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Phenotypic and Genotypic Characterization of Probiotic Bacteria Isolated from Gir Cow Milk for their Fortification in Soya Food

Ankur C. Thummar and Vimal M. Ramani*

Dairy Microbiology Department, College of Dairy Science, Kamdhenu University, Amreli - 365 601, Gujarat, India.

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Soy foods are more and more accepted as healthy food due to its numerous health benefits. However, the one of the unfavorable property of soy food is its incomplete digestion, due to the presence of soy indigestible oligosaccharides, which resulting in undesirable symptoms, such as flatulence, cramping and bloating. One of the solution of above problem is use of probiotic microorganism and use of such probiotic to soy food would help on solution of digestion and may confer additional health benefits too to consumers of soy food. In present study raw milk samples collected from different taluka of Amreli district, Gujarat. Total 157 bacteria were isolated. Among 157 bacteria, 67 were selected on the basis of preliminary tests which included Gram-positive, catalase negative and non-motile. The carbohydrates fermentation was determined by modified MRS agar containing bromocresol purple as a pH indicator and supplemented with 1 % of raffinose instead of dextrose. These organisms were characterized for their bile salt and pH tolerance too. Furthermore, base on carbohydrate fermentation, bile salt tolerance and acid tolerance result, two bacterial isolates DHA_DHR_MR _12 and DHA_JRA_M_13 were selected for 16s-rRNA identification. After identification confirm that the isolate DHA_DHR_MR _12 confirmed as *Lactobacillus fermentum* and DHA_JRA_M_13 confirmed *Enterococcus faecium*.

Keywords: Raffinose, Probiotic, Soya food, Acid tolerance, Bile salt tolerance.

Glycine max is the terms referring to the domesticate crop of soybean (Carl Linnaeus, 1737). Swedish botanist gave the genetic name "*Glycine max*" to soyabean. Glycine means to "sweet" and max means "large", large nodule on soybean plant (Merrill, 1917). Soy materials have a fiber, minerals, oil, vitamins and well-balanced protein composition (Liu, 1997). Food and Drug Administration (FDA,1999) has announced in 1999 that soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease by lowering blood cholesterol level. Among protein sources, soy products have an excellent nutritional status based on their high protein content and soy protein contain enough of all the essential amino acids to meet biological requirements, when consumed at the recommended level. Soy protein intake is also considered to have a potential role in the prevention of chronic diseases such as osteoporosis, atherosclerosis, cancer and menopausal disorders (Liu *et al*., 1999). However, the one of the unfavorable property of soy food is its incomplete digestion, due to the presence of soy indigestible oligosaccharides, which resulting in undesirable symptoms, such as flatulence, cramping and bloating (Anderson *et al*., 1991).

One of the solution of above problem is use of probiotic microorganism and use of such probiotic to soy food would help on problem of digestion and may confer additional health benefits too to consumers of soy food. Moreover, soy indigestible oligosaccharides can also act as prebiotic and support the growth of probiotics and

* To whom all correspondence should be addressed.
Tel: +91 9426422883. Fax: 02792 229456;
E-mail : vimalmramani@gmail.com

collectively both can be utilized as a synbiotic. Probiotics are living microbial food supplements that beneficially affect the host by improving its intestinal microbial balance (Gibson and Roberfroid, 1995). Probiotic microorganisms favourably alter the intestinal micro flora balance, promote intestinal integrity and mobility, inhibit the growth of harmful bacteria and increase resistance to infection. Probiotics are increasingly used in nutraceuticals, functional foods and in microbial interference treatment (Kaushik *et al*., 2009).

During their journey to lower intestinal track from mouth, probiotic bacteria face low acidic environment in stomach while high bile salt environment in duodenum so it is necessity that probiotic strain should have ability to resist against low pH (Conway *et al*., 1987) as well as bile salt (Gilliland *et al*., 1984). It is reported that probiotic bacteria from its first entrance i.e. mouth to release to the stomach takes 3 hours and they need resistance from low pH environment as well as high bile salt environment to sustain themselves in the stressful condition of stomach (pH-1.5 to 3.00) and upper intestine which contain bile.

