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Probiotic efficiency of lactic acid bacteria from milk treated against gastro intestinal pathogens

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ABSTRACT: The *Lactobacillus* is rich in whey it can be easily isolated from the whey sample. In the present study, lactobacillus sp was isolated from whey sample collected from Aavin Dairy industry, Madurai. The raw milk sample was enriched in peptone water. It was then plated on MRS medium by spread plated method. The selection of acid producing organism was done in MRS medium supplemented with calcium carbonate and L-cystein. The selected isolate was identified based on morphological and biochemical characterization tests and it was confirmed that the isolate belonged to the genus lactobacillus in accordance with the bergey's manual of determinative bacteriology. Before evaluating the isolated lactobacillus as probiotics against selected pathogens, Lactobacillus was tested for its tolerance against sodium chloride, acidic pH, Ox bile tolerance etc. Bacteriocin was extracted using MRS medium and it was then partially purified using salt aggregation method. The partially purified bacteriocin was the mixed with SDS containing phosphate buffer for testing the antimicrobial activity. The partially purified bacteriocin protein was tested against clinical pathogens like *E.coli, P.aeruginosa, P.fluoresence* and *Klebsiella pneumonia* using agar well diffusion method. Bacteriocin is proteinaceous in nature. The molecular weight of partially purified bacteriocin was detected by SDS-PAGE. The quantitative estimation of organic acid was determined for the isolated *Lactobacilli.* Antioxidant property of the lactobacillus was detected by reducing power assay method.

Keywords: *Lactobacillus, pH, Ox-bile, Nacl, Bacteriocin and SDS-PAGE;*

1. INTRODUCTION

Today's consumers are increasingly aware of the importance of the maintenance of their environment health and nutrition. With the growing interest in self-care and integrative medicine coupled with health. Embracing increasing population, recognition of the link between diet and health has never been stronger [1].

Probiotics are a live microbial food supplement that has helps in balancing the host intestinal microbial system. The term "probiotics" was originally used to mean a substance that stimulates the growth of other microorganisms [2]. The meaning of this term has been redefined as a viable microbial agent which when used in animal man beneficially affects the host possibly by improving the balance of the indigenous micro flora [3]. Based on this meaning several terms such as friendly-beneficial or healthy bacteria are commonly to probiotics. In order to assess the properties of probiotics, the food and agriculture organization and world health organization (AO/WO) suggested that the following guidelines be used. Probiotics should be able to survive passage though the digestive tract, proliferate in the gut, gram positive organism and are include primarily two genera Lactobacillus and Bifido bacterium.

Lactic acid bacteria are gram positive, facultative anaerobes, these are catalase negative, non-spore forming rods, cocci and cocco-bacilli generally present a indigenous micro flora in raw milk and produce lactic acid as a major end product during fermentation of carbohydrates. The lactic acid bacteria (LAB) are characterized by an increased tolerance to a lower p^H range. These aspects partially enable LAB to out compete other bacteria in a natural fermentation, as they can withstand the increased acidity from organic acid production (e.g., lactic acid) [4]. A Probiotic lactic acid bacterium with antimicrobial activity has attracted much attention as they can be used to enhance the hygienic quality of the product by inhibiting the growth of pathogenic bacteria. Bacteriocins from LAB are natural antimicrobial peptides or proteins that have many potential applications in food preservation and health care industrie. [5].

Bacteriocins from gram-positive bacteria have are frequently found in many commercially useful lactic acid bacteria and regarded as safe for human consumption. Also, these bacteria are non-toxic to eukaryotic cells, and they

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have much broader inhibitory spectra compared to bacteriocins from the gram-negative bacteria. Whey is considered as wastewater obtained as byproduct of cheese production process after the separation of fat and casein from milk. Whey is a rich source of lactose, nitrogenous substance including vitamins and other essential nutrient for the growth of certain bacteria. Whey is rich in lactic acid bacteria. The purpose of this study was to produce the bacteriocin from probiotics lactic acid bacteria and to evaluate the potential effects of bacteriocins against clinical pathogens.

