

Full Length Research Paper

***In vitro* characterization of bacteriocin producing *Bacillus subtilis* from milk samples**

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Bacillus subtilis was isolated from milk samples. Antibiotic resistance and the antimicrobial activity of *B. subtilis* were studied. All the four isolates of *B. subtilis* were sensitive to antibiotics such as streptomycin (25 g/ml), ampicillin (10 g/ml), penicillin (10 g/ml), erythromycin (15 g/ml), amoxicillin (10 g/ml). But they were resistant to bacitracin (10 g/ml). *B. subtilis* shown antibacterial activity against the selected human pathogens such as *Salmonella* spp, *Streptococcus* spp, *Klebsiella* spp and *E. coli*. The antimicrobial substance from *B. subtilis* extracted with organic solvent such as ethyl acetate have also shown antibacterial activity against the human pathogens. The proteinaceous nature of the *B. subtilis* exerted antimicrobial activity. The amount of protein varied between 0.05 - 0.55 mg/ml and the protein was qualitatively analyzed by SDS-PAGE. The entire samples have shown peptide < 62 kDa.

Key words: *Bacillus subtilis*, antibiotics, *Salmonella* spp, human pathogens, ethyl acetate, SDS PAGE.

INTRODUCTION

Milk is a complex biological fluid probably containing about 100,000 different molecular species in several states of dispersion. It is often the sole source of food for the very young mammal. The role of milk is to nourish and provide immunological protection. The major component of milk is water, the remainder consists of fat, lactose and protein (casein and whey proteins). Milk also contains smaller quantities of minerals, vitamins (vitamin A and vitamin C), specific blood proteins, enzymes (lactoperoxidase and acid phosphatase) and somatic cells (Richard, 2002).

Milk by its very nature is a natural growth medium for microorganisms. Various physio-chemical properties influence the growth of micro-organisms in milk. The other source of contamination begins from the farm to the manufacturing level. The type of microorganisms present in raw milk includes *Micrococcus*, *Staphylococcus*, *Bacillus* spp, *Pseudomonas*, *Enterobacter*, *Klebsiella* and *Serratia* etc. They can thrive under various conditions also. Giffel (1998) detected *Bacillus cereus* in 35% of the raw milk analysed. Pathogenic bacteria also may be

present in raw milk, which includes *Streptococcus* spp, *S. aureus* and *E. coli*. Donkor et al. (2007) estimated the presence of *Yersinia* spp (19.8%), *Klebsiella* spp (16.7%), *Proteus* spp (7.3%), *Enterobacter* spp (6.3%), *E. coli* (2.1%) and *Staphylococcus* spp (14.6%), *Bacillus* spp (11.5%) and *Mycobacterium* spp (1%). But the microorganisms isolated from milk sample possess antimicrobial activity.

Antimicrobial activity by most of the microorganisms are due to the antimicrobial peptides synthesized by most of the microorganism. Antimicrobial peptides are cationic peptides that display hydrophobic or amphiphilic properties. Dave and Shah (1997) characterized bacteriocins produced by *Lactobacillus acidophilus* using protein profile.

Aktypis et al. (1998) characterized thermophilic T, a novel bacteriocin produced by *Streptococcus thermophilus*. *B. subtilis* produce bacteriocin subtilin (Banerjee and Hansen, 1998). Lactic acid bacteria can produce antagonist compounds that vary in their spectra of activity. Abo-Amer (2007) characterized bacteriocin like inhibitory substance produced by *Lactobacillus planetarium* isolated from Egyptian homemade yogurt. The antimicrobial agent was active against wide range of gram positive and gram-negative pathogens. 10% SDS

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PAGE analysis of the antimicrobial agent indicated two peptides with high molecular size of < 45 kDa (Abo Amer, 2007). The milk borne pathogen pose serious threats and illness to humans. But many bacteria of different taxonomic branches and residing in various habitats such as soil, water, milk and fermented food products produce antimicrobial substance that are active against other bacteria.

