

Antagonistic Mechanism of Probiotic *Lactobacillus* against Sea Food and Human Pathogenic Bacteria

M. Jayaprakashvel^{1, 2}, R.Guru³, G.Surendiran⁴, Yashika Chelvan¹,
P. Ashok Kumar⁴, K.Selvanayagi⁴ and A. Jaffar Hussain^{1,2}

¹Department of Marine Biotechnology, AMET University (u/s 3 of UGC Act 1956),
135, East Coast Road, Kanathur, Chennai-603112, India.

²Centre for Marine Bioprospecting, AMET University (u/s 3 of UGC Act 1956),
135, East Coast Road, Kanathur, Chennai-603112, India.

³PG and Research Department of Microbiology,
Sengunthar Arts and Science College, Tiruchengode-637 205, India.

⁴Department of Pharmacy, University of Manitoba, Winnipeg, Canada.

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Probiotic bacteria are finding newer and wider applications in the food and allied industries. They were increasingly used as biopreservatives. Seafood processing and packaging is emerging as a strong industry now a days. In this context, probiotic organisms having the capacity to inhibit the pathogens in seafood shall have immediate applications. Hence, we have made a study on exploring potential probiotic lactobacillus and their antagonistic mechanisms against sea food pathogens and few human pathogens. Thirty different bacterial strains were isolated from commercial and house hold curd samples from Chennai. They were found to be *Lactobacillus* spp. based on their microscopic and biochemical properties. They were tested for their antimicrobial potential against common sea food pathogens viz., *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. and human pathogens such as *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*. While screening for bactericidal activity, out of thirty, a strain *Lactobacillus* sp. AMET2017 exhibited remarkable inhibitory activity against one seafood and one human pathogens (maximum of 14 mm zone of inhibition) followed by *Lactobacillus* sp. AMET2025 in comparison with the other strains against both sea food and human pathogens. Crude ethyl acetate extracts (EAE) and crude bacteriocin (CB) were separated from the broth culture of *Lactobacillus* sp. AMET2017 and their Inhibitory Concentration (IC50) was tested using MTT 3-(4, 5-Dimethylthiazol-2-yl) - 2, 5-Diphenyltetrazolium bromide) assay. It has been found that the comparatively the EAE had better performance and hence it alone subjected to further studies. The antibacterial mechanism of EAE from *Lactobacillus* sp. AMET2017 was analyzed. There has been no considerable change in protein synthesis but the DNA integrity was found to be affected in the tested pathogens. Hence, it is concluded that the EAE of *Lactobacillus* sp. AMET2017 inhibits the sea food and human pathogens by affecting their DNA integrity and thus they can be effectively characterized for the use as biopreservatives of sea foods.

Keywords: Probiotic bacteria, lactobacilli, sea food pathogens, biopreservatives, DNA integrity.

Under certain set of conditions, perishable foods such as Seafood has the potential to cause diseases from viral, bacterial, and parasitic

* To whom all correspondence should be addressed.
E-mail: jayaprakashvel@gmail.com

pathogens. Naturally occurring marine *Vibrio* species¹ are accountable for many reported cases of infection from the utilization of raw molluscan shellfish which are delicacy in many countries, but certain species such as *V. vulnificus*² can be associated with high mortality in individuals who

are immune-compromised or have underlying liver disease³. The problem mainly arises at the food packaging (post-processing) level, which is common to all foods and not specific for seafood products. Meticulous cooking of seafood products would almost eliminate all microbial and parasitic pathogens; it will not destroy some microbial toxic metabolites (e.g., *Staphylococcus* toxins). A probiotic is any microbial (but not necessarily living) preparation or the components of microbial cells with a beneficial effect on the health of the host. Use of probiotics is getting momentum in recent years for the effective prevention and management of microbial infections⁴. Lactic Acid Bacteria (LAB) are widely known to inhibit food borne pathogens and spoilage microorganisms, hereby they can be used as biopreservative for extending the shelf-life and enhancing the safety of food products¹⁸. The majority of the new probiotic products contain *Bifidobacterium*, *Lactobacillus* or closely related species (*Lactobacillus acidophilus*). Strains of the *Lactobacillus casei* group comprising the species *L. casei*, *L. paracasei* subsp. *paracasei* and subsp. *tolerans* and *L. rhamnosus* are also being increasingly applied in novel-type yoghurts to enhance shelf life and increase food security⁵.