MATERIALS AND METHOD

Sample collection

The sterilization of glassware's such as sampling bottle, Petri plate and flask after washing with detergent was carried out in autoclave (121 °C at 15 psi for 15 min). Gir cow raw milk sample collection carry out from different taluka of Amreli district. In Saurashtra area of Gujarat, Gir cow is famous for their domicile nature and high milk production. Gir cow milk reach in nutrients like highest mineral level i.e. calcium, protein, vitamin A, vitamin B12. In Amreli taluka we collect milk sample from village like Devaliya, Sarbhanda, Jaliya, Chandgadh, Nana Gokharvala, Jesingpara, Satipara, Nava khijadiya, Vankiya and Tarvada as a sample collection site (Total 10 sample collection site). In Dhari taluka we collect milk sample from village likewise Rampara, Hudli, Khicha, Chalala, Nagadhra, Kuvdi, Dharangni, Jay Ranchod and Chalala danev (Total 9 sample collection site). In Bagasara taluka we collect milk sample from Deri pipaliya, Mota munjiyasar, Juni Halvad, Manekvada, Kagadadi, Pipali, Ghantiyal, Rafala, Junavaghaniya, Jethiyavadar, Mavjinjva, Vaghaniya and Nava Vaghaniya as a sample collection site (total 13 sample collection site). All the milk samples were collected in 10 ml sterile glass screw cap tube and these samples were brought to the bacterial isolates laboratory at 4 °C temperature in cool box and processed within 4 h.

Isolation of bacteria

Isolation of bacteria from collected raw milk samples was done using serial dilution technique. 0.5 mL of milk sample was added in 4.5 mL of sterile saline water (0.97 % NaCl) and mixed properly then 0.5 mL aliquot from test tube was transferred to 10^{-1} test tube containing 4.5 mL of sterile saline water and subsequent dilutions were done up to 10^{-4}.

After proper mixing, 0.1 mL from each dilution was spread on 1) deMan, Rogosa and Sharpe MRS agar (HiMedia, India). 2) M17 agar medium components (HiMedia, India). 3) Elliker agar medium (HiMedia, India). The plates were incubated at 37 °C for 24 h in anaerobic condition (Anaerobic System Mark-II, HiMedia-India). The obtained isolated colonies were repeatedly sub-cultured to obtain pure cultures. The pure cultures were maintained on respective broth at 4 °C in 5 mL vial.

Preliminary Screening of bacterial isolates

Lactic Acid Bacteria isolates preliminary screen out by Grams reaction, catalase activity and motility test.

Gram reaction and Morphology

Cultures were grown in suitable mediums at 37 °C for 24 h under anaerobic condition. Cells from fresh cultures were used for Gram staining test. Perform Gram staining reaction using HiMedia kit (HiMedia, India).

Catalase test

Catalase is an enzyme produced by many organisms that can hydrolysis the hydrogen peroxide into water and oxygen and it causes gas bubbles formation. The formation of gas bubbles indicates the presence of catalase enzyme (Yavuzdurmaz, 2007).

Catalase test was performed for all isolates. Isolates were streak on MRS, M17 and Elliker medium slant and incubate it for 24 h to 48 h at 37 °C an anaerobic condition (Anaerobic System Mark-II HiMedia-India). After 24 h incubation add 3 % (w/v) hydrogen peroxide (Merck, India)

solution into slant to check catalase enzyme presence. The isolates which did not formed gas bubbles recorded as catalase negative.

Motility test (Hanging drop technique)

This test is used to express the motility of bacterial cells. Using a sterile wire loop, a small drop of bacterial culture was placed on the centre of the cover slip. The cavity slide (Concave slide) was inverted over the cover slip and first examined under the 40X then motility of isolate observed in oil immersion lens. The isolates, which did not possess motility were recorded as non-motile organisms.

Modified agar plate screening

0.1 mL of inoculums of all bacterial isolates were plated on MRS analogue agar medium supplement with bromocresol purple (MRS–BCP Agar) and raffinose instead of dextrose. Then plates were incubated for 24 h to 48 h at 37 °C in anaerobic condition (Anaerobic System MarkII, HiMedia-India). The bacterial isolates produce acid that developed yellow colour zone on the agar, only those which exhibited a yellow zone were preserved on 25 % (w/v) glycerol (HiMedia, India) for storage at -20°C (Mo chen and azlin, 2008).

Classification criteria for three level of yellow colour zone formation: excellent (+++) if the isolate form yellow colour zone more than 24 mm diameter; very good (++) if the isolate form yellow colour zone between 12 mm-24 mm diameter; good (+) if the isolate form yellow colour zone between 1 mm-12 mm diameter.

Specific substrate screening

All bacterial isolates were checked for their α-galactosidase enzyme production by streaking on MRS analogue medium containing 40 µg mL^{-1} 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) (Sigma Aldrich) and 1 % (w/w) of the raffinose instead of dextrose. X-α-Gal is a specific chromogenic substrate for α-galactosidase. The X-α-Gal stock solution (20 mg mL^{-1}) was prepared in N-dimethyl formamide (HiMedia, India). After sterilization of 100 mL analogues medium add 0.2 mL stock solution of X-α-Gal, specific isolates were streaked on MRS analogue plate, after streaking plates were incubated an aerobically at 37 °C for 24 h to 48 h and stored for 3 h to 4 h at 4 °C. After incubation periods colony turns to blue colour which indicate the production of α-galactosidase enzyme (Mo chen and azlin, 2008).

