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Isolation and identification of antagonistic *Lactobacillus acidiophilus* from curd

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Abstract- *Lactobacillus acidophilus* comes under the genus Lactobacillus which comprises of large group of beneficial bacteria that have similar properties and all produce lactic acid as an end product of the fermentation process. Previous studies shows that *L.acidophilus* has its own significant importance as it improves gastrointestinal function, boosts immune system, decreases the frequency of vaginal yeast infection and helps in reducing serum cholesterol levels. The present study is directed towards isolation and identification of antagonistic *L. acidophilus* from different regions of Allahabad. A total of 50 curd samples were collected from different regions of Allahabad. After careful examination of morphological and biochemical characteristics, all 74 isolates of different Lactobacillus species were found to be present in which 7 strains of *L. acidophilus* were found. Further these 7 strains were subjected to antagonistic test against selected bacterial pathogens and results revealed that all strains of *L.acidophilus* were found to be good antagonistic activity against selected bacterial pathogens. This study concluded that curds contain strains of *L.acidophilus* which shows good antagonistic property; thus revealing that it is safe and beneficial for consumption.

Index Terms- *Lactobacillus acidophilus*, curd, Antagonistic, Antibiotic sensitivity.

I. Introduction

Since the ancient times India has been traditionally rich when it comes to microbial diversity. Due to increasing population and food insecurity there has been rapid increase in the consumption of fermented food products. For ensuring the nutritional quality and safety of these fermented products a lot of research should be done.

The Lactobacilli comprises a large and diverse group of Gram (+ve), non sporulating, non motile rods or cocci that are associated to produce lactic acid as the major metabolic end products of carbohydrate fermentation (Pelinesco *et al*., 2009). Lactic acid bacteria are divided in to five genus, which are *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Bifidobacteria*, and *Pediococcus*. Out of which *Lactobacillus*, *Bifidobacteria* are the most common bacteria that are categorized under prebiotics (Espirito Santo *et al*., 2003). Lactic acid bacteria and their metabolites play vital role in improving the quality and shelf life of many fermented food products and used for biopreservation (Zottola *et al*., 1994), starter cultures for fermented food products and some of them are also component of intestinal micro flora (Fuller, 1992 and Holzapfel *et al*., 2001). Various strains of Lactobacilli provide additional health benefits by acting as bile tolerance (Walker and Gilliland, 1983), gastric juice tolerance (Kilara, 1982) and antibiotic resistance (Curragh and Collins, 1992)

Lactobacillus acidophilus gets its name from *lacto*-meaning milk, *-bacillus* meaning rod-like in shape, and acidophilus meaning acid-loving. This bacterium thrives in more acidic environments than most related microorganisms (pH-4-5 or lower) and grows best at 45°C celsius. *L. acidophilus* occurs naturally in the human and animal gastrointestinal tract, mouth, and vagina. *L.acidophilus* is a homofermentative microorganism which ferments lactose and produces only lactic acid. The traditional Indian fermented product is curd which is prepared by fermentation of the milk by lactic acid bacteria. Lactic acid bacteria convert milk sugar lactose into lactic acid and they suppress spoilage bacteria because of the production of antimicrobial substances. Consumption of curd has been reported to cure diarrhea (Agarwal and Bhasin, 2002); and reported to have anti-diabetic (Yadav *et al*., 2007), anticarcinogenic (Arvind *et al*., 2010) and few other benefits (Prajapati *et al*., 2014). It also plays an important role in preserving milk and milk products (Mutlag *et al*., 2013). In the present investigation the *L.acidophilus* bacteria has been isolated from curd from different localities of Allahabad (U.P). In a study similar as this one the lactic acid bacteria was isolated from vegetables and traditional fermented foods including dhokla batter, jalebi batter, idli, lassi, curd and cabbage (Patel *et al*., 2012 and Neha *et al*., 2014). In other study lactic acid bacteria was isolated from Appam batter and vegetable pickle (Vijai Pal *et al*., 2005).

Therefore the present study was carried out to evaluate the therapeutic values of curd with respect to the presence of antagonistic *L.acidophilus*.

II. Materials and Methods

1-Collection of curd samples: Curd samples were collected from the different localities of Allahabad city. The collection of curd samples was done in sterile, wide mouthed autoclavable sample bottles. After collection, the samples were immediately transported to the lab and were kept at 4°C until used.

2-Isolation and Maintenance of *L.acidophilius*: The intial isolation of *L.aciodoplhilus* strains from different curd samples was done on de Mann, Rogosa, Sharpe (MRS) medium. Samples were directly streaked on MRS media and incubated aerobically at 37°C for 24 hrs. After complete incubation obtained colonies were subcultured and were subjected to various identification parameters. The isolated and identified *L.acidophilus* strains were inoculated in MRS broth and after proper growth they were stored at 4°C for further study. Regular sub culturing of the isolates was done at every tenth day throughout the course of study.

