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Screening and evaluation of *Lactobacillus* spp. for the development of potential probiotics

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Probiotics are live microbial food ingredients that have beneficial effects on consumption. The present study searched fermented food samples for potential lactobacilli that could be used for the development of novel gastrointestinal probiotics. Fifty five (55) isolates were obtained including 46 bacterial and 9 yeast isolates. On the basis of morphological and biochemical properties, 21 were identified as *Lactobacillus* spp. Systematic procedures were used to evaluate the probiotic properties of the *Lactobacillus* isolates including carbohydrate fermentation, autoaggregation, acid tolerance, bile resistance and pancreatin tolerance test. 85% of the examined *Lactobacillus* spp. showed the ability to form auto-aggregate, 75% can withstand bile while all the strains were able to tolerate pancreatin. Commercially available probiotic strain *Lactobacillus casei* was isolated and tested for functional criteria required to be a beneficial probiotics and results were compared.

Key words: *Lactobacillus*, probiotics, fermented food, auto-aggregation, bile tolerance.

INTRODUCTION

The concept of probiotics evolved at the turn of 20th century from a hypothesis first proposed by Nobel Prize winning Russian scientist Elie Metchnikoff (Bibel, 1988), who suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products because the fermenting bacilli (*Lactobacillus*) positively influenced the microflora of the colon, decreasing toxic microbial activities (Sanders and Huisin't, 1999). Probiotics can be classified as "live microorganisms that, when consumed in an adequate amount, confer health benefit on the host" (Join FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Foods. London, Ontario, April 30 and May 1, 2002:1-11). The major bacterial species that have been considered as probiotics over the years are *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium longum* and some other *Bifidobacterium* species (Arvanitoyannis and Houwelingen-Koukaliaroglou, 2005).

The potential and established health benefits associated with the usage of probiotics are: prevention of enterocolitis, sepsis (in very low birth weight infants),

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Abbreviations: IBS, Irritable bowel syndrome; **MRS,** Man Rogosa Sharpe; **OHOL,** obligately homofermentative lactobacilli; **FHEL,** facultatively heterofermentative lactobacilli; **OHEL,** obligately heterofermentative lactobacilli; **µL,** micro litre; **MR-VP,** methyl red VogesProskauer; **WHO/FAO,** World Health Organization/Food and Agriculture Organization; **w/v,** weight/volume.

diarrhea and *Helicobacter pylori* infections and prevention of cancer, heart disease, irritable bowel syndrome (IBS), ulcerative colitis (in adults), rheumatoid arthritis, nasal and food allergies, atopic dermatitis by competitive exclusion of pathogens, stimulation of mucus secretion and modulation of immune response (Leroy et al., 2008).

The main factors that promote growth in the global market of probiotics are increasing levels of health-consciousness and the availability of probiotics in the form of dietary supplements. The market of probiotic products generated US$15.9 billion in 2008 and is forecasted to reach US$ 28.8 billion in 2015. The major factors that have facilitated market growth of probiotics are the appropriate components for formulation and the scientific knowledge of the provided benefits (Markets and Markets, 2009; Global Industry Analystic, 2010).

Probiotic microorganisms are host specific; thus a strain selected as a probiotic for one animal may not be suitable in another animal. Furthermore, microorganisms selected for probiotic use should exhibit certain characteristics, they must be able to adhere to intestinal mucosa, easily cultivable, should be non-toxic, non-pathogenic to the host and moreover exert beneficial effect on the host. The microorganisms must be able to produce useful enzymes or physiological end products that can be used by the host. They should remain viable for long time and withstand acidic pH of the stomach (Leroy et al., 2008). Hence the current investigation was planned to isolate best suited microorganisms from the fermented food stuffs.

MATERIALS AND METHODS

Isolation of lactobacilli strains

Twenty seven fermented food samples like home made curd, dosa batter, idli batter, jalebi batter, lassi and yogurt were collected in pre-sterilized containers aseptically and stored at 4°C till further processing. One gram of appropriately diluted sample was spreaded on freshly prepared Man RogosaSharpe (MRS) agar and incubated at 30°C for 24-72 h. Isolated colonies of distinct morphology were sub-cultured on fresh MRS plates. All the isolates were preserved as glycerol stocks at -20°C for further use.