2. MATERIAL AND METHODS

2.1 Collection and Enrichment of Samples

The whey samples were collected in a sterile tube from Aavin dairy industry, Madurai. 1ml of whey sample was enriched in 100ml of peptone water. It was then incubated for 24 hrs at 37^0 C.

2.2 Isolation of Lactic Acid Bacteria from Enriched Raw Milk Sample

Spread plate technique was used to isolate the organisms. For that 1ml of enriched sample was serially diluted up to 10^{-8} dilutions. And spread plated (0.1ml) into MRS (Mann Rogoso and Sharpe) agar plate incubated for 24 hrs at 37^0C. After incubation individual bacterial colonies were selected and re-streaked on the MRS agar plate to obtain the pure culture of the isolates. The pure cultured strains were maintained in 20% glycerol stock.

2.3 Identification and characterization of isolated organisms

2.3.1 Morphological characterization

Morphological characteristics such as abundance of growth, pigmentation, optical characteristics, size and shape were studied on MRS agar plates.

2.3.2 Bio-chemical characterization

The biochemical characterization test of probiotics producing microorganisms such as Indole test, MR-VP test, citrate utilization test, Triple sugar iron test, starch hydrolysis test, Nitrate reduction test, Gelatin hydrolysis test, and Carbohydrate fermentation test were carried out.

2.4 Probiotic Properties of Isolates

The determination of probiotic properties of isolates were performed by the following methods.

2.4.1 Selection of Acid Producing Bacteria

The isolation of acid producing bacteria, was carried out by MRS medium supplemented with $CaCo_3$ and L-cystein. The isolated organisms were tested for acid production by streaking on the plate. Incubate the plates at 37^0C for 24-48 hours. A clear zone around the isolates indicates the positive result [6].

2.4.2 Tolerance to Sodium Chloride

MRS broth was prepared with different concentration of NaCl i.e., 4, 5, 6, 6.5% and it was then inoculated with the isolated organism and incubated at 37^0C for 48 hours. The turbidity was measured at 600 nm for the ability to tolerate NaCl.

2.4.3 Tolerance to Acidic pH

Determinations of resistance to P^H were used invitro. The tolerance to acidic pH was tested by inoculating 1% (v/v) fresh over night culture of *lactobacillus* into the MRS broth with varying pH ranging from 2.5-9.0. The pH were adjusted with concentrated acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated at 37^oC for 24 hours. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD) against the un-inoculated broth.

2.4.4 Tolerance to Bile Salt

Bile salt resistance was performed by using ox bile at different concentration (0.0, 0.3, 0.5, and 1.0%). The MRS medium containing different concentrations of ox bile was inoculated with the isolates and then it was incubated at 37^0C for 24 hrs. After incubation the survival of the organism was confirmed by the turbidity of medium [7].

2.5 Partial purification of Bacteriocin

The isolates are grown on MRS broth (pH 6) seeded with 5% inoculum of overnight culture and maintained at 30^oC for 48 hours. Partial purification of bacteriocin was done by salt saturation method. The culture of Lactobacillus was saturated with 70% ammonium sulphate and was stored at 4^0C to precipitate out the protein. After 24 hrs, centrifugation was done at 10,000 rpm for 15 minutes. The pellet was then purified by repeated washing method. 1000 µl of partially purified bacteriocin was taken in the eppendorf tube and centrifuged at 10,000 rpm for 10 minutes. The pellet was washed with phosphate buffer six times, followed by centrifugation at 10,000 rpm for 10 minutes every time. It was finally dissolved in phosphate buffer containing SDS (0.6%) and the activity of bacteriocin was checked by well diffusion method [8].

