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Isolation And Identification Of *L. Acidophilus* Isolated From Milk And Curd And Its Antagonistic Activity Against Pathogens.

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Abstract

Lactobacillus is a genus of gram-positive, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria. *Lactobacillus acidophilus* plays a most important role in the fermented products of the dairy industry and delivers the therapeutic characteristics of human health. As the greater quantity of antibiotics was used for the treatment of humans and farm animals as well and even for fish in aquaculture, that will result in the selection of pathogenic bacteria resistant to multiple drugs. Several research a great focus on the Identification of Lactic Acid Bacteria, that include conventional biochemical examinations like carbohydrate fermentation outlines using commercially accessible kits, physiological tests, and complex techniques by using molecular-based biology methods. More recently, Bio-log and API Kit have been applied to an enormous amount for Lactic Acid Bacteria Identification. The current study is carried out to isolate, identify, and evaluate the antagonistic activity of *L. acidophilus.* obtained from curd and milk samples as well. The antimicrobial action of this species was assessed with the agar well diffusion method against some pathogenic bacteria (*Staphylococcus aureus, Escherichia coli, and Salmonella typhi*). L. acidophilus. isolated and identified based on biochemical characterization as catalase-negative, motility negative, citrate negative indole negative, and fermentation of carbohydrates. In the present study, the total number of six isolates were detected as *Lactobacillus sp.* The isolated and identified *L. acidophilus* strains (n=6) were examined for their antagonistic activity against MDR strains of *S. aureus, E. coli.* and *S. typhi.*In the study, maximum inhibition was detected by LA1and LA4 against *S.aureus* (18mm) and *S.typhi* (18mm), respectively. The bare minimum inhibition was noticed in the case of LA3 against *S. typhi* i.e., 4.0mm. Other Lactobacillus isolates exhibited modest or nil antagonistic activity against tested pathogenic bacteria.

Keywords- *Lactobacillus acidophilus*, Curd, Antimicrobial Activity, Bacterial Pathogens (*S. aureus, E. coli, and S. typhi*).

Introduction

Probiotics are living organisms as vitals that are utilized as foodstuff additives with positive impacts on the healthful body by establishing microbial equilibrium in the gastrointestinal tract. (Hassanzadazar etal.,2012). The most vivacious group of acid production by bacteria in the food trade is the lactic acid bacteria (LAB), which are employed for dairy articles as starter culture (Gharaei-Fathabad and Eslamifar,2011) LAB as protective cultures are the most common of probiotics that are reflected as safe due to having a very specific type of features. Major genera of LAB are *Lactobacillus, Streptococcus. Lactococcus, Pediococcus, Enterococcus, Leuconostoc,* and *Bifidobacterium,* (Tafvizi etal., 2012) These types of bacteria trigger a decrease of diseases of the gastrointestinal tract by improving helpful microorganisms' growth and decreasing pathogens' inhabitant processes. (Hawaz,2014)) Lactic acid Bacteria are generally disseminated in the real environment that can prevent the development of pathogenic microbes by creating specific elements. (Hawaz,2014) Lactobacilli are gram-positive, catalase-negative microaerophilic or facultatively anaerobic, on-spore forming, rod-shaped bacteria (Pelinescu etal.,2009) that are recognized as the most essential probiotics and appropriate microflora. of