3 Identification of *L.acidophilus*: Lactobacillus strains isolated in the study was identified on the basis of cultural, morphological and biochemical characteristics by the procedure described in Bergey's manual of systematic bacteriology (Holt *et al*., 1994, Williams and Wilkins, 1945).

3.1 Cultural Characteristics: Various cultural characteristics were studied for the identification of the isolates viz. color, surface, size, shape, centre, optical feature and elevation. The colonies appeared on MRS plates were separate, creamish white, smooth, small, round, flat, opaque and convex with no pigmentation.

3.2 Morphological Characteristics: Morphological examination of the isolates was done with the help of Gram staining (Holt *et al*., 1986). It was done for identifying and classifying bacteria as gram positive or gram negative. The bacteria, which retain primary stain (appear dark blue or violet) was named as gram-postive, whereas those that lose crystal violet and counter stain by safranin (appear red) were reffered to as gram-negative (**Fig -1**).

3.3 Biochemical Characteristics: The following biochemical tests were performed to identify the isolates like Slide Catalase test, Oxidase test, Carbohydrates fermentation test, Nitrate reduction test, Starch hydrolysis test, Litmus milk test, Aesculin hydrolysis test, Growth at 45°C and 15°C as mentioned in (**Table -1)**

III. Probiotic Pattern of *L.acidophilus*

4.1 Antagonistic activity of *L.acidophilus* against selected bacterial pathogens: It was done by agar well diffusion method on nutrient agar medium (Tagg and Mcgiven, 1971). Overnight (18hrs) broth culture of test pathogens (**Table -2**) was swabbed uniformly on the surface of nutrient agar (N.A) plates using sterilized cotton swabs. One plate of N.A. was kept as media control. For pathogen control, 6 N.A. plates were swabbed with the 6 pathogens. For treatment control, 6 N.A. plates were swabbed with 6 pathogens, single well was cut in each plate and only broth of 0.1ml of supernatant inoculums of *L.acidophilus* was inoculated in it. Plates were incubated at 37°C for 24-48hrs. Zone of inhibition formed around the wells was observed and measured in millimeters and results were recorded (**Fig-2**).

4.2 Antibiotic sensitivity pattern of *L.acidophilus*: The antibiotic sensitivity pattern of *L.acidophilus* was done on MRS medium. Overnight (18hrs) broth cultures of L.acidophilus were swabbed uniformly on the surface of media using sterilized cotton swabs. The following antibiotic discs (Penicillin, Ampicillin, Neomycin, Erthromycin, Tetracycline and Nalidixic acid) were placed on media with the help of forceps and plates were incubated at 37°C for 24-48 hrs. Zone of inhibition formed around the antibiotic was observed and measured in millimeters and results were recorded (**Graph -1**).

5 Statistical analyses: The obtained data from the study was statistically analyzed using "Two way Anova" (Fisher and Yates, 1968).

IV. Results

Isolation and Identification of *L.acidophilus* from curd: A total of 74 bacterial strain of *Lactobacillus* were obtained from 50 curd samples of 4 different zones of Allahabad like Naini (n=13), Behraina (n=13), Kareli (n=12) and Civil lines (n=12). The incidence of *L.acidophilus* was 7 (5.18%), *L.amylovorous* was 9 (6.66%), *L.yamanashensis* was 15(11.1%), *L.jensenii* was 3 (2.22%), *L.delburkii* was 9 (6.66%), *L.amylophilus* was 22 (16.28%), *L.salivaris* was 3 (2.22%), *L.bulgaricus* was 17 (12.58%), *L.helveticus* was 4 (2.96%), *L.vitulinus* was 8 (5.92%), *L.lactis* was 2(1.48%) and *L.crispatus* was 2(1.48%) as given in (**Fig-3**).

Antagonistic activity of *L.acidophilus*:

