Fetalclone II (Hyclone, Germany) at 37℃ in 5% CO_2 atmosphere. Two week old monolayers were inoculated with exponentially grown isolates (approximately 10^8 cfu)) followed by 1 h incubation at 37℃ in 5% CO_2 atmosphere. Bacteria were detached by treatment with 0.5% triton or trypsin for 10 min and enumerated by plate counting and real time PCR, respectively. Lactobacilli genus specific primers (Table 1) were used for real time PCR enumeration as described by Candela et al. (2005). Exact numbers of lactobacilli used in each experiment was also enumerated by plate counting on MRS agar.

Survival and retention in BALB/C mice

Eight week old female BALB/c mice (6 to 8 mice per group) were purchased and maintained in University animal centre, housed at 12 h light/dark cycle. Use of animals was reviewed and approved by the Animal Use and Care Committee of the School of Medicine, Xi'an Jiaotong University. Mice were orogastrically inoculated with a single dose (100 µl) of lactobacilli containing 2×10^9 CFU in PBS. Feacal samples were collected at 3, 6, 9 18, 24, 36, 38, 60 and 72 h and lactobacilli were enumerated by plate counting on MRS agar containing vancomycin (100 µg/ml). Single colonies were confirmed by microscopy and genus specific PCR.

RESULTS

Isolation and Identification of isolates

All 61 isolates were identified to be *Lactobacillus* by genus specific PCR. BLAST result of 16sRNA and 16sRNA-23sRNA spacer region by genebank database showed that NWS19 is *L. rhamnosus* while; NWS09, NWS11, NWS14 and NWS 29 belong to *Lactobacillus fermentum* species. The GenBank accession numbers of sequences submitted to gene bank are from HQ026755-HQ026759 and HQ111079- HQ111083.

Resistance to low pH and survival in gastrointestinal juices

Seventeen isolates (28%) were able to grow well (O.D ≥ 0.800 at 600 nm) in acidic conditions at pH 2.5 after 48 h incubation. These seventeen isolates were subjected to treatment with gastro-intestinal juices and 80% survival in gastric juice at pH 2.5 for 3 h. This was used as the standard to select potential probiotic organisms. Five strains survived more than 80% in simulated gastrointestinal juices (Table 2). NWS29 showed highest survival in gastric and intestinal juice (92 and 82%, respectively) on pH 2.5. Furthermore, NWS29 tolerated simulated gastric and intestinal juices (pH 2) for more than 6 and 24 h, respectively (Figure 1). None of the other strain survived for more than 12 h in gastrointestinal juices at pH 2.

Tolerance to ox-gall

Delay in growth (lag time) in 0.3% ox-gall, for 6 isolates (NWS09, NWS11, NWS14, NWS19, NWS29 and NWS53) was less than 2 h. NWS53 was poorly represented by tolerance to low pH and consequently was not selected for further evaluation. Highest tolerable bile concentrations for NWS9, NWS11, NWS14, NWS19 and NWS29 are presented in Figure 2. NWS09, NWS19 and NWS29 tolerated highest percentage (1.8%) of ox-gall, while NWS11 and NWS14 showed growth on 1 and 1.2% of ox-gall, respectively.

Table 2. Survival of selected strains and LGG in gastrointestinal juices.

Isolate	Gastric juice at pH 2.5 log cfu/ml			Intestinal juice (pH 8.0) log cfu/ml		
	0 H	03 H	Survival (%)	0H	6H	Survival (%)
NWS09	7.345 ±0.039	5.954 ±0.009	81.0[a]	5.954 ±0.009	3.901 ±0.053	65[a]
NWS11	7.781 ±0.042	6.321 ±0.033	81.2[a]	6.321 ±0.033	3.256 ±0.067	51.5[b]
NWS14	7.123 ±0.070	6.113 ±0.040	80[b]	6.113 ±0.040	2.081 ±0.054	34[c]
NWS19	7.633 ±0.032	6.932 ±0.031	90.8[c]	6.932 ±0.031	3.951 ±0.060	57[d]
NWS29	7.715 ±0.054	7.139 ±0.042	92.5[d]	7.139 ±0.042	5.914 ±0.049	82[e]
LGG	7.823 ±0.074	6.991 ±0.063	89.3[e]	6.991 ±0.063	5.456 ±0.063	77.8[f]

Values presented are means of duplicate determinations. ± Indicates standard deviation from the mean, [a,b,c,d,e,f] Within the same column followed by different superscript letters differ significantly ($P < 0.05$).

