

Isolation of lactic acid bacteria from *Allium cepa* var. *aggregatum* and study of their probiotic properties

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ABSTRACT

The shallot (*Allium cepa* var. *aggregatum* or the *A. cepa* *Aggregatum* Group) is a botanical variety of the species *Allium cepa*, to which the multiplier onion also belongs. Shallots are called "small onions" in South India and are used extensively in cooking. The scientific use of shallots as a source of Lactic Acid Bacteria (LAB) has not yet been examined. Indigenous knowledge revealed shallots as a good health source. An attempt has been made to find out the possibilities of LAB in fresh shallots. Four isolates were identified on the basis of their morphological, cultural, physiological and biochemical tests and their probiotic properties were evaluated. These isolates were screened for resistance against bile salt, gastric juice, intestinal juice, different NaCl concentrations, acidic pH, ability to inhibit pathogens, antibiotic resistance, adherence capacity as well as survival under different storage temperatures. Isolated strains *Bacillus coagulans* (*Lactobacillus sporogenes*), *Lactobacillus brevis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* showed satisfactory probiotic potentials.

Keywords: *Allium cepa* var. *aggregatum*, *Bacillus coagulans* (*Lactobacillus sporogenes*), *Lactobacillus brevis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis*, Lactic acid bacteria,

INTRODUCTION

Lactic Acid Bacteria (LAB) have been involved in fermentations and food preservation techniques for thousands of years. First signs of LAB utilizations date back to 6000BC, describing the fermentation of milk and fermentation of meat 1500BC and vegetable products 300BC [1]. LAB are found in a large variety of nutrient rich environments, including milk and dairy products, plants, cereals, meat and meat products [2]. Several studies have isolated LAB from fruit and vegetable [3-8]. Farther the effectiveness of LAB fermentation produces many products; some of them have an antimicrobial activity, such as hydrogen peroxide, organic acid, diacetyl and bacteriocin [9-12]. The activity of compounds produced by LAB has been reported by many researches against different microorganisms [13-17]. In the recent years Probiotic based products have gained a lot of attention due to their health promoting prospects. Probiotics are commonly recognized as viable microorganisms that exhibit a beneficial effect on the health of the host when they are ingested [18]. Today, there is a growing need for new strains of lactic acid bacteria that carry the probiotic traits mentioned above and with favourable health effects on human and animals. This can be obtained from other natural ecological niches which remain unexploited.

Shallot (*Allium cepa* var. *aggregatum*) belongs to the onion family that formed as a cluster of bulbs attached to single disc. Shallots are small in size but sweeter and tastier than onions. Shallots have better nutritional value than onions. On weight per weight basis, they have more anti-oxidants, minerals, and vitamins than onions. They are rich source of flavonoid anti-oxidants such as quercetin, kemferfol...etc. Further, they contain sulphur anti-oxidant compounds such as diallyl disulfide, diallyl trisulfide and allyl propyl disulfide. These compounds convert to allicin through enzymatic action following disruption of their cell surface while crushing, and chopping. Research studies show that allicin reduces cholesterol production by inhibiting the HMG-CoA reductase enzyme in the liver cells. Further, it also found to have anti-bacterial, anti-viral, and anti-fungal activities. Many studies have revealed [19-21] that regular consumption *Allium* species has a health effects on the human health. The phyto-chemical compounds allium and Allyl disulfide in onion have anti-mutagenic (protects from cancers) and anti-diabetic properties (helps lower blood sugar levels in diabetics). Shallots hold several fold more concentration of vitamins and minerals than in onions, especially vitamin A, pyridoxine, folates, thiamin, vitamin C etc. Pyridoxine (B-6) raises GABA chemical level inside the human brain that helps soothe nervous irritability. In addition, 100 g fresh shallots carry 1190 IU (35% RDA) of vitamin A. The protective effect of vegetables from *Allium* family against certain diseases such as cancers, cardiovascular disease, has been attributed to the presence of organosulphur compounds as well as polyphenol substances which are located in onion [22]. The present study aimed to investigate the presence of LAB in *Allium cepa* var. *aggregatum* and screen their probiotic properties.