Phenotypic/physiological identification of isolates

Provisional identification of *Lactobacillus* spp. was based on their ability to grow in MRS broth, positive Gram reaction, absence of endospore and rod shaped cells. Biochemical characterization of the isolates was carried out by evaluating the production of catalase, oxidase, arginine hydrolysis, methyl red Voges Proskauer (MR-VP) reactions, nitrate reduction test, and starch hydrolysis test (Hammes and Hertel, 2009). The *Lactobacillus* was categorized on the basis of fermentation group and physiological properties of isolates. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies obligately homofermentative lactobacilli (OHOL); growth both at 15 and 37°C without gas production is characteristic of facultatively heterofermentative lactobacilli (FHEL), whereas gas production at 37°C and variable growth at 15°C are characteristic of obligately heterofermentative lactobacilli (OHEL) (Koll et al., 2010).

Testing of auto-aggregation ability

Auto aggregation ability is the indicator of adhesion ability and tolerance of gastrointestinal environmental conditions, which is considered as prerequisite for screening lactobacilli for their functional properties. Lactobacilli were grown for 48 h at 37°C on MRS agar plates. 10 µL of culture was suspended on a glass microscope slide in 1 ml of 0.9% saline solution (pH 6.7). Auto-aggregation was then determined by the ability to form aggregates (clearly visible sand-like particles) within 2 min at room temperature. The results were expressed as: score 0- no auto-aggregation, score 1- intermediate autoaggregation (presence of some flakes), and score 2- strong auto-aggregation (Pascual et al., 2008).

Testing of acid tolerance

The effect of low pH on the survival of lactobacilli was examined by inoculating 1% of freshly prepared seed culture in 20 mL MRS broth with pH 2.0, 5.0 and 7.0 followed by incubation at 37°C and 200 rpm for 24-48 h. Growth was measured by taking OD after a time interval of 24 and 48 h at 620 nm (Systronics, Japan). Increase in optical density was considered as reflection of growth at 37°C (Koll et al., 2008).

Testing of bile tolerance

The effect of bile on the survival of lactobacilli was examined by inoculating 1% 24 h old culture in MRS broth containing oxbile (2% w/v) followed by incubation at 37°C and 200 rpm (Koll et al., 2010). Growth was measured by taking OD after a time interval of 24 and 48 h at 620 nm (Systronics, Japan). Increase in optical density was considered as reflection of growth at 37°C.

Testing of pancreatin tolerance

The effect of pancreatin on the survival of lactobacilli was examined by inoculating 1% 24 h old culture in MRS broth containing pancreatin (0.5% w/v) followed by incubation at 37°C and 200 rpm for 24-48 h (Koll et al., 2010). Growth was measured by taking OD after a time interval of 24 and 48 h at 620 nm (Systronics, Japan). Increase in optical density was considered as reflection of growth at 37°C.

RESULTS AND DISCUSSION

Isolation of lactobacilli strains

Fifty five (55) strains were isolated from 27 fermented food items. Of these, 46 were bacterial isolates and nine were yeast isolates. Based on colony morphology, 55 isolates were selected for further study. The purified colonies of isolates were stored on slants and glycerol stocks at 4 and -20°C, respectively. Fermented food samples are considered as good source of *Lactobacillus* spp. The samples used for the study are indigenous part of diet of the Indian people. Fermented foods including fermented dairy products are known to have beneficial health effects in addition to improving food safety. Many