Classification criteria for three level of blue colour intensity: excellent (+++), if the isolate form dark blue colour colony: very good (++), if the isolate form normal blue colour colony: good (+), if the isolate form light blue colour colony.

Acid tolerance

Preliminary selection of acid tolerant lactobacilli using rapid method was determined according to slightly modified methods as described by Pelinescu *et al*. (2009). 100 µL overnight cultures of the 25 bacterial isolates were inoculated into 10 mL MRS broth with pH -3, and pH -7 as a control. The inoculated broths were then incubated in anaerobic condition for 24 h at 37 °C. Growth of the bacterial isolates was measured by streaking bacteria on MRS plate after incubation and optical density using a spectrophotometer at 600 nm after 24 h incubation (Gilliland, 1984).

The percent difference between the variation of optical density (O.D) at pH-7.0 (O.D pH-7) and the variation of optical density (O.D) at pH-3 (O.D pH-3) would give an index of isolates surviving that can be expressed as follows (Zambou *et al*., 2011).:

$$\text{Surviving (\%)} = \frac{\text{O.D pH (7)} - \text{O.D pH (3)}}{\text{O3.D pH (7)}} \text{ X } 100 \quad \text{...(1)}$$

Classification criteria included three arbitrary level of acid condition tolerance: excellent (+++) if the isolate survived pH-3 after 24 h (81 %-100 %); very good (++) if the isolate survived at pH-3 after 24 h (66 %-80 %); good (+) if the isolate survived at pH-3 after 24 h (50 %-65 %).

Bile salt tolerance

The tolerance of lactic acid bacteria to bile salts was performed in MRS supplemented with bile salts using a modified method described by Dora and Glenn (2002).

25 bacterial isolates were tested for their bile salt tolerance by determining their growth in MRS broth containing levels (0.3 % w/w) of bile salts (Ox-gall, HiMedia-India) and normal MRS medium take as a control. Freshly prepared 0.1 mL cultures were inoculated into 10 mL MRS broth with contain respective amount of ox-gall and incubated at 37 °C for 24 h under anaerobic condition. After incubation in anaerobic condition

all 25 organism streak on MRS agar plate for checkout bile tolerance capacity, bacterial isolates are grow in plate which consider as a bile tolerance. Optical densities were measured using a spectrophotometer at 600 nm after 24 h incubation.

The percent difference between the variation of optical density (O.D) of culture without bile salts (O.D 0.0 % BS) and the variation of optical density of culture containing 0.3 % bile salts (O.D 0.3 % BS) would give an index of isolates surviving that can be expressed as follows (Zambou *et al*., 2011):

$$\text{Surviving (\%)} = \frac{\text{O.D 0.0 \%BS} - \text{O.D 0.3 \%BS}}{\text{O.D 0.3\%BS}} \text{ X } 100 \quad ...(2)$$

Classification criteria included three arbitrary level of bile salt tolerance: excellent (+++) if the isolate survived bile 0.3 % after 24 h (81 %-100 %); very good (++) if the isolate survived at bile 0.3 % after 24 h (66 %-80 %); good (+) if the isolate survived at bile 0.3 % after 24 h (50 %-65 %). An isolate survived if it demonstrated a surviving percentage equal or greater than 50 %.

Genomic DNA preparation

Extraction of genomic DNA was carried out from culture DHA_DHR_MR _12 and DHA_JRA_M_13 cultivated in MRS broth for 24 h at 37 ^{0}C. 2 mL of culture was centrifuge (REMI CM12 plus) for 10 minutes at 10,000 rpm. Cell pellet was resuspended 700 µL of lysis buffer (50 mM glucose, 25 mM tris-HCL (pH-8), 10 mM EDTA (pH-8), 1 % gaunidiumthiocynate and 1 % SDS (Sodium Dodecyl Sulphate). 20 µL of proteinase K was added, vortexed for 10-15 second and incubated it at 37 ^{o}C for 5 minutes. Same step was repeated for RNA removal by using RNase A enzyme. Then centrifuge were carried out at 10,000 rpm for 10 minutes. Supernatant was transferred in new sterile vial without disturbed the small white pellet.