MATERIAL AND METHODS

Isolation of Bacteria

Fresh shallots were purchased from local market; outer dry papery skin was removed and the fleshy bulbs were pasted in mixer grinder using sterile saline. 1ml of the paste was serially diluted to 10^{-3} - 10^{-4} using sterile saline (0.85% NaCl), and 0.1ml was spread on to sterile de-Mann, Rogosa and Sharpe (MRS) agar plates. The plates were incubated at 37°C for 48 hours anaerobically. Morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking. Finally, pure colonies were obtained. Only gram +ve colonies were selected and inoculated on fresh media for further identification.

Morphology and growth

Colony size, shape, margin and colour on solid medium, growth pattern in broth and agar slants was recorded. Bacterial cellular morphology, size, shape and arrangement of the cells have been recorded. Motility, Indole test, spore formation, type and arrangement of cells, Starch Hydrolysis, Arginine hydrolysis, Indole test has been done. Normal growth rates were assessed by taking the OD at 660nm. The growth rate at different pH, NaCl and at different temperature was carried out and recorded.

Sugar fermentation tests

The isolates were examined for their ability to ferment different sugars like Fructose, Galactose, Cellobiose, Esculin, Inulin, Rhamnose, Melibiose, Mannitol, Maltose, Mannose, Ribose, Trehalose, Arabinose, Lactose, Sucrose, Xylose, Salicin, Cystein, Sorbitol, Raffinose and Glycerol as described by Harrigan (1998). Enteric fermentation (SRL) broth was prepared with 1% of each sugar along with Bromocresol purple indicator (0.01%) solution. Durham's tubes were inverted in to test tubes containing 5 ml of MRS broth and then sterilized. The temperature sensitive sugars were sterilized using syringe filters (25mm/0.2um Himedia India). Tubes were inoculated with active culture and incubated at 37°C for 48-72hrs. Results were observed by change in the media color and gas formation.

Study of Probiotic Properties:

Resistance to acidic pH

Resistant to acidic pH is considered as another major selection criterion for potential probiotic strains. Microbes should pass through the acidic environment of stomach [23] to reach small intestine. Stomach, pH is as low as 1.0 but in vitro assays pH 3.0 has been preferred. The isolates were inoculated into MRS broth maintained at pH 2 and pH 3. The rate of survival of the isolates has been examined by plating 0.1ml of cultures at 0, 1.5 and 3 hours.

Bile Tolerance

Bile plays an important role in the survival of bacteria in the small intestine. Food remains in the small intestine for around 4 hours [24] till it gets absorbed. All the strains were screened for their survival at different bile concentrations. Cultures were inoculated into 10 ml MRS broth in test tubes and incubated at 37°C overnight in anaerobic condition. 100µl of active culture was inoculated into fresh MRS broth tubes with pH 6.5 containing 0.3%, 0.5% and 1.0% bile (CDH India). The bacterial survival was measured by MRS agar colony count with taking 100µl culture for 0, 30, 60, 90 and 180 min and aliquots spread onto MRS agar plates to calculate the CFU/ml. The experiment was determined in triplicate to calculate intra-assay variation. CFU/ml was recorded.

Gastric Juice Tolerance

The simulated gastric juice was prepared freshly by suspending pepsin 1:10000 (3g/L) (SRL) in sterile NaCl (0.5%) and the pH was adjusted to 2.0 and 3.0 respectively. This was filter sterilized using 0.45µm filter. The isolates were grown in de Man, Rogosa and Sharpe (MRS) broth at 37°C for 24 h and centrifuged at $2,500 \times g$ at 4°C for 10 min. The collected cells were resuspended in sterile saline (0.5% NaCl) and inoculated into the simulated gastric juice (pH 2.0 and 3.0) at 10^8 cfu/ ml. The test was done in triplicates. Because the pH in the human stomach ranges from 1 (during fasting) to 4.5 (after a meal) and food ingestion can take up to 3 h, tolerance was assayed by determining the total viable count at 0, 1.5 and 3-h incubation in simulated gastric juice.