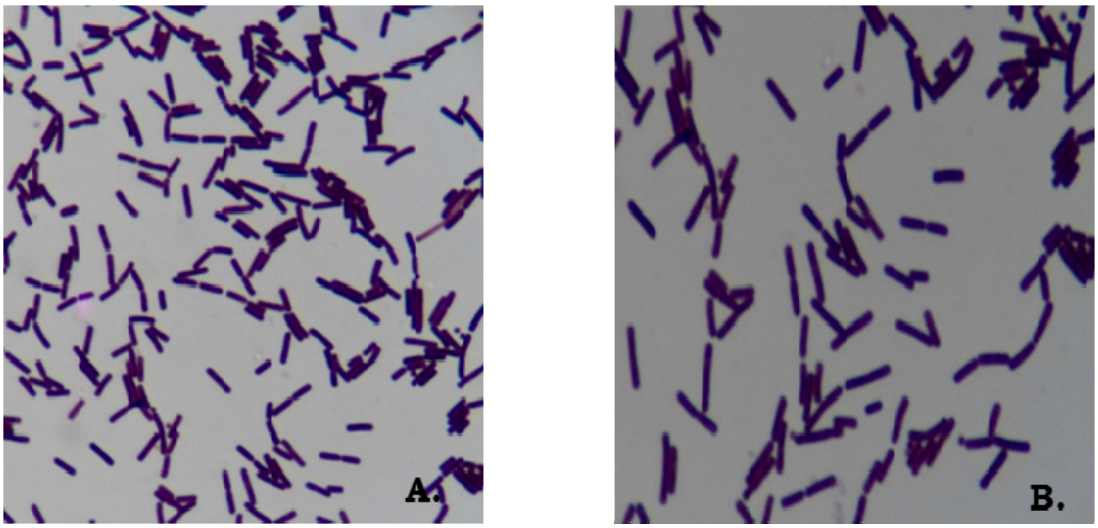

Figure 1. Morphological features of isolates under light microscope after Gram's reaction (100x) A. NKC17(3B); B. NKC18(5C).

Lactobacillus spp. have been used for the fermentation and as natural means of preserving perishable foods. Erdogrul et al. (2006) isolated 21 strains from ten fermented food samples. *Lactobacillus* spp. has a long history of natural occurrencein a variety of food products and has GRAS status. Clinical studies underline the safe use of *Lactobacillus* in humans (Todorov and Dicks, 2008).

Morphological and biochemical identification of isolates

The colonies with pure white glistening, convex, small (2-3mm diameter) with regular margins were subcultured on MRS agar to obtain pure culture. The provisional identification of lactobacilli was based on the growth of Positive Gram's reaction, non endospore forming rods on MRS agar with negative catalase reaction (Hammes and Hertel, 2009). Out of 46 bacterial isolates, 40 were found to be Gram positive, 26 isolates were without endospore and among them 21 gave negative catalase reaction (Figure 1) and hence considered as lactobacilli.

Catalase and oxidase tests indicated the negative nature of the isolates (MacFaddin, 1980). All twenty-one isolates including the control strain (*Lactobacillus casei*) isolated from yakult were found to be oxidase and catalase negative. Similar findings were recorded by Erdogrul et al. (2006). Arginine hydrolysis was performed in MRS broth containing 0.3% arginine (Spano et al., 2002). In the current study, sixteen strains out of twenty-one were able to hydrolyze arginine. *Lactobacillus* vary in their ability to degrade arginine and those able to derive energy from arginine catabolism may be more competitive in the stressful environment of wine (presence of acid and alcohol) than those stains that are unable to degrade arginine (Liu and Pilone, 1995; Spano et al., 2002).

All the 21 isolates were found to be MR-VP negative, and were unable to hydrolyze starch and reduce nitrate. Hammes and Hertel (2009) have reported that *Lactobacillus* spp. are unable to reduce nitrate and hydrolyze starch. Findings of the current study are comparable to that of Forouhandeh et al. (2010) in which they reported that none of their isolates exhibited nitrate reduction and starch hydrolysis.

For detecting fermentation group, some physiological properties were assessed using glucose as the test sugar. In the present study out of 21 isolates, eight were identified to be OHOL; seven were identified to be FHEL; six were identified to be OHEL (Table 1).

Testing of auto-aggregation ability

Aggregation is important for biofilm formation and may assist probiotic bacteria to adhere to mucus and epithelial cells and survive harsh conditions in the gastrointestinal tract (Lepargneur and Rousseau, 2002; Reid and Burton, 2002). Adhesion ability is considered as a prerequisite for screening intestinal lactobacilli for their functional properties (Collado et al., 2007; Mercenier et al., 2008; Sathyabama et al., 2012). In the current study, three strains showed no auto-aggregation ability, nine strains showed intermediate auto-aggregation ability and nine strains including NKY1H1 (commercially available probiotic strain) showed strong auto-aggregation ability (Table 2). Koll et al. (2010) have reported that 59% of their test strains showed auto-aggregation and 30 strains were strongly autoaggregative. Auto-aggregationis strain-

Table 1. The *Lactobacillus* strains showing acid and gas production from glucose-MRS broth at 15 and 37°C.