Biopreservation is an innovative and efficient way of extending the shelf-life of food products and reducing the risks from microbial spoilage. Biopreservation employs the inoculation of food products with selected bacterial strains which are having the ability to inhibit the growth of undesirable bacteria⁶. The production of bacteriocins by lactic acid bacteria has been known for many years. According to the original definition, the term bacteriocin refers to proteins of the colicin type, characterized by lethal biosynthesis, intra specific activity, and adsorption to specific receptors. Those produced by gram-positive bacteria fit closely to the classical colicin model⁷. *Lactobacillus* bacteriocins are found within each of the four major classes of antimicrobial proteins produced by lactic acid bacteria. Class I (lantibiotics)⁸ bacteriocins are small membrane active peptides (<5 kDa) containing the unusual amino acid lanthionine. Class II bacteriocins are small, heat-stable, non-lanthionine containing membrane-active peptides (<10 kDa). The class III bacteriocins which have to date only, been found

in *Lactobacillus*, include heat labile proteins of large molecular mass. Class IV is a complex bacteriocin group of proteins associated with other lipid or carbohydrate moieties, which appear to be required for activity and are relatively hydrophobic and heat stable⁹. Probiotic *lactobacilli* as biopreservative for sea food is gaining momentum in recent years. Attempts were made to isolate and evaluate lactic acid bacteria from seafoods and characterize them as biopreservatives¹⁰(Matamoros *et al.*, 2009)⁴¹. In this context, the present work has been undertaken to isolate probiotic lactobacilli and characterize their inhibitory metabolites against few sea food and human pathogenic bacteria with the ultimate aim to use them as biopreservatives of seafoods.

MATERIALS AND METHODS

Isolation of *Lactobacillus*

Five different commercially sold curd samples were collected as in their original packages. Their brand names are Hatsun, Dodla, Heritage, Dairy fresh and Curd prepared at AMET University. The test sample was diluted up to 10⁻⁶ dilution and spread plated on MRS agar (Proteose peptone 10.000 g/L, Beef extract 10.000 g/L, Yeast extract 5.000 g/L, Dextrose 20.000 g/L, Polysorbate 80 1.000 g/L, Ammonium citrate 2.000 g/L, Sodium acetate 5.000 g/L, Magnesium sulphate 0.100 g/L, Manganese sulphate 0.050 g/L, Dipotassium phosphate 2.000 g/L, Agar 12.0 g/L, Final pH (at 25°C) 6.5±0.2) and the plates were incubated at 37°C for overnight¹¹. Thirty different strains of *lactic acid bacteria* (LAB) were isolated. The axenic cultures were routinely sub-cultured and maintained in sterile distilled water in eppendorf tubes at 4°C and preserved until for further use.

Morphology and biochemical characterization of LAB

Pure cultures of bacteria were streaked on MRS agar plates to obtain single colonies for observation of colony morphology. Colony characteristics such as size, shape, consistency and pigmentation were observed and recorded after two days of incubation. Further these LAB were characterized for their cellular, physiological and biochemical properties using various staining and tests such as Gram staining, Oxidase test, Catalase test, Simple staining, Endospore Staining,

Motility test, Indole Test, Methyl Red (MR) Test, Voges Proskauer (VP) test and Citrate utilization test following various methods described by Cappuccino and Sherman¹² and the results were interpreted using identification keys provided in Bergey's Manual of Determinative Bacteriology¹³.

Screening of *Lactobacillus* for antimicrobial activity against seafood and human pathogens

Preparation of *Lactobacillus* culture filtrate

All the 30 lactobacilli were grown for 48 h in MRS broth. Two ml of the cultures were then centrifuged in sterile eppendorf tubes at 8000 rpm for 5mins. The cell free culture supernatant was collected in fresh sterile eppendorf tube and the extracellular culture filtrate was used to screen for antibacterial activity against sea food pathogens.