Intestinal Juice Tolerance

The simulated intestinal juice was prepared freshly by suspending pancreatin (1g/L) in sterile NaCl (0.5%) and adjusted the pH to 8.0. This was again filter sterilized by using 0.45µm filter. 1ml each of the suspension of the isolates were inoculated into 9ml of simulated intestinal juice (pH 8.0) and incubated at 37°C. The tests were done in triplicates. The survival rate was assessed by determining the total viable count at 0, 2, 4 and 6hrs of incubation.

Auto aggregation

Auto aggregation assay was performed by growing the isolates [25] in MRS broth for 24 hours anaerobically at 37°C. The cells were harvested by centrifugation at 5000 rpm for 15 min, at 4°C. The cells were washed twice

and re-suspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU/ml. Four ml of the cell suspension was mixed for 10 seconds in a sterile tube to determine auto aggregation during 5h of incubation, at room temperature. The upper suspension was used in each hour by transferring 0.1ml to another 3.9ml of phosphate buffer solution, and the optical density at 660nm was measured. Tests were carried out in triplicate and the results were averaged.

The auto aggregation percentage was calculated by the formula: $1 - (A_t/A_0) \times 100$, where, A_t represents the absorbance at time $t = 1, 2, 3, 4$ or 5 , and A_0 the absorbance at $t = 0$. Aggregation abilities of microorganisms were screened by visual observation.

Co-aggregation

The bacterial cells were harvested by centrifugation at 5000 rpm for 15 min after incubation at 37°C for 18h, washed twice and resuspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU/ml. Equal volumes (2 ml) of each cell suspension was mixed together in pairs by vortexing. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own. The absorbances at 660 nm of the suspensions were measured after mixing and after 5 h of incubation. The percentage of co-aggregation was calculated using the equation [26]

$$\text{Co-aggregation (\%)} = [(A_x + A_y) / 2] - A(x+y) / [(A_x + A_y) / 2] \times 100$$

Where x and y represent each of the two strains in the control tubes, and $(x + y)$ the mixture of isolate tested for co-aggregation.

Resistant to Antibiotics

Disc diffusion method has been used to determine the antibiotic susceptibility of the isolates. In this method the standardized bacterial isolate is spread on an agar plate and then paper disc containing specific concentration of antibiotics are placed and incubated at 37°C overnight. If the isolate is susceptible to the antibiotic it does not grow around the disk thus forming a zone of inhibition. Strains resistant to an antibiotic grow up to the margin of disk. The diameter of zone of inhibitions was measured using antibiotic zone scale (Himedia India) and result was read from the Kirby Bauer chart as sensitive, intermediate or resistant.

Antimicrobial activity

Agar well diffusion method (27) was used to determine the inhibitory capacity of the isolated LAB against pathogenic strains such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Bacillus subtilis* and *A. niger*. The cell free extract of the isolates and pathogenic strains were incubated in MRS agar medium at 37°C for 24 to 48 h and the results were recorded as the size of their inhibition zones.

RESULTS AND DISCUSSION

Physiological and Biochemical Characterization

The isolates ON1, ON3, ON4 were Gram positive and catalase negative, whereas the isolate ON2 was gram +ve and catalase +ve. MRS broth tubes containing Durham's tubes were inoculated and incubated for 5 days for gas production from glucose (Table 1). Isolate ON2 has shown gas production while ON1, ON3 and ON4 showed no gas from glucose. Ability to grow at different temperatures is also used for the identification of the isolates.

