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Evaluation of Probiotic Characteristics of Bacteria Isolated from Fermented Foods

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ABSTRACT

Probiotics have beneficial effects on the host's health and can be isolated from many food sources. Fermented foods are rich source of lactic acid bacteria that are beneficial microorganisms which help to decrease the levels of potentially pathogenic microorganism in the gastrointestinal tract. These lactic acid bacteria should possess certain characteristics to be used as a probiotic. The objectives of the study were to isolate, screen and evaluate the probiotic characteristics of lactic acid bacteria from fermented foods (Idli, Dosa, Dhokla, Jalebi, Kanji, and Shrikhand). Twenty one isolates were obtained out of which fourteen of them were gram positive and Catalase negative that are the characteristics of lactic acid bacteria which were selected for further probiotic evaluation. Five isolates showed tolerance to acidic conditions at pH 2, 3 and 4, such as prevailing in the stomach. Bile tolerance test disclosed that eleven isolates were able to tolerate 0.3% mean intestinal bile concentration. NaCl tolerance test revealed that four isolates tolerated NaCl concentrations (ranging from 4%,6%,8%,10%,12%). Five isolates were able to adhere to mucosal surface indicated by the hydrophobicity test. All isolates were able to produce lactic acid which was confirmed by the lactic acid estimation test. Three isolates were sensitive to tetracycline and kanamycin but resistant to ampicillin and rest others were resistant to ampicillin, tetracycline and kanamycin. DO4 which is a bacterial isolate obtained from batter of dosa was able to qualify all the above tests and can be used as a suitable probiotic candidate in future applications related to health.

KEY WORDS: Probiotic, lactic acid bacteria, fermented food.

1. INTRODUCTION:

The favorable attributes associated with consumption of fermented foods was stipulated in the beginning of the 20th century by Metchnikoff (1907) [1]. Since then, these organisms have become pioneers of health foods in human nutrition. These were termed as 'probiotics' by Fuller (1989) who defined probiotics as "live microbial feed supplement that beneficially affects the host by improving its intestinal microbial balance[2] . The definition of probiotics has been altered and reformed several times and a unanimous definition has been accepted that is "Probiotics are live microorganisms, which when administrated in adequate amounts confer health benefits upon the host" given by FAO/WHO (2001)[3]. Probiotic bacteria are able to curb potentially pathogenic microorganism in the gastrointestinal tract and improve the proliferation of beneficial microorganisms. Probiotic microorganisms have been reported to decrease lactose intolerance, improve immune function, help in cholesterol reduction, show antimutagenic activity and decrease diarrhea[4].

Probiotic bacteria compete with pathogens for nutrients and mucosal adherence, produce antimicrobial substances and modulate mucosal immune functions[5].

A potential probiotic strain must fulfill a set of beneficial/probiotic characteristics including acid tolerance, bile tolerance, antibiotic resistance etc. and must be accurately identified to achieve these selection criteria.

Lactic acid bacteria with exceptional as well diverse probiotic characteristics may be present in fermented foods. LAB is considered to be safe for consumption as they are generally recognized as safe (GRAS) microorganisms. It is also expected that the diverse fermented food products that exist in different geographical locations may yield equally prospective lactic acid bacteria. Thus exploration of lactic acid bacteria (LAB) with superior and strong probiotic attributes need to be studied in fermented foods.

Therefore, the present study is aimed to characterize bacteria from fermented food of Indian origin and evaluate their probiotic properties.

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2. MATERIALS AND METHODS

2.1 Sample collection:

Batters of six fermented food products (Idli, Dosa, Dhokla, Jalebi,

Kanji, and Shrikhand) were prepared and collected in sterile containers and brought to the lab for further analysis[6].