1 mL of isopropanol was added in to the lysate and mixed it gently till white precipitation was seen, tube were centrifuged at 10,000 rpm for 10 minutes. Supernatant was carefully discarded without disturbing the pellet. Add 500 µL of 70 % chilled alcohol for washing, this procedure was repeated for twice and resuspended pellet by pipetting and then tubes were centrifuged at 10,000 rpm for 10 minute, Supernatant was discard without disturbing white pellet. Tubes were kept open for air dry the pellet for 10 minute at the 37 ^{o}C. Pellet was resuspended in 100 µL of elution buffer (10 mM Tris-HCL (pH-8) and 1 mM EDTA (pH-8)), incubated it at 55 ^{o}C for 10 minutes. Genomic DNA was purified and stored at -20 ^{o}C and checked in 0.8 % agarose gel electrophoresis and further used for polymerize chain reaction.

PCR reaction protocol

The DNA sequences (5' to 3') for the primers used in this study and their corresponding specificities were as follows: - 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). Add 1 µL of template DNA in 20 µL of PCR reaction solution. Primer, molecular grade water, template DNA, 25mM $MgCl_2$, 2.5 mM dNTP mix and Taq polymerase concentration used in both PCR reactions were 1 µL, 31.5 µL, 1 µL, 5 µL, 5 µL and 0.5 µL respectively. The final volume was 50 µL. 35 amplification cycles were carried out of this PCR mixture. Each cycle was for 45 second at 94 ^{0}C for denaturation of genomic DNA, followed by annealing for 60 second at 55 ^{0}C and polymerization was carried out at 72 ^{0}C for 60 second. The PCR reaction was performed in 96 well gradient thermal cycler (Merk, India). Unincorporated PCR primers and dNTPS (deoxy nucleotides) were removed from PCR product by using Montage PCR clean up kit (Millipore).

Agarose gel electrophoresis of PCR product

PCR products were separated by electrophoresis on 1.0 % (w/v) agarose gel (HiMedia, India) containing 2 µL ethidium bromide (10 mg mL^{-1}). 10 µL of each PCR product and 5 µL of 6X loading dye (HiMedia, India) were loaded into agarose gel and run in 1X TAE buffer (HiMedia, India). The gel was run for 60 min at 50 V. After electrophoresis, PCR product was visualized in UV Transilluminator (GeNie, India).

Sequencing of Amplified 16s r-RNA gene

The purified PCR product of approximately 1400-1500 base pair were sequenced by using 2 primers as described earlier. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystem, USA). Sequencing products were resolved on Applied Bio system model 3730XL automated DNA sequencing system (Applied BioSystem, USA). After sequencing, the 16s rDNA sequence was used to carried out basic local alignment search tool (BLAST) alignment search tool of National Centre for Biotechnology Information (NCBI)

GenBank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.02905816 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Sample collection and isolation of bacteria

Collections of 126 raw milk samples were carried out from total 32 village and 3 taluka of Amreli district. Total 157 organisms isolated using de Man, Rogosa and Sharpe (MRS), M17 and Elliker medium. Among 157 bacteria, 67 were selected on the basis of preliminary tests which included Gram staining reaction, catalase test and motility test. Total 67 LAB isolated from three taluka of Amreli district includes 28 LAB isolates from Amreli, 14 LAB isolates from Dhari and 25 bacterial isolates from Bagasara which shows in Figure-1.

Preliminary Screening of Lactic Acid Bacteria

For preliminary screening of lactic acid bacteria, Gram staining reaction, motility and catalase test were performed. The Gram's staining reaction, motility test and catalase test could support the characterization of *Lactobacilli* genus. In Gram staining all 67 LAB, all the bacteria gave blue-purple colour in the staining reaction and thus all were Gram positive. Similarly, all the 67 bacterial isolates were found non-motile and showed the absence of catalase enzyme too. Size, shape and arrangement of isolates show in Figure-2. Among 67 bacterial isolates 20 rod shaped and 47 were cocci shaped. In rod shape isolates, single rod-18, cluster of rod-2, while in cocci shape isolate, single cocci-10, diplococci -5, bunch of cocci-8 and chain of cocci-24 isolates.

Joshi *et al* (2012) isolated bacteria form curd sample, they reported that all the strains were non-motile. Lactic acid bacteria are facultative anaerobes with a preference of anaerobic conditions. Oxygen is sometimes used for formation of hydrogen peroxide, which is toxic for lactic acid bacteria and do not contain catalase to break it down. Our results of motility and catalase test are at par to this.

Modified agar plate screening

Modified MRS-BCP agar plate was used for screening of raffinose fermentation by LAB. The dye bromocresol purple, worked as a pH indicator, the colour of which changes from purple at pH 6.8 to yellow at pH 5.2. Raffinose utilized by bacterial isolates and produce acid, which turns the modified MRS agar medium purple to yellow after incubation. Positive result was recorded as yellow colour zone diameter surrounded to well. All 25 LAB isolates form yellow colour zone in diameter show in Table-1.