After 5 days observation it has been found that the optimum temperature for the growth is between 30-37 °C. The isolate ON1 has shown excellent growth at 45°C and it has survived at 60°C too but no growth has been observed at 15°C. ON2 has shown good growth between 15°C-45°C. The isolate ON3 could not grow at 15°C but could grow at 45°C. The isolate ON4 was unable to grow at 45°C but shown good growth at 15°C (Table 2). Growth at different NaCl concentrations was observed. All of the isolates have shown growth at 2-6% NaCl concentration. Isolate ON1 shown least growth at 6% NaCl. The isolates ON2, ON3 and ON4 showed good growth at 2% - 6% of NaCl. ON2 and ON3 have shown very less growth at 8% NaCl, but isolate ON4 was unable to survive at 8% NaCl.

Arginine hydrolysis by the isolates is another criterion used to identify them. The bright orange colour indicated the positive arginine hydrolysis where as the yellow colour indicated negative test. Only isolate ON4 has shown positive result for arginine hydrolysis, where as remaining isolates were negative for the reaction. Hydrolysis of starch was negative in all isolates except ON1. All of the isolates were non motile, non spore forming except ON1 which has shown motility and presence of endospore. Gelatin liquification was +ve only for ON1, where as remaining isolates were -ve for this test. The carbohydrate fermentation test is the most useful test for the identification of different strains. Twenty one (other than glucose) different carbohydrates were used for identification. They gave different fermentation patterns which are shown in Table 3.

Cultural, morphological, physiological and biochemical characteristics showed that the following genera and species of LAB were present in the *Allium cepa* var. *aggregatum* examined. They are ON1- *Bacillus coagulans* (*Lactobacillus sporogenes*), ON2- *Lactobacillus brevis*, ON3- *Lactobacillus delbrueckii* subsp. *bulgaricus* and ON4- *Lactococcus lactis*

Growth curve

During incubation of isolates the viable cell count was monitored. The results revealed that *Lactobacillus sporogenes*, *Lactobacillus brevis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were in stationary phase till 60 hours (Figure 1), whereas in *Lactococcus lactis* the rate of multiplication was very slow compared to other isolates and decline phase started soon after 48 hours of growth.

Resistance to Low pH

All the isolates survived 3 hours of incubation at pH 3, but the survival rate reduced to <10%. In case of *L. sporogenes* the survival rate was around 40% after 3rd hour of incubation. All the isolates were sensitive to pH 2. The survival rate reduced to 1% after 3rd hour of growth. *L. sporogenes* has shown 10% survival after 3 hours of incubation at pH 2 (Figure 2).

Bile Tolerance

The strains were screened for their ability to survive at different concentration of bile salt. Strains were inoculated in 0.3%, 0.5% and 1.0% bile salt and allowed to grow till 3 hours. From the results it has been found that, all the isolates were resistant to 0.3% and 0.5% bile salt. At 1% of the bile salt the survival rate suddenly decreased to <20% at 30 minutes of incubation. The survival rate further reduced to <10% after 3rd hour of growth. *Lactobacillus sporogenes* and *Lactococcus lactis* have shown more resistant to the bile than the other two isolates here (Figure 3).

Tolerance to Gastric juice

The degrees of gastric juice resistance exhibited by isolates were determined and the results (Figure 4) showed 40-60% of survival at pH 3 for 1.5 hours of incubation, whereas in pH2 the survival rate was 10-20% for 1.5 hours of incubation. At 3rd hour of incubation the survival rate reached to 15-35% for pH 3 and <5% for pH 2.

Tolerance to Intestinal Juice

The isolates were tested for their ability to grow in the artificial intestinal juice. It appeared that the all the strains exhibited good resistance to intestinal juice at pH 8 for four hours of growth (Figure 5). Good multiplication of all the isolates has been found at 6th hour of incubation.

Aggregation

On the basis of sedimentation characteristics aggregation capability of the isolate was tested. The isolates exhibited good amount of aggregation during the test time of 5 hours (Figure 6). *Lactobacillus delbrueckii* subsp. *bulgaricus* showed a maximum aggregation of 84% and *Lactobacillus brevis* shown 48% of aggregation. Whereas *Lactobacillus sporogenes* and *Lactococcus lactis* shown 62% of aggregation after 5 hours of incubation.

