PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA HAVING LIPOLYTIC ACTIVITY

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Abstract **:** Among 24 LAB isolates from 32 various dairy samples, seven strains were screened by lipolytic activity using tributyrine agar plates. All seven selected LAB isolates possesses lipolytic activity are characterized by Biochemical and morphological tests identified the character resemblance to *Lactobacillus sp.* These isolates are designated as L_{13}, L_{14}, L_{15}, L_{16}, L_{17}, L_{18}, and L_{19}. All isolates were studied for major four probiotic attributes such as acid tolerance, bile salt tolerance, antibacterial activity and antibiotic resistance. Acid tolerance was performed at pH 3 and pH 4, while bile salt tolerance was performed at 0.15% and 0.3% concentration of bile salt, both tests were assessed by turbidometric growth measurement as optical density at 600 nm. Antibacterial activity was performed by Agar well diffusion against *S. aureus, E. Coli, B. cereus* and *Salmonella typhi*. Antibiotic sensitivity was accessed by disc diffusion test using standard antibiotic discs (Hi media) of novobiocin (NV) and erythromycin(E), chlortetracycline(CT), gentamycin, tobramycin(TOB), collistrin(CL), carbenicilin(CB), nitrofurantoin(NIT), lincomycin(L), cefuroxime(CXM), Kanamycin(K). In present study, among seven lipolytic LAB isolate L_{17} and L_{18} have good probiotic potential with efficient lipolytic activity, while L_{15} and L_{16} have efficient probiotic activity with less lipolytic activity.

Keywords: LAB, MRS, Probiotic, GI tract, Lipolytic activity etc.

I. INTRODUCTION

According to World health organization (WHO) Probiotics are "Live microbial food supplements which when administered in adequate amount confer health benefit on host (M. G. Shehata*et.al.* 2016). Lactic acid bacteria (LAB) are gram positive , non-spore forming, catalase negative, cocci or lactobacilli which produce lactic acid from fermentation of carbohydrates. LAB are major component as component as starter culture in dairy industries. Lactic acid bacteria are regarded as major group of Probiotic bacteria and have been successfully treated in various diseases like acute infantile diarrhoea and intestinal disorder. *Lactobacillus* is one of the most important genera of lactic acid bacteria (Eid *et.al*.2016).

Lactic acid bacteria shows competition with harmful gut flora, adhesion to epithelium of GI tract, enhancing host immunity, producing antibacterial substances, Probiotic and antagonistic activity, cholesterol assimilation (Rashmi B. Gayathri D.2014).The ideal Probiotic strains have special properties such as resistant to bile, hydrochloric acid and pancreatic juice, the ability to tolerate stomach and duodenum condition and gastric transport, stimulation of immune system thereby improving intestinal function (Suneeti*et.al.* 2017).

Lipase have broad biotechnological application as they hydrolyse fat in to fatty acid and glycerol. Several study showed that Probiotic isolates producing lipase can be used to reduce cholesterol level and overcome malabsorption in aids in proper digestion of fat in diets. Some study showed Probiotic with lipase producer bacteria have broad application in food industry for hydrolysis of milk fat, lipolysis of butterfat and cream, improving cheese flavour, enhance rate of cheese ripening (Rashmi B. Gayathri D. 2014). According to guidelines for evaluation of probiotic in food reported by joint FAO/WHO working group the most widely used *in vitro* tests are resist ant to gastric acidity and bile salt, antimicrobial activity, cholesterol assimilation and antibiotic sensitivity (M. G. Shehata*et.al.* 2016).

The aim of the present study is to isolate is to isolate best possible Lactic acid Bacteria (LAB) having lipolytic activity and select the most suitable strains of probiotic bacteria by evaluating major probiotic characteristics. However the beneficial effect of probiotics are strain specific pointing to need to use various natural resources to identify new probiotic candidates therefore the selection of the LAB candidates from fermented local dairy product is of great significance.

II. MATERIAL AND METHODS

2.1 Collection of source sample:

Total 32 samples, among which ten milk samples, ten curd samples, five cheese samples and seven samples of Calves infant faces were collected in sterilized test tubes. All samples were collected from nearby villages from Nanded district of Maharashtra, India. Samples were kept at refrigerated (-4^{o}C) condition until microbiological analysis for isolation of Lactic acid Bacteria.

