ORIGINAL ARTICLE

Prevalence of bacteriocin-producing *Lactobacillus*, food spoilage, and bovine mastitis-causing bacteria in commercial foodstuffs

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Abstract

Introduction: Present research investigation was focused for the prevalence of bacteriocin-producing, food spoilage, and bovine mastitis causing bacteria in commercial foodstuff. **Materials and Methods:** A total of 389 commercial food samples comprising meat, fish products, milk and dairy products, raw vegetables and products, bakery products, beverage, and fermented rice products were investigated from various randomly selected local retail shops and supermarkets of Salem, Erode, Tirupur, Namakkal, and Coimbatore districts of Tamil Nadu. All the food samples were serially diluted and aseptically inoculated on various enrichment broth and selective media and incubated aerobically and anaerobically. Streaking and re-streaking were performed on various selective agar media until pure bacterial cultures developed. All the bacteria were subjected for various morphological and biochemical tests useful for identification up to genus and species level. **Results:** Microbiological investigation confirmed the occurrences of 688 bacteria comprising of *Escherichia coli* (86, 22.1%), *Listeria* (82, 21.1%), *Aeromonas* sp. (72, 18.5%), *Clostridium* sp. (70, 17.9%), *Staphylococcus* sp. (64, 16.5%), *Lactobacillus* sp. (62, 15.9%), *Streptococcus* sp. (54, 13.9%), *Bacillus* sp. (53, 13.6%), *Enterobacter* sp. (43, 11.1%), *Salmonella enterica* (39, 10%), *Klebsiella* sp. (33, 8.5%), and *Enterococcus* sp. (30, 7.7%) from various commercial food products. The *Lactobacillus* sp. isolated were showing antagonistic activity against the tested indicator organisms. **Conclusion:** Bacteriocin isolated from some *Lactobacillus* sp. showed good antibacterial property against food spoilage and bovine mastitis-causing bacteria and can further be studied for its applications and mode of action. The prevalence of other Gram-negative and Gram-positive bacteria from the commercial food samples is an impending danger for transfer of foodborne infections to human and animals

Key words: Antibacterial, bacteriocin, bovine mastitis, commercial foodstuff, *Lactobacillus* sp.

INTRODUCTION

One of the concerns of the food industry is the contamination by pathogens, which are a frequent cause of foodborne diseases. The consequences of quality loss of food product caused by microorganisms are a consumers' risk. The U.S. Food and Drug Administration (FDA) gives a high priority to protecting the people from microbial contamination of the food supply (Hill 1996). The FDA concerned foodborne bacteria are listed in Bacteriological Analytical Manual, FDA (FDA 2012). These include *Aeromonas hydrophila, Aeromonas sobria, Bacillus cereus, Escherichia coli, Salmonella, Shigella, Campylobacter, Yersinia enterocolitica, Yersinia pseudotuberculosis, Listeria monocytogenes, Staphylococcus aureus, Streptococcus agalactiae, B. cereus, Clostridium perfringens, Clostridium botulinum,* and *Cronobacter, Klebsiella oxytoca* India is presently second in milk