Co-aggregation

The co-aggregations of the isolates with five pathogenic bacteria were examined. Results were expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension. Different patterns of co-aggregation with pathogenic bacteria have been observed. It is found to be related to the anti pathogenic activity of the isolates (Figure 7).

Antibiotic Sensitivity Test

The determination of antibiotic sensitivity of the isolate is an important prerequisite prior to considering it safe for human and animal consumption. The isolates were subjected to antibiotic susceptibility test. The results are given in Table 4. The isolates were resistant to most of the antibiotics used. According to earlier reports, specific antibiotic resistance traits among probiotic strains may be desirable [28-29].

It has been said by many authors that probiotics should be resistant to certain antibiotics when used along with antibiotics to prevent gastrointestinal disorders. Whereas others claim that antibiotics resistant probiotics used may serve as host of antibiotic resistance genes, which can be transferred to pathogenic bacteria.

Anti Bacterial Activities

Antimicrobial activity helps to select the potential probiotics strains. Antimicrobial activity usually targets the intestinal pathogens [30]. The isolates were examined for their antibacterial activity. The antibacterial effect on the indicator microorganisms was determined by diameter of inhibition zones. *L. sporogenes* inhibited the growth of all the pathogens used in the test in different degrees except *K. pneumoniae*. *L. brevis* could not inhibit the growth of *B. subtilis* and *A. niger*. *Lactobacillus delbrueckii* subsp. *bulgaricus* inhibited the growth of all the pathogens except *B. subtilis*. *Lactococcus lactis* was able to inhibit only the growth of *B. subtilis*, *Staphylococcus aureus* and *A. niger*. In this case the degree of inhibition was very less for *B. subtilis* and *A. niger* (Table 5).

CONCLUSION

The present research revealed the presence of *Bacillus coagulans* (*Lactobacillus sporogenes*), *Lactobacillus brevis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* in the shallots. All these isolates are potential probiotics strains. Their acid, bile, and alkaline stability will allow them to survive in the stomach and proliferate in the intestine. This will help strains to reach the small intestine and colon and contributing to the balance of intestinal microflora. All the strains also possessed high antibacterial activity, thus might potentially help to alleviate diarrhoea and other intestinal infections.

ACKNOWLEDGMENTS

The authors are thankful to Indian Council of Medical Research (ICMR) for financial assistance. No. 3/1/2/40/Nut./2013 dated 20.11.2013

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Table 1: Morphological, cultural and physiological characteristics of the isolates

Sl. No.	Isolate No.	Catalase test	Size(mm)	Shape	Margin	Gram's staining	Shape	Motility	Gas from glucose	Spore formation	Arginine utilization	Gelatin liquefaction	Starch Hydrolysis	Growth in broth	Growth on slants	NaCl-2%	NaCl-4%	NaCl-6%	NaCl-8%	NaCl-10%	Indole test
1	ON 1	-ve	> 1	Circular	Entire	+ve	Rods, pairs	+	-ve	+ve	-ve	+ve	+	Sediment	Smooth flat	+	+	+	-	-	-
2	ON 2	+ve	< 1	Circular	Entire	+ve	Rods short	-	+ve	-ve	-ve	-	+	Turbidity	Smear / faint	+	+	+	+	-	-
3	ON 3	-ve	< 1	Slightly convex	Entire	+ve	Thin long rods	-	-ve	-	-ve	-ve	-	+	flat	+	+	+	+	-	-
4	ON 4	-ve	< 1	Circular	Entire	+ve	Diplococci, small rods	-	-ve	-ve	+ve	-ve	-	+	Beaded	+	+	+	-	-	-

(+++ Luxurious growth, (++) Moderate growth, (+) less growth, (-) No growth

Table 2: Physiological characteristics of the isolates

Sl. No.	Isolate No.	Growth at different temperature (°C)							Growth at different pH							
		15	30	37	45	50	55	60	2	3	4	5	6	7	8	9
1	ON1	-	+	++	++	++	++	+	+	+	+	+++	++	++	++	++
2	ON2	+	++	++	++	-	-	-	-	+	+	+++	++	++	++	+
3	ON3	-	++	++	++	+	-	-	-	+	+	++	++	++	++	++
4	ON4	++	++	++	-	-	-	-	-	+	+	++	++	++	++	++

