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Isolation of Lactic Acid Bacteria from Different Dung Samples and *In Vitro* Screening for Certain Probiotic Properties

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ABSTRACT

Lactic acid bacteria as probiotics are a center of attention for the manipulation of human gastrointestinal (GI) micro biota as a means of introducing them into digestive tract. Whether or not the probiotic strains employed shall be of human origin is a matter of debate but this is not a matter of concern as long as strains shown to survive the transport in the human GI and to colonize human intestine. This includes survival in the stressful environment of the stomach such as high pH. The objective of present study was to isolate and characterize different lactic acid bacteria having probiotic properties from different animal dung samples viz., cow, horse, buffalo, cow infant, goat, buffalo infant. The selected 6 bacterial strains from each sample were investigated for tolerance for bile salt (0.3%, 0.5%, 1% and 1.5% concentrations), survival in acidic and alkaline condition, susceptibility to antibiotics, and salt tolerance of 1 to 10% concentrations, arginine hydrolysis ability and catalase activity. The selected strains were able to survive up to 1.0% bile salt, pH value 5 and 9 and salt tolerance up to 6%. Among all the strains, buffalo dung isolate was able to survive at 1.5% bile salt and was found to be most efficient. All the strains show positive results for arginine hydrolysis and negative results for catalase test. These results suggest that isolated strains are thought to survive stressful intestinal environment and are considered to be suitable for probiotic applications.

KEY WORDS: probiotics, lactic acid bacteria, dung, bile tolerance.

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INTRODUCTION:

The isolation and screening of microorganisms from natural sources is always been an important aspects of research. So far animal health is concern, probiotics are gaining important consideration for their role they play in alleviate certain disease condition. In present study we have used various animal dung samples to isolate and screen out certain probiotic properties. The term Probiotics comes from the Greek word 'pro bios' which means 'for live'[1]. Mention of dietary use of cultured dairy products containing live microorganisms is found in the Bible and sacred books of Hinduism. Probiotics are live micro-organisms that when administered in adequate amounts confer a health benefit on the host by improving microbial balance in intestine[2]. According to Fuller (1991) the belief in the beneficial effects of the probiotic approach is based on the knowledge that the intestinal microflora provides protection against various diseases[3]. The expansion of new probiotic products has produced new scientific achievements and a strong demand for improved and scientifically-based selection criteria[4]. However when the great variety of species, strain characteristics, and the habitat specifics are considered, it becomes apparent that a established probiotic effect on a one strain or species cannot be transferred to other strains or species[5].

Among the probiotic microorganisms, Lactic acid bacteria (LAB) are regarded as the major group. Most probiotic microorganisms belong to Lactic Acid Bacteria, such as *Lactobacillus* sp, *Bifidobacterium* sp and *Enterococcus* sp[6]. LAB as non pathogenic in nature have been essential in food and feed fermentation for centuries, because they add the nutritional value to the products. Cattle dung and poultry faeces represent sources from which potentially useful lactic acid bacteria could be isolated and exploited for biotechnological applications[7]. Oral administration of LAB is well tolerated and proven to be safe in 143 human clinical trials and no adverse effects were reported[9]. The aim of present study is to isolate lactic acid bacteria from dung samples from six animals and representative six isolates investigated for probiotic properties.

MATERIAL AND METHODS:

Isolation of lactic acid bacteria from different dung samples

Dung samples from six different animals' viz., cow, horse, buffalo, cow infant, goat & buffalo infant were collected from the area around Bardoli, District Surat, Gujarat. Dung samples were collected in early morning in sterile plastic bag and then taken to lab for further work. Isolation of lactic acid bacteria was carried out on MRS agar medium supplemented with bromo cresol purple as indicator. One

gm of each dung sample was added into 10 ml of sterile distilled water followed by centrifugation at 2500 rpm for 5 min. The supernatants were then diluted up to 10^6 dilution factor and 0.2 ml from last 3 dilutions were spread on MRS agar plates. The plates were incubated for 48 hr in CO_2 incubator at 37 °C. The colonies with yellowish zone indicate the acid production on MRS agar. The selected isolates from each dung samples were the purified on new sterile MRS agar plate and stored at -4 °C for further work.

Identification tests for Lactic Acid Bacteria

All the six isolates were subjected to microscopic observation for gram staining reaction. Following biochemical test were performed.