Among 25 isolates, all were producing yellow colour zone while 2 isolate produce excellent (+++), 12 isolate produce very good (++) and 11 isolate produce good (+) which show in Figure-3. While 2 bacterial isolate having maximum acid production i.e. raffinose utilize were show in Figure-4.

Mo chen (2008) reported the raffinose fermenting bacteria shows yellow colour zone in MRS analogue medium supplement with oligosaccharide likewise raffinose. Our results are in affirmation to this.

Result of the MRS-BCP plate screening showed that all these probiotic strains ferment raffinose which regarded as prebiotic which indicates that they might transform in to simple form of sugar during their metabolism. This might also indicate that the soy raffinose and inulin could work as prebiotic for these probiotic bacteria and collectively both can be utilized as a symbiotic.

Specific substrate screening

Among 25 isolates, all are producing blue colour while 2 isolate produce excellent (+++), 14 isolate produce very good (++) and 9 isolate produce good (+) which show in Figure-5. Blue colour intensity generated during specific substrate method were show in Table-1 and two isolate producing maximum blue colour colony show in Figure-6.

Mo chen (2008) reported that the some

LAB produce α-galactosidase, In presence of α-galactosidase X-α-Gal hydrolysis and convert in to blue colour colony in MRS analogue medium supplement with oligosaccharide plus X-α-Gal as a substrate. Our results are in affirmation to this

Classification criteria for three level of blue colour intensity: excellent (+++), if the isolate form dark blue colour colony: very good (++), if the isolate form normal blue colour colony: good (+), if the isolate form light blue colour colony.

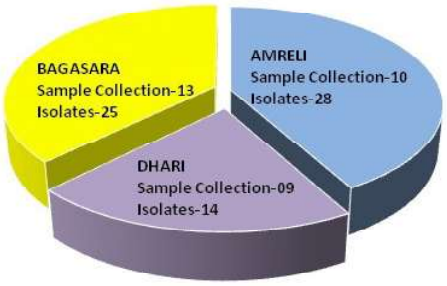


Fig. 1. Bacterial isolated from different taluka of Amreli district

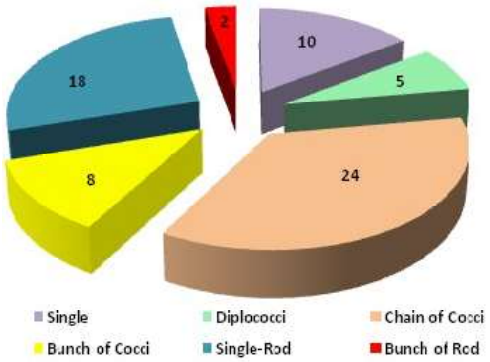


Fig. 2. Shape and arrangement of bacterial Isolates

Table 1. Yellow colour zone of acid production by bacterial isolates using raffinose in modified agar plate screening and Blue colour formation using specific substrate screening.

Sr. No	Culture number	Yellow colour zone in diameter	*Raffinose fermenting ability	Blue colour generated by colony	Blue colour intensity of X-α-Gal hydrolysis
1	AMR_NKH_M_22	7 mm	+	Light blue colour	+
2	AMR_NKH_MR_23	12 mm	++	Normal blue colour	++
3	AMR_NKH_MR_24	8 mm	+	Light blue colour	+
4	AMR_VAN_MR_25	8.5 mm	+	Normal blue colour	++
5	AMR_TAR_M_26	9 mm	+	Light blue colour	+
6	AMR_TAR_M_27	12 mm	++	Normal blue colour	++
7	AMR_SAR_MR_28	16 mm	++	Normal blue colour	++
8	DHA_NAG_M_7	9 mm	+	Light blue colour	+
9	DHA_KUV_MR_8	8.5 mm	+	Light blue colour	+
0	DHA_HUD_MR_9	12 mm	++	Normal blue colour	++
11	DHA_KUV_MR_10	12.5 mm	++	Normal blue colour	++
12	DHA_HUD_MR_11	15 mm	++	Normal blue colour	++
13	DHA_DHR_MR_12	31 mm	+++	Dark blue colour	+++
14	DHA_JRA_M_13	30 mm	+++	Dark blue colour	+++
15	DHA_CHA_MR_14	11 mm	+	Normal blue colour	+
16	BAG_DEP_MR_1	13 mm	++	Normal blue colour	++
17	BAG_MOM_MR_2	17 mm	++	Normal blue colour	++
18	BAG_KAG_MR_7	8.5 mm	+	Light blue colour	+
19	BAG_PIP_MR_8	4 mm	+	Light blue colour	+
20	BAG_MAN_MR_20	16 mm	++	Normal blue colour	++
21	BAG_VAG_MR_21	12 mm	++	Normal blue colour	++
22	BAG_PIP_MR_22	13.5 mm	++	Normal blue colour	++
23	BAG_NVA_MR_23	5.5 mm	+	Normal blue colour	++
24	BAG_NVA_MR_24	10 mm	+	Light blue colour	+
25	BAG_RAF_MR_25	21 mm	++	Normal blue colour	++