(+++ Luxurious growth, (++) Moderate growth, (+) less growth, (-) No growth

Table 3: Biochemical characteristics of the isolates by utilization of carbon sources

Sl. No	Isolate No.	Fructose	Galactose	Cellobiose	Esculin	Inulin	Rhamnose	Melibiose	Mannitol	Maltose	Mannose	Ribose	Trehalose	Arabinose	Lactose	Sucrose	Xylose	Salicin	Cystein	Sorbitol	Raffinose	Glycerol
1	ON 1	+	+	-	+	-	-	-	+	+	+	-	+	+	+	+	-	-	-	-	+	-
2	ON 2	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+
3	ON 3	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-
4	ON 4	+	+	-	-	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-

Positive reaction (+), negative reaction (-)

Table 4: Antibiotic resistance of the isolates

Antibiotics	<i>Lactobacillus sporogenes</i>	<i>Lactobacillus brevis</i>	<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	<i>Lactococcus lactis</i>
Ampicillin	R	S	R	S
Chloramphenicol	S	I	S	R
Erythromycin	S	S	S	S
Gentamycin	S	R	R	S
Kanamycin	S	S	R	R
Lincomycin	I	I	S	S
Nalidixic acid	R	R	R	R
Neomycin	I	R	R	I
Norfloracin	S	S	R	R
Penicillin	R	S	I	R
Streptomycin	S	R	R	S
Tetracycline	S	R	S	R
Vancomycin	I	R	S	R

S-Sensitive, R-Resistance, I-Intermediate

Table 5: Antimicrobial activity of the isolates

Strain	<i>Lactobacillus sporogenes</i>	<i>Lactobacillus brevis</i>	<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	<i>Lactococcus lactis</i>
<i>B. subtilis</i> MTCC 8605	+	-	-	++
<i>E. coli</i> MTCC7410	++	++	+++	-
<i>K.pneumonia</i> MTCC 9751	-	+	++	-
<i>P. aeruginosa</i> MTCC 9499	++	++	+++	-
<i>S.aureus</i> MTCC 9760	+++	++	++	+++
<i>A. niger</i> MTCC 8652	++	-	++	++

(+++ Excellent, (++) very good, (+) good, (-) no action

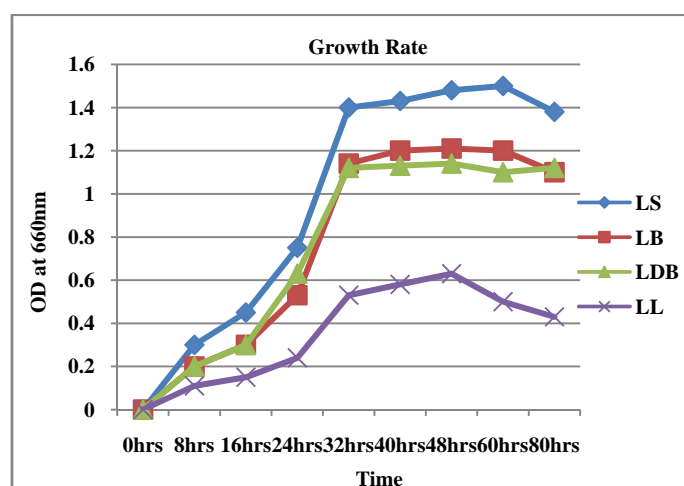


Figure 1: Growth rate of the isolates. LS- L. sporogenes, LB- L. brevis, LDB- L. delbrueckii subsp. bulgaricus, LL- Lactococcus lactis