The antagonistic activity of isolated *L.acidophilus* strains were screened against 6 pathogenic bacteria viz. *B.cereus*, *B.subtilis*, *S.aureus*, *E.coli*, *S.typhi* and *S.dysenteriae* and showed different inhibitory spectrum **(Fig-2)**. Among these *L.acidophilus* strains *LA1* showed 12.5mm inhibition zone against *B.cereus*, 5.5mm against *B.subtilis*, 0.0mm against *S.aureus*, 7.5mm against *E.coli*, 5.5mm against *S.typhi* and 0.0mm against *S.dysenteriae*. *LA2* showed 16.0mm against *B.cereus*, 15.0mm against *B.subtilis*, 10.0mm against *S.aureus*, 6.5mm against *E.coli*, 8.5mm against *S.typhi* and 16.0mm against *S.dysenteriae*. *LA3* showed 6.5mm against *B.cereus*, 10.5mm against *B.subtilis*, 7.5mm against *S.aureus*, 6.5mm against *E.coli*, 17.0mm against *S.typhi* and 12.5mm against *S.dysenteriae*. *LA4* showed 9.5mm against *B.cereus*, 0.0mm against *B.subtilis*, 5.0mm against *S.aureus*, 13.0mm against *E.coli*, 13.5mm against *S.typhi* and 8.5mm against *S.dysenteriae*. LA5 showed 17.0mm against *B.cereus*, 0.0mm against *B.subtilis*, 0.0mm against *S.aureus*, 12.5mm against *E.coli*, 5.0mm against *S.typhi* and 10.5mm against *S.dysenteriae*. LA6 showed 18.5mm against *B.cereus*, 17.0mm against *B.subtilis*, 12.0mm against *S.aureus*, 13.5 mm against *E.coli*, 14.5mm against *S.typhi* and 6.0mm against *S.dysenteriae*. *LA7* showed 6.5mm against *B.cereus*, 0.0mm against *B.subtilis*, 6.0mm against *S.aureus*, 6.5mm against *E.coli*, 0.0mm against *S.typhi* and 5.5mm against *S.dysenteriae*. The study reveals that all the *L.acidophilus* showed inhibitory spectrum against the tested pathogens. Among studies *L.acidophilus* isolated *LA6* was found to be most antagonistic strain. However, *LA7* was found to be least antagonistic strain. Among the pathogens *B.cereus* was most inhibited strain followed by *E.coli*.

Antibiotic sensitivity pattern of *L.acidophilus*

The seven antagonistic strains of *L.acidophilus* was also screened against different antibiotics *viz.* Ampicillin (A), Neomysin (N), Erthromysin (E), Penicillin (P), Tetracyclin (T), and Nalidixic acid (Na). The study reveals that some strains of *L.acidophilus* were found to be sensitive, some were resistant and some were found to be intermediate to these antibiotics. Among these 7 strains *LA1* was found to be resistant to A, P, Na, and intermediate to N, E, T. *LA2* was found to be resistant to A, P, Na, T and intermediate to N, E. *LA3* was found to be resistant to A, N, E, P, Na and intermediate to T, *LA4* was found to be resistant to A, P, Na and intermediate to E and sensitive to T, *LA5* was found to be found resistant to A, P, Na, and intermediate to N, E and sensitive to T, *LA6* was found to be resistant to A, N, P, Na and sensitive to E, T. and *LA7* was found to be resistant to A, P, Na, T and intermediate to N and sensitive to E (**Graph -1**)

Statistical analysis of Antagonistic activity of *L.acidophilus* strains

The obtained data from the study was statistically analyzed using "TWO way ANOVA" (Fisher and Yates, 1968).No significant values were found as presented in (**Table -4).**

V. Conclusion

In the present study 7 antagonistic strains of *L.acidophilus* was isolated from 50 curd samples, collected from different regions of Allahabad. Primary study reveals that 7 strains of *L.acidophilus* have good antagonistic activity against selected bacterial pathogens. It is thus concluded that strains of *L.acidophilus* can be used as biopreservative agents and for manufacturing of fermented milk products. But detailed study on their role in human body, bile tolerance, acid tolerance, resistance against protolytic enzymes and gastric juices is needed to keep them in probiotic category. Last but not least, this study shows that Indian curd contains strains of *L.acidophilus* with good antagonistic property; hence it reveals that it is safe and beneficial for consumption.

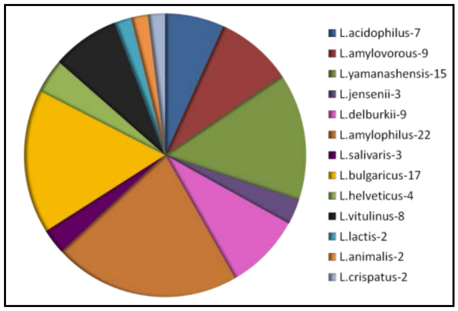
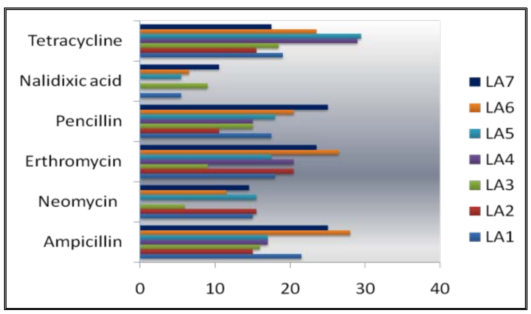


Fig 3- Incidence of *Lactobacillus spp.* in curd sample



Graph 1 - Antibiotic senstivity pattern of isolated *L.acidophilus*(LA) strains.
*** values includes diameter of well *i.e.* 5mm**