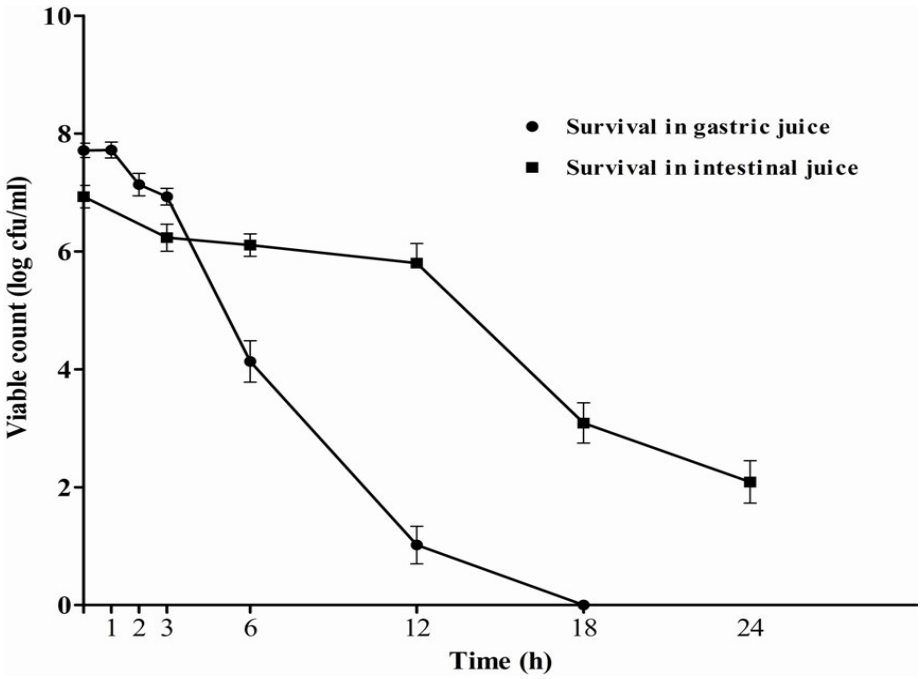

Figure 1. Effect of simulated gastric juice (pH 2.0) and simulated intestinal juices (pH 8.0) on *L. fermentum* NWS29 as evaluated by viable counting after different time intervals.

Antimicrobial activity

Inhibition of five pathogenic bacteria by 5 isolates and *L. rhamnosus* GG is shown in Table 3. Isolates showed strain-specific antibacterial potential against pathogenic strains to varying degrees, from no inhibitory activity to activity against one or two bacteria. NWS29 showed highest antimicrobial activity against all tested pathogenic strains. Bacteriocins produced by all these strains were resistant to low pH and 100°C, except the bacteriocin of NWS19, which lost activity on 100°C for one hour.

Antibiotic resistance

Selected strains (NWS11, NWS14, NWS19 and NWS29) were susceptible to penicillin (MICs 0.06-0.5), erythromycin (MICs 0.06-0.5), clindamycin (MICs 0.06-0.5), gentamicin (MICs 0.06-1.5), tetracycline (MICs 0.06-0.5), chloramphenicol (MICs 0.5-8), and streptomycin (MICs 2-8), except *L. fermentum* NWS09 and NWS14, which were resistant to erythromycin (MICs >256) and tetracycline (MIC 64), respectively. By implying specific PCR, the *erm*(B) gene was successfully amplified from *L. fermentum* NWS09, while no tetracycline resistant genes was detected from *L. fermentum* NSW14. All isolates had intrinsic resistance for vancomycin (MICs > 256).