Arginine hydrolysis test

In order to see ammonia production from arginine, MRS broth supplemented with arginine and Nessler's reagent were used. Sterile MRS broth containing 0.3% L arginine hydrochloride was inoculated with 1% overnight grown isolates. Inoculated tubes were incubated at 37°C for 48 hour. After incubation, ammonia production was detected by adding 100µl of cultures with same amount of Nessler's reagent on a white background. The change in colour was recorded. Bright orange color indicates positive reaction while yellow colour indicates negative result. A negative control was also maintained which did not contain arginine.

Catalase test

The presence of catalase activity can be confirmed by visible formation of gas bubbles from hydrogen peroxide as it breakdown the hydrogen peroxide into water and gas bubbles. The test was performed on overnight growth isolates on nutrient agar slant. After 24 hr 3% hydrogen peroxide was added drop wise on culture in tube. Results were recorded by the production of bubbling on slant.

Bile tolerance

Starting culture was prepared by inoculating single colony of each isolates into sterile MRS broth and incubated at 37°C for 24 hr. Bile tolerance was checked on MRS agar prepared with different concentration of bile salts. 0.1ml of overnight grown culture were spreaded on MRS agar plate with 3%, 0.5%, 1.0% and 1.5% (w/v) bile salts (Himedia). Results were recorded after 24 hr in terms of presence or absence of colonies.

Acidic and alkali tolerance

Acid tolerance was determined using method described by Yeong-Soo Park[9]. The overnight grown culture was used to inoculate MRS broth (v/v). 0.1ml culture was inoculated into 9.9ml of sterile broth of various pH such as 3, 4, 5, 6, 7, 8, 9. The pH of all broth were adjusted with 1N HCl and 1N NaOH. Inoculated broths were incubated at 37°C for 24 hr. The results were recorded and tabulated in terms of presence and absence of growth.

NaCl tolerance

Each isolates were checked for their ability to tolerate different NaCl concentrations. MRS broths (9.9ml) with various concentrations (1 to 10%) of NaCl were inoculated with active culture of each isolate (0.1ml). Results were recorded after 48 hr of incubation at 37°C.

Antibiotics susceptibility

Antibiotic susceptibility of each isolates was checked and recorded in terms of resistant or sensitive. All the isolates were inoculated with soft nutrient agar on previously poured and solidified 3% agar. Antibiotic discs (C- Chloramphinicol, R- Rifampicin, P-Penicillin, and A-Ampicillin) were kept circularly at regular interval in plate. Observations were taken after 24hr of incubation.

RESULT AND DISCUSSION:

In the present study attempt were made to isolate and characterize lactic acid bacteria from six animal dung samples to analyze its probiotic properties. The salient feature of findings is outlined as below.

Isolation and Identification of lactic acid bacteria from different dung samples

Petrof (2009) mentioned that the isolation of probiotics is not limited to the human tract[10]. The guts of several animal species, including pigs, rats and even poultry and other animals are good sources of probiotics. Recently, *L. johnsonii* CRL 1647, isolated from the *Apis mellifera* L. bee gut, was shown to exhibit a beneficial effect on honeybee colonies[11]. *In vitro* selection is the first approach used to select a few strains that can be evaluated *in vivo*[12]. In present work, by direct method of isolation 37 lactic acid producing bacterial isolates were obtained from all the six animal dung samples. All of them produced acid on MRS medium containing bromo cresol purple. M ROGOSA (1951) developed a selective medium for the isolation and enumeration of oral and faecal lactobacilli and Bifidobacterium that contains a Columbia agar base supplemented with propionic acid[13]. The low pH of this medium, which

is tolerated by lactobacilli and bifidobacteria, inhibits the growth of other organisms in sample. The morphological characteristics was also investigated and showed that most of the isolates were gram positive rod. Buffalo dung sample was found to be rich in the terms of microbial diversity with over 9 isolates obtained. Out of total 37 isolates, 6 representative isolates from each sample were selected for further studies. These isolates viz., CD3, BD7, GD4, HD2, CID4 and BID6 were selected on the basis of size of clear yellow color zone produced surrounding the colony within 24 hr.

In present study all the isolates were found to hydrolyze arginine. Furthermore all the isolates were catalase negative, which indicates that all are lactic acid bacteria. Results of Cullimore (2000) support our data[14]. Lactic acid bacteria constitute an essential part of the healthy gastrointestinal microceology and are involved in the host metabolism[15]. Lactic Acid Bacteria are a group of Gram-positive, non- spore forming, cocci or rod shaped, catalase-negative and fastidious organisms, considered as 'Generally Recognized as Safe' (GRAS) organism[16].