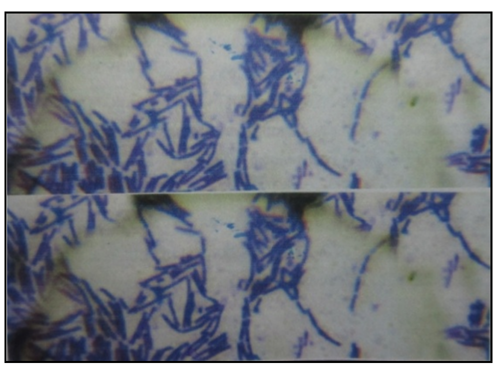


Fig 1:- Morphological characteristic of *L.acidophilus* (X100)

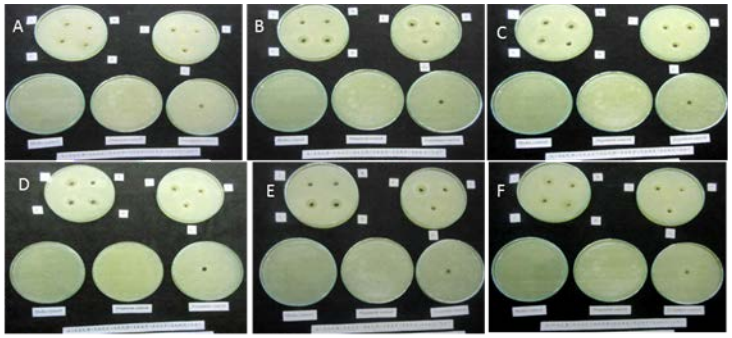


Fig 2- Antagonistic activity of *L.acidophilus* against selected pathogens.
A to *F - Antagonistic activity of *L. acidophilus* isolates against *B. subtilis, Bacillus cereus, Staphylo coccus aureus,* Escherichia *coli, Salmonella typhi, Shigella dysenteriae

Table no-1:- Biochemical characteristics of isolated *Lactobacillus spp*

Lactobacillus spp.	**No. of isolates**	**Catalase Test**	**Oxidase Test**	**Glucose**	**Glycerol**	**Lactose**	**Maltose**	**Mannitol**	**Sucrose**	**Nitrate reduction test**	**Starch Hydrolysis Test**	**Litmus Milk Test**	**Growth at 45^{0}C**	**Growth at 15^{0}C**	**Aesculin Hydrolysis**
L. acidophilus	7	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve
L. amylovorous	9	-ve	-ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve
L. yamanashensis	15	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
L. jensii	3	-ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve
L.delburkii	9	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve
L. amylophilus	22	+ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
L. salivaris	3	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
L. bulgaricus	17	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
L. helveticus	4	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
L. vitulinus	8	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve
L. lactis	2	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
L. animalis	2	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve
L. crispatus	2	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve

*+ve- presence, -ve-absence

Table no-2 Antagonistic activity of *L.acidophilus* against selected bacterial pathogens

Gram +ve	**Catalogue No.**	**Gram -ve**	**Catalogue No.**
Bacillus subtilis	MCCB-0064	*Escherichia coli*	MCCB-0017
Bacillus cereus	MCCB-0005	*Salmonella typhi*	MCCB-038
Staphylococcus aureus	MCCB-0046	*Shigella dysenteriae*	MCCB-0043

Table no-3 Antibiotic sensitivity pattern of isolated *L.acidophilus* strain

Strain	**Ampicillin**	**Neomycin**	**Erythromycin**	**Penicillin**	**Nalidixic acid**	**Tetracycline**
LA1	21.5 mm	15.0 mm	18.0 mm	17.5 mm	5.5 mm	19.0 mm
LA2	15.0 mm	15.5 mm	20.5 mm	10.5 mm	0.0 mm	15.5 mm
LA3	16.0 mm	6.0 mm	9.0 mm	15.0 mm	9.0 mm	18.5 mm
LA4	17.0 mm	0.0 mm	20.5 mm	15.0 mm	0.0 mm	29.0 mm
LA5	17.0 mm	15.5 mm	17.5 mm	18.0 mm	5.5 mm	29.5 mm
LA6	28.0 mm	11.5 mm	26.5 mm	20.5 mm	6.5 mm	23.5 mm
LA7	25.0 mm	14.5 mm	23.5 mm	25.0 mm	10.5 mm	17.5 mm

Table no 4 - Anova table of antagonistic activity of *Lactobacillus acidophilus* strains

Source of Variance	**d.f**	**S.S**	**M.S.S**	**F (cal)**	**F (tab) 5%**	**Result**
Due to pathogens	5	437.125	87.42	5.11	2.53	Non significant
Due to isolates	6	182.67	30.44	1.78	2.42	Significant
Due to error	30	513.20	17.10			
Total	41					

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