2.2 Isolation of Lactic acid bacteria:

Curd samples and calves infant faeces were diluted serially from 10^{-1} to 10^{-10} using sterile physiological saline (0.85% NaCl) then 0.1 ml of aliquot of higher dilution were spread on MRS (deMan Rogosa Sharpe) agar plates and incubated anaerobically at 37°C for 48 h. Milk samples were incubated until coagulation at 37°C, while cheese samples were cultured in sterilized reconstituted skim milk and incubated until coagulation at 37°C. Coagulated samples were streaked on over agar surface of MRS medium and were incubated anaerobically at 37°C for 48h.White and creamy colonies were picked up randomly and purified transferring on MRS agar medium thrice to get a pure culture.The culture were routinely checked for purity by microscope examination (M.G. Shehata*et.al*2016).

The pure cultures were characterized by Grams staining, cell morphology and catalase reaction using standard protocol (K.R. Aneja.2003). Gram positive and catalase negative lactobacilli were selected, maintained on MRS agar slants and preserved in MRS broth and 20% glycerol and stored at -20°C. (Eid, *et. al.* 2016).

2.3 Screening of Lipase producer:

All isolated Lactobacilli strains were screened using tributyrin agar. All were inoculated on to tributyrin agar and kept for incubation in microaerophilic condition at 37°C for 72 h. Lipase producer bacterial colonies showed opaque zone around the colony (potentially positive for lipase activity) were selected for Morphological ,biochemical characterization and further study. (Rashmi B S and Gaythri D. 2014).

2.4 Qualitative determination of Lipolytic activity by disc diffusion method:

The isolate capable for lipase production were further screened to isolate the best possible *Lactobacilli* based on agar disc diffusion method. Sterile Methyl red agar plate with olive oil is used for disc diffusion method. Each bacterial isolate were taken one loopful and cultured in to 5 ml liquid medium with composition NaCl 1%; Yeast extract 1%; peptone 2%; Tween-80 1% and sterile olive oil 2%. Paper disc of 5mm was dipped into incubated bacterial cultural for 10-15 min and put into the methyl red agar plate with composition (gm/lit): peptone 10gm; NaCl 5gm; $CaCl_2$ 0.1gm; Agar 20gm; 2.5% tween-80; 5% olive oil and 0.01% methyl red. The culture was incubated for 3 to 5 days. The clear zone was measured in mm and used for further study (Niken Candra Bestari, Suharjono, 2015).

2.5 Probiotic Characterization:

2.5.1 Acid Tolerance

Acid tolerance test was performed using MRS broth medium. MRS broth of pH 3, pH 4 and pH 6 (Control) were prepared using 1N HCl. MRS broth with pH 6 was used as Control. All broth including control were autoclaved at 121°C for15 min and inoculated with 1% overnight grown culture of selected LAB isolates in MRS broth. All broths were incubated at 37°C. Optical density as growth rate was measured by spectrophotometer at 600 nm after 2 and 4h of incubation (P. Muthukumar and C. Kandeepan 2015).

2.5.2 Bile Tolerance

Bile salt tolerance was further tested in MRS broth which contains 0.0%, 0.15% and 0.3% concentration of bile salt. 1% overnight grown culture of each isolates was inoculated in each broth and incubated at 37°C. Growth was assessed by measuring optical density at 600 nm after 2 and 4h of incubation (P. Muthukumar and C. Kandeepan 2015).

2.5.3 Antimicrobial Activity

Antimicrobial activity of LAB isolates against pathogenic strains was assessed by Agar well diffusion assay. Test organisms used were *Escherichia coli, Staphylococcus aureus, salmonella typhi*, and *Bacillus cereus*. Test organisms were grown in nutrient broth and LAB isolates were cultured in MRS broth. 200 µl suspension of the pathogen was spread on to surface of Muller Hilton Agar (MHA) plates. Agar wells of 5mm in size were made on MHA plate using cork borer. A 100µl of overnight grown culture of LAB isolate was poured in towel on plates. Plates were allowed to dry and incubated 37°C for 24-48 h (Ruby yadav *et. al.* 2016) (Suneeti *et.al.* 2017).