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production in the world with an annual production of 60.60 million metric tons of milk. Tamil Nadu dairy industries play a key role in this accomplishment. However, even though they are producing the required milk, the dairy industries are facing a problem in the name of bovine mastitis. This bovine mastitis is a multifactorial disease and is one of the most difficult to control. It can be caused by many different bacterial species, the most common of which are *Staphylococcus* and *Streptococcus* species.[1] The prevalence of different species varies geographically, temporally, and also due to control measures adopted in herds. In addition, different pathogens are typical of different types of mastitis (clinical, subclinical, or heifer mastitis). It causes swelling and redness of the udder tissue of the cow damaging all the milk-producing cells, thereby reducing the milk production. Dairy industries have been consulting many research institutes to overcome from this problem. Lactic acid bacteria (LAB) are characterized as Gram-positive cocci or rods, non-aerobic but aerotolerant, ability to ferment carbohydrates for energy and lactic acid production.[2,3] The metabolic pathway of glucose may be homo fermentative or heterofermentative. In the first case, two molecules of lactate are generated (as in *Streptococcus* and *Lactococcus*), and in the second, lactate, ethanol, and carbon dioxide are produced, as in *Leuconostoc* and some *Lactobacilli*.[4] LAB are also able to produce small organic substances that contribute with aroma and give specific organoleptic attributes of the products.[5] LAB include various major genera: *Lactobacillus, Lactococcus, Carnobacterium, Enterococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella*. Other genera are *Aerococcus, Microbacterium, Propionibacterium,* and *Bifidobacterium*.[6] LAB are usually known as safe (Generally recognized as safe) and have an important role in the preservation of foods and fermented products. They can be used as the natural competitive microbiota or as specific starter cultures under controlled conditions.[7] Some of these bacteria produce antagonistic substances, called bacteriocins that in small amounts are very active against pathogens.[8-10] The review of literature clearly revealed that there are bacteria that are useful in industry, but many of them are involved in food spoilage, and some turned out to be pathogenic to animals and humans. The objective of the present research is to check the prevalence of various Gram-negative and Gram-positive bacteria from various commercial foodstuffs used for human consumption and to identify a lactic acid bacterium with a hyper bacteriocin activity.

MATERIALS AND METHODS

Sample Collection

A total of 389 samples comprising meat, fish products, milk and dairy products, raw vegetables and products, bakery products, beverage, and fermented rice products were procured from various randomly selected local retail shops, local markets and supermarkets in Salem, Erode, Tirupur, Namakkal, and Coimbatore districts of Tamil Nadu, India, and immediately transferred to the Molecular Diagnostics and Bacterial Pathogenomics Research Laboratory for bacterial isolation and identification.

Isolation of Bacteria

All the solid food samples (1 gm) were initially crushed in a sterilized mortar with pestle and were serially diluted between 10^{-2}/ml and 10^{-8}/ml in distilled water and finally inoculated in brain, heart infusion broth (HiMedia Laboratories, Mumbai) and nutrient (NA) broth (HiMedia Laboratories, Mumbai), Robertson's cooked-meat broth (HiMedia Laboratories, Mumbai), de Man, Rogosa and Sharpe (MRS) broth (HiMedia Laboratories, Mumbai), and incubated at various temperatures. All the liquid food samples (1 ml) were also serially diluted between 10^{-2}/mL and 10^{-8}/mL in various broths mentioned above and incubated at specific temperatures. Inoculum from broth samples was also aseptically inoculated on various enrichment broths and selective agar media such as NA agar, blood agar, Luria-Bertini agar, tryptic soy agar, and MacConkey agar xylose lysine deoxycholate agar (HiMedia Laboratories, Mumbai) as and when required for the isolation of specific bacteria and incubated aerobically and anaerobically at various temperatures.

As the major focus is on LAB, the samples from NA broth were then inoculated into culture-specific medium, and the most likely media are MRS (HiMedia Laboratories) and are incubated aerobically at an optimum temperature for 16–24 h. From the inoculated plates preferred that the colonies showing different morphological characters were randomly selected, streaking and re-streaking were carried out each time in fresh media plates until the pure culture was obtained. The pure cultures were grown in MRS agar plates and preserved at 4°C. Glycerol stocks were made, and the pure cultures are preserved at Deep freezer (−80°C). All the bacteria specific culture media and growth temperatures details are given in Table 1.

Identification of Bacteria

Bacterial colonies from selective agar were subjected to various morphological, cultural, and biochemical characters as per the Bergey's manual of determinative bacteriology with little modification.[5] These tests included motility of the bacteria, Gram's and endospore staining, gelatin liquefaction, litmus milk fermentation, and triple sugar iron agar for the fermentation of sugar such as glucose, lactose, and sucrose. All the isolated bacteria were evaluated for the phenotypical tests like Nitrate reduction (NR), Starch hydrolysis (SH), Voges-Proskauer (VP), Citrate-hydrogen Sulfide (CT), Methyl Red CAMP- CAMP Test (MR), Indole (IN), Novobiocin resistance (NOV-R), Hemolysis