gut (Nsofor,2014). These are most of the general probiotic bacteria useful proposed for the food commerce as the acids produce by them inhibit pH under the growth range which causes metabolic quietness of most pathogens (Savadogo,2006) and are homofermentative bacteria that grow voluntarily at low pH (below pH 5.0) (Thakkar,2015) Lactobacilli have an essential role in limiting unwanted microflora present in the gut and can avoid the increase of pathogenic bacteria by making antimicrobic metabolites. These can be exploited as biotic preservers and are designed easily in foods. (Oyetyo 2004). Most Lactic Acid Bacteria despite their origin, can hinder the growth of pathogenic bacteria, including antibiotic-resistant bacteria because of their ability to produce many antimicrobial metabolites. Antimicrobial action, acid tolerance, and bile salinities are three key qualities for assessing the probiotic capability of bacteria that are efficient to be used as therapeutic. LAB generates hydrogen peroxide, diacetyl, organic acids, bacteriocins, and antifungal mixtures like fatty acids through lactic acid fermentation. Bacteriocins are protein composites with the ability of growth inhibition of sensitive pathogenic bacteria and different degradation systems in the digestive system compared with antibiotics. (Hawaz,2014). LAB are resilient to gastrointestinal juice, bile salts, lysozyme, and gastric acid. Different antimicrobial composites are also prepared from LAB to have battled and prevented pathogenic microbes.(Hawaz,2014)) Such types of combinations might influence the metabolic rate or toxins of different pathogenic bacteria.(Rushdy Abeer and Gomaa Zakaria,2013, Osuntoki and korie ,2010)Lactobacillus acidophilus, primarily isolated and identified from baby feces, has gone through many makeovers in the depiction of its metabolic, functional, and taxonomic characteristics. L. acidophilus got its name from two words- lacto=milk, bacillus=rod-like, and acidophilus stands for acid-loving. Several strains of this species are commercially utilized in several dairy artifacts. This bacterium also supports our gastrointestinal system for breaking down sugars, like lactose into Lactic acid. *L.acidophilus*, like many probiotic supplements, when consumed gives a great health benefit normally by improving or restoring microflora of the gut (Hawaz,2014) *L.acidophilus* is also connected with antagonistic actions ahead growth for *Staphylococcus aureus, Escherichia coli, Salmonella typhi,* and *Clostridium perfringens*. The S. aureus, out of all the four pathogenic organisms, was the most affected by *L. acidophilus. Lactobacillus acidophilus* is the type of Lactic acid bacteria that have been receiving considerable notice as probiotics since they have the innate ability to put forth antagonistic activity. A considerable number of investigations have been done on Lactobacilli which prominence on their health-promoting properties and method of antimicrobial action. Several functional foods are consumed as part of a normal diet, and they provide consumers with well-documented and physiological benefits such as probiotic bacteria. Corresponding to different scientific reports, ever-increasing fat loss and immune response of the host, antibiotic-induced diarrhea heightening symptoms of irritable bowel syndrome, antiallergic and anticancer effects, and intestinal inflammation, are some other helpful impacts of probiotics. (Shokryazdan etal 2014 and Nsofor,2014) The connection between assured food and health profits has been explored for numerous years. In present years, there have been several active studies in probiotics, because of that of the growing commercial attention to probiotic food. Currently, probiotics are utilized not only as a main part of growth but additionally as a stimulator of the immunity and inhibition of many illnesses. (Piano Ballare etal.,2004) Probiotic foods as of their nutritive value and healthiness sector throughout the previous time like the therapeutic impacts are undertaken into concern (Smid, 2005 and Islam etal., 2012) Probiotics are living microbes and multiply in the human guts that give a healthiness advantage by altering the enteric microflora. Milk is the crucial source of food for infants in mammals before any other type of food digestion. It encloses many extra nutrients, as well as protein and lactose (Pehrsson,2000) Curd, is a very trendy menu at the last of almost every meal in India. The present study was intended to screen the antagonistic and antimicrobial activity of *Lactobacillus acidophilus isolated* from milk and curd samples against selected pathogenic bacteria.

Materials and Methods

Sample collection and processing

The present study was carried out at Biogen Laboratory Prayagraj. For the isolation of *Lactobacillus acidophilus*, two dairy products were used, milk and curd. The antagonistic action was examined against three pathogenic bacteria i.e. (*Staphylococcus aureus, Escherichia coli, and Salmonella typhi).* For the study, raw samples of milk and curd were gathered from the local market of Prayagraj city in pre-sterilized sample bottles. After collection, samples were immediately transported to the laboratory and were kept under controlled conditions.

Isolation of Lactobacillus acidophilus

Isolation was done by the pour plate and streak plate method in aerobic as well as the microaerophilic environment in the following way.

Isolation by pour plate method.