Agar well diffusion assay

Seafood pathogens *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. were obtained from the culture collection of Department of Marine Biotechnology, AMET University, Chennai. The selected human pathogens *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* were procured from Infectious Diseases Laboratory, YRG Care, Chennai. Actively growing broth cultures of these pathogens were prepared in nutrient broth which was obtained after 24 hours incubation. The respective pathogen was swabbed on nutrient agar plate. Wells were made with 8 mm diameter cork borers in the pathogen inoculated plates. The cell free culture filtrates at 100 µl concentration of selected lactobacilli strains were pipetted in each well and antimicrobial activity as zone of inhibition was observed after 2 days of incubation at 37°C.

Extraction of bioactive metabolites from the culture filtrate of LAB

About 200 ml of MRS^{15,48} (meg, lizz et al.) broth was prepared and the selected strains (*Lactobacillus* sp. AMET2017 and *Lactobacillus* sp. 2025) were inoculated and kept in the incubator shaker for 72 hours with 170 rpm and 37°C. After incubation they were centrifuged and the supernatant was collected in a clean separate flask for each culture. To this filtrate equal volume of ethyl acetate was added in a separating funnel and shaken vigorously and intermittently at every 15 min interval for 2 hours. The separated ethyl acetate layer was collected and concentrated using slow evaporation method in watch glasses. Then,

these concentrated extracts were re-dissolved in 2 ml of ethyl acetate, filter sterilized and stored in sterile eppendorf tubes until further use.

Extraction of bacteriocin from the culture filtrate of LAB

Cell free culture filtrate of selected two strains was obtained as described above. About 200 ml of thus obtained cell free supernatant was subjected to ammonium sulphate precipitation (4°C) at 80% saturation level and resulting precipitate was re-suspended in 20 mM Phosphate buffer (pH 7 ± 0.5) and used for further dialysis process. Pre-treated dialysis membrane (immersing the membrane into the warm 20 mM Phosphate buffer (pH 7 ± 0.5) for 10 minutes) was filled with precipitated protein and tied appropriately. This pack was dialyzed for 12 h against 20 mM Phosphate buffer (pH 7 ± 0.5) at 4°C. After dialysis the proteinous suspension containing bacteriocin was carefully collected, centrifuged and the pellet obtained was re-suspended in 2 ml of Phosphate buffer and stored at 4°C for further use.

Screening the LAB extracts for Antibacterial Activity

Antimicrobial activity of ethyl acetate extracts and bacteriocin preparations of lactobacilli were tested against seafood pathogens namely *Salmonella* sp., *Vibrio* sp. and *Shigella* sp. and human pathogens such as *P. aeruginosa*, *E. faecalis* and *S. aureus* by disc diffusion assay. Nutrient Agar plates were prepared and the test pathogens were swabbed to obtain lawn cultures. Sterile filter paper discs at a size of 6 mm were immersed to saturation level with Lab extracts and air dried in a laminar air flow chamber. The discs were placed at equidistance in respective pathogen lawn along with appropriate controls and the plates were incubated at 37°C for 24 h. The antimicrobial activity was determined by measuring the diameter of zone of inhibition around the disc, if any.

Minimal Inhibitory Concentration of ethyl acetate extracts and bacteriocins Pathogens using Broth dilution method

Minimum inhibitory concentration of ethyl acetate extract (EAE) of LAB strains AMET2017 & AMET2025 was tested against selected sea food pathogen *Shigella* sp. and human pathogen *S. aureus* in concentration 50, 75 and 100 µl and bacteriocin in concentration of 25, 50, 75 and 100 µl. Add 5 ml nutrient broth in each

tubes. Different concentrations of crude ethyl acetate extracts and bacteriocin preparations were introduced in respective pathogen inoculated test tubes. Control tubes with solvents as well as untreated pathogens were also maintained. All tubes were incubated for 24 h at 37°C.

MTT dye binding Assay

One ml of actively growing culture was taken from the respective treatments explained above were performed its absorbance at 600 nm in UV-Vis Spectrophotometer was measured. To this culture 1 ml of MTT reagent was added incubated for 10 minutes. Then, above mixture was centrifuged at 8000 rpm for 5 minutes. Then, the pellet was collected and re-suspended in 1 ml of ethanol. Then, absorbance of ethanolic wash was measured at 575 nm in UV Vis Spectrophotometer¹⁴.