Table 1: Growth conditions for the isolated organisms

Name of Bacteria	Growth media name of bacteria and culture conditions			
	Broth media	**Culture conditions**	**Agar media**	**Culture conditions**
Aeromonas sobria	BHI Broth	Incubated Aerobically at 30°C for 24 h	NA Agar	Incubated Aerobically at 30°C for 24 h
Bacillus cereus	NA Broth	Incubated Aerobically at 30°C for 24 h	NA Agar	Incubated Aerobically at 30°C for 24 h
Bacillus subtilis	NA Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 30°C for 24 h
Bacillus subtilis		Incubated Aerobically at 30°C for 24 h	NA Agar	Incubated Aerobically at 30°C for 24 h
Clostridium perfringens	RCM Broth	Incubated Anaerobically at 37°C for 24 h (Gas pack inside gas jar) and also CO_2 incubator with 5% CO_2	Blood Agar	Incubated Anaerobically at 37°C for 24 h (Gas pack inside gas jar) and also CO_2 incubator with 5% CO_2
Clostridium perfringens	RCM Broth	Incubated Anaerobically at 37°C for 24 h (Gas pack inside gas jar) and also CO_2 incubator with 5% CO_2	Blood Agar	Incubated Anaerobically at 37°C for 24 h (Gas pack inside gas jar) and also CO_2 incubator with 5% CO_2
Enterobacter aerogenes	BHI Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 37°C for 48 h
Enterococcus faecalis	BHI Broth	Incubated Aerobically at 37°C for 24 h	Tryptic Soy Agar+5% sheep blood	Incubated Aerobically at 37°C for 24 h
Escherichia coli	NA Broth	Incubated Aerobically at 37°C for 24 h	Macconkey Agar	Incubated Aerobically at 37°C for 24 h
Klebsiella oxytoca	NA Broth	Incubated Aerobically at 30°C for 24 h	Semisolid nitrogen-free malate (NFB Medium)	Incubated Aerobically at 30°C for 24 h
Klebsiella pneumonia		Incubated Aerobically at 30°C for 24 h	Semisolid NFB Medium	Incubated Aerobically at 30°C for 24 h
Lactobacillus acidophilus	MRS Broth+ Tween 80	Incubated Aerobically at 37°C for 24 h	MRS Agar+ Tween 80	Incubated Aerobically at 37°C for 96 h
Lactobacillus plantarum	MRS Broth+ Tween 80	Incubated Aerobically at 37°C for 24 h	MRS Agar+ Tween 80	Incubated Aerobically at 37°C for 96 h
Lactococcus lactis subsp. Lactis	MRS Broth+ Tween 80	Incubated Aerobically at 37°C for 24 h	MRS Agar+ Tween 80	Incubated Aerobically at 37°C for 24 h
Lactococcus lactis subsp. Lactis	MRS Broth+ Tween 80	Incubated Aerobically at 37°C for 24 h	MRS Agar+ Tween 80	Incubated Aerobically at 37°C for 24 h
Listeria monocytogenes	NA Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 37°C for 24 h
Salmonella enterica serovar Paratyphoid	BHI Broth	Incubated Aerobically at 37°C for 24 h	Xylose lysine deoxycholate agar (XLD agar)	Incubated Aerobically at 37°C for 24 h
Salmonella enterica serovar Typhi	BHI Broth	Incubated Aerobically at 37°C for 24 h	XLD	Incubated Aerobically at 37°C for 24 h
Salmonella enterica serovar Typhimurium	BHI Broth	Incubated Aerobically at 37°C for 24 h	XLD	Incubated Aerobically at 37°C for 24 h
Salmonella enterica serovar Typhimurium	BHI Broth	Incubated Aerobically at 37°C for 24 h	XLD	Incubated Aerobically at 37°C for 24 h
Salmonella enterica serovar Enteritidis	BHI Broth	Incubated Aerobically at 37°C for 24 h	XLD	Incubated Aerobically at 37°C for 24 h

(*Contd...*)

Table 1: (*Continued*)