The milk samples were diluted to 10^4 levels in Ringer's solution., 1ml aliquot was taken and was transferred in sterilized Petri plates in triplicates. After this molten and cooled MRS agar was poured (15-20ml) in the Petri plates and the plates were allowed to solidify at room temperature. All the total plates were incubated at 37 ^{0}C for 24-48 hours under aerobic and microaerophilic conditions using the candle jar extension method and observations were carried after 24-48 hours (Charteris,2007)

Pure culture preparation by streak plate method

Bacterial colonies were purified by subsequent subcultures using the streak plate method. MRS Agar media was poured onto sterilized plates and left for solidification. After solidification, plates were streaked with sample and curd samples in triplicates with the help of a sterilized inoculation loop. The plates were incubated at 37^{0}C for 24-48 hours.

Identification of *Lactobacillus acidophilus*

The identification of isolated *L. acidophilus* was done by cultural, morphological, physiological, and biochemical analysis.

Identification by cultural characteristics

The obtained colonies on MRS agar plates were observed for the various colony and cultural characteristics such as colony shape, size, margin, elevation, texture, odor, and color. Along with it, various growth conditions (aerobic and microaerophilic conditions) were also observed. After proper growth, the suspected colonies were selected and screened for their morphological physiological, and biochemical characteristics.

Identification by morphological characteristics

The morphological examination of the isolated bacteria was performed by Gram's staining. For this, a single colony of every isolated bacterium was taken and stained according to the standard protocol of gram staining and observed under an oil immersion microscope.

Identification by biochemical characteristics

To study the biochemical characteristics and to confirm the Lactobacillus acidophilus following biochemical experiments were performed.

Catalase test: One drop of hydrogen peroxide was taken on a clean glass slide and very little amount of isolated colony was transferred on it with a clean glass rod. Immediate production of effervescence indicated a positive result i.e., the culture can produce catalase enzyme.

Motility test: A straight needle was touched to a colony of isolated bacteria in and stabbed once to a depth of half an inch in the middle of the tube and incubated at 35-37˚C and examined daily for up to 7 days. The needle was kept in the same line it entered as it was removed from the medium.

Citrate Utilization test: The isolated Lactobacilli were inoculated in Simmons Citrate Agar and incubated at 37˚C for 24 hours. After 24 hrs., the change of media from green to blue was recorded accordingly for the isolated bacteria.

Indole Production test: The suspected Lactobacilli were inoculated in peptone broth and incubated at 37˚C for 48 hours. After incubation, Kovac's reagent (1ml in each tube) was added and looked for the formation of the red precipitate.

Gelatin Hydrolysis test: In a set of test tubes Gelatin media was poured and autoclaved. After autoclaving, a straight needle was touched to a colony of isolated bacteria and stabbed once to a depth of the tube and removed the needle all through the same line as it went inside. The tubes were then incubated at 37˚C for 48 hours. After incubation, the tubes were kept in the refrigerator to check the solidification of gelatin (in case gelatin was liquified) and the gelatin hydrolase enzyme activity of the isolates.

Carbohydrate Fermentation test: For carbohydrate fermentation test where neutral red was used as an indicator in the basal media Neutral red broth base or the basal media was prepared, poured into tubes and Durham tube was inserted in each tube for gas detection followed by the autoclaving at 15lbs/inch^2 for 15-20 mins. Different sugar substrates – Glucose, Mannitol, Sorbitol, Arabinose, and Salicin were prepared 1% and autoclaved at 10lbs/inch^2 for 10 mins. After autoclaving, 1ml of respective sugar and isolated bacteria was transferred in the tubes and kept back inside the incubator for 48 hours at 37˚C.

From each sugar set, i.e., from the set of Glucose, Mannitol, Arabinose, Sorbitol, and Salicin, one tube was kept as control (without inoculation of isolated bacteria) inside the incubator at the same temp. and time.

Identification by physiological characteristics

After Gram staining, isolated bacteria were further checked for their growth at three different temperatures and three different sodium chloride (NaCl) concentrations.

Growth at 10˚C,15˚C, and 45˚C

The isolated suspected Lactobacilli were streaked on MRS Agar plate and tested for their ability to grow at three different temperatures that were 10˚C and 15˚C incubated for one week and 45˚C kept inside the incubator for 24-48 hours.