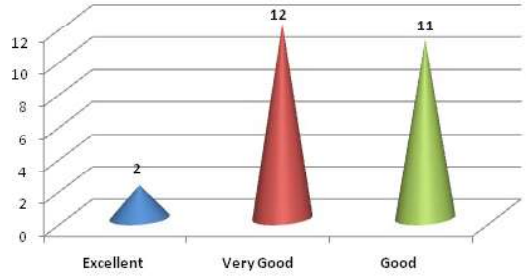


Fig. 3. Yellow colour zone formation by bacterial isolates in modified agar plate screening.

Acid and Bile tolerance

During their journey to lower intestinal track from mouth, probiotic bacteria face low acidic environment in stomach while high bile salt environment in duodenum so it is necessary that probiotic strain should have ability to resist against low pH as well as bile salt. It is reported that probiotic bacteria from its first entrance i.e mouth to release to the stomach takes 3 h and they need resistance from low pH environment as well as high bile salt environment to sustain themselves

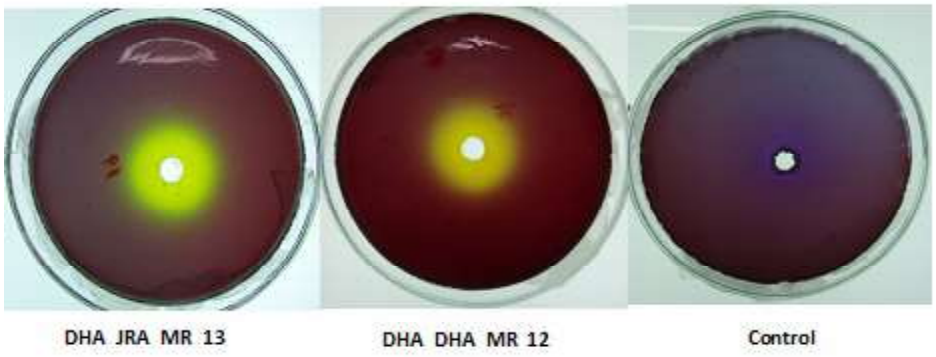


Fig. 4. Show positive and negative raffinose fermenting plate of LAB isolates.

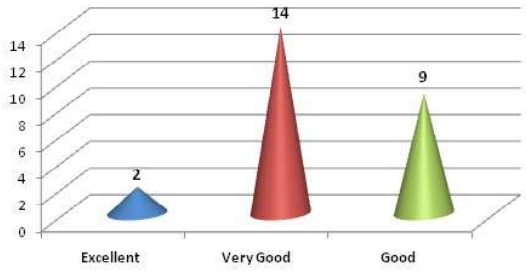


Fig. 5. Blue colour formation by bacterial isolates culture in specific substrate screening

in the stressful condition of stomach (pH-1.5 to 3.00) and upper intestine which contain bile.

Maximum LAB isolates were check ability to grow and survive at different bile salt concentration after three hours. The survival at different bile salt concentration is one of the main criteria for *in vitro* selection of potentially probiotic bacteria and critical points for the microbes because some of LAB are able to hydrolyzes of bile salt and it have potential to decreases level of

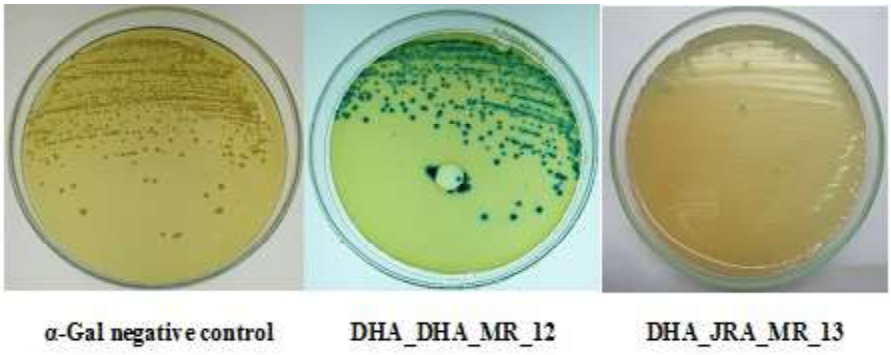


Fig. 6. Specific substrate screening by hydrolysis of X-á-Gal by LAB Isolates.

medium or eliminates the toxic effect of the bile salt to the LAB.