Name of Bacteria	Growth media name of bacteria and culture conditions			
	Broth media	Culture conditions	Agar media	Culture conditions
Staphylococcus aureus	NA Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 37°C for 24 h
Staphylococcus chromogenes	NA Broth	Incubated Aerobically at 37°C for 24 h	Medium 92	Incubated Aerobically at 37°C for 24 h
Staphylococcus epidermidis	NA Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 37°C for 24 h
Staphylococcus haemolyticus	NA Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 37°C for 24 h
Staphylococcus sciuri	NA Broth	Incubated Aerobically at 37°C for 24 h	NA Agar	Incubated Aerobically at 37°C for 48 h
Staphylococcus simulans	NA Broth	Incubated Aerobically at 37°C for 24 h	LB Agar	Incubated Aerobically at 37°C for 24 h
Staphylococcus xylosus	NA Broth	Incubated Aerobically at 37°C for 24 h		Incubated Aerobically at 37°C for 48 h
Streptococcus agalactiae	MRS Broth+ Tween 80	Incubated Aerobically at 37°C for 24 h	MRS Agar+Tween 80	Incubated Aerobically at 37°C for 96 h

RCM: Robertson's cooked-meat, BHI: Brain, heart Infusion, NFB: Nitrogen-free malate, MRS: de Man, Rogosa and Sharpe, NA: Nutrient

(HE), Catalase (CA), Oxidase (OX), Coagulase (CO), Alkaline dehydrogenase (ALD), Alkaline phosphatase (PA), Gelatinase (GE), Esculinase (ES), Lecithinase (LE), beta - glactosidase (BG), Arginine dehydrogenase (ARD), Lysine decarboxylase (LYD), Ornithine decarboxylase (ORD), Urease (UR), Hippuricase (HI) as per the standard protocol.

Indicator Bacteria

The indicator organisms were procured from Microbial Type Culture Collection, Chandigarh and National Collection of Industrial Microorganisms, Pune, for antibacterial testing. The indicator microbes are listed in Table 2.

Extraction of Genomic DNA for Polymerase Chain Reaction (PCR)

Genomic DNA from each Lactobacillus isolates was extracted using a Wizard genomic DNA extraction kit (Promega, USA) following its technical manual's instructions. The DNA was finally dissolved in 80 µl of buffer (10 mM Tris-HCl, 1m M EDTA, pH 8.0) and quantified at 260 nm using DNA Nanodrop (Thermo Fischer). This genomic DNA was used as a source of template DNA in PCR.

Screening of *Lactobacillus* Isolates by PCR

The polymerase chain reaction was carried out with all the isolates of *Lactobacillus* and *Lactococcus* use species-specific primers to confirm the prevalence of *Lactobacillus acidophilus, Lactobacillus plantarum* (LP), and *Lactococcus lactic* subsp *lactis* using standard profile condition specific for each isolate. These were used at the optimized concentration to amplify the species-specific genes such as 23S rRNA gene, recA, and gadB gene, respectively. All the species-specific primers LacidoF 5'-CACTTCGGTGATGACGTTGG-3' LacidoR 5'-CGATGCAGTTCCTCGGTTAAGC-3' (23SrRNA; 575bp),[11] planF 5'-CCGTTTATGCGGAACACCTA-3' and planR 5'-TCGGGATTACCAAACATCAC-3' (recA; 318bp)[12] and gadB21 5'-CGTTATGGATTTGATGGATATA AAGC-3'and GAD7 5'-ACTCTTCTTAAGAACAAGTTT AACAGC3' (gadB; 602bp)[13] were commercially synthesized. The amplification products were analyzed by electrophoresis on a 2% agarose gel containing 0.5µg/ml of ethidium bromide and the bands were visualized.[14] The assay conditions for each species are mentioned below.

Species-specific PCR for *L. acidophilus, Lactobacillus,* and *Lactococcus lactis*

Each PCR product was amplified by the following conditions: Initial denaturation step for 5 min at 95°C, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 63°C for LacidoF and LacidoR; 46°C for 15 s for planF and pREV; 30 s at 50°C for gadB21; and GAD7 and elongation for 60 s at 72°C with an additional extension step was 5 min at 72°C after the last cycle.

Screening of Isolates of Bacteriocin Producing Bacteria

The crude bacteriocin was produced by growing *L. acidophilus* (18 isolates