Growth at different NaCl (2%,4% and 6.25%) concentrations

The isolates were streaked on three different concentrations of NaCl in MRS plates and incubated at 37˚C for 48 hours. After the incubation period, the plates were examined for the proper growth of bacteria at the concentration of NaCl.

Detection of antagonistic activity of isolated *L. acidophilus*

All Gram-positive and catalase-negative bacilli were selected for the assessment of antimicrobial ability. An Antimicrobial effect of isolates was evaluated by a well diffusion test on the Nutrient Agar medium. plated with three pathogens. For this purpose, the fresh culture of isolates was centrifuged (8000 rpm, 15 min) and supernatants were removed. Pathogens (*S. aureus E. coli, and S. typhi*,) were swabbed in four plates evenly Nutrient Agar Plates by using sterilized cotton swabs, while one plate was kept as media control (without swabbing of any pathogen). For control 1 well was cut in one swabbed plate and filled with 1ml of MRS broth only. For treatment 3 wells were cut in two swabbed plates and filled with 0.1 ml of cell-free extract of L. acidophilus obtained from centrifugation of 24 hrs. old, isolated L. acidophilus and labeled as LA1, LA2, and LA3on one plate and LA4, LA5, and LA6 on the other. One swabbed plate was kept without any treatment to evaluate any other reason for inhibition of microbes All the plates were then incubated at 37˚C for 24-48hrs. After incubation, a zone of inhibition was observed, measured in millimeters, and then results were recorded.

Results

Isolation and Identification of *Lactobacillus acidophilus*

In the present study, a total of nine isolates were isolated and identified from milk and curd samples .As per the results of gram staining it was evident that all nine isolates were gram-positive rod-shaped and could be identified as *Lactobacillus.* Out of these four-six isolates were suspected as *L. acidophilus,* based on biochemical, cultural, morphological, physiological characteristics. Out of these 6 isolates, two were obtained from milk while four were isolated from curd. The isolates were non- catalase-negative, non-motile, Indole negative, nitrate negative, and Gelatin Hydrolase negative (Table-1). The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to its simplicity. On performing the catalase test, there was no bubble observed on the slide showing that the isolated bacteria were catalase-negative and could not decompose hydrogen peroxide to produce oxygen. It supports the specific features of *L. acidophilus* as catalase negative. The motility test was also negative which showed that the isolated bacteria were non-motile. Non-motility is the main characteristic of *L. acidophilus.* The green color of Simmons Citrate Agar media was not changed in the Citrate Utilization test showing the isolates could not utilize Citrate, it helps in the identification of *L. acidophilus* as it is citrate negative. The Indole producing test and Gelatin Hydrolase test were also negative indicating that the isolates could not produce indole and has no hydrolase enzyme synthesis in them. These are the well-known characteristics of *L. acidophilus.* Among all the nine isolates, six were fermenting the Glucose, Mannitol (without any production of gas) and could not ferment Sorbitol and Arabinose while the three isolates could ferment Sorbitol and Arabinose and were not fermenting Mannitol (Table 2). These results of carbohydrate fermentation were evident for the confirmation of *L. acidophilus.* Among the nine isolates, no isolate grew at 10˚C while six of them were able to grow at 15˚C and all the nine were grown at 45˚C (Table 3). these results again confirm the presence of *L.acidophilus.* Among all the nine isolates all have growth at 2% and 4% of salt concentration but six isolates which were identified as L. acidophilus were not able to grow at 6.25% of salt concentration (Table 4)

The antagonistic activity by L. acidophilus against selected pathogens

The isolated and identified *L. acidophilus* strains (n=6) were screened for their antagonistic action against MDR strains of *S. aureus, E. coli, and S. typhi.* where they showed inhibitory actions by forming clear inhibition zones (Table-5) after using the agar well diffusion method. In the study Maximum zone of inhibition was observed by LA1 against *S. aureus*