Determination of mechanism of antibiotic activity

Broth cultures of pathogens were prepared and grown along with ethyl acetate extract and bacteriocin preparations. Then, they were grown for 24 h and the following parameters were tested in these cultures. Controls were prepared without any solvents or bacteriocins. Cell free culture filtrates were collected and subjected to estimation of proteins (extracellular, intracellular and membrane bound proteins) using the Bradford Method. The cell pellets from the respective treatments were collected and subjected to DNA extraction using Qia-quick DNA extraction kit following the instructions of the manufacturer (Qiagen). DNA integrity and banding pattern was observed in a 0.7% agarose gel following standard protocols.

RESULTS

Isolation of *Lactobacillus*

Lactobacillus was isolated from 5 different curds. The maximum number of colonies was observed in AMET curd then others (Figure 1). Thirty morphologically distinct colonies including 2 strains from AMET Curd, 1 from Hatsun Curd, 22 from Dodla Curd, 3 from Heritage Curd and another 2 from Dairy Fresh Curd were isolated and sub cultured to purity on MRS Agar. Though AMET curd has more number of colonies, the diversity was not rich. However, though numbers of colonies are less in Dodla curd, the diversity of colonies is rich and hence more number of strains

were isolated from that. The strains were coded with unique accession number with a prefix of AMET followed by Arabic numbers.

Morphology of the isolated bacterial strains

The morphologies of the obtained strains and their characteristics are summarized below. The colonies are tiny, small, medium, large, white chocky, convex colonies. The colonies are raised, flat, serrated. The nature is dry, rough, and mucoid-slimy. The cells are gram positive rods, non-motile, non-spore forming bacteria, oxidase negative, catalase positive, indole positive, MR positive, VP negative and citrate negative. The above results have proved that the bacterial strains were *Lactobacillus* spp.

Screening for *Lactobacillus* for antimicrobial activity against seafood pathogens;

Agar well diffusion assay

Among, 30 different strains of *Lactobacillus* only two strains namely AMET2017 and AMET2027 exhibited inhibition against one sea food and one human pathogen (Figure 2). AMET2017 showed the maximum inhibitory activity against *Shigella* sp. (1.4 ± 0.07 cm) and *S. aureus* (1.8 ± 0.5 cm) followed by strain AMET2025 which exhibited considerable zone of inhibition against *Shigella* sp. (1.3 ± 0.1 cm) and *S. aureus* (1.5 ± 0.4 cm). The strain AMET2017 was chosen for further studies because of its high inhibitory activity compared to other.

Growth inhibition assays

The crude extract and crude protein extracted from the culture filtrate of the AMET2017 was tested for its antibacterial activity by both well diffusion and broth dilution assay. It was inferred that the crude protein did not inhibited the growth of the bacterial pathogen whereas the ethyl acetate crude extract effectively inhibited the growth of the pathogens as mentioned in Figure-3.

Determination of inhibitory concentration 50 (IC₅₀) using MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay

For further confirmation, the MTT assay was performed. In this assay MTT was used as indicator for the determination of live cells. The effective Inhibitory concentration was observed at 0.5 µg/ml. The IC₅₀ was ranged from 0.5 µg/ml. This was characterized based on color change. When MTT was added it will be converted to