Both gram-negative and gram-positive bacteria produce bacteriocin, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides. Risoen et al. (2004) characterized an antimicrobial substance from the *Bacillus cereus* strain ATCC 14579. Even though various micro organism are evidenced in milk samples due to improper handling and sanitary measures while some are pathogenic and pose serious threats and illness on humans. A large number of studies have been carried out only using *Lactobacillus* spp. Therefore in the present study, being a friendly microorganism *Bacillus subtilis* has been targeted to isolate from milk samples and to characterize based on their potential as antimicrobial agent.

MATERIALS AND METHODS

Glass wares were first soaked in chromic acid solution (10% potassium dichromate in 25% sulphuric acid) for a few hours and washed thoroughly in tap water. After second washing in detergent solution, they were washed thoroughly in tap water, rinsed in distilled water and air-dried. Media and glass wares were sterilized in an autoclave at 121°C at 15 lbs/sq. inch pressure for 15 min.

Collection of sample

Milk samples such as farm milk and market milk (TEJA) were collected once from Pallavaram, Chennai, India. They were immediately brought to the laboratory and used for the present study.

Isolation of bacteria

Raw, boiled (100°C for 15 min) and pasteurized (72°C for 15 s) farm and market milk samples were serially diluted and spread on nutrient agar medium. All the plates were kept for 24 h incubation at 37°C. The isolated bacterial colonies were purified to homogeneity by quadrant streaking, stored in nutrient agar slants at 4°C and sub-cultured periodically.

Identification of the organism

The bacteria isolated were identified based on physical characterization and the biochemical tests outlined in Bergey's Manual of determinative Bacteriology (Williams and Wilkins, 1994).

Antibiotic resistance/susceptibility screening

The sensitivity and resistance of isolates to various antibiotics such as ampicillin (10 g/ml), streptomycin (25 g/ml), penicillin (100 g/ml), bacitracin (100 g/ml), erythromycin (15 g/ml) and amoxycillin (10 g/ml) were studied using microbial sensitivity disc

(Hi-media) by disc diffusion method. 24 h culture of *B. subtilis* isolated from milk samples were swabbed on nutrient agar plates under sterile conditions in laminar air flow and the antibiotics discs of appropriate concentrations were placed on nutrient agar plates and incubated at 37°C. After incubation for 24 h, the plates were observed for growth. A clear zone around the disc was evidence for antibiotic resistance and susceptibility of the isolate. Diameter of the zone of inhibition was measured in millimeters. Nutrient agar plates without antibiotics served as control.

Antibacterial activity of bacterial isolates

Antibacterial activity of four bacterial isolates from milk samples were tested against the four human pathogenic bacteria such as *Salmonella* spp, *Klebsiella* spp, *E. coli* and *Staphylococcus* spp. Isolate bacteria were grown in Luria Bertani (LB) broth kept for 48 h. After 48 h incubation, samples were transferred to sterile centrifuge tubes and centrifuged at 6000 x g for 5 min at 4°C. Then the supernatant was collected and it is filtered using 0.45 m (pore size) membrane filter. Filtered supernatant was stored in refrigerator for further study. Human pathogens such as *Salmonella* spp, *Klebsiella* spp, *E. coli* and *Staphylococcus* spp were grown in LB broth (Hi media) and incubated for 24 h in orbital shaking incubator at 37°C. After 24 h, pathogens were stored in refrigerator for further study. LB agar plates were prepared and pathogens were swabbed on it and well were made on the plates with the help of borer, 100 l of filtered supernatant of bacterial isolates was added into the wells and kept for incubation for 48 h. After 48 h plates were observed for antibacterial activity.

Antibacterial activity of isolates with organic solvents

The supernatant of the bacterial isolates obtained above was taken. To that equal volume of ethyl acetate, a universally proved polar organic solvent that could dissolve many compounds was added and kept in orbital shaker for one hour or more preferably overnight. Then transfer the organic layer and distribute it equally into different conical flasks and cover the flask with cheese clothes to prevent contamination. And after complete drying, ethyl acetate was added to the residue present in the flask.

