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ORIGINAL ARTICLE

Isolation and *In Vitro* Antibacterial activity of Lactic acid bacteria from cow dung

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ABSTRACT
Our research is mainly composed of isolation of lactic acid bacteria from cow dung. Lactic acid bacteria are generally normal flora of the human body present in our intestine and helpful in providing various health benefits, such as helpful in curing irritable bowel syndrome, gastroenteritis, helpful in providing immunity and having cholesterol reducing properties .The purpose of this study was to investigate lactic acid bacteria with probiotic and antibacterial potential isolated from cow dung. Total 15 isolates were obtained from cow dung. On the bases of primary screening, 2 isolates were selected and characterized morphologically, physiologically, and biochemically as lactic acid bacteria. These selected isolates were further tested for their antimbacterial potential against food borne pathogenic bacteria (Bacillus cereus, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa) by using agar well diffusion assay. The antibacterial potential of the strains were analysed on the basis of zone of inhibition (mm) around the well containing cell free supernatant of each isolate . Strain CD1 was found to most effective against Bacillus cereus and bacillus subtilis . On the basis of biochemical characterization, and Bergey's manual CD1 and CD11 were identified as Lactobacillus plantarum and Enterococcus mundtii .
Keywords: Probiotics, antimicrobial activity. Lactic acid bacteria.

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INTRODUCTION

Cattle rearing in India has been a tradition and intimately limited to the agricultural economy. Different products obtained from cow milk, ghee, curd, urine, and dung are used widely in a number of Ayurvedic formulations. Cow dung is traditionally used as organic fertilizer in Indian sub-continental farming for centuries. The addition of cow dung increases the mineral status of soil, enhances resistance of plants against pests and diseases; stimulate plant growth [1] and other beneficial activities such as sulphur oxidation and phosphorus solubilization. Normally,Composition of cow dung is about 80% water and supports a matrix of undigested plant material that is rich in nutrients, micro-organisms, and their byproducts.

Probiotics are live microorganisms which are beneficial to both humans and animals by promoting intestinal tract health. The major beneficial properties of probiotics include their ability to compete with enteric pathogens, contribution in increasing digestive capacity, the enhancement of mucosal immunity, and the reduction of intestinal pH. As a result of all these properties, probiotics are also helpful in creating unfavorable environment for the growth of enteric pathogens and act as barrier for disease-causing bacteria and prevent them to colonizing in the intestine.

Probiotic bacteria are helpful in reducing host's inflammatory responses by stabilizing the gut microbial environment, fortifying the intestinal permeability barrier, facilitating the degradation of antigens, and altering their antigenicity and immunogenicity [1]. *Lactobacillus* is the most commonly used genera as probiotic generally referred to as lactic acid bacteria (LAB) .Lactic acid bacteria are mainly Gram-positive, rod-shaped, acid-tolerant, facultative anaerobic or microaerophilic, fermentative,and non spor forming. The effectiveness of LAB as a probiotic agent is linked to an ability to survive in stomach acid and

bile salt (BS) and to adhere to and colonize the intestinal lining [2]**.** Like many probiotic bacteria, LAB exhibit either bactericidal or bacteriostatic properties. Direct antimicrobial activity by Lactobacillus species is derived from the production of organic acids, hydrogen peroxide, bacteriocins, and low-molecularweight compounds [3]**.** Lactic acid bacteria isolates from dairy cow dung samples have been shown to possess both the survivability and antimicrobial properties of effective probiotics [4] .

MATERIAL AND METHODS

Collection of samples

cow dung samples were collected from different regions of Kurukshetra and Yamunanagar district, aseptically in sterile poly bags and transported to the Microbiology laboratory of the Department for the evaluation of microbial analysis.

Strain isolation

1 g of dung sample was added into 9 ml of normal saline. After homogenization, serial dilutions were prepared upto 10^{-9} with 0.85% (w/v) normal saline and 0.1 ml decimal of appropriate dilutions were plated onto de Man, Rogosa, Sharpe (MRS)agar medium (Himedia, India). The agar plates were incubated at 35°C for 24 h under anaerobiosis. Morphologically different colonies were picked and re-streaked onto MRS agar plate's upto purity. Glycerol stocks of strains were preserved at -20°C.

Identification of strain

Phenotypic characterization

The morphological, cultural and biochemical characteristics including gram staining and colonial appearance was determined.