Acid tolerance

In acid tolerance among 25 bacterial isolate, 21 isolates tolerate between 81%-100% (Excellent), 1 isolates tolerate between 66 %-80 % (Very good) and 2 isolate tolerate between 50 %-65 % (Good) while 1 were not tolerant more than 50 % which show in Figure-7 and Table-2.

Sirilun *et al.* (2010) reported that a viable rate of more than 90 % of 43 out of 114 strains at pH-3 in their study, over observation also in affirmation to that.

Table 2. Acid and Bile salt tolerance of bacterial isolates.

Serial number	Culture number	Acid Tolerance		Bile Tolerance	
		Percentage(%) of tolerance	Acid tolerance ability	Percentage(%) of tolerance	Bile tolerance ability
1	DHA_DHR_MR_12	54	+	99	+++
2	DHA_JRA_M_13	68	++	98	+++
3	AMR_NKH_M_22	98	+++	56	+
4	DHA_NAG_M_7	95	+++	60	+
5	AMR_NKH_MR_23	96	+++	55	+
6	AMR_NKH_MR_24	97	+++	72	++
7	AMR_VAN_MR_25	92	+++	51	+
8	BAG_MAN_MR_20	96	+++	56	+
9	BAG_DEP_MR_1	96	+++	61	+
10	BAG_KAG_MR_7	50	+	56	+
11	AMR_TAR_M_26	99	+++	79	++
12	BAG_MOM_MR_2	97	+++	82	+++
13	AMR_TAR_M_27	98	+++	63	+
14	BAG_VAG_MR_21	99	+++	59	+
15	BAG_PIP_MR_8	95	+++	50	+
16	AMR_SAR_MR_28	95	+++	66	++
17	DHA_KUV_MR_8	99	+++	52	+
18	BAG_PIP_MR_22	38	-	79	++
19	BAG_NVA_MR_23	96	+++	53	++
20	BAG_NVA_MR_24	97	+++	22	-
21	DHA_HUD_MR_9	95	+++	95	+++
22	DHA_KUV_MR_10	94	+++	92	+++
23	BAG_RAF_MR_25	99	+++	56	+
24	DHA_HUD_MR_11	96	+++	67	++
25	DHA_CHA_MR_14	99	+++	95	+++

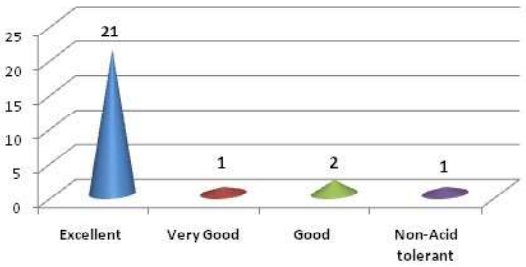


Fig. 7. Acid and Bile tolerance by bacterial isolates

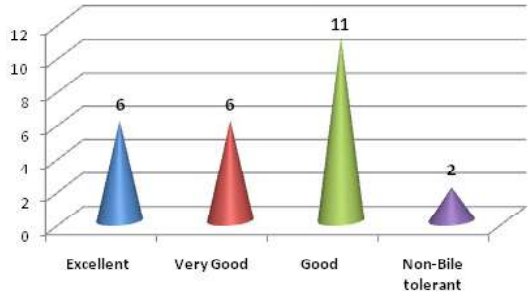


Fig. 8. Genomic DNA electrophoresis on 0.8% agarose gel. Lane 1-2: Genomic DNA from two isolates, Lane C: Control DNA from reference strain. 1% agarose gel is showing single 1.5 kb of 16S rDNA amplicon. Lane 1: 1 Kb DNA ladder; Lane 2: 16S rDNA amplicon

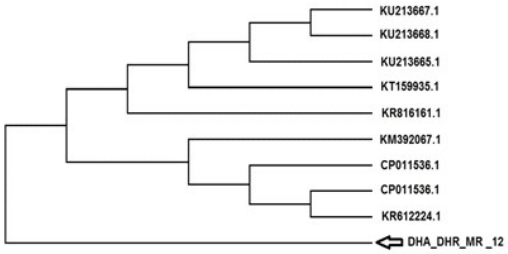


Fig. 9. Phylogenetic tree of selected 16S rDNA region sequence of the genus *Lactobacillus fermentum* obtained from BLAST search of the isolate DHA_DHR_MR _12 sequence for phylogenetic inference