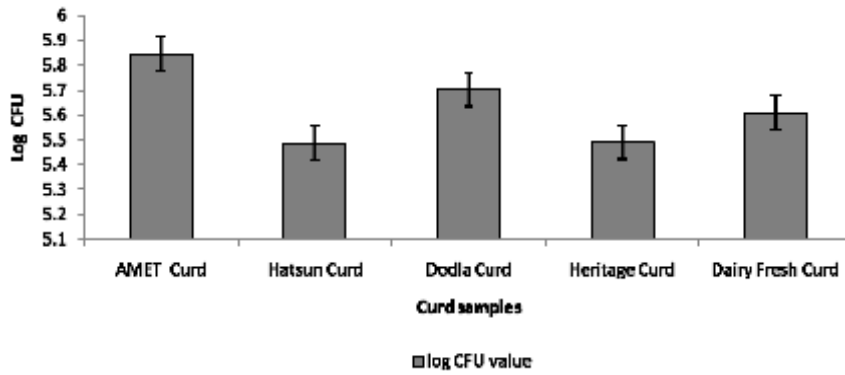
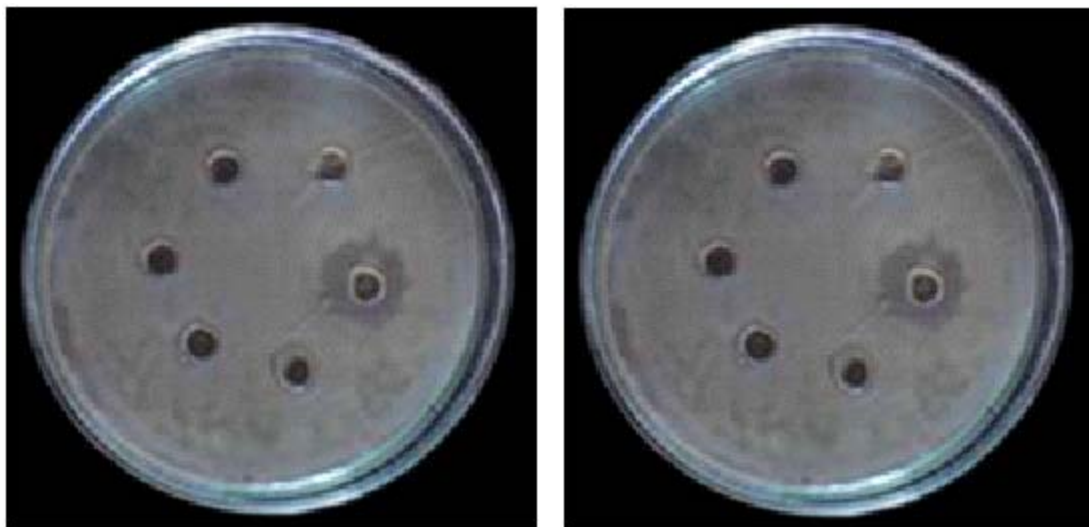


Fig. 1. Population of lactobacilli from different curd samples

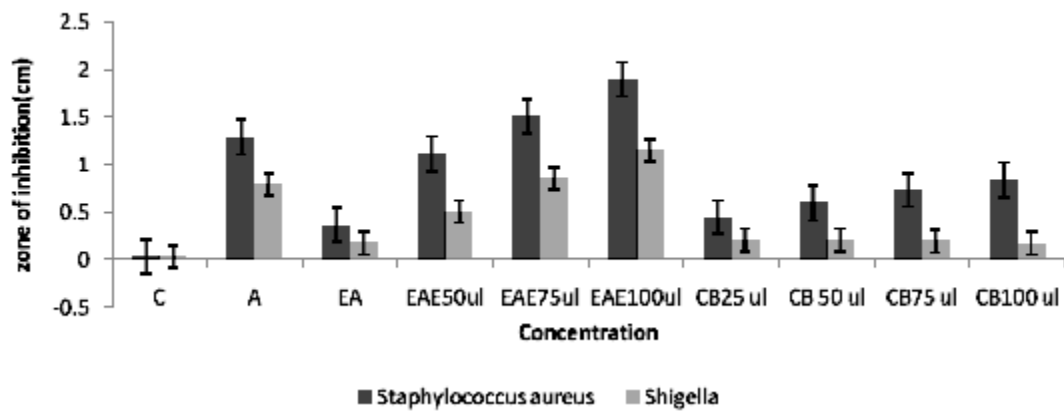


Shigella sp.

S. aureus

Fig. 2. Antibacterial activity of metabolites from LAB using well diffusion assay

Growth Inhibition Assay



A-Antibiotic ,C-Control,EA- Ethyl Acetate,EAE- Ethyl Acetate Extract and CB- Crude Bacteriocin

Fig. 3. Growth Inhibition Assay

Formide. This will be bringing a color change from yellow to violet. The positive tubes were selected for protein (Extracellular, intracellular, membrane bound) inhibition assay. For this assay *Shigella* spp and *s. aureus* strain were tested against various extracts from AMET2017 the results are indicated in Figure-4.