Biochemical characterization

Indole test, MR-VP test, citrate utilization test, sugar fermentation test, gelatin hydrolysis test, lipase activity, hemolytic activity were employed to identify the isolated lactic acid bacteria.

Safety assessment of LAB

Safety is the important criteria for bacterial strains intended to use in the food industry.

Antibiotic sensitivity test

Study of antibiotic susceptible is important for selection and evaluation of safe probiotic strain. The antibiotic susceptibility assay was analyzed by Kirby Bauer method or Disc diffusion method. In this test bacterial isolate were inoculated uniformly into the surface of MRS agar plate. A filter disc impregnated with a standard amount of an antibiotic is applied to the surface of the plate and the antibiotic is allowed to diffuse into the adjacent medium. The result is a gradient of antibiotic surrounding the disc. Following incubation, a bacterial lawn appears on the plate. Zones of inhibition of bacterial growth may be present around the antibiotic disc. The size of the zone of inhibition is depending on the diffusion rate of the antibiotics, the degree of sensitivity of the micro-organisms and the growth rate of bacterium. Discs with very small zones or no zones of inhibition means that the bacteria are not susceptible to the antibiotic. Large zones indicate the levels of susceptibility: Susceptible (S), Intermediate (I), or Resistance (R).

Antibiotic used in this study were: Ampicillin (10**µg)**, Gentamicin (10**µg)** and Tetracycline (30**µg)**, Penicillin (30**µg)**, Streptomycin (30**µg)**, Erythromycin (30**µg)**, chloroamphenicol (30**µg)**, vancomycin (30**µg)**

Hemolytic and enzymatic activity

Hemolytic activity of both the strains were determined by spot inoculating overnight bacterial cultures on Blood agar plates followed by incubation of 24 h at 35°C.Recorded characteristics of heamolysis on blood agar were β-heamolysis (clear zone around colonies) , α-heamolysis (no clear zone around colonies). To analyse the enzymatic activity of the isolates gelatinase and lipase test were carried out . Gelatinase production was determined by streaking both the isolates on the MRS agar plates supplemented with 3% gelatin and the plates were incubated at 35°C for 24 h and the Lipase enzyme production was evaluated by streaking the 24 h old culture of both the isolates on the MRS agar plates supplemented with olive oil as a source of fatty acids. Plates were incubated at 35°C for 24 h. presence of clear zone around the streak indicates that the isolates were lipase and gelatinase enzyme positive respectively [5].

Antibacterial activity of selected isolate

Antimicrobial activity of the lactobacilli isolates was checked by disk diffusion method. Isolates were screened against *Bacillus subtilis* MTCC 1143, *Escherichia coli* MTCC 433, *Enterococcus faecalis* MTCC 439, Pseudomonas aeruginosa MTCC 6642, and *Staphylococcus aureus* MTCC 9886, as the indicator microorganisms. All the strains were procured from the MTCC culture collection centre, Chandigarh (India).

Both the Isolates CD1 and CD11 were sub cultured in sterile test tubes containing MRS broth at 37°C for 24 hrs and transferred into a sterile flask containing MRS broth and the flasks were incubated at 37°C for 48 hr. The isolates were centrifuged at 10,000 rpm at 4ºc for 15 min. The culture supernatant was collected in sterilized test tubes and was neutralized to pH 6.5 with 1N NaOH and catalase was added at the rate of 0.1 mg/ml. Inhibitory activity was observed by well diffusion method. The wells in the pre-swabbed nutrient plates were cut with sterile borer and 20 µl of neutralized culture supernatant was placed into the well.

Analysis of probiotic propery

Both the isolates CD1 and CD11 were tested for various probiotic properties such tolerance to acid and bile, cell surface hydrophobicity and auto aggregation ability.

Acid and bile salt tolerance

Acid tolerances of selected lactobacilli were determined by the method described by [9]. Lactobacilli isolates were cultured for 6 hrs in MRS broth at 37°C. 100 ml fresh MRS broth was prepared, and pH had been adjusted to 2, 4 or 6 using 1N HCL or NaOH. Add 1 ml of the 6 hrs old culture in flasks. Optical density was recorded at 620 nm after 6 and 24 hrs incubation periods at 37°C. Surviving (%) can be calculated by following formula:

$$\text{Survivalability \%} = \frac{\text{loglog CFU 2,4,6}}{\text{loglog CFU 6.5}} \text{ X } 100$$