2.2. Screening and isolation of bacteria from fermented foods

The isolation sources were fermented food products (Idli, Dosa, Dhokla, Jalebi, Kanji, and Shrikhand). Samples were diluted to using 0.9% NaCl. Dilutions were spread on deMan, Rogosa and Sharpe (MRS) medium agar plates. The plates were incubated at 37 °C for 24 hrs. After incubation, isolation of selected colonies was done using streak plate technique[7]. Pure culture of the isolated colonies was maintained. The isolates were examined according to their colony morphology, Catalase reaction and gram reaction[8]. Lactic acid bacteria are known to be gram positive (bacilli or cocci) and catalase negative[9]. Catalase test was performed by dropping 3% hydrogen peroxide solution onto the single colonies on glass slide and formation of bubbles was observed. Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. Lactic acid bacteria are Catalase negative as they do not contain Catalase enzyme and thus do not produce any bubbles[10] .

2.3 Evaluation of Probiotic Characteristics

2.3.1. Tolerance to Low pH:

Gram positive and Catalase negative isolates were selected. Equal aliquots of the active overnight grown culture were inoculated in MRS broth tubes with set pH levels (2, 3, 4, 5, and 6). These tubes were incubated at 37 °C for 24 hrs. After 24 hours, the growth was monitored at OD600. Acid or pH tolerance test was performed to observe the growth of selected isolates at lower pH levels (2, 3, and 4) present in the stomach. Acid present in the stomach can degrade the lactic acid bacteria[11].

2.3.2. Tolerance to Bile:

Gram positive and Catalase negative isolates were selected. Equal aliquots of the active overnight grown cultures were inoculated in MRS broth tubes with set bile levels (0.3, 0.5, 0.6, 0.8, and 1%). These tubes were incubated at 37 °C for 24 hrs. After 24 hours the growth was monitored at OD600. Bile tolerance test was performed to observe the growth of selected isolates at 0.3% mean intestinal bile concentration. Bile present in small intestine can degrade lactic acid bacteria as it is a biological detergent[12].

2.3.3. Tolerance to NaCl:

Gram positive and Catalase negative isolates were selected. Equal aliquots of the active overnight grown culture were inoculated in MRS broth tubes with set NaCl levels (4, 6, 8, 10, and 12%). These tubes were incubated at 37 °C for 24 hrs. After 24 hours the growth was monitored at OD600[13].

2.3.4. Hydrophobicity of Isolates:

Gram positive and Catalase negative isolates were selected. Equal amount of the active cultures were inoculated in MRS broth tubes. These tubes were incubated at 37 °C for 24 hrs and were centrifuged at 8,000×g for 15 min. The cell pellet was washed and resuspended in equal volume of Ringer solution (6% NaCl, 0.0075% KCl, 0.01% CaCl2 and 0.01% NaHCO3) and absorbance at 600 nm was measured. Cell suspension was then mixed with equal volume of n-hexadecane and vortexed for 3 min. The two phases were allowed to separate for 60 minutes and the absorbance of lower phase was measured at 600 nm[13]. The percentage hydrophobicity of strain was calculated using the following equation:

Hydrophobicity(%) = [OD600(initial) – OD600(with hexadecane) / OD600(initial)] * 100

Hydrophobicity test was performed to observe the adhesion of lactic acid bacteria to mucosal membrane. Lactic acid bacteria compete with pathogens that cause diseases by blocking the pathogenic adhesion sites and thus decrease the levels of pathogens in human gut.

2.4. Lactic Acid Estimation:

Gram positive and Catalase negative isolates were selected. Equal amount of the active cultures were inoculated in MRS broth tubes. These tubes were incubated at 37 °C for 24 hrs. The lactic acid production was determined by the standard titration procedure for total titratable acidity (TTA) (A.O.A.C, 1990). Titration using 25ml of the sample was done on addition of few drops phenolphthalein as indicator. 0.1M Sodium hydroxide (NaOH) was slowly added from a burette into the samples until the appearance of pink color. Each ml of 0.1M NaOH is equivalent to 90.08mg of lactic acid.