Bile tolerance

The ability to survive the action of bile salts is an absolute need of probiotic bacteria, and it is generally included among the criteria used to select potential probiotic strains[12]. Investigation on bile tolerance shows that only BD7 isolate from buffalo dung could grow in the range of 0.3 to 1.5%. Furthermore CD3, CID4 and BID6 could able to grow up to 0.5% bile salt. GD4 and HD2 were the sensitive to bile salt and could not able to survive after 0.3%. These data agreed with the report of D.O. Darilmaz (2012), who reported that tolerance of lactobacillus spp. varied at different pH[17]. When evaluating the potential of using lactic acid bacteria as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids[18].

Table No. 1: Growth of LAB isolates on Bile salt containing media.

Bile salt tolerance				
Isolate	**0.3%**	**0.5%**	**1%**	**1.5%**
CD3	+	+	-	-
BD7	+	+	+	+
GD4	+	-	-	-
HD2	+	-	-	-
CID4	+	+	-	-
BID6	+	+	-	-

\+ Growth occur - No Growth occur

pH tolerance

According to FAO/WHO (2002) acid tolerance is one of the general criteria that are considered during the selection of potential probiotic strains to guarantee their viability and functionality[19]. Resistance to low pH is one of the important factors for selection of potential probiotics[20]. The survival at low pH is important if intended to use in gastrointestinal tract for health improvement. The results of Acid tolerance are shown in Table no 2. Determination of acid tolerance of Lactic Acid Bacteria was investigated for pH of 3.0, 5.0 and 9.0. Isolate CD3, BD7 and HD2 could grow at low pH 3, but their growth was lower as compare to growth between pH5 and pH7. Among all the six isolate studied, although at variable degree, only BD7 and HD2 isolates could grow in entire pH range studied. It indicate that these isolates are resistance to broad pH range and able to survive at low ph and can be used in probiotic applications. B. Hyronimus (2000) reported that *Bacillus laevolacticus* DSM 6475 and all Sporolactobacillus strains tested except *Sporolactobacillus racemicus* IAM 12395 were resistant to pH 3[21]. Generally the survival rate was low at pH3 and pH9, moderate at pH7 and good at pH5.

Table No. 2: Growth of LAB isolates at pH 3, 5, 7 and 9

pH tolerance				
Isolates	**3.0**	**5.0**	**7.0**	**9.0**
CD3	+	+	+	-
BD7	+	+	+	+
GD4	-	+	+	+
HD2	+	+	+	+
CID4	-	+	+	+
BID6	-	+	+	-

\+ Growth - No Growth

NaCl tolerance

The result of NaCl tolerance presented in Table no.3. Determination of NaCl tolerance of Lactic Acid Bacteria was investigated for NaCl tolerance of 1% to 10% in MRS broth. Initially the growth was recorded in low salt concentration containing MRS broth. But later on most of the isolates were able to grow up to 6% and visible growth recorded after 3 days of incubation at 37°C. Only BD7 isolate could grow up to high salt concentration of 8%. Although the growth of CD3 and HD2 was recorded less but these isolates could grow up to 7% NaCl.

Table No. 3: Growth of LAB isolates at 1 to 10% NaCl containing MRS broth

NaCl tolerance										
Isolates	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
CD3	+++	+++	+++	+++	+++	++	+	-	-	-
BD7	+++	+++	+++	+++	+++	+++	++	+	-	-
GD4	+++	+++	+++	+++	+++	++	-	-	-	-
HD2	+++	+++	+++	+++	+++	++	+	-	-	-
CID4	+++	+++	+++	+++	++	+	-	-	-	-
BID6	+++	+++	+++	+++	++	+	-	-	-	-

+ Less growth ++ Good growth +++ Very good growth - No Growth

Antibiotic susceptibility

Lactic acid bacteria used as probiotics may serve as hosts of antibiotic resistance genes, which can be transferred to pathogenic bacteria, so it is therefore important to verify that the single bacterial isolates (strains) do not contain transferable resistance genes[22]. In present study antibiotic susceptibility was check for all six isolates. In present study we have used four antibiotics namely chloramphenicol, rifampin, penicillin and, ampicillin.. LAB isolates were found sensitive to Chloramphenicol, rifampin and ampicillin and resistant to penicillin. Consistent with previous reports by D'Aimmo, Modesto, and Biavati (2007) the susceptibility to different antibiotics could variable and depending on the species[23].

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