Human pathogens such as *Salmonella* spp, *Klebsiella* spp, *E. coli* and *Staphylococcus* spp were grown in LB broth and incubated for 24 h in orbital shaking incubator at 37°C. After 24 h pathogens were stored in refrigerator for further study. LB agar plates were prepared and pathogens were swabbed on it and wells were made on the plates with the help of borer, 100 l of filtered ethyl acetate supernatant was added into the wells and kept for incubation for 48 h. After 48 h, plates were observed for antibacterial activity.

ESTIMATION OF PROTEIN

Sample Preparation

Upto 1 ml of the bacterial supernatant isolated from milk samples 1 ml of 20% Tri chloroacetic acid (TCA) was added and kept for incubation for half an hour. It was then centrifuged at 6000 x g for 20 min. The pellet was washed with acetone twice and again centrifuged it. The supernatant was discarded and the pellet was dissolved in 0.2 M phosphate buffer. The sample was stored at 4°C for further study (Lowry et al., 1951).

Assay

The protein assay was done to determine the total protein content present in the bacterial isolates prior to loading into the

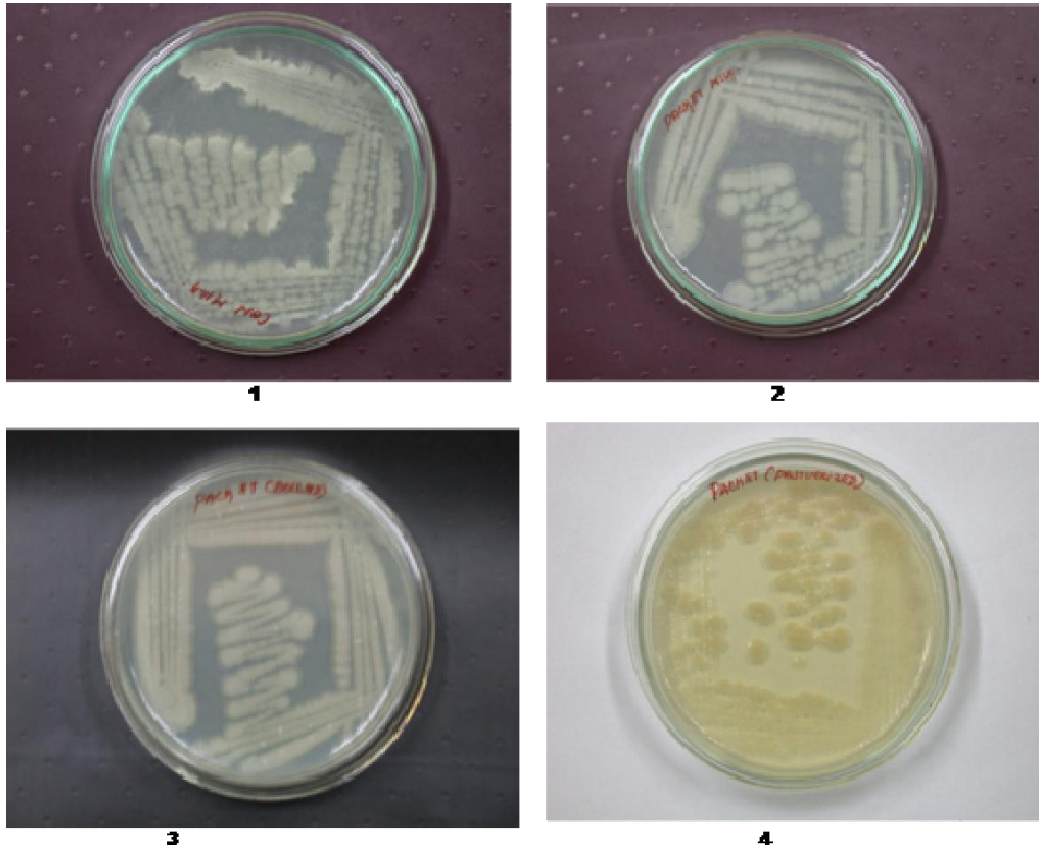


Plate 1. *B. subtilis* isolated from milk samples. 1. *B. subtilis* (Farm milk), 2. *B. subtilis* (Market milk), 3. *B. subtilis* (Boiled Market milk), 4. *B. subtilis* (Pasteurized market milk).

polyacrylamide gel electrophoresis (PAGE) wells. Protein concentrations were determined using BSA (bovine serum albumin) as standard. It was done using 0.5 ml Folin's reagent and 4.5 ml alkaline copper reagent. 50 l of sample was made up to 1 ml with distilled water. Then it was mixed with alkaline copper reagent followed by Folin's reagent. Then it was read at 660 nm in a spectrophotometer. The amount of protein present in the sample was expressed in mg/ml.