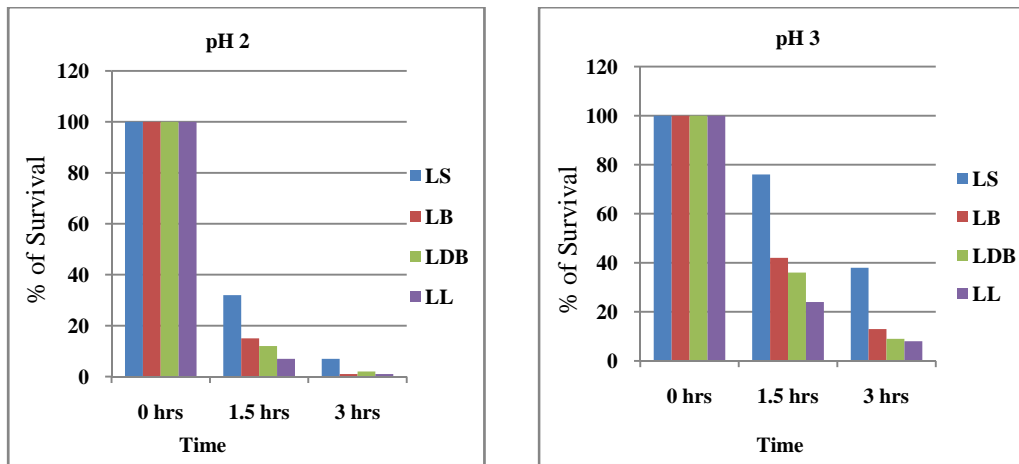


Figure 2: Tolerance to acidic pH by the isolates. LS- *L. sporogenes*, LB- *L. brevis*, LDB- *L. delbrueckii* subsp. *bulgaricus*, LL- *Lactococcus lactis*

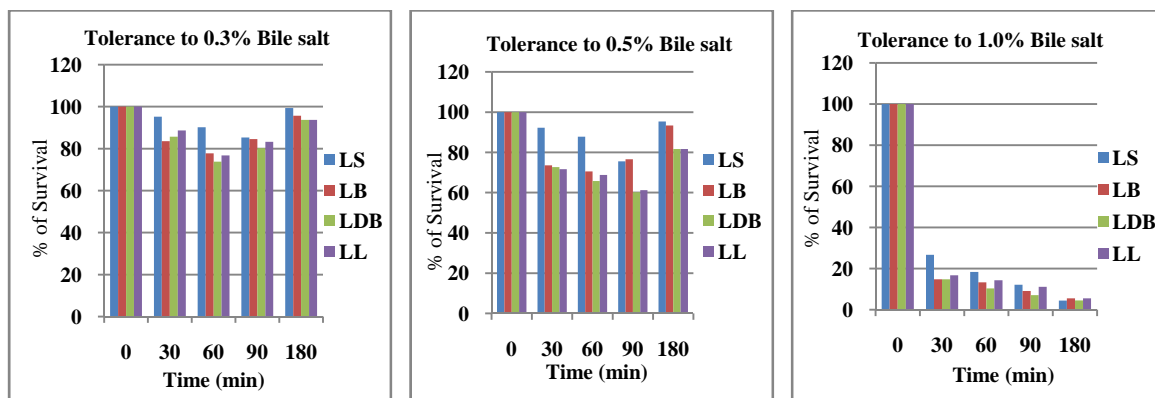


Figure 3: Tolerance to Bile salt by the isolates. LS- *L. sporogenes*, LB- *L. brevis*, LDB- *L. delbrueckii* subsp. *bulgaricus*, LL- *Lactococcus lactis*