2.5.4 Antibiotic Sensitivity Test

According to standard procedure disc diffusion assay was performed to study the antibiotic sensitivity assay using Muller Hilton (MHA) agar. The isolates of LAB were propagated in MRS broth for 24 hrs at 37°C anaerobically for 24 h. Each isolate was inoculated on MRS agar using sterile cotton swab. The antibiotics were supplied in the form of DODECA disc (Hi media) which includes novobiocin (NV) and erythromycin(E), chlortetracycline(CT), gentamycin, tobramycin(TOB), collistrin(CL), carbenicilin(CB), nitrofurantoin(NIT), lincomycin(L), cefuroxime(CXM), Kanamycin(K). All this discs were placed on previously inoculated MRS agar plates and incubated at 37°C for 24 hrs. After incubation the zone of inhibition was observed and recorded in millimeter (Suneeti*et.al*, 2017).

III. RESULT AND DISCUSSION

3.1 Isolation Lipase producer LAB:

Twenty four Lactic acid bacteria (LAB) were isolated from 32 different source samples as milk, curd, cheese and calves infant faeces. All LAB isolates were gram positive rod shaped and catalase negative in nature. Among 24 LAB isolates seven isolates exhibited lipolytic activity on tributyrine agar. These seven Lipase producer LAB isolates were characterized by morphological, biochemical tests (Table 1) and carbohydrate fermentation test (Table 2)and designated as L_{13}, L_{14}, L_{15}, L_{16}, L_{17}, L_{18} and L_{19}. Qualitative assay on olive oil agar media showedthat all seven isolate have lipolytic acivity among which L_{13}, L_{17} and L_{18} have maximum Lpolytic activity, while L_{15} and L_{16} have less lipolytic activity (Graph.1). In present study, dairy samples were used for isolation of Lipase producing Lactic acid Bacteria from area of Nanded, Maharashtra, India. Lactic Acid Bacteria were isolated using MRS agar and Lipase producing Strains were primarily screened on tributyrine agar. Total 24 isolates were obtained on MRS agar as gram positive and catalase negative, among which seven isolates were showed zone of clearance on tributyrine agar and identified as lipase producer. All Lipase producer are designated as strain L_{13}, L_{14}, L_{15}, L_{16}, L_{17}, L_{18} and L_{19} (Rashmi B and Gaythri G 2014). All strains were examined for morphological and Biochemical test. All selected isolates resembles to *Lactobacillus sp.* as

per Bergay's Manual of Systematic Bacteriology. All seven lipase producer LAB isolates were further examined for qualitative lipolytic activity assay using disc diffusion method on olive oil agar containing methyl red. Among Seven lactobacilli strain L_{13}, L_{17} and L_{18} showed maximum lipolytic activity, while L_{15}, L_{16} ad L_{19} showed minimum lipolytic activity (Graph 1) (P. Patel B. Desai, 2018). All isolates were propagated for Probiotic characterization.

3.2 Acid tolerence:

All seven selected isolates were studied for survival and growth inacidic condition at pH 3 , pH 4 and turbidometric growth was analysed by spectrophotometric analysis after 2h and 4h of incubation at 37ºC. L_{13}, L_{14} and L_{18} can survive and grow moderately while L_{17} showed efficient growth at pH 3 and pH 4(Graph 2 & 3). The low pH is known to provide an effective barrier against the entry of bacteria in to intestinal tract, pH of the stomach generally ranges from 2.5 to 3.5 so the viability of probiotic bacteria in the gut is most important therapeutic parameter.(G. Sigroha *et. al.*2017).

3.3 Bile salt tolerence:

The lactobacilli are capable of surviving high bile salt concentration and therefore be adapt to GI tract condition (Ramirez chavarin *et.al.* 2013). Among all seven isolates strain L_{17},and L_{18} grow effectively at 0.15% and 0.3% bile salt concentration, while strains L_{14}, and L_{19} showed more growth at 0.15% and 0.3% bile salt concentration.(Graph 3 & 4). L_{13}, L_{15}, and L_{16} Show less growth. In present study all isolated LAB could survive at high bile salt concentration. (Graph 4 & 5). Bile entering the duodenal section of small intestine has been reported to reduce the survival of bacteria hence tolerant to bile salt is considered to be prerequisite for metabolic activity of bacteria in small intestine (G. Singroha*et.al*. 2017) tolerance to bile salt help Probiotic bacteria to reach the small intestine and colon and contribute in balancing the intestinal microfloara (M.G. Shehata*et.al.*2016).