SDS-PAGE

Discontinuous SDS-PAGE was carried out according to the method described by Laemmli (1970). This was performed by using 12% resolving gel (pH 8.8) and 5% stacking gel (pH 6.8) in Tris- Glycine buffer (pH 8.3) . Polyacrylamide gel was stained with silver nitrate. After staining, the molecular weight of protein samples were determined using molecular weight marker.

RESULTS AND DISCUSSION

Milk plays an important role in day to day life. Because it provides all the essential nutrients for the very young mammal. Milk being a source of contamination various types of microorganisms such as *Staphylococcus* spp and *Bacillus* spp will be present in the raw milk, but

pathogenic bacteria in milk has been a matter of public health concern. Therefore, the objective of the present study was to find out the quality of the farm milk and market milk and also the prevalence of *B. subtilis* organisms in the milk sample and to characterize *Bacillus subtilis* based on their potential as antimicrobial agent.

The gram-positive rod shaped aerobic or facultative anaerobic spore forming bacteria have been assigned to the genus *Bacillus*. Even though bacteriocinogenic strains have been encountered amongst pathogenic *Bacillus* spp such as *B. cereus* and closely related *Bacillus thuringiensis*, *B. subtilis* is considered as safe. Therefore *B. subtilis* can be exploited for application in food industry.

B. subtilis was isolated from milk samples such as farm milk, market milk (boiled and pasteurized) by using crowded plate technique. With reference to colonial morphology as mentioned by Jonathan (2004), physical characterization and biochemical test such as catalase, oxidase, methyl red, voges proskauer, indole, nitrate reduction, sugar fermentation, citrate utilization, urease and starch hydrolysis as outlined in the Bergey's manual of determinative bacteriology, the bacteria was identified as *B. subtilis* (Plate 1). All the isolates were