Strain ID	At 15°C (48 h)		At 37°C (48 h)	
	Acid	Gas	Acid	Gas
NKI1A1	+	-	+	-
NKC1F2	+	+	+	+
NKY1H1	-	-	+	-
NKC5J2	-	-	+	-
NKJ1K1(L)	+	+	+	+
NKC6L2	+	-	+	-
NKC7M1	+	-	+	-
NKC9O3	+	+	+	+
NKJ1K2(S)	+	+	+	+
NKC11R1	-	-	+	-
NKC12S2	-	-	+	-
NKC13T2	-	-	+	-
NKD2V1	+	-	+	-
NKC17(1B)	+	+	+	+
NKL1(2B)	-	-	+	-
NKL1(2C)	+	-	+	-
NKC17(3A)	+	-	+	-
NKC17(3B)	-	-	+	-
NKC18(4A)	+	-	+	-
NKC18(5B)	+	+	+	+
NKC18(5C)	-	-	+	-

NKY1H1 commercially available probotic strain (*Lactobacillus casei*); OHOL obligately homofermentative lactobacilli (- - + -); FHEL facultatively heterofermentative lactobacilli (+ - + -); OHEL obligately heterofermentative lactobacilli (+ + + +).

Table 2. Auto-aggregation ability of isolates.

Strain ID	Auto-aggregation score
NKI1A1	1
NKC1F2	1
NKY1H1	2
NKC5J2	0
NKJ1K1(L)	1
NKC6L2	2
NKC7M1	1
NKC9O3	2
NKJ1K2(S)	2
NKC11R1	0
NKC12S2	1
NKC13T2	1
NKD2V1	2
NKC17(1B)	1
NKL1(2B)	2
NKL1(2C)	0
NKC17(3A)	1
NKC17(3B)	2
NKC18(4A)	1
NKC18(5B)	2
NKC18(5C)	2

NKY1H1 commercially available probotic strain (*Lactobacillus casei*); Score 0- no auto-aggregation; score 1- intermediate auto-aggregation; score 2- strong auto-aggregation.

specific and most probably involves species-specific surface proteins (Todorov and Dicks, 2008).

Testing of acid tolerance

Probiotic strains have to survive harsh conditions in the gastrointestinal tract (GIT), e.g. pH values ranging from 1.0 to 3.0 in the stomach (Mainville et al., 2005). Resistance to low pH is thus important criteria for the selection of probiotic bacteria (Havenaar et al., 1992).The effect of low pH was studied to determine the ability of twenty-one isolates to survive at low pH of stomach during their passage through gastrointestinal tract (Koll et al., 2010). In this study, at pH 2 isolate NKC12S2 showed good growth after 48 h of incubation. At pH 5, isolates NKC5J2, NKC17(3B) andNKL1(2B) showed good growth after both 24 h and even after 48 h of incubation. NKC17(1B) showed good growth after 24 and 48 h of incubation. NKC17(3A) and NKC18(5C) showed good growth after 24 h incubation but growth declined after 48 h of incubation. Strain NKY1H1 showed fair growth at both pH after 24 h of incubation but this growth declined significantly after 48 h of incubation (Table 3).

In some reported cases, none of the strains grew at pH 2.5 and only very few were reasonably acid tolerant (Jacobsen et al., 1999; Awasthy et al., 2008). This difference in acid tolerance of strains from two species within the same genus may be due to differences in the cell wall structure (Conway et al., 1987).

Testing of bile tolerance

Before reaching the distal part of the intestinal tract and exerting their probiotic effect, these bacteria must survive during transition through the stomach and upper part of the intestinal tract (Bao et al., 2010). Resistance to elevated concentration of bile salt is an important selection for probiotic bacteria in order to survive harsh environment of gastrointestinal tract (Todorov and Dicks, 2008). Lan-Szu and Bart (1999) have substantiated that strains selected as probiotic bacteria should tolerate bile for at least 90 min, which is the time needed to cross the barrier; in the present study, tolerance was checked for 24 and 48 h of incubation. Although the bile concentration of human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v (Sjovall, 1959; Gilliland et al., 1984). In the present study, bile

Table 3. Effect of pH on the growth of selected isolates (OD at 620 nm).