Adhesion to caco-2 cells

Before the adhesion assay, the number of caco-2 cells

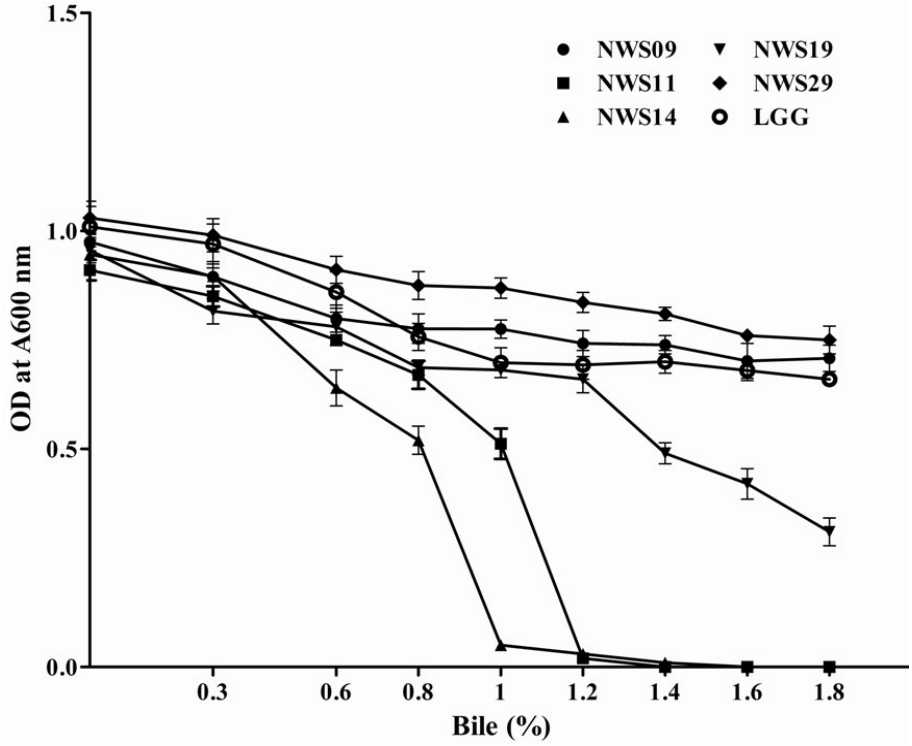

Figure 2. Effect of different concentrations ox-gall in MRS broth on *L. fermentum* NWS09, *L. fermentum* NWS11, *L. fermentum* NWS14, *L. rhamnosus* NWS19, *L. fermentum* NWS29 and *L. rhamnosus* GG as determined by optical density at 600 nm.

Table 3. Antimicrobial activity of selected strains against pathogenic bacteria.

Isolate	*E. coli*	*Sal. typhi*	*Shig. dysenteriae*	*S. aureus*	*L. monocytogenes*
NWS09	+	-	++	+	+
NWS11	+	++	+	-	-
NWS14	+	+	+	-	+
NWS19	++	++	+	+	-
NWS29	++	+	++	++	+

+: Diameter of inhibition zone between 2-4mm; ++: diameter of inhibition zone 4mm or above; -: no inhibition zone.

was calculated in four wells and an average number of 4.32×10^5 Caco-2 cells/ well were used for all calculations. The pH specific considerable variation was observed among the bacteria regarding their ability to adhere to caco-2 cells *in vitro*. NWS 29 and NWS11 were adhesive, while NWS19 showed moderate to low adhesion on pH 4.5 and 7 (Table 4). Difference between PCR and culture method was not statistically significant ($P < 0.05$).

Survival and retention in BALB/c gnotobiotic mice

NWS11, NWS19 and NWS29 were isolated in high numbers ($> 10^8$ cfu) from BALB/c mice faeces after 6 and9 h of inoculation. After 24 h NWS29 was in significantly higher numbers as compared to NWS11 and NWS19. NWS 11 was not detected after 36 h, while NWS19 was in fairly low numbers. NWS29 was retained and survived well in BALB/c gastrointestinal tract as shown by its isolation after 72 (Figure 3).

DISCUSSION

Before reaching the intestinal tract and exerting beneficial effect on host, probiotic bacteria must first survive in the stomach. Stomach pH is known to fluctuate; when an individual is fasting the stomach pH may be as low as 1.5 to 2. Ability to grow at low pH in MRS broth is often used

Table 4. Adhesion of selected strains and LGG to caco-2 cells.

Isolate	pH	Log added cfu ±S.D	Attached cfu/caco-2	
			Plate counting	Real time PCR
NWS11	4.5	8.206 ±0.031	3.9[a]	4.1[a]
	7.0		1.41[b]	1.96[b]
NWS 19	4.5	8.161±0.037	1.02[c]	0.98[c]
	7.0		0.785[d]	0.88[d]
NWS 29	4.5	8.1875±0.029	21.4[e]	19.2[j]
	7.0		2.92[f]	3.10[j]
LGG	4.5	8.230±0.018	9.38[g]	11.43[k]
	7.0		2.33[h]	1.67[l]

Presented values are means of duplicate determinations. [a,b,c,d,e,f,g,h,i,j,k,l] Within the same or right column followed by different superscript letters differ significantly ($P < 0.05$).