3.4 Antimicrobial activity:

Antimicrobial activity is one of the main feature of probiotic bacteria .This study examines the antibacterial activity of isolated strains against test organisms *E coli, S aureus Salmonella typhi* and. *B cereus.* All seven LAB isolates were examined for their potential inhibitory activity against four test pathogenic strains by agar well diffusion method. The result exhibited that all seven isolates have inhibitory activity agaist *E coli, S aureus Salmonella typhi* and. *B cereus*. Among all, L_{14} strain showed very high activity agaist *S.aureus* while L_{17}, and L_{18} showed very high activity against *B.Cereus*, L_{18} and L_{19} showed slight inhibitory activity against *E. Coli* while L_{13} and L_{17} showed slight inhibitory activity against *Salmonella typhi*.(Table 3).

3.5 Antibiotic sensitivity test:

Antibiotic sensitivity test is performed by disc diffusion method using DODECA disc (Hi media) of novobiocin (NV) and erythromycin(E), chlortetracycline(CT), gentamycin, tobramycin(TOB), collistrin(CL), carbenicilin(CB), nitrofurantoin(NIT), lincomycin(L), cefuroxime(CXM), Kanamycin(K). Among all seven isolates each isolate was resistant to colistin and intermediarry resistant to kanmycin. L_{14} was resistant to cefuroxime and novobiocin, L_{13} and L_{14} were resistant to chlortetracyclin, while L_{16} and L_{19} were resistant to Linomycin and L16 and L19 are resistant to Erythromycin. Among all seven LAB isolate L15 and L16 were resistant to majority of antibiotics. L13, L14 and L19 isolates intermediary resistant to majority of antibiotic, while L17 and L18 are susceptible to most of the antibiotics. It had reported that majority of lactobacilli showed the resistant against kanamycin, erythromycin, tetracycline and gentamycin. The resistant to antibiotic means that the Probiotic can be given at the same time when antibiotic treatment is required. Secondly, microfloara of intestine can recover more quickly (p.muthukumar and C.kandeepan 2015).

Those probiotic bacteria that can tolerate low pH and bile salt means they not only can transit through stomach and can be active in intestine but also are able to alive and survive in stress condition (P. Muthukumar and C. Kandeepan 2015). Lactobacilli may antagonize pathogen by several mechanisms which involve production of antimicrobial compound such as Lactic acid, acid, hydrogen peroxide and bacteriocin, competition for substrate and co-aggregation with pathogen. Among many mechanisms organic acid production is the main mechanisms mediating antimicrobial activity. Furthermore, the degree of inhibition is specific to particular strains that depends upon amount of organic acid production (Anna Belicova*et.al.* 2013). There were different bacilli present in milk of of catals which contains bacteriocin. The bacteriocin producing *Lactobacillus* strains was examined for growth inhibition of gram positive *S. Aureus, S. Xylosus*and gram negative bacteria *E. Coli* and *Y. Enterocolitica* (Eid R. Et. al. 2016).

V. CONCLUSION

In present study, 24 LAB strains were isolated from different samples. Among which seven isolates showed lipolytic activity. All seven lipolytic LAB isolates were propagated for probiotic characterization. Two isolate designated as L_{17} and L_{18} showed good acid, bile tolerance, and antibacterial activity with efficient lipase activity. As lipase is extensively used enzyme in food industry probiotic bacteria with lipolytic activity have much significance. Although as per this study the selected strains have good potential for probiotic bacteria, further it is required to exploit the study of probiotic properties and optimization and substrate affinity for lipase activity of strain in appropriate formulation.

VI. FIGURES AND TABLES

Table 1: Morphological and biochemical and physiological characteristics of LAB isolates

Biochemical/ Morphological Test	LAB isolate						
	L13	L14	L15	L16	L17	L18	L19
Gram staining	+	+	+	+	+	+	+
Microscopic observation	Rod shaped with rounded ends	Cocobacilli, single, pair or short chain	Rods single,chais	Rods rouneded end, single pairs	Short rods single pairs	Rods single pairs	Small rods single pair short chains
Motility test	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-
Gelatin liquification	-	-	-	-	-	-	-
Indole production	-	-	-	-		-	-
MR test	+	+	+	+	+	+	+
VP test	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	-	+	+	-
Growth at different temp.							
10°C	+	+	+	+	+	+	+
37°C	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
45°C	+ +	+ +	+ +	+	+	+	+

+ (positive); -(Negative); + +(moderately positive) + + + (Highly positive)

Table 2: Carbohydrate fermentation of different Lipolytic LAB isolates.