Determination of mechanism of antibacterial activity

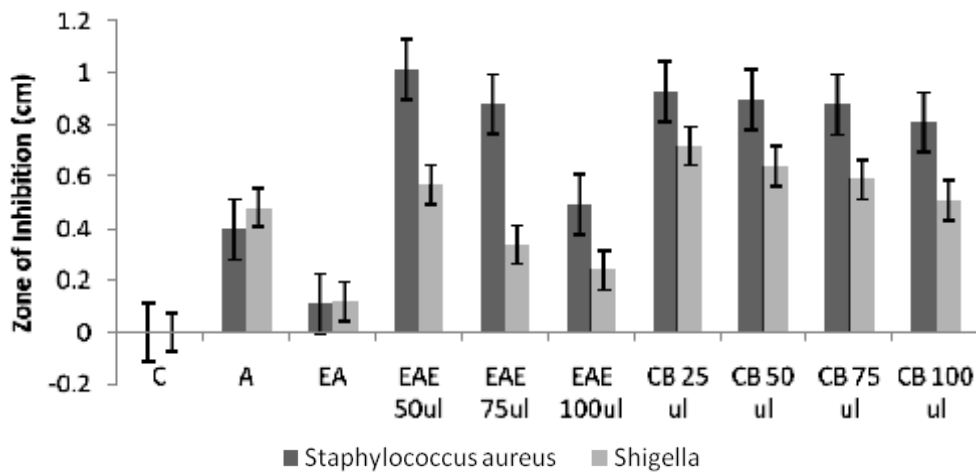
Protein inhibition assay

The ethyl acetate crude extract of *Lactobacillus* AMET- 2017 not inhibited the total

soluble extra cellular, intra cellular and membrane bound proteins of selected pathogens at the tested concentrations. This revealed that the mechanism of action of the crude extract was not through the inhibition of protein synthesis (Figure 5).

DNA fragmentation assay

DNA fragmentation assay was carried out, in order to determine the mode of action of ethyl acetate crude extract of *Lactobacillus* it inhibit the DNA integrity. The crude extract effectively induces DNA breaks at the concentration of 1 µg/ml. which inferred that



A-Antibiotic ,C-Control,EA- Ethyl Acetate,EAE- Ethyl Acetate Extract and CB- Crude Bacteriocin

Fig. 4. Determination of inhibitory concentration 50 (IC 50) using MTT 3- (4,5- Dimethylthiazol-2-yl)- 2, 5- Diphenyltetrazolium bromide) assay

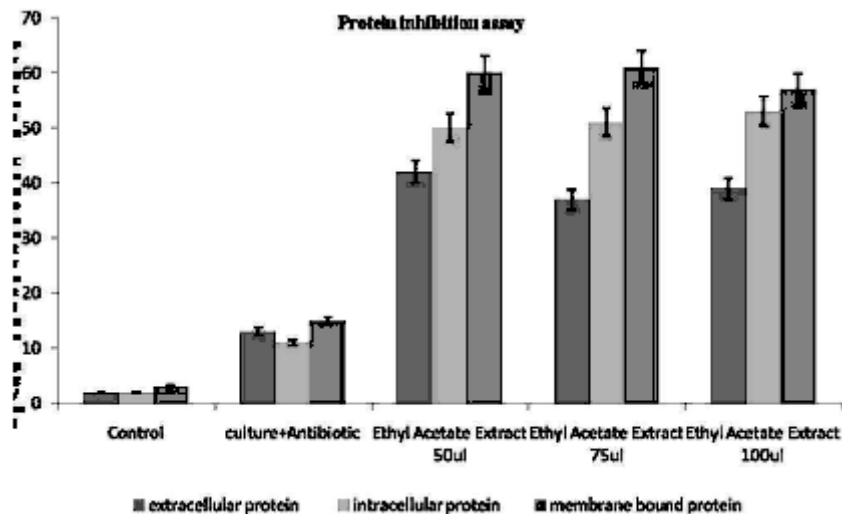
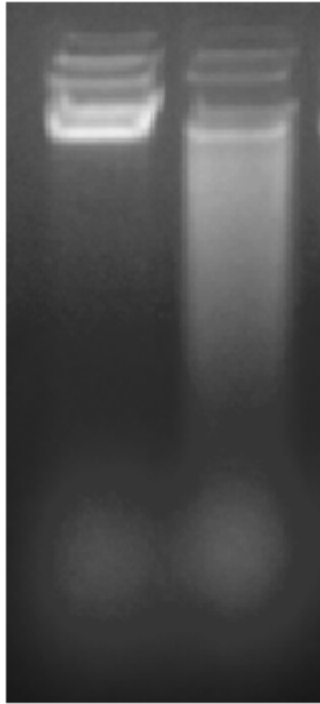