Total titratable acidity of lactic acid (mg/ml) = (Volume of NaOH used in ml x Molarity of NaOH used x Equivalent factor) / Volume of sample used in ml

The total titratable acidity of lactic acid was performed to determine the amount of lactic acid produced by lactic acid bacteria. Lactic acid is an inhibitory substance that degrades cell wall of pathogens that cause diseases and thus decrease their levels in human gut[14].

2.5. Antibiotic resistance test:

Gram positive and Catalase negative isolates were selected. Equal amount of the active cultures was inoculated in MRS broth tubes. These tubes were incubated at 37 °C for 24 hrs. Lactic acid bacterial

culture was spread on Mueller Hinton (MH) a gar plates. Resistance of the isolates to 3 types of antibiotics was performed by the disc diffusion method. Ampicillin (25 µ g), tetracycline (10 µg) and kanamycin (30 µg) discs were placed on the surface of the agar plates containing the bacterial culture. The plates were incubated at 37 °C for 24 hrs and presence of zone of inhibition was observed [15]. Antibiotic resistance test was performed to see whether lactic acid bacteria were resistant to the selected antibiotics. If there is an activity of antibiotics prevailing in human body, it can degrade lactic acid bacteria and decrease their survival in human gut. The absence of zone of inhibition indicates that lactic acid bacteria are able to grow in the presence of antibiotics and are resistant to antibiotics whereas the presence of zone of inhibition indicated that lactic acid bacteria are susceptible to the antibiotics.

3. RESULT AND DISCUSSION:

3.1. Isolation and screening of bacteria from fermented foods:

6 food samples (Idli, Dosa, Dhokla, Jalebi, Kanji, Shrikhand) batters were prepared and allowed to ferment and were collected. Lactic acid bacteria were isolated on MRS agar at 37 °C. 21 isolates were obtained which were further screened. Pure cultures of these isolates were prepared. Out of the 21 isolates 17 gram positive and 4 gram negative colonies were observed after gram staining procedure under microscope. Among 17 gram positive bacteria 6 were cocci and 11 were bacilli. Lactic acid bacteria are gram positive that give blue-purple color. Out of 21 isolates 7 Catalase positive and 14 Catalase negative colonies were obtained after Catalase test. Lactic acid bacteria are Catalase negative as they do not have Catalase and are not able to break down hydrogen peroxide (no gas bubbles are formed). 14 isolates were gram positive and Catalase negative that are the characteristics of lactic acid bacteria were selected for further probiotic evaluation **(table 1).**

Table 1. Total 21 isolates obtained from fermented foods out of which 14 isolates were gram positive and catalase negative

Sample	No. Of colonies obtained After Isolation	Gram positive and Catalase negative Colonies
DHOKLA	3	3
JALEBI	4	0
SHRIKHAND	3	1
IDLI	3	3
KANJI	4	3
DOSA	4	4

3.2. Probiotic Characteristics of isolated bacteria:

3.2.1. Tolerance to Low pH:

To reach the small intestine lactic acid bacteria have to pass from the stressful conditions of stomach. pH tolerance test was performed to assess the ability of selected isolates to survive at low pH levels, since it is known that the stomach acidity can affect lactic acid bacteria viability. The isolates that showed growth at lower pH (2,3,4) were K3, K4, DO1, DO2 and DO4 **(table 2)**.

3.2.2. Tolerance to bile:

The mean bile concentration of small intestine is 0.3% and the strains should tolerate these bile levels for their survival. The isolates that showed growth at 0.3% bile concentration were DH2, S2, I1, I2, I3, K3, K4, DO1, DO2, DO3 and DO4 **(table 2)**.