A modified method given by Dora and Glenn [10] is used for estimation of bile salt tolerance in a similar method of acid tolerance. MRS broth supplemented with different concentration 0.2 and 0.4% of bile salts were used for the experiment. Surviving (%) in bile salt can be calculated by following formula:

$$\text{Survivalability \%} = \frac{\text{loglog CFU 0.2,0.4,0.6}}{\text{loglog CFU0}} \text{ X } 100$$

Auto aggregation ability

Selected isolates were grown in MRS broth at 37°C overnight. After incubation, the broth was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet obtained was washed twice with PBS buffer solution and re-suspend in the same solution, followed by incubation at 37°C for 5 h. Equal amount of aliquot was taken and absorbance was measured at OD_{600} at 0, 1, 2, 3, 4 and 5 h.(11)

Autoaggregation % = $1- (A_t / A_0) \times 100$

Where A_t =Absorbance after incubation at 1, 2, 3, 4 and 5 h

A_0 = Absorbance at 0 h

Adhesion property: Hydrophobicity

Selected isolates were grown in MRS broth at 37°C overnight. After incubation, the broth were centrifuged at 10,000 rpm at 4°C for 10 min. The pellet obtained was washed twice with PBS buffer solution and re-suspend in the same solution. 3 ml of cell suspension was added to 1 ml of each hydrocarbon (xylene, chloroform and ethyl acetate). Absorbance (OD_{600}) was taken at 0 h and after vortexing both phases for 2 min. Incubation was done for 2 h and absorbance was taken again.

Hydrophobicity % = $\{(A_0 - A_t) / A_0\} \times 100$

RESULT AND DISCUSSION

Total 11 lactic acid bacteria(CD1, CD2 ,CD3, CD4, CD 5,CD6 ,CD 7,CD 8 CD9 , CD10) were isolated from cow dung . All the isolates were gram-positive as examined by Gram's staining .out these isolates five isolates were (CD9,CD2 ,CD4, CD6 and CD8) coccus and five were rod shape (CD1 ,CD3, CD7, CD9 and CD 10)as revealed by microscopic examination .all the isolates were catalase negative and non spore forming isolate CD1 and CD11 gave best antimicrobial activity against test organism and was selected for further study.

Phenotypic characterization

CD1 appeared as white, punctiform colony with flat and entire margin whereas CD11 gave cream circular colonies with entire and raised margin on MRS agar medium.

Biochemical characterization

The selected isolate CD1 and CD11 is further identified by various biochemical test as shown in Table 1 and Table 2.

Table 1: Biochemical characterization of isolate .

S.no.	**Test**	**CD1**	**CD11**
1	**Gram staining**	Rod (+ve)	Coccus (+ve)
2	**Sugar fermentation test**	A^+ G^-	A^+ G^-
3	**Indole test**	-	-
4	**MR-VP test**	+, -	+, -
5	**Citrate utilization test**	-	-
6	**H_2S production**	-	-
7	**Casein hydrolysis**	-	-
8	**Lipase production**	-	-
9	**Gelatin hydrolysis**	-	-
10	**Hemolytic activity**	-	-
11	**Catalase reaction**	-	-

(-) no growth, (+) growth and A^+ (acid producing) G^- (non gas producing)

Carbohydrate fermentation

The sugar fermentation patterns were studied and the results were compared with Bergey's Manual of Determinative Bacteriology and the screened isolates were tentatively identified as *Lactobacillus plantarum* and *Enterococcus mundtii* .as showed Table :

Table :2Carbohydrate fermentation results of isolate CD1

Isolates	Sugars											Identification
	Lactose	L.arabinose	Dextrose	Raffinose	Sucrose	Xylose	sorbitol	Maltose	Mannose	Glactose	Rhamnose	
CD1	+	+	+	+	+	+	-	+	+	+	-	*L.plantarum*
CD11	+	-	+	-	+	+	+	+	+	+	+	*E.mundtii*

(+) = able to ferment sugar; (–) = not able

Antibacterial activity of Lactobacillus species

The result showed that the supernatant obtained by centrifugation of isolates broth inhibited the growth of all the test organisms . The results were expressed in terms of Zone of inhibition (mm).The maximum zone of inhibition was recorded as 18.25±1.02mm and 17.83±0.62mm for *Bacillus subtilis* by both the isolates CD1 and CD11 respectively .while the minimum zone of inhibition was recorded against *Salmonella typhimurium* 8.17±0.05mm and 10.17± 0.04mm by both the isolates .(Table 3)