(18.0 mm), LA 3 against *E.coli* (17.0 mm), and LA 4 against *S.typhi* (18.0 mm).(Fig 1,2,3.4.5,6 and Table 5) .Moderate inhibitory effect was observed by LA1 against E.coli (13.0 mm),LA2 against *E.coli* (15.0 mm), and LA 5 against *S typhi* (12.0 mm) (Fig 1,2,3,4,5,6 and Table 5) .Mild Moderate antagonistic effect was observed by LA2 against *S typhi* (7.0 mm),LA4 (9.0 mm), LA5 (6.0) and LA 6 (5.0 mm) against S.aureus (Fig 1,2,3,4,5,6 and Table 5) .The minimum or mild effect was observed by LA 3 against *S typhi* (4.0 mm) (Fig 3,6 and Table 5.).Almost nil antagonistic effect was recorded by LA1 against *S typhi*, LA 2 and LA 3 against *S. aureus*, LA4 and LA5 against *E.coli* and LA 6 against *E coli* and *S typhi* (Fig 1,2,3,4,5,6 and Table 5)

Discussion

In the present study, 6 isolates were identified as almost the same results were interpreted by earlier studies (Guessas and Kihal 2004) as Lactic acid bacteria were obtained from goat's milk in Algerian arid zone and reported the isolates as gram-positive, catalase-negative, and non-sporing bacteria. According to some results of studies, it is evident that isolated bacteria were pragmatic by light microscope (Cortés-Zavaleta, Tal 2014) The gram staining outcomes indicated that the isolated bacteria can be identified as lactobacilli One study showed isolation and detection of Lactobacillus from local yogurt and by using carbohydrate fermentation test (Chowdhury and Islam 2016). It was reported that glucose (used as the carbon source in the test) was fermented without the formation of any gas in Durham's tube i.e., the only acid was produced. In the current study, all six isolates were able to produce acid by changing the color from red to yellow but did not form any gas bubble (Table-2). Phenol red broth basal media was used as an indicator to distinguish the bacteria according to their outlines of carbohydrate utilization, showing that the isolated bacteria can ferment mannitol and glucose, but could not ferment sorbitol and arabinose. No bubble was observed inside the Durham tube in glucose and mannitol which indicated that no gas production could be associated with the growth. The results of the present study matched with *L. acidophilus* strain characteristic the present study some of the screened isolated of *L. acidophilous* showed inhibitory effect from maximum, moderate, mild and moderate, mild and nil that is also evident from various studies showing that isolated Lactobacillus species with inhibitory metabolites which were diffusible and extracellular because of which they were able to reduce the growth of the microbes. It is documented that several mechanisms have been credited to explain antagonistic activities of LABs (Hawaz,2014) These mechanisms may be lowering the pH of the harboring environment by producing lactic and acetic acid, competition for nutrients, and adhesion sites with other inhabiting bacteria in surroundings (Gupta and Bajaj 2017). Production of antioxidants and bacteriocins are some of the enunciated mechanisms of antimicrobial exploits. It is reported that lactic acid bacteria are having tolerance against high salt concentrations as it permits the bacteria to initiate metabolism, which generates acid that further reduces the growth of undesirable microorganisms. (Pathak and Dutta 2016) In the present study the isolates tolerating high salt concentration showed maximum inhibition production of some antimicrobial compounds such as organic acids, short-chain fatty acids, and bacteriocins are responsible for the inhibition of the growth of pathogenic microorganisms (Khalil ,2015). The antimicrobial effect of lactobacilli is linked to the production of organic acid, hydrogen peroxide, and antimicrobial peptides with a variable range of actions were observed (Forouhandeh, 2010 The sensitivity of Gram-negative pathogens can be connected to their thin peptidoglycan cell walls and their susceptibility concerning with acidic metabolites. (Cortes-Zavaleta etal.,2014) A significant number of studies have been completed on Lactobacilli which prominence on their health-promoting properties and for the antimicrobial action (Thakkar etal.,2015). Lactic acid bacteria with gastric and intestinal juice and intestinal absorption capacity can be used as potential probiotics for further development (Chowdhury and Islam,2016) Single strain of *Lactobacillus helveticus* MB2-1 was isolated from traditional fermented milk and recognized numerous mechanisms which have been certified to enlighten antagonistic activities of Lactic Acid Bacteria. (Li etal., 2014).