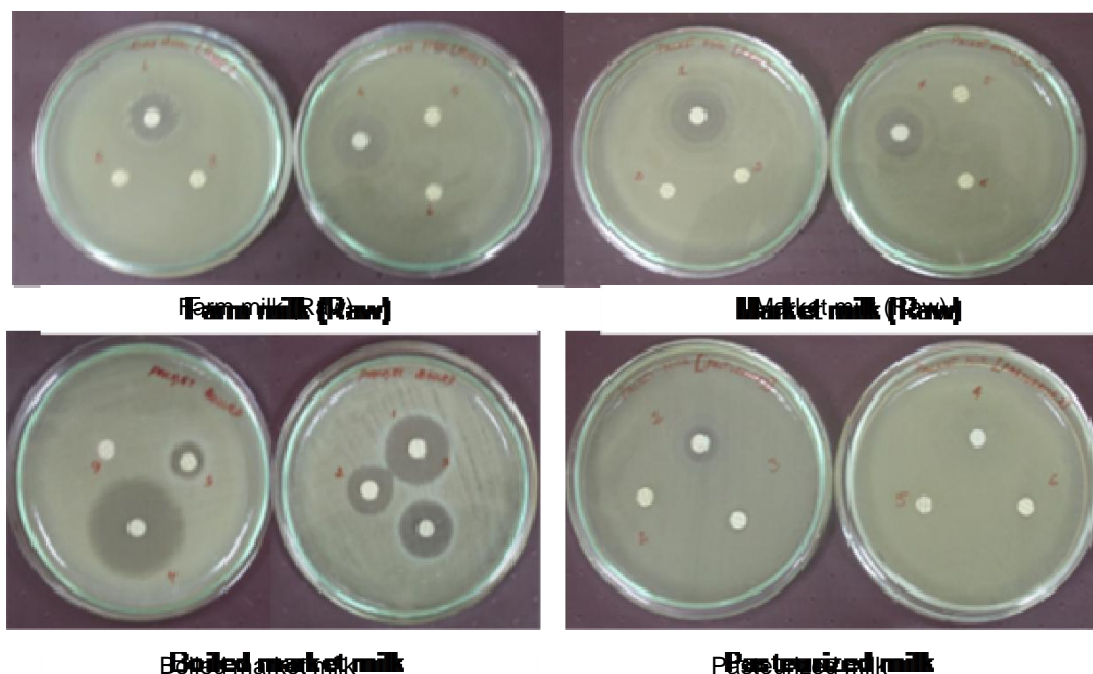


Plate 2. Antibiotic resistance/sensitivity spectrum of *B. Subtilis* from milk samples. 1-Streptomycin, 2-Ampicillin, 3-Penicillin, 4-Erythromycin, 5-Amoxycillin and 6-Bacitracin.

Table 1. Measurement of zone of inhibition of antibiotics against *B. subtilis* isolates from milk samples.

Samples	Antibiotics (g/ml)					
	Streptomycin (25 g/ml) (mm)	Ampicillin (10 g/ml) (mm)	Penicillin (10 g/ml) (mm)	Erythromycin (15 g/ml) (mm)	Amoxycillin (10 g/ml) (mm)	Bacitracin (10 g/ml) (mm)
Farm milk (Raw)	8	-	-	9	-	-
Market milk (Raw)	9	-	-	8	-	-
Market milk(Boiled)	5	6	9	13	4	-
Marketmilk(pasteurized)	5	-	5	10	-	2

B. subtilis from boiled market milk sample have maximum zone of inhibition against erythromycin

morphologically different in their growth in nutrient agar medium at 37°C but they are biochemically similar.

B. subtilis isolated from milk samples were sensitive to antibiotics such as streptomycin (25 g/ml) and erythromycin (15 g/ml). The resistance/sensitivity spectrum of different *B. subtilis* to various antibiotics are represented in plate 2. The zone of inhibition of streptomycin, ampicillin, penicillin, erythromycin, amoxycillin and bacitracin against *B. subtilis* from milk samples were measured in millimeters using standard recommendations and their results are shown in Table 1. Except bacitracin (10 g/ml) all the isolates were sensitive to antibiotics such as streptomycin (25 g/ml), ampicillin (10 g/ml), penicillin (10 g/ml), erythromycin (15 g/ml), amoxycillin (10 g/ml) and erythromycin showed the maximum zone of inhibition (13 mm) against *B. subtilis*

isolated from boiled market milk sample which is followed by streptomycin and penicillin (9 mm).

B. subtilis are sensitive to maximum antibiotics (83%) under study. Therefore the antibiotics selected for the present study can be recommended for infections with *B. subtilis*. *B. subtilis* have produced various antibiotics of various chemical structure such as *Mycosubtilisin* (Duitman et al. 1999). But they have shown resistance towards bacitracin (10 g/ml). This could be attributed to the presence of plasmid DNA in the genetic makeup. *B. subtilis* isolated from milk samples showed antibacterial activity against the human pathogens such as *Salmonella* spp, *Streptococcus* spp, *Klebsiella* spp and *E.coli* (Table 2; Plate 3). The maximum zone of inhibition was observed in *E.coli* (10 mm). The antibacterial activity of pure *B. subtilis* with organic solvent (Ethyl Acetate)

Table 2. Measurement of zone of inhibition of antibacterial activity of *Bacillus subtilis* against human pathogens.

Samples	Human pathogens			
	<i>Staphylococcus spp</i> (mm)	<i>Salmonella spp</i> (mm)	<i>Klebsiella spp</i> (mm)	<i>E. coli</i> (mm)
Farm milk (Raw)	4	7	6	7
Market milk (Raw)	-	-	-	5
Market milk(Boiled)	5	5	-	5
Market milk (Pasteurized)	4	9	4	10

Bacillus subtilis from pasteurized milk samples have shown the maximum zone of inhibition against *E. coli*.