Table :3 **Antibacterial activity of isolates**

Indicator organisms	**CD1**	**CD11**
Bacillus subtilis MTCC1143	18.25±1.02	17.83±0.62
Escherichia coli MTCC433	10.4±0.33	14.83±0.5
Enterococcus feacalis MTCC 439	13.15±0.9	14.9±0.5
Pseudomonas aeruginosa MTCC 6642	9.77±2.07	10.4±0.33
Staphylococcus aureus MTCC 9886	15.18±0.1	12.9±0.5
Salmonella typhimurium MTCC1255	8.17±0.04	10.17±0.04

Antibiotic Susceptibility Assay

Antibiotic susceptibility test is usually carried out to determine which antibiotic will be most successfully in treating a bacterial infection in vivo. Testing of antibiotic sensitivity is often done by Kirby-Bauer method. isolates CD1 were sensitive to all antibiotics i.e. Penicillin,, vancomycin , Gentamicin Chloramphenicol, Erythromycin, Streptomycin and Tetracycline, whereas CD11 strain is resistance to Penicillin and Streptomycin. Antibiotic resistance may occur due to natural processes such as transformation, transduction and conjugation, or due to human mediated activity such as antibiotics mediated activity such as antibiotics abuse, particularly in farming and agricultural industry[7]. There are several evidences to show that fresh cow dung and cow urine are antifungal and antiseptic in nature, which might be due to secretion of antimicrobial metabolites by cow dung micro flora. [8]

Table : 4 Antibiotic susceptibility assay for LAB isolated from cow dung

Antibiotics	Isolates	
	CD1	CD11
Ampicillin (10**µg**),	**S**	**S**
Gentamicin (10**µg**)	**S**	**S**
Tetracycline (30**µg**),	**S**	**S**
Penicillin (30**µg**),	**S**	**R**
Streptomycin(30**µg**)	**S**	**R**
Erythromycin (30**µg**)	**S**	**S**
chloroamphenicol (30**µg**),	**S**	**S**
vancomycin (30**µg**)	**S**	**s**

Probiotic properties

Tolerance to low acid conditions

A successful probiotic possess the property of tolerating harsh acidic conditions. Both the strains were tested for survival in acidic conditions at different pH 2, 4 and 6. Both grew well at minimum tested pH of 1.0 after 60 and 120 min of incubation. Lactic acid is produced by lactic acid bacteria during fermentation metabolism thus revealed its ability to survive in acidic environment of stomach.(Table:5)

Table 5. Acid tolerance of CD1 and CD11

pH	*Isolates*	Abs. at 0 h	log cfu/ml	Abs. at 60 min	log cfu/ml	Abs. at 120 min	log cfu/ml	Abs. at 180 min	log cfu/ml	Mean±SD of log cfu/ml	Survival Rate
2	Cd1	0.056	8.089	0.055	8.079	0.055	8.056	0.050	7.989	8.053 ± 0.07	89.45
	Cd11	0.051	8.037	0.052	8.008	0.046	7.903	0.029	7.627	7.893± 0.18	88.84
4	Cd1	0.062	8.173	0.060	8.127	0.052	8.089	0.051	8.033	8.105 ± 0.05	90.03
	Cd11	0.059	8.113	0.054	8.053	0.048	7.948	0.042	7.894	8.002 ± 0.09	88.31
6	Cd1	0.094	8.741	0.093	8.696	0.090	8.672	0.091	8.660	8.692 ± 0.03	96.55
	Cd11	0.078	8.475	0.077	8.447	0.075	8.429	0.073	8.409	8.440 ± 0.02	94.07

Tolerance to bile salts

Bile salts are surface active agents having potent antimicrobial activity. They act as detergent thus disrupts the cell membranes. Small intestine have low concentration of bile salts between 0.2-2 percent. Both the strains CD1 nd CD11 showed good survival after 8 h of incubation. The result of assessment are summarized in Table 6.