3.2.3. Tolerance to NaCl:

NaCl concentrations from 4-12% were selected to assess the tolerance of the strains against this inhibitory substance. The isolates that showed growth at all NaCl concentrations (4%,6%,8%,10%,12%) were DH1, DO1, DO3 and DO4 **(table 2).**

Table 2. Tolerance to inhibitory substances

Isolates → Inhibitory Substances↓		DH1	DH2	DH3	S2	I1	I2	I3	K1	K3	K4	DO1	DO2	DO3	DO4	POS
		Growth of bacteria (OD at 600 nm)														
pH LEVEL	2	0.026	0.028	0.012	0.036	0.037	0	0.035	0	0.024	0.044	0.024	0.06	0.046	0.016	0.36
	3	0	0	0	0	0	0	0	0.012	0.012	0.028	0.022	0.028	0	0.024	0.01
	4	0	0.013	0	0	0	0	0	0	0.05	0.06	0.05	0.06	0.04	0.021	0.036
	5	0.033	0.038	0.027	0.058	0.04	0.012	0.042	0	0.023	0.029	0.161	0.147	0.103	0.14	0.038
	6	0.23	0.226	0.355	0	0.215	0.168	0.339	0.253	0.285	0.161	0.394	0.429	0.493	0.38	0.02
BILE CONC.(%)	0.3	0	0.01	0	0.026	0.055	0.012	0.052	0	0.133	0.04	0.012	0.061	0.038	0.022	0.03
	0.5	0	0	0	0	0	0	0.028	0	0.015	0	0	0	0.011	0.016	0.013
	0.6	0	0	0	0	0	0	0	0	0.164	0.011	0	0.019	0	0	0.01
	0.8	0	0.013	0	0.033	0.028	0	0.03	0	0.015	0.025	0.018	0.04	0.019	0	0.025
	1	0	0	0.027	0	0.35	0.071	0.071	0.085	0	0	0	0	0	0	0.01
Nacl CONC. (%)	4	0.017	0.013	0.02	0.044	0.138	0.101	0.174	0.07	0.254	0.262	0.028	0.061	0.051	0.027	0.034
	6	0.011	0.018	0	0	0.167	0.094	0.205	0.131	0.22	0.123	0.043	0.05	0.027	0.03	0.016
	8	0.028	0	0	0	0	0	0	0.033	0.061	0.053	0.053	0.06	0.041	0.023	0.03
	10	0.022	0.033	0.019	0.045	0.029	0	0.022	0	0.034	0.033	0.021	0.063	0.056	0.023	0.049
	12	0.02	0.016	0.012	0	0.014	0	0	0.028	0	0	0.016	0	0.018	0.025	0.01

3.2.4. Hydrophobicity of Isolates

Adhesion of bacteria to the mucosal surface is another criteria for selection of probiotic microorganisms. Bacterial adhesion to variety of substrates is also associated with hydrophobicity that can determine colonisation capability of bacteria that in turn can prevent pathogens from attaching to mucosal membrane by binding and blocking the sites for pathogenic adhesion. The level of adhesion increases with increase in hydrophobicity that is depicted in **table 3**. S2, I1, I2, DO2, DO4 adhere to mucosal surface with I2 showing maximum hydrophobicity of 80.7%.

Table 3. Hydrophobicity of isolates

Isolates↓	Initial O.D. at 600nm	Hexadecane O.D. at 600nm	Hydrophobicity(%)
DH1	0.05	0	-
DH2	0.053	0	-
DH3	0.046	0	-
S2	0.632	0.481	23.9
I1	0.212	0.073	65.6
I2	0.14	0.027	80.7
I3	0.07	0	-
K1	0.034	0	-
K3	0.064	0	-
K4	0.087	0	-
DO1	0.041	0	-
DO2	0.162	0.057	64.8
DO3	0.025	0	-
DO4	0.261	0.078	70.1
POS	0.025	0.021	16

3.3. Lactic Acid Estimation:

Lactic acid (organic acid) produced by lactic acid bacteria is essential for inhibition of pathogenic microorganisms by degrading their cell wall. The total titratable acidity of lactic acid is given in **figure 1**. All isolates produced lactic acid. Maximum lactic acid production of 165.74 mg\ml was observed in S2.