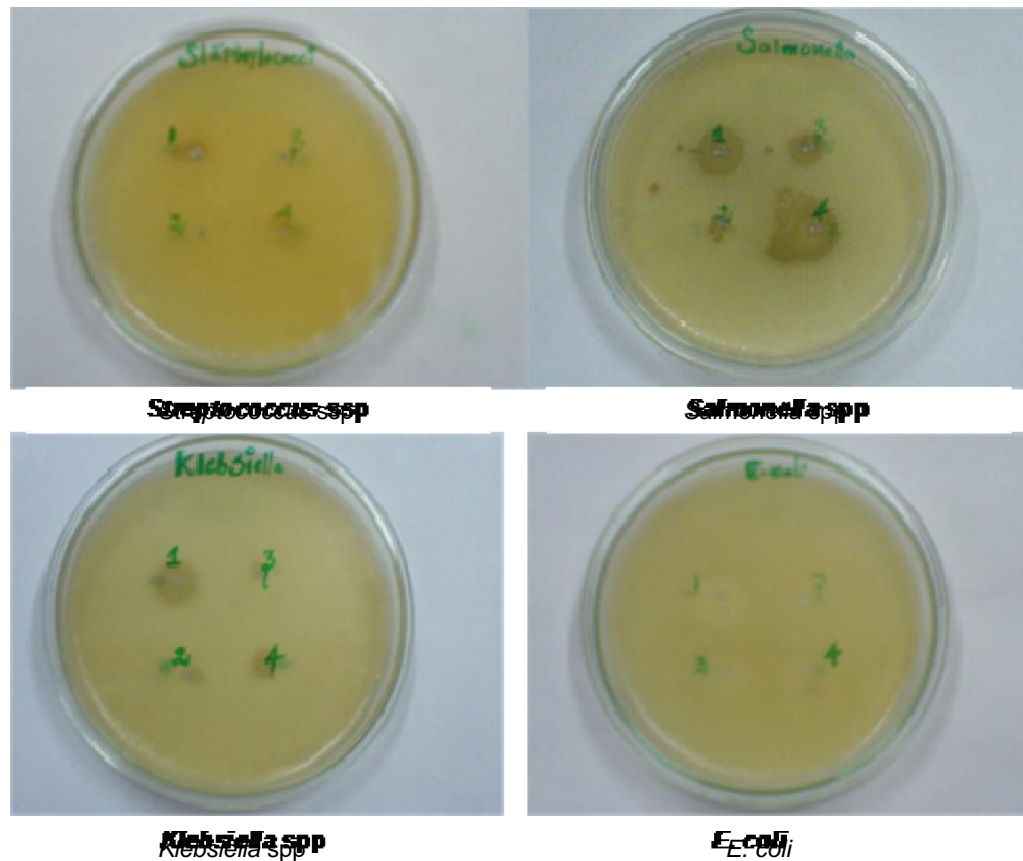


Plate 3. Antibacterial activity of *Bacillus subtilis* from milk samples against human pathogens. 1-*B. subtilis* (Farm milk), 2-*B. subtilis* (Market milk), 3-*B. subtilis* (Boiled Market milk) 4-*B. subtilis* (Pasteurized market milk)

against human pathogens such as *Salmonella spp*, *Streptococcus spp*, *Klebsiella spp* and *E. coli* was also studied. The ethyl acetate fractions of bacterial isolates have shown maximum zone of inhibition against *Salmonella spp* and *E. coli* (Table 3; Plate 4), but the pure culture of *B. subtilis* have shown maximum zone of inhibition than with the solvent extraction. This is because *B. subtilis* might have secreted antimicrobial substance to its surrounding during growth. They have shown broad spectrum of inhibition against selected human pathogens.