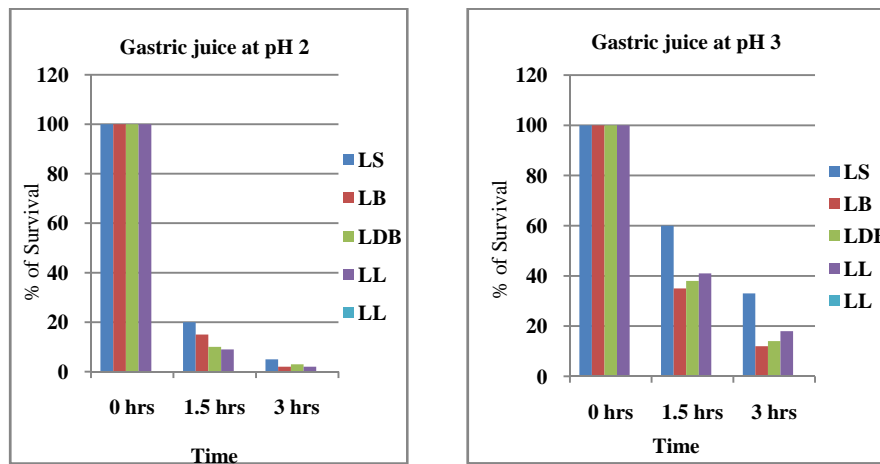


Figure 4: Tolerance to Gastric juice by the isolates. LS- *L. sporogenes*, LB- *L. brevis*, LDB- *L. delbrueckii* subsp. *bulgaricus*, LL- *Lactococcus lactis*

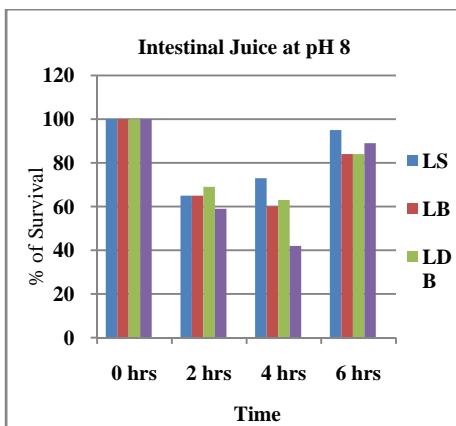


Figure 5: Tolerance to Intestinal juice by the isolates. LS- *L. sporogenes*, LB- *L. brevis*, LDB- *L. delbrueckii* subsp. *bulgaricus*, LL- *Lactococcus lactis*

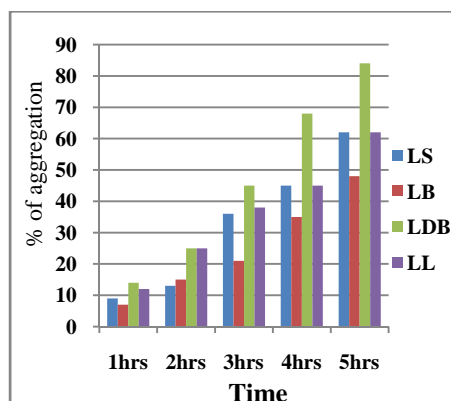


Figure 6: Auto-aggregation of the isolates. LS- *L. sporogenes*, LB- *L. brevis*, LDB- *L. delbrueckii* subsp. *bulgaricus*, LL- *Lactococcus lactis*

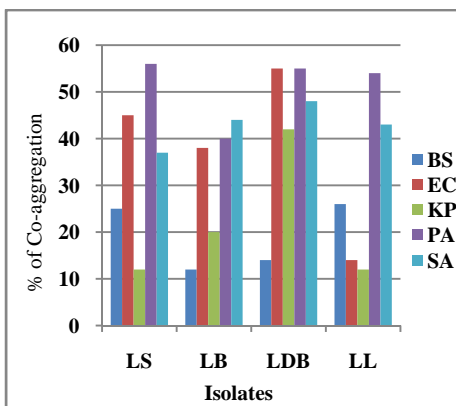


Figure 7: Co-aggregation of the isolates. LS- *L. sporogenes*, LB- *L. brevis*, LDB- *L. delbrueckii* subsp. *bulgaricus*, LL- *Lactococcus lactis*. BS- *Bacillus subtilis*, EC- *E. coli*, KP- *Klebsiella pneumonia*, PA- *Pseudomonas aeruginosa*, SA- *Staphylococcus aureus*