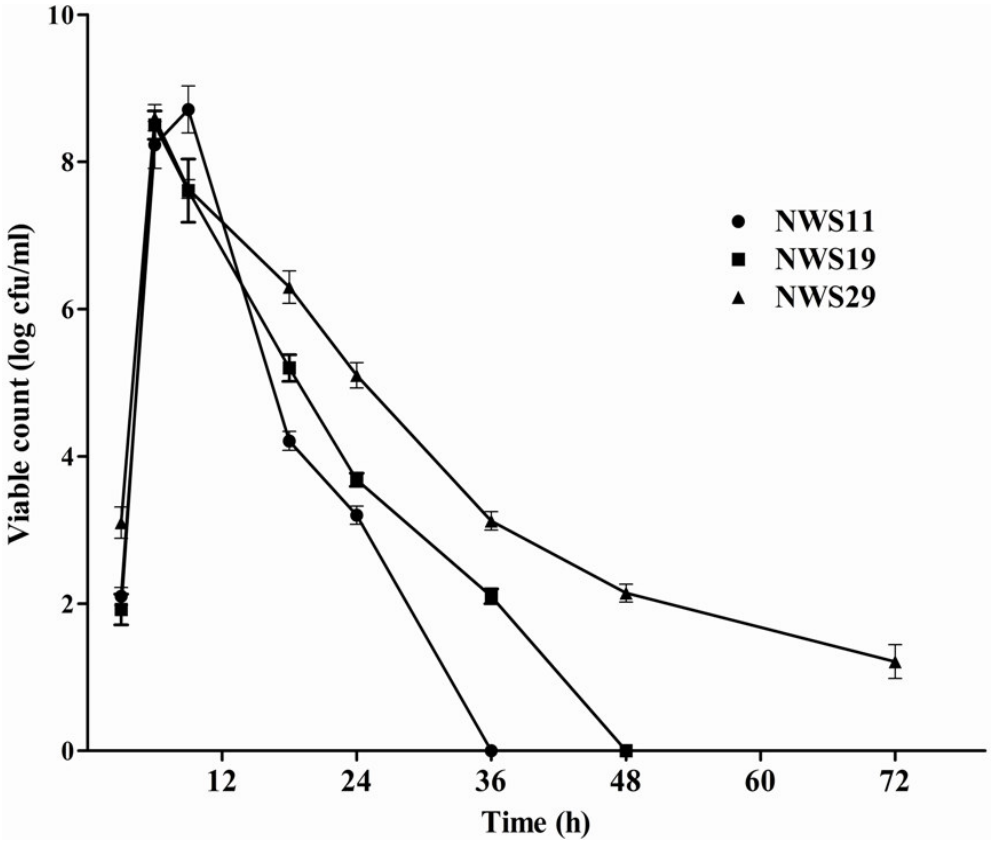

Figure 3. Survival of *L. fermentum* NWS11, *L. rhamnosus* NWS19 and *L. fermentum* NWS29 as determined by viable counting from faeces of BALB/C mice, collected at different time intervals, on MRS agar supplemented with vancomycin.

for probiotic selection. However, survival of bacterial strains in human gastric juice is a more accurate indication of the ability of strains to survive passage through the stomach (Dunne et al., 2001). NWS29 was highly tolerant to pH and simulated gastrointestinal tract, making it an excellent candidate for potential probiotic. In previous reports, Dunne et al. (2001) showed that *L. fermentum* KLD could survive well in human gastric juice

at pH 2.5. Coeuret et al. (2004) and Bao et al. (2010) also reported high rate of survival of different probiotic strains of lactobacilli at pH 2.5 but not at pH 1.2. *L. fermentum* strains have shown almost 100% survival under simulated stomach acidic conditions (Mathara et al., 2008).

Concentration of bile salt can be as high as 1.5 to 2% (w/v) in the first hour of digestion, and may decrease afterwards to around 0.3% (w/v) (Noriega et al., 2004). Resistance to bile salt is species and strain specific (Bao et al., 2010; Morelli, 2000) and a *Lactobacillus* strain that can grow in physical bile concentration could survive in gastrointestinal tract (Sanders et al., 1996). Micro-organisms tolerate bile by activity of bile salt hydrolase enzyme or some components of food can reduce the toxic effect of bile on microorganisms (Bao et al., 2010; du Toit et al., 1998). A prior exposure to lower pH for three hours could also result in the induction of resistance to bile.