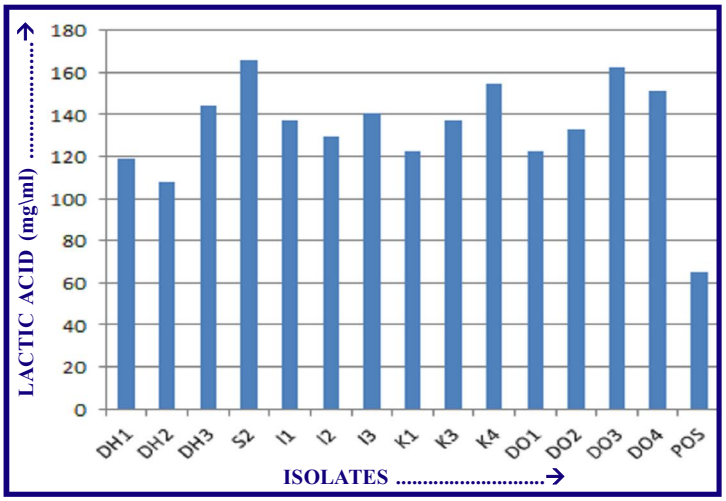

Figure 1. TTA of Lactic acid production

3.4. Antibiotic resistance test:

The bacteria should be resistant to the effect of antibiotics inside body. The presence of inhibition zone indicated that lactic acid bacteria are susceptible to the tested antibiotics. **Table 4** shows bacterial resistance to antibiotics. All isolates are resistant to kanamycin. All isolates are resistant to ampicillin and tetracycline except S2, K1 and K3.

Table 4. Antibiotic resistance of isolates to ampicillin, kanamycin and tetracycline (zone of inhibition)

Isolates↓	Ampicillin	Tetracycline	Kanamycin
DH1	-	-	-
DH2	-	-	-
DH3	-	-	-
S2	0.5cm	0.6cm	-
I1	-	-	-
I2	-	-	-
I3	-	-	-
K1	0.5cm	0.3cm	-
K3	0.6cm	0.2cm	-
K4	-	-	-
DO1	-	-	-
DO2	-	-	-
DO3	-	-	-
DO4	-	-	-
POS	-	-	-

4. CONCLUSION:

Fermented foods are one of the richest sources for isolation of lactic acid bacteria. Lactic acid bacteria are typically involved in a large number of spontaneous food fermentations but they are also closely associated with the human environment. The intake of probiotic bacteria shows various health benefits. Determination of probiotic properties of lactic acid bacteria isolated from fermented foods (Idli, Dosa, Dhokla, Jalebi, Kanji, and Shrikhand) was the aim of this study. 21 isolates were obtained on MRS agar. 14 isolates were found out to be gram positive and Catalase negative. For the microbial strains to be used as probiotics, certain selection criteria must be used for safety. To determine the probiotic properties of these isolates different tests were employed namely acid tolerance test, bile tolerance test, NaCl tolerance test, hydrophobicity test, lactic acid estimation test and antibiotic resistance test. The isolates that showed growth at lower pH (2,3,4) were K3, K4, DO1, DO2 and DO4. The isolates that showed growth at 0.3% bile concentration were DH2, S2, I1, I2, I3, K3, K4, DO1, DO2, DO3 and DO4. The isolates that showed growth at all NaCl concentrations (4%,6%,8%, 10%,12%) were DH1, DO1, DO3 and DO4. S2, I1, I2, DO2, DO4 adhere to mucosal surface with I2 showing maximum hydrophobicity of 80.7%. All isolates produced lactic acid. Maximum lactic acid production of 165.74 mg\ml was observed in S2. All isolates were resistant to kanamycin. All isolates were resistant to ampicillin and tetracycline except S2, K1 and K3. DO4 isolated from fermented dosa

batter was able to qualify all the above tests and was selected as a suitable probiotic candidate. There is a high possibility that this would be able to reach the intestinal tract in good number and can be used as a starter culture for probiotic preparations.

Conflict of Interest: The authors declare that they have no conflict of interest.

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