2.6 Collection of clinical pathogen

The clinical pathogens (*E.coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and pseudomonas fluoresence*) were collected from Bose Clinical laboratory, Madurai.

2.6.1 Determination of Antimicrobial Activity by Well Diffusion Method

The antibacterial spectrum of the bacteriocin from Lactobacillus sp was determined by well diffusion method. Mueller Hinton agar plates were prepared and the test pathogens were swabbed on the surface of the agar plates. The well was cut with the help of cork borer. Aliquots of partially purified bacteriocin (50µl) were added into the well. The plates were incubated at 37^0C for 48 hrs. After incubation, the diameters of the zones of growth inhibition were measured. The zone formed was compared with standard antibiotic disc [9].

2.7 Determination of Protein

Using Lowry *et al* (1951) [10] methods, the protein concentration of the bacteriocin in supernatant was determined while bovine serum albumin was used as standard.

2.8 Molecular weight determination in SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [11] was used to determine the molecular weight of the bacteriocin. Polyacrylamide gel electrophoresis was done to separate proteins. The partially purified bacteriocin was separated using SDS-PAGE.

2.9 Quantification of Organic Acid and Determination of pH Value

1% of 24 hrs culture of LAB was inoculated on 10% sterilized skimmed milk and it was then incubated at 37^{0}C for 72hrs. Sample was collected at every 24, 48, 72hrs. Liquid of coagulated milk was separated by filtration. The pH of the separated milk was recorded using pH meter. Quantification of organic acid was performed through titration with 0.1N NaOH using phenolphthalein as indicator. Standardization of NaOH was done using 0.1N NaOH was taken in a conical flask and titrated against 0.1N standard oxalic acid using phenolphthalein indicator [12].

2.10 Antioxidant Property

Antioxidant effects were determined by reducing power assay method. The reducing power was determined according to the procedure followed by Finlay, etal., 1990. Different concentrations of sample solution (1 mL) were mixed with 0.2 mM of sodium phosphate buffer (pH 6.6, 2.5 ml) and 1% potassium ferricyanide (2.5 ml) and incubated at 50°C for 20 min. Followed by the incubation, 10% trichloro acetic acid (w/v) was added (2.5 ml) and centrifuged at 3,000 rpm for 10 min. The upper layer aliquot (2.5 ml) was mixed with deionized water (2.5 ml) and 0.1% ferric chloride (0.5 ml), and measured at 700 nm absorbance.

3. RESULTS:

3.1 Isolation and Characterization of Microorganisms from Whey Sample

Lactobacillus species was isolated from the whey sample collected from Aavin dairy industry Madurai, using MRS agar medium. This medium seems to be specific for the isolation of lactobacillus species (Fig 1a). The isolated strain was identified based on its physiological and biochemical characteristics features (Table-1). On MRS agar medium, the isolate showed abundant growth and it produced large, white, irregular colonies. The isolate was a gram positive long rod, non motile organism and it does not produce spores. Indole was not produced by the organism. It produced red color in methyl red test. It utilized citrate as its sole carbon source. It revealed negative test results for voges proskauer test, nitrate reduction test and gelatin hydrolysis. Besides starch also found to be negative.

From the results, it was confirmed that, the isolated strain belonged to the genus lactobacillus. The results were compared with the bergey's manual determinative bacteriology. (Table: 2).

Table 1. Morphological Characterization of the isolate

S. No	Characterization	Observation
1.	Colony morphology	Colonies are large, white, irregular colonies.
2.	Grams staining	Gram positive, rods
3.	Spore staining	Endospore absent
4.	Motility	Non-motile

Table 2:Biochemical characterization of Pobiotic isolate

Sl.No	Characterization	Observation
1.	Indole production test	Negative
2.	Methyl red	Positive
3.	Voges proskauer	Negative
4.	Citrate utilization test	Positive
5.	Triple sugar iron test	Alkaline slant
6.	Gelatin hydrolysis	Negative
7.	Nitrate reduction test	Negative
8.	Starch hydrolysis test	Negative
9.	**Carbohydrate fermentation tests**	
10.	Lactose	Negative
	Sucrose	Positive
	Dextrose	Positive
	Mannitol	Positive

3.2 Selection of isolated lactobacillus for its Probiotic property

3.2.1 Selection of strain for acid production

The isolated lactobacillus grown on MRS medium supplemented with 0.05% L-cystine and 0.5% calcium carbonate for its acid production. A clear zone was produced around the colony indicated the production of acid (Fig 1b).

3.2.2 Tolerance of acidic p^H

Maximum growth (OD=2.20) of isolated lactobacilli from whey sample was observed at pH 5.0. The OD value was the average value of two readings, where a control OD was 0.16. The results were depicted in Figure:2. And statistics analyses were found to be significant. .

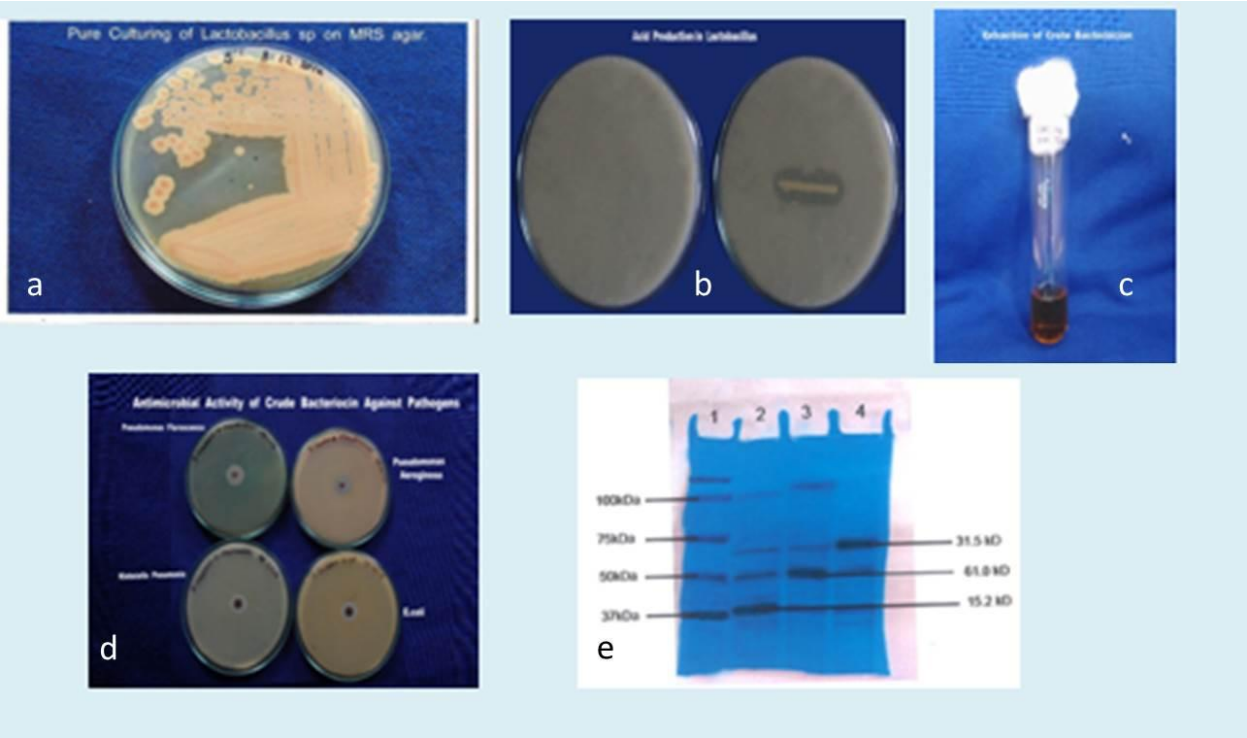

Figure.1 : Isolation and Characterization of Microorganisms from Whey Sample a. isolation of lactobacillus species,b. A clear zone indicating the production of acid.c. Partial purification of bacteriocin from cell free supernatant with 70% ammonium sulphate. d. Inhibitory activity against *E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa and P. fluorescence.* E. SDS-PAGE gel electrophoresis for bacteriocin molecular weight determination.

3.2.3 Tolerance of sodium chloride

Lactobacillus species revealed variation in the NaCl tolerance. It depends upon the species. The isolated lactobacillus sp tolerate up to greater saline condition. From table-4 it was found that the isolated lactobacillus showed maximum tolerance at 3% and 4% and it was found to show an optimum growth up to 6.5% of sodium chloride. (Table:3).

3.2.4 Tolerance of Ox-bile

After incubation at 37^{0}C, the test organism exhibited the resistance against different concentration of ox bile. The isolated lactobacillus was able to survive in 0%, 0.3%, 0.5%, and 1% bile salt. The isolated lactobacillus sp was also able to multiply in the above mentioned concentrations of bile salt (Table: 4).

3.3 Partial Purification of Bacteriocin

Partial purification of bacteriocin was performed by precipitation of cell free supernatant with 70% ammonium sulphate. Precipitation was found to increase the size of zone of inhibition of bacteriocin (Fig 1c).

3.4 Determination of Antimicrobial Activity of Partially Purified Bacteriocin

The susceptibility of various clinical pathogens to growth inhibition by the partially purified bacteriocin was presented in table 5. The antimicrobial activity of the

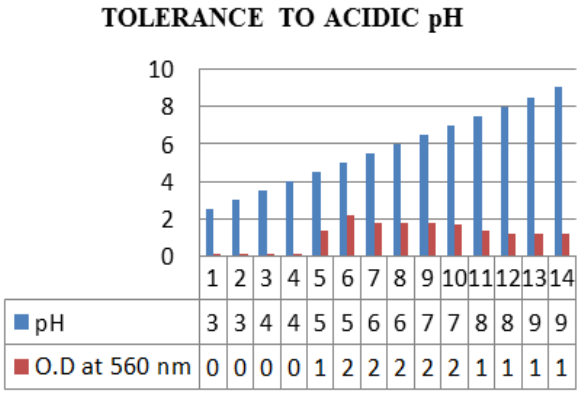

Figure.2: Tolerance to Acidic pH

Table 3 : sodium chloride tolerance of isolated Lactobacillus sp

S. No	Concentration of NaCl	*Lactobacillus* sp
1.	3%	++
2.	4%	++
3.	5%	+
4.	6%	+
5.	6.5%	+

Note: ++ good growth, + visible growth

Table: 4.Tolerance of Ox-bile

S. No	Ox-bile	OD Value(560nm)
1	0%	0.36
2	0.3%	0.38
3	0.5%	0.39
4	1%	0.41

organism was detected by adding 50µlof supernatant into the well. After incubation, zone formation was measured. It showed inhibitory activity against *E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa and P. fluorescence.* Among these, maximum activity observed against *P. aeruginosa, E. coli and P. fluorescence* and minimum activity was observed against *Klebsiella pneumonia* (Table: 5 and Fig 1d).

Table: 5. Antimicrobial activity of partially purified bacteriocin against clinical pathogens

S. No	Organisms used	Zone of inhibition in mm
1.	*E. coli*	11
2.	*K. pneumoniae*	9
3.	*Pseudomonas aeruginosa*	19
4.	*Pseudomonas fluoroscence*	17

3.5 Determination of Protein by Lowry et al., Method

The amount of protein present in the given sample was quantitatively estimated by lowry et al method using bovine serum albumin as standard. The amount of protein present in the given sample was found to be 220 mg protein/ml.

3.6 Molecular Weight Determination of Bacteriocin in SDS-PAGE

The molecular weight of the bacteriocin was determined by SDS-PAGE gel electrophoresis (Fig 1e). A single protein band was observed when stained with coo-massive blue and it clearly indicated the purity of the protein. The molecular weight of the partially purified protein was calculated to be about 90 kDa.

3.7 Acidification and Determination of pH

The speed of acidification for Lactobacillus was slow. The Lactobacillus sp lead to produce higher titratable acidity which revealed 0.067 N after 96 hrs of incubation with a pH of 4.7. (Table: 6).

Table: 6. Organic acid production and pH value

S. No	Hours	Acidification	pH value
1	24	0.023	5.11
2	48	0.036	4.99
3	72	0.054	4.5
4	96	0.067	4.7

3.8 Antioxidant Property

The anti oxidant property of lactobacillus was determined by the reducing assay method. For determining the reducing power, absorbance was taken at 700 nm. Increased absorbance, lead to enhanced power.. The isolated strain showed O.D value of 0.68.

4. DISCUSSION

Day by day, the dramatic significant increase in the usage of probiotics encounters the advantages of this biological product and also it has been put forth that this can be used in the prevention of human cancers such as colon cancer [13]. In the present investigation, whey sample was collected from aavin dairy industry, Madurai since whey is a rich source of lactose, nitrogenous substances including vitamins and other essential nutrient for the growth of certain bacteria. Whey is also rich in lactic acid bacteria. The samples were spread plated on MRS agar medium (selective medium for isolating lactic acid bacteria). The isolated strain was subjected to biochemical tests and it was confirmed to be lactobacillus in accordance with the Bergey's manual of determinative bacteriology. Before evaluating the isolated lactobacillus as probiotics against selected pathogens, important characteristics of these Lactobacilli were studied. Bacteria must tolerate gastrointestinal stress conditions for their metabolic activity, as well as to colonize in the gastrointestinal tract. Therefore it is necessary to evaluate the resistance ability of bacteria to gastrointestinal stress, before their use as probiotics.

Evaluation of probiotic potential of lactobacillus was carried out by stimulating the environment in the gastrointestinal tract (GIT): subjecting the isolates to acidic pH, exposure to bile salt etc. The typical transit time of food in the stomach is approximately 20 minutes to 3 hours. Among other factors, stomach acidity varies from person to person naturally and whether an individual has fasted prior to ingestion or not [14]. High acidity in the stomach and the high concentration of bile components in the proximal intestine are the major host factors that a probiotic strain should tolerate to express probiotic effect on the host. The isolated lactobacillus sp was screened in skim milk agar medium supplemented with $CaCO_3$ for its acid production. A clear zone was found around the isolate which confirms the production of acid which was similar to the work of Kaizu [15]. pH is an important factor which can dramatically affect the bacterial growth.

In our experimental design it was observed for the growth of the isolated *Lactobacillus* spp. in various pH values ranges from 2.5 to 9.0. The reason for choosing this pH range was to determine whether Lactobacillus species can grow in acidic and alkaline conditions and also to predict the optimum pH value for good growth. From the experimental results, it was found that the isolated *Lactobacillus* spp. From whey sample was able to survive in

extreme acidic pH (pH 2.5 to 3.5) and basic pH (pH 7.5 to 8.5). Maximum growth (OD= 2.20) of isolated lactobacilli was observed at an acidic pH of 5. This finding coincides with the findings of Hammes [16]. In which they observed maximum growth (OD= 2.054) of isolated lactobacilli from Bogra yoghurts was observed at pH 5.0 and for lactobacilli isolated from Khulna yoghurt maximum growth (OD= 1.93) was observed at pH 6.5. NaCl is an inhibitory substance which may inhibit the growth of certain types of bacteria. The current results showed that *Lactobacillus* sp. was able to tolerate upto 6.5 % of NaCl and good growth was observed at 3% and 4% of NaCl. The findings of Hoque [17] showed that *Lactobacillus* sp. isolated from yoghurts was able to tolerate 1-9% of NaCl and good growth was observed at 1% NaCl.

The experimental results also have the similarities with the findings of [18], in case of lactobacilli isolated from gastrointestinal tract of swine that were tolerable to 4-8% NaCl. Resistance to bile salt of the isolates could be attributed to their ability to produce bile hydrolase. Bile salt hydrolase (BSH) protects the cells that produce it from the toxicity of conjugated bile salts by deconjugating the bile acids. In the present investigation the efficacy of the lactobacillus sp was tested against the different concentration of oxbile. But the isolate found to tolerate upto 1% concentration of oxbile. This result revealed that this probiotic bacteria can survived even in the gastric juice of stomach. The results of resistance to bile salt was supported by the findings of [19] reported that the viable number of *Lactobacillus sp* decreased significantly at 1.0% of bile salt.

The various advantage of lactobacillus strains including its antimicrobial activity against bacterial pathogens, H2O2 production, lactic acid, bacteriocin and unknown heat stable, non lactic acid molecules [20]. Lactobacilli produce antibiotic like proteins called bacteriocins which may help to restrict the growth of some intestinal pathogens. Bacteriocins have been reported to be inhibitory against several other bacteria [21]. The present investigation highlights the production of bacteriocin from *lactobacillus* sp. MRS medium was seemed to be more suitable for the bacteriocin production. The obtained bacteriocin was tested for antibacterial activity against *E.coli, Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *P. fluorescence*. The highest activity was demonstrated against *P. aeruginosa, E. coli* and *P. fluorescence* whereas the least inhibitory effect was observed in Klebsiella pneumonia. Our results are in total conformity with the work of Laemmli [11], wherein he emphasized the role of antibacterial activity of bacteriocin isolated from *Bacillus mycoides* (whey sample). The bacteriocin produced by them showed strong activity against food borne pathogens Listeria monocytogenes and Leuconostoc mesenteroides. Todorov and Dicks [22] suggested that bacteriocin production was strongly dependent on pH, nutrients source and temperature. Maximum bacteriocin activity was noted at pH 6.0, temperature 30°C and 1.5% NaCl [23]. The molecular weight of the bacteriocin was determined by SDS-PAGE. Partially purified bacteriocin from the lactobacillus species revealed a single protein band. Its molecular weight was estimated at about 90 kDa by SDS-PAGE. Similarly Guarner et al., [24], also obtained a single protein band with the molecular weight of 94 KDa for Lactobacillus lactis.

The present experiment indicated that organic acid production was increased with the incubation time. On the other hand, the pH of the media decreased with the increasing acid production. Highest acidity (0.067) and lowest pH (4.7) was observed after 72 h incubation at 37°C for probiotic Lactobacillus sp. The speed of acidification was slow for the Lactobacillus sp. These results were supported by the findings of Finlay and Falkow [25]. Wherein he suggested that highest acidity (6.53%) and lowest pH (3.65) was observed after 72 h incubation at 37°C for probiotic LAB isolated from Bogra yoghurt in organic acid production by *Lactobacilli*.

5. CONCLUSION

Probiotics are live microbial food supplements that are beneficial to the host in balancing the intestinal microbial system. Lactic acid bacteria are considered as probiotic bacteria in which *Lactobacillus* play a major role. In conclusion, the experimental results showed that isolated *Lactobacillus* sp. were able to tolerate inhibitory substances such as 0 – 1% bile acid, 3-6.5% NaCl and also able to survive in simulated gastric (pH 5) condition, as well as in alkaline (pH 9.0) condition. The isolated *lactobacilli* were able to produce organic acid in milk. The lactobacillus obtained in this study presented some probiotic properties and the bacteriocin produced by this isolate exhibited a broad spectrum of antimicrobial activity against four clinical pathogens.

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