Antimicrobial activity is an important mechanism to exert probiotic properties, and considerable research has focus on the production of antimicrobial substances from lactobacilli in last decade (Bao et al., 2010; Batdorj et al., 2007; Servin, 2004). Growth of pathogenic bacteria is inhibited by production of antimicrobial compounds such as organic acids, hydrogen peroxide, diacetyl and bacteriocins by lactobacilli, as well as their competition for nutrients (Loessner et al., 2003; Makras et al., 2006). Ability of bacteriocin of NWS29 to inhibit the pathogenic bacteria and its resistance to heat and low pH, indicate the probiotic potential of *L. fermentum*. On the basis of resistance to low pH, gastrointestinal juices and bile, 5 selected strains were further evaluated.

Lactobacilli are generally susceptible to penicillin, erythromycin, clindamycin, gentamycin, chloramphenicol and tetracycline. Resistance to erythromycin and tetracycline in *L. fermentum* is acquired resistance and has also been reported, previously (Cataloluk and Gogebakan, 2004; Fons et al., 1997; Gfeller et al., 2003). All isolates in our study also had species-dependent intrinsic resistance to vancomycin, which is not transferable and can be exploited in probiotic therapies coupled with antibiotics (Ammor et al., 2007; Klare et al., 2007). Presence of *erm*(B) gene in *L. fermentum* indicates that a careful evaluation must be done before giving probiotic status to any strain. Any acquired resistance present in nutritional or probiotic strain may act as a reservoir for antibiotic resistance, and transfer that resistance to other microorganisms, including pathogens (Mathur and Singh, 2005; Nawaz et al., 2011). NWS09 and NWS14 were not evaluated further because of acquired resistance in these strains.

The adhesion ability to epithelial cells and mucosal surfaces is considered an important property for probiotics. It is a multistep process, which depends on composition, structure and forces of interaction between bacterial and intestinal epithelial cells (Bao et al., 2010; Dunne et al., 2001). Caco-2 is a human intestinal cell line, which expresses morphological and physiological characteristics of normal human enterocytes. Caco-2 cell lines have been exploited to elucidate the mechanisms of adhesion of enteropathogenic and probiotic bacteria in human gut (Dunne et al., 2001; Schillinger et al., 2005).

Adhesion ability of probiotic to Caco-2 cells can be detected by enumerating the attached bacteria by plate counting or real time PCR (Candela et al., 2005; Matijasic et al., 2003). We used both of these methods and did not find a statistically significant difference. Many of the previous studies have shown that a direct relation exists between the number of attached bacteria and the number of added bacteria (Lee et al., 2000; Matijasic et al., 2003; Tuomola and Salminen, 1998), which explain a little lower number of *L. rhamnosus* GG 2.33 cfu/Caco-2 attachment as compared to previously described as 3.25 cfu/Caco-2 (Matijasic et al., 2003). Comparison of the adhesion ability of these isolates with *L. rhamnosus* GG was made as the number of added bacteria in adhesion assay, was identical or in a close range.

Significantly, higher adhesion of LGG and NWS29 on pH 4.5 as compared with pH 7, demonstrate that pH can influence the attachment of bacteria on cultured epithelial cells (Matijasic et al., 2003). Although, adhesion to Caco-2 cells is an important criterion for selection of probiotics, it can be misleading too, as the strongly adhesive strains *in vitro* can have with poor survival *in vivo* and vice versa. We established the survival of NWS29 in BALB/c gnotobiotic for more than 72 h, which provide it sufficient time to exert its probiotic effect on host.

In conclusion, all 61 lactobacilli isolates were subjected to different parameters for the evaluation of their probiotic properties. Taking in account all the results, *L. fermentum* NWS29 was identified as probiotic strain for human use, with strong antimicrobial activity and ability to survive in GI tract. Further studies are needed before the incur-poration of this strain in human food Chain as a probiotic. Currently, we are analyzing the immunomodulatory and antiallergic potential of this strain.

ACKNOWLEDGMENT

This work was sponsored by Higher Education Commission (HEC) of Pakistan.

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