The inhibitive substances produced by bacteria can be generally proteins (Klaenhammer, 1993; Jimenez Diaz et

al., 1993; Vandenberg, 1993). The proteinaceous nature of the *B. subtilis* antimicrobial substance was clearly understood by the quantitative analysis of protein present in it. The amount of extracellular protein content present in each *B. subtilis* was tabulated in Table 4. The amount of protein in the entire sample varied between 0.05 - 0.55 mg/ml. These proteins were qualitatively analyzed by SDS-PAGE (Plate 5).

The entire protein molecules have separated according to their molecular weight. The separation of proteins of *B. subtilis* isolated from farm milk have shown band corresponding to 62,000 to 25,000 Dalton; Specific band

Table 3. Measurement of zone of inhibition of antibacterial activity of *B. subtilis* with organic solvent (Ethyl Acetate) against human pathogens.

Samples	Human pathogens			
	<i>Staphylococcus spp</i> (mm)	<i>Salmonella spp</i> (mm)	<i>Klebsiella spp</i> (mm)	<i>E. coli</i> (mm)
Farm milk (Raw)	2	4	2	4
Market milk (Raw)	2	1	5	-
Market milk (Boiled)	-	2	-	-
Market milk (pasteurized)	-	2	2	3

B. subtilis from raw farm milk have shown maximum zone of inhibition against *Salmonella spp* and *E. coli*.

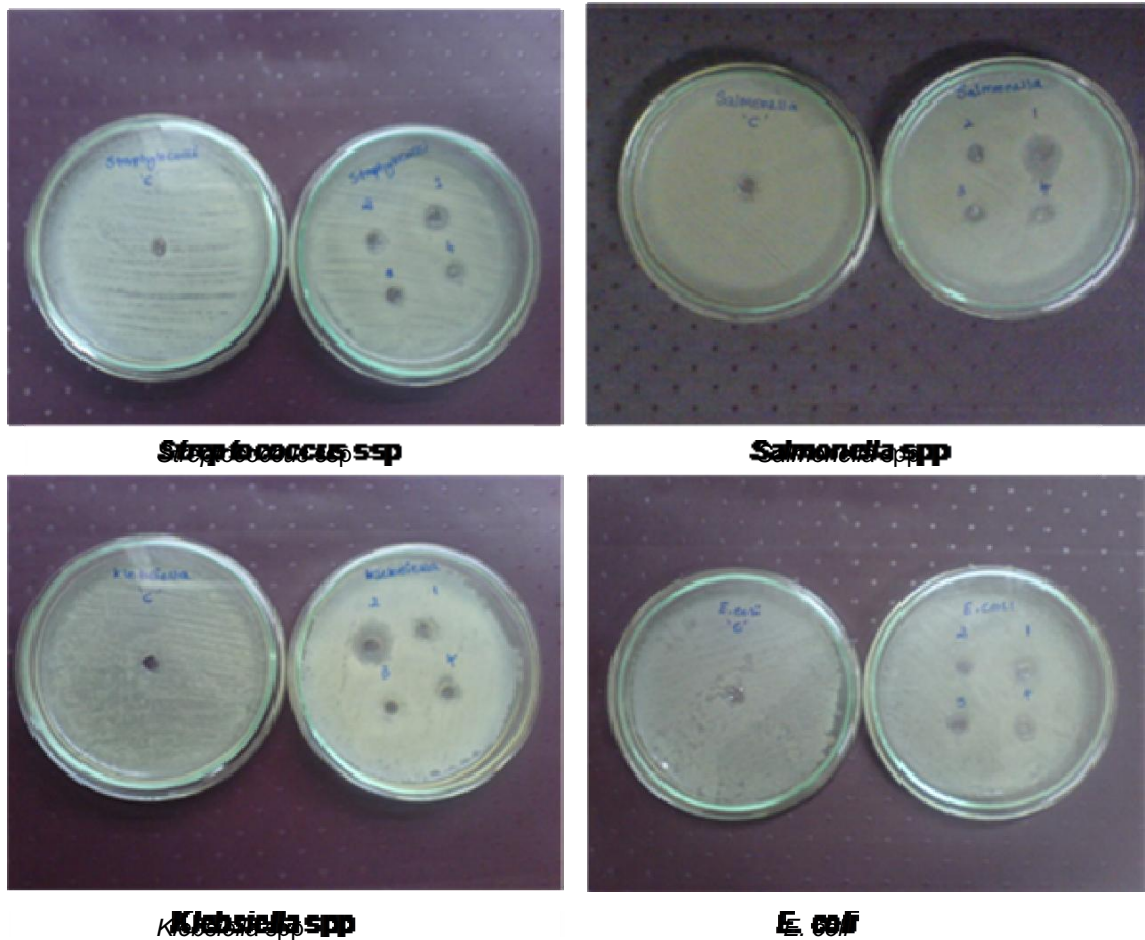