Strain ID	pH 2		pH 5	
	O. D. After 24 h	O. D. After 48 h	O. D. After 24 h	O. D. After 48 h
NKI1A1	0.490	0.150	0.896	0.671
NKC1F2	0.465	0.440	0.788	0.650
NKY1H1	0.422	0.115	0.731	0.688
NKC5J2	0.118	0.118	1.052	1.209
NKJ1K1(L)	0.437	0.120	0.744	0.810
NKC6L2	0.336	0.635	0.842	0.680
NKC7M1	0.203	0.712	0.812	0.679
NKC9O3	0.174	0.600	0.726	1.312
NKJ1K2(S)	0.379	0.461	0.767	0.701
NKC11R1	0.120	0.117	0.733	0.676
NKC12S2	0.572	1.030	0.729	0.665
NKC13T2	0.796	0.668	0.619	0.634
NKD2V1	0.589	0.159	0.679	0.712
NKC17(1B)	0.472	0.704	0.770	1.564
NKL1(2B)	0.628	0.408	1.465	1.404
NKL1(2C)	0.153	0.136	0.754	0.651
NKC17(3A)	0.959	0.128	1.061	0.750
NKC17(3B)	0.753	0.135	0.957	1.801
NKC18(4A)	0.118	0.393	0.774	0.626
NKC18(5B)	0.538	0.143	0.713	0.921
NKC18(5C)	0.858	0.157	2.040	0.737

NKY1H1 commercially available probotic strain (*Lactobacillus casei*).

Table 4. Tolerance of the selected strains towards bile and pancreatin (OD at 620 nm).

Strain ID	Bile		Pancreatin	
	O. D. after 24 h	O.D. after 48 h	O. D. after 24 h	O.D. after 48 h
NKY1H1	0.325	0.263	0.356	0.811
NKC6L2	0.421	0.221	0.548	6.020
NKC9O3	0.334	0.730	0.835	7.347
NKJ1K2(S)	0.689	6.120	0.339	0.705
NKD2V1	0.786	8.13	0.228	0.683
NKL1(2B)	0.267	0.611	0.321	0.657
NKC17(3B)	0.498	0.668	0.483	5.610
NKC18(5C)	0.372	0.641	0.532	6.021

NKY1H1 commercially available probotic strain (*Lactobacillus casei*).

concentration of 2% w/v was taken. In this study, eight strains were tested for bile tolerance. Interestingly, six strains NKJ1K2(S), NKD2V1,NKC9O3, NKL1(2B), NKC17(3B) and NKC18(5C) not only survived high bile concentration but showed equally good growth after both 24 and 48 h. Commercially available probiotic strain NKY1H1 showed poor growth after incubation period of 24 h and the growth declined even more after 48 h incubation (Table 4).

This property may provide these strains with an advantage *in vivo* because upon exposure to bile acids, cellular homeostasis disruptions causes the dissociation of lipid bilayer and integral protein of their cell membranes, resulting in leakage of bacterial content and ultimately cell death. Resistance to ox-bile has been recorded for *Lactobacillus acidophilus* (Park et al., 2006), *Lactobacillus salivarius*241, *Lactobacillus plantarum*423 and *Lactobacillus curvatus*DF38 (Brink et al., 2006).

Testing of pancreatin tolerance

According to FAO/WHO (2006) and Mercenier et al. (2008) tolerance of gastrointestinal environmental conditions are considered as a prerequisite for screening intestinal lactobacilli for their functional properties. The effect of pancreatin was examined to determine the ability of eight isolates to survive during their passage through gastrointestinal tract (Koll et al., 2010). All tested strains were resistant to pancreatin at a concentration of 0.5% w/v and strain NKC9O3, NKC17(3B), NKC6L2 and NKC18(5C) showed excellent growth after 48 h incubation. Strain NKY1H1 showed average growth after 24 h incubation but the growth improved significantly after 48 h (Table 4). Koll et al. (2010) have reported resistance of all the test strains to pancreatin at 0.5% w/v.

Conclusion

This study was carried out for the isolation and identification of *Lactobacillus* species from traditional fermented foods that could be a means of ensuring safety. The probiotic properties of the isolates were comparable to commercially available *L. casei* (NKY1H1). In particular, strain NKL1(2B), NKC17(3B) and NKC18(5C) fulfilled the functional criteria required to be used as novel and beneficial probiotic. These three strains may be regarded as good candidate for *in vivo* studies and can be identified by specific molecular biology techniques.

Conflict of Interests

The authors declare that they have no conflict of interest.

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