Table 6. Bile tolerance of CD1 and CD11

Bile salt concentration (%)	*Isolates*	Abs. at 0h	log cfu/ml	Abs. at 4h	log cfu/ml	Abs. at 8h	log cfu/ml	Mean±SD of log cfu/ml	Survival Rate
0.2	Cd1	0.094	8.717	0.093	8.688	0.092	8.664	8.689 ± 0.02	96.67
	Cd11	0.076	8.445	0.074	8.411	0.070	8.346	8.400 ± 0.05	93.86
0.4	Cd1	0.087	8.522	0.079	8.495	0.077	8.451	8.489 ± 0.03	94.44
	Cd11	0.071	8.397	0.068	8.334	0.063	8.294	8.341 ± 0.05	93.20
0.6	Cd1	0.048	7.948	0.033	7.716	0.023	7.049	7.571 ± 0.46	84.23
	Cd11	0.040	7.842	0.041	7.515	0.021	6.903	7.420 ± 0.47	82.91

Autoaggregation ability

Adhesive properties are essential for probiotic bacteria, as these provide protection of host mucosal surfaces against entry of pathogens. Aggregation inhibits adherence of pathogen via forming a barrier, which prevents colonization of pathogen thereby limiting their infection [12]. It can also increase the concentration of excreted inhibitory substances [13].both isolates CD1 and CD11 were good

autoaggregation percentage showing that they can be helpful in preventing host from pathogenic disease by preventing colonization of patohogenic bacteria intestinal membrane and may be act as barrier .

Table 7: Autoggregation ability of isolates

Isolates		OD_{600}					Autoaggregation (%)				
	0h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
CD1	0.53	0.03	0.03	0.02	0.01	**0.03**	94.0	94.0	96.0	98.0	94.0
CD11	0.72	0.02	0.05	0.06	0.01	**0.02**	97.0	93.0	83.0	98.0	97.0

Bacterial adhesion to hydrocarbons (BATH) test

The result of this study showed that the probiotic strains exhibited strong hydrophobicity towards non-polar solvents viz., chloroform, xylene, whereas low hydrophobic towards polar solvent, ethyl acetate. It was hypothesized that the presence of S-layer proteins on the cell wall of *lactobacilli* which have high isoelectric points showed strong affinity towards non-polar solvent. The results of microbial adhesion to hydrocarbons are mentioned in table 8.

Table 8: Bacterial adhesion test to hydrocarbon of isolates

Isolates	OD_{600}			% Hydrophobicity		
	Ethyl acetate	**Chloroform**	**Xylene**	**Ethyl acetate**	**Chloroform**	**Xylene**
CD1	0.93	0.85	**0.95**	81.0	77.0	84.0
CD11	0.98	0.24	**0.94**	80.0	71.0	74.0

DISCUSSION

In the present study, Lactic acid bacteria were isolated from cow dung . Various biochemical tests were performed for identification. None of the strains showed positive result towards gelatinase production, lipase production and hemolytic activity. Positive hemolytic activity (ability to breakdown red blood cells) halt the underlying epithelial layer whereas positive gelatinase activity (ability to hydrolyse gelatin) breakdown the protective lining of the GIT. Bile salts are surface active agents and amphipathic molecules. Bile acids are products of cholesterol metabolism and synthesized in liver. It is secreted in conjugated form (either with glycine or taurine) from gall bladder to duodenum (500-700 ml/day). Bile acids play an important role in digestive process (emulsification of fat). Bile concentration of intestine is 0.3% w/v. The average time of food transit through the small intestine varies generally from 1-4 hour. pH of small intestine is about 8.0. Presence of bile salts and pancreatin makes adverse conditions for survival in small intestine. Thus, strains selected for use as probiotic bacteria should possess acid-tolerant and bile-resistant qualities for providing health benefits. The presence of bacteriocin in both strains against indicator organisms showed having a probiotic potential. The multi-drug resistance needs to be solved with bacteriocin producing lactic acid bacteria. Interaction of the bacterial strain with itself (clumping of the cell) determines the auto-aggregation capability. Probiotic bacteria should adhere to the enterocytic cellular lines of oral cavity and GIT in order to exhibit their beneficial effects. Bacterial aggregation depends on the amount of biofilm production which helps in adhesion of the cell. Exact mechanism is not known of autoaggregation. It has been suggested that cell surface properties play key role in autoaggregation as well as hydrophobicity. Adherence to epithelia helps in evaluating the surface hydrophobicity towards the non-polar and polar solvent. A good probiotic must possess high autoaggregation and strong hydrophobicity. All the probiotic attributes tested in this study revealed the safe status of both the isolates for further use in food and fermentation industry. However, further evaluation of their beneficial effects on human beings will promote the application of both the strains in pharmaceutical and cosmetic industry.

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