Plate 4. Antibacterial activity of *B. subtilis* with organic solvent (Ethylacetate) against human pathogens. 1-*B. subtilis* (Farm milk), 2-*B. subtilis* (Market milk), 3-*B. subtilis* (Boiled Market milk) and 4-*B. subtilis* (Pasteurized market milk) C – Control.

Table 4. Protein content of *B. subtilis* isolated from milk samples.

S.No	Sample	Protein (mg/ml)
1.	Farm milk	0.06
2.	Raw	0.05
	Pasteurized	0.55
	Boiled	0.50

corresponding to 47,500 kDa was present in farm milk and boiled farm milk samples. Raw market milk have shown band corresponding to 25,000 Dalton; Boiled market milk have shown band corresponding to 62,000 to 25,000 Dalton; Pasteurized market milk also have shown band corresponding to 25,000 Dalton. In the present study, the entire sample have shown peptide < 62 kDa.

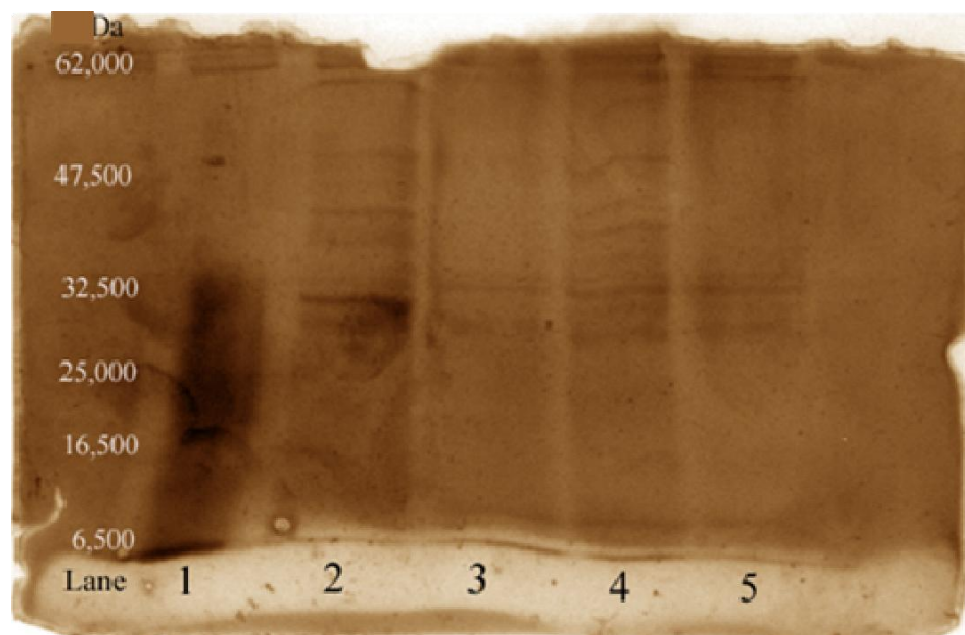


Plate 5. Protein profiling of *Bacillus subtilis* isolates from milk samples, Lane 1: Molecular marker, Lane 2: Farm milk (Raw), Lane 3: Market milk (Raw), Lane 4: Boiled Farm milk, Lane 5: Pasteurized Market milk.

Conclusion

In future, *B. subtilis* can be used as starter culture in fermentation technology. *B. subtilis* will be of potential interest in food safety and may have future application as food preservative. The application of these peptides as antimicrobial substance can be extruded to use in clinical studies too.

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