Sr no	Strain	Carbohydrate fermentation among all selected seven lipolytic LAB isolates							
		Glucose	Galactose	Lactose	Arabinose	Xylose	Maltose	Sucrose	mannitol
1	L_{13}	A+/G+	A+/G-	A+/G-	A-/G-	A+/G+	A+/G-	A+/G-	A-/G-
2	L_{14}	A+/G+	A+/G-	A+/G-	A+/G-	A+/G+	A+/G+	A+/G-	A-/G-
3	L_{15}	A+/G-	A-/G-	A+/G-	A-/G-	A-/G-	A+/G-	A+G-	A-/G-
4	L_{16}	A+/G+	A-/G-	A-/G-	A-/G-	A-/G-	A+/G+	A+/G-	A-/G-
5	L_{17}	A+/G+	A+/G-	A-/G-	A-/G-	A-/G-	A+/G-	A+/G-	A-/G-
6	L_{18}	A+/G+	A+/G-	A+/G-	A-/G-	A-/G-	A+/G+	A+/G-	A-/G-
7	L_{19}	A+/G+	A-/G-	A+/G-	A+/G-	A+/G-	A+/G+	A+/G-	A-/G-

A+ (Acid positive); A-(Acid Negative); G+(Positive for gas production); G- (negative for gas production.

Table.3: Antimicrobial Activity of LAB isolates against Indicator Test organisms

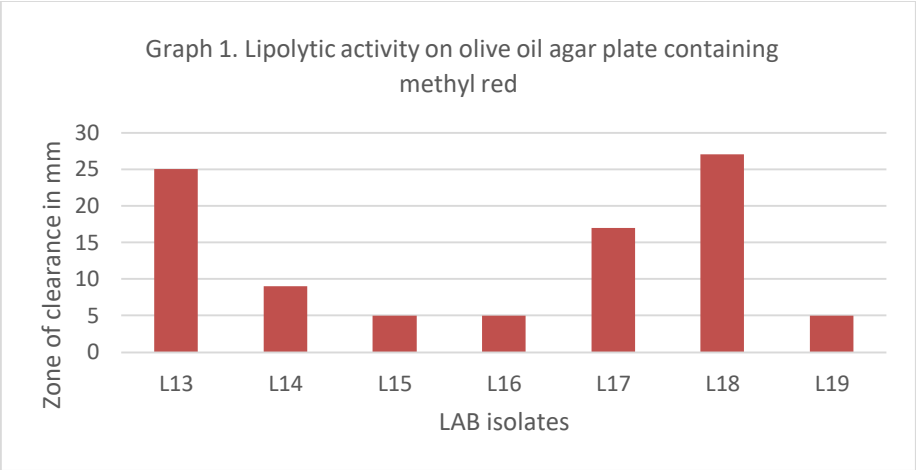
Indicator organisms	**Inhibitory activity of Lab Isolates against test organisms agar well diffusion assay.**						
	L 13	**L 14**	**L15**	**L 16**	**L 17**	**L 18**	**L 19**
S.Aureous	+ +	+ + +	+ +	+ +	+ +	+ +	+ +
E.coli	+ +	+ + +	+ +	+ +	+ +	+	+
Bacillus cereus	+ +	+ +	+ +	+ +	+ + +	+ + +	+ +
Salmonella tuphi	+	+ + +	+ +	+ +	+	+ +	+ +

(Zone with diameter: 4-8 mm (slight activity): + ,8-12 mm(medium activity): + +, more than 12 mm(very high activity): + + + .