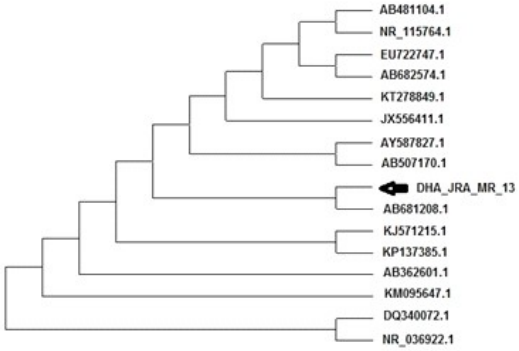


Fig. 10. Phylogenetic tree of selected 16S rDNA region sequence of the genus *Enterococcus faecium* obtained from BLAST search of the isolate DHA_JRA_M_13 sequence for phylogenetic inference

Bile salt tolerance

In bile tolerance among 25 isolate, 6 isolates tolerate between 81 %-100 % (Excellent) and 6 isolates tolerate between 66 %-80 % (Very good) and 11 isolate tolerate between 50 %-65 % (Good) while two were not tolerant more than 50 % which show in Figure-7 and Table-2.

Yavuzdurmaz *et al* (2007) reported that all of the isolates are also able to grow in 0.3 % bile salt as they survive, our observation also affirmation to that.

Two bacterial isolates *viz*. DHA_DHR_MR_12, DHA_JRA_M_13 gave best acid and bile tolerance which were selected further for genotypic characterization and its identification.

16s-RNA identification of isolates

Genomic DNA was extracted from the isolated bacteria like DHA_DHR_MR _12 and DHA_JRA_M_13 and Respective primer like 27F and 1492R were used for the amplification and sequencing of 16s rRNA gene region shown in Figure-8. A total number of bp found after amplification and sequencing of 16s rRNA gene region were 1098 in DHA_DHR_MR _12 while in case of DHA_JRA_M_13 isolates, a total number of bp were 1488 by using ABI 3730xl DNA Analyzer. Both bacterial isolates sequenced data were used for identification of bacteria against nonredundant collection data of GenBank database and this identification carried out using BLAST.

The BLAST result showed that the 16S rDNA sequence of isolate DHA_DHR_MR _12 had 99% sequence similarity and having maximum score i.e. 2342 bits with *Lactobacillus fermentum* strain K5 while in case of DHA_JRA_M_13 had 99 % sequence similarity and having maximum score i.e. 2736 bits with *Enterococcus faecium* strain NK8. The result also showed that isolate DHA_DHR_MR _12 had 99 % sequence similarity with *Lactobacillus fermentum* strain K16, *Lactobacillus fermentum* strain K7, *Lactobacillus fermentum* strain KFC, *Lactobacillus fermentum* strain KF3, *Lactobacillus fermentum* strain BT1-11 and *Lactobacillus fermentum* strain PD2 (GenBank accession numbers KU213667, KU213665, KT159935, KR816161, KM392067 and KR612224 respectively).

While isolate DHA_JRA_M_13 *Enterococcus faecium* strain TS4E2, Bacterium Te58R, *Enterococcus faecium* strain JCM 20313, *Enterococcus faecium* strain NRIC 0112, *Enterococcus faecium* strain PSB 5 and *Enterococcus faecium* strain AT15 (GenBank accession numbers KJ571215.1, AY587827.1, AB507170.1, AB362601.1, KM095647.1 and KP137385.1 respectively).

Further relationship between isolate DHA_DHR_MR _12 and selective genera of *Lactobacillus fermentum* were use to construct phylogenetic tree which shown in Figure-9. Same way prepared phylogenetic tree of DHA_JRA_M_13 and selective genera of

Enterococcus faecium which shown in Figure-10. All these results confirm that the isolate DHA_DHR_MR _12 belongs to genera *Lactobacillus fermentum* and DHA_JRA_M_13 belongs to genera *Enterococcus faecium*.

Bacterial Isolate DHA_DHR_MR _12 was of *Lactobacillus fermentum* and prepare its sequin file with help of sequin tool and submitted data at GenBank and received GenBank accession number is KU720301 and same procedure was followed for second bacterial isolate DHA_JRA_M_13 of *Enterococcus faecium* and got GenBank accession number is KU695260. The evolutionary divergences between the numbers of base substitute per site from between sequence were conducted using maximum composite Neighbor-Joining method for DHA_DHR_MR _12 and DHA_JRA_M_13 respectively

CONCLUSION

In our present studies it is conclude that among 67 bacterial isolates, 2 bacterial isolates exhibited potential probiotic characteristics like acid tolerance, bile tolerance and transform raffinose, a non-digestible oligosaccharide (prebiotic) to simple form of sugar using specific enzyme. Based on their performance, two bacterial isolate were selected for its molecular identification through DNA preparation, PCR based amplification of 16s rRNA and sequencing. After sequencing of 16s rDNA confirmed as a *Lactobacillus fermentum* and *Enterococcus faecium*. These results collectively suggest that *Lactobacillus fermentum* and *Enterococcus faecium* have promising properties for potential probiotics.

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