Conclusion

The result of the current study showed that different strains of *L. acidophilus* isolated from milk and curd by serial dilution pour plate technique prove antimicrobial activity against three selected pathogenic bacteria *S. aureus, E. coli.* and *S. typhi*. The tested isolated strains of *L. acidophilus* showed an inhibitory zone against these pathogens. Among all the six strains of L. acidophilus, LA1 showed the highest inhibitory action against *S. aureus* and *E. coli* and LA4 against *S. typhi.* In the current study, four strains of *L. acidophilus* were isolated from curd while only two isolates were obtained from milk, it can be concluded that mostly the curd and up to some extent milk also have excellent antagonistic properties. Therefore, this study confirms that taking milk and consumption curd is safe and beneficial for humans as it protects or provides resistance to the human body against these tested pathogens. the results of this study, antagonistic effects of produced substances by the bacteria on the pathogens, have an important role in food preservation and human health. The study on their role in the human body, bile tolerance, acid tolerance, resistance against proteolytic enzymes, and gastric juice is required to keep them in a separate probiotic category. These bacteria can be raised to produce various kinds of food and pharmaceutical products. They can also be used to produce new functional foods. Therefore, increasing the use of dairy products containing probiotics, identification, and production of foods containing the highest and most

effective lactobacilli are recommended in daily diet. Milk and curd (particularly Indian ones) are fit for health and the consumption of both is safe.

Table 1: Results of biochemical identification of Lactobacillus isolates

Test	Number of Isolates of Lactobacillus								
	1	2	3	4	5	6	7	8	9
Catalase	-	-	-	-	-	-	-	-	-
Motility	-	-	-	+	-	+	-	-	+
Citrate	-	-	-	-	-	-	-	-	-
Gelatin Hydrolase	-	-	-	-	-	-	-	-	-
Indole Production	-	-	-	+	-	+	-	-	+

Table 2: Results of carbohydrate fermentation of *Lactobacillus* isolates

Type of Carbohydrates	Number of Isolates of Lactobacillus								
	1	2	3	4	5	6	7	8	9
Glucose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	-	+	-	+	+	-
Sorbitol	-	-	-	+	-	+	-	-	+
Arabinose	-	-	-	+	-	-	-	-	+

Table 3: Growth at different temperatures of *Lactobacillus* isolates

Temperatures	Number of Isolates of Lactobacillus								
	1	2	3	4	5	6	7	8	9
10˚C	-	-	-	-	-	-	-	-	-
15˚C	+	+	+	-	+	-	+	+	+
45˚C	+	+	+	+	+	+	+	+	+

Table 4: Growth at different salt concentrations of *Lactobacillus* isolates

Salt concentrations	Number of Isolates of Lactobacillus								
	1	2	3	4	5	6	7	8	9
2%	+	+	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+	+	+
6.25%	-	-	-	+	-	+	-	-	+

Table 5: Inhibitory effect of isolates on *L. acidophilus* against pathogens *S. aureus, E. coli* and *S. typhi.*

S. No.	L.acidophilus strains	Zone of inhibition against pathogens (mm)		
		S. aureus	E. coli	S. typhi
1	LA1	18mm	13.0mm	0.1mm
2	LA2	0.2mm	15.0mm	7.0mm
3	LA3	0.1mm	17.0mm	4.0mm
4	LA4	9.0mm	0.1mm	18.0mm
5	LA5	6.0mm	0.2mm	12.0mm
6	LA6	7.0mm	0.1mm	0.1mm

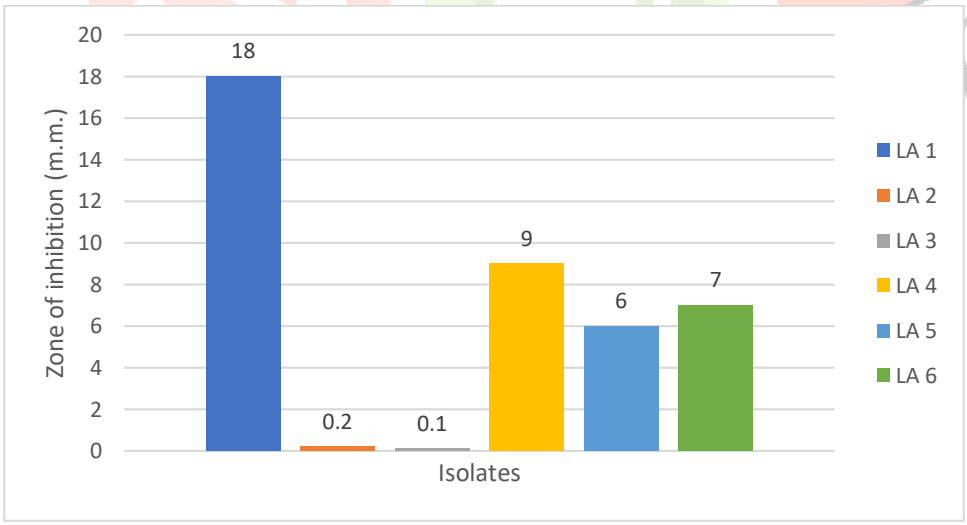

Fig 1 : Inhibitory effect of isolates of *L.acidophilus* against *S. aureus*

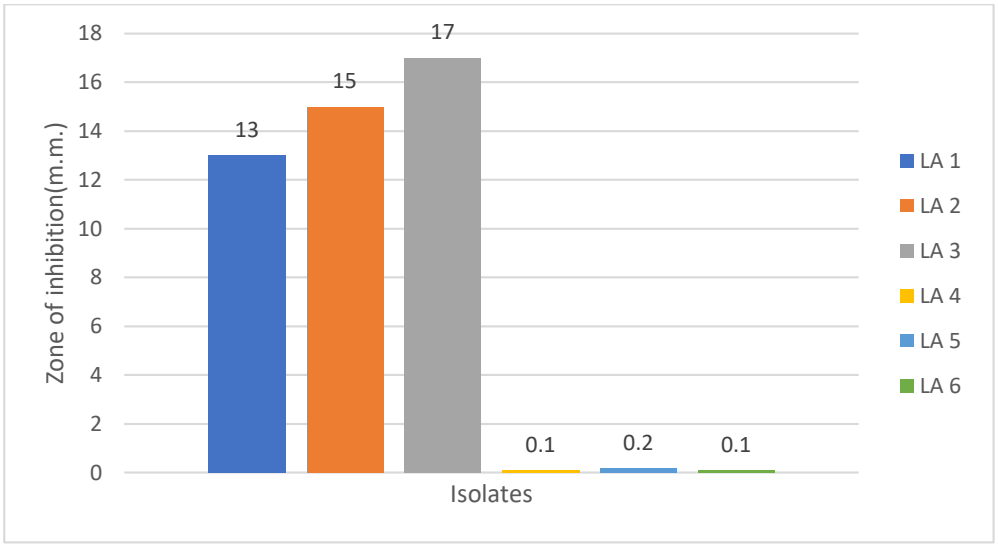

Fig 2 : Inhibitory effect of isolates of *L.acidophilus* against *E. Coli*

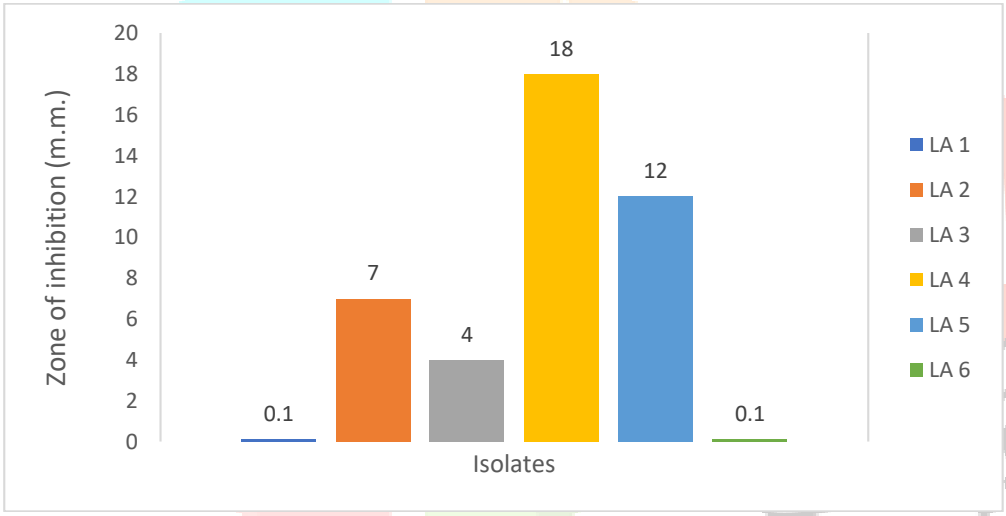

Fig 3 : Inhibitory effect of isolates of *L.acidophilus* against *S.Typhi*

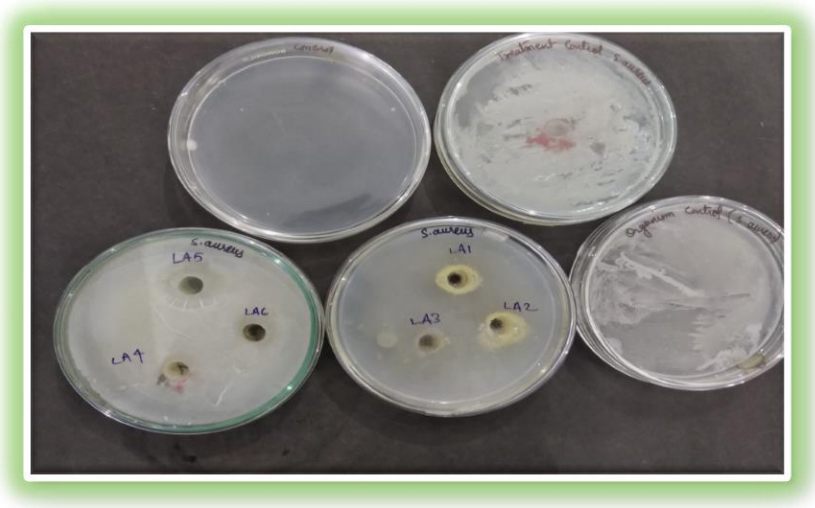

Fig 4 - Antagonistic activity of L. acidophilus against S. aureus

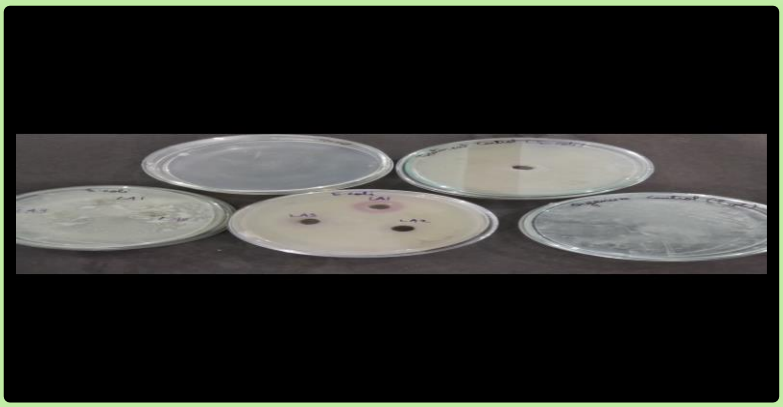

Fig 5: Antagonistic activity of L. L. acidophilus against E. coli

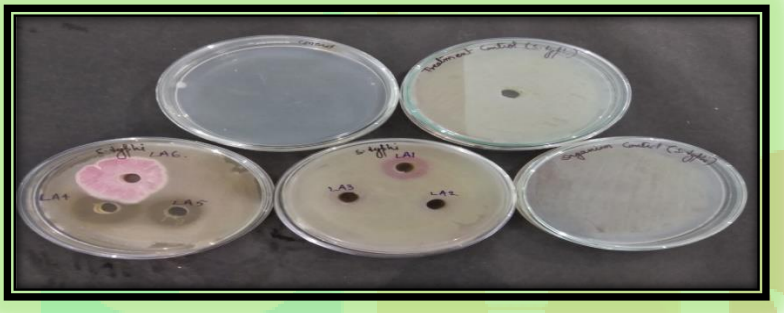

Fig 6: Antagonistic activity of L. acidophilus against S. typhi

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