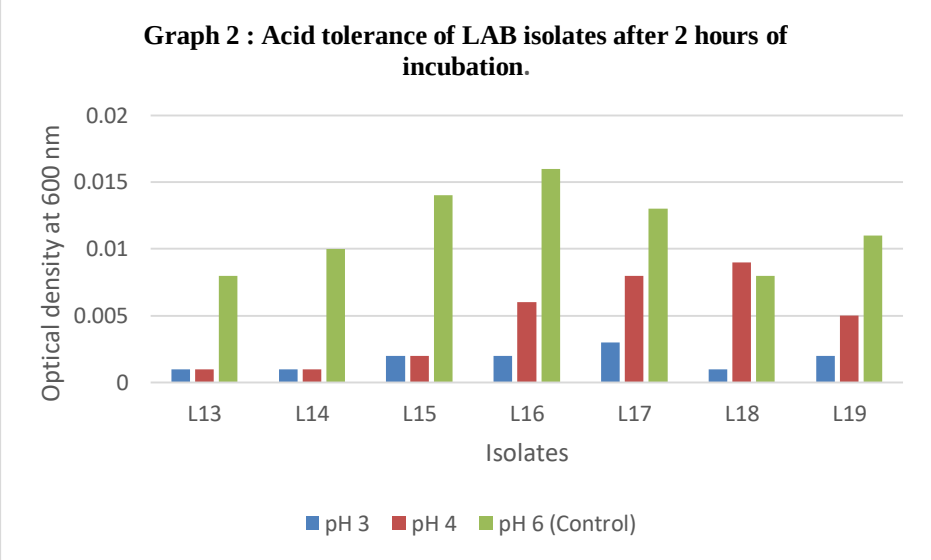
Table 4: Antibiotic sensitivity pattern of different seven LAB isolates

(Resistant: + + +, Intermediate: + +, Susceptible: +)

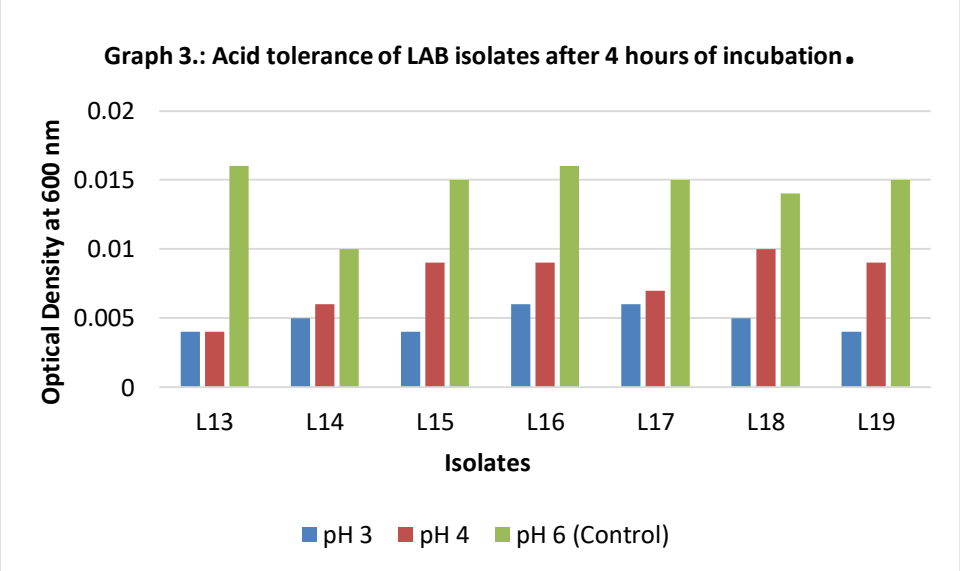
Antibiotic	**Antibiotic sensitivity test of LAB strains against antibiotics by disc diffusion assay**						
	L 13	**L 14**	**L15**	**L16**	**L17**	**L18**	**L19**
Nitrofurantoin	+ +	+	+ +	+ +	+ +	+	+
Lincomycin	+ +	+	+ +	+ + +	+ +	+	+ + +
Chlortetracycline	+ + +	+ + +	+	+ +	+	+	+
Novobiocin	+	+ + +	+	+ +	+	+	+
Kanamycin	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Colistin	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Gentamicin	+	+	+ + +	+ +	+	+	+ +
Carbenicilin	+	+ +	+	+ +	+	+	+
tobramycin	+	+	+ +	+ +	+	+ +	+
Erythromycin	+ +	+	+	+ + +	+ +	+ + +	+ +
Cefuroxime	+	+ + +	+ +	+ +	+	+ + +	+ +



Graph 1: Qualitative assay by Disc diffusion for lipolytic activity of different LAB isolates: L_{13}, L_{17} and L_{18} showing maximum Zone of clearance.



Graph 2: Acid tolerance of Lipolytic LAB isolates after 2 hours of incubation by turbidometric growth measurement (O.D. at 600 nm).



Graph 3: Acid tolerance of Lipolytic LAB isolate after 4 hours of incubation by turbidometric growth measurement (O.D. at 600 nm).

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