Fig. 5. Effect of crude metabolites and ethyl acetate extracts on protein content in pathogenic bacteria

mechanism of action of the extract was through DNA breakage (Figure 6).



From Left Lane 1: *S. aureus* treated; Lane 2: *Shigella* sp. treated

Fig. 6. DNA Integrity test on agarose gel

DISCUSSION

Lactic acid bacilli were routinely isolated from fermented food such as curd and were characterized to have probiotic and prebiotic activity⁵. In the present study, 30 morphologically different strains were isolated from five curd samples. The colonies are Tiny, Small, Medium, Large, white chocky, convex colonies. The cells are gram positive rods. The shapes of colonies are raised, flat, serrated. The nature is dry, rough, mucoid slimy. The above results have proved that the bacterial strains were non-motile *Lactobacillus* spp. In the well diffusion assay, among thirty different strains of *Lactobacillus* AMET- 2017, showed the maximum inhibitory activity against seafood pathogen and human bacterial pathogens followed by strain AMET-2025. The strain AMET-2017 was chosen for further studies because of its high inhibitory activity compared to other strains.

In the growth inhibition assays, the crude extract and crude protein extracted from the culture filtrate of the AMET- 2017 was tested for its antibacterial activity by both well diffusion and broth dilution assay. It was inferred that the crude protein did not inhibit the growth of the bacterial pathogen whereas the ethyl acetate crude extract effectively inhibited the growth of the pathogens. For further confirmation, the MTT assay was performed. In this assay MTT was used as an indicator for the determination of live cells¹⁶. The effective Inhibitory concentration was observed at 0.5µg/ml. The IC₅₀ was ranged from 0.5µg/ml. When MTT was added it will be converted to Formide. This brings color change from yellow to violet. The positive tubes were selected for protein (extracellular, intracellular, membrane bound) inhibition assay. For this assay *Shigella* sp strain AMET-2017 was selected for this study. In the protein inhibition assay, The ethyl acetate crude extract of *Lactobacillus* AMET- 2017 inhibited the total protein of extra cellular, intra cellular and membrane bound, at the concentration of 1µg/ml as assayed by Bradford method which revealed that the crude extract was not found to inhibit the protein synthesis. Studies were conducted before to test the DNA damage activity of biomolecules in agarose gels¹⁴. The crude extract effectively damages the DNA of the pathogen by fragmentation at the concentration of 1µg/ml. This was determined by DNA profile on Agarose gel electrophoretic technique. This can be concluded that the probiotic activity of LAB is more efficient in controlling bacterial pathogens at DNA level. Probiotic bacteria having the ability to produce antimicrobial principles can be effectively used as biopreservatives⁶.

The above results clearly indicate that crude protein products have the potential to control pathogenic bacteria and ensure safety to enhance shelf life of seafood products. The antibiotic products of *Lactobacillus* spp. can be used as biopreservatives against sea food pathogens in an eco-friendly manner.

CONCLUSION

The selected probiotic *Lactobacillus* sp. AMET2017 was found effective against two human pathogens. The crude ethyl acetate extracts of

Lactobacillus sp. AMET2017 is more potential antibacterial than the crude bacteriocin preparation. The crude ethyl acetate extracts of *Lactobacillus* sp. AMET2017 inhibited *Pseudomonas aeruginosa*, *Enterococcus faecalis* by affecting the DNA integrity. This study increases the scope of using *Lactobacillus* sp. AMET2017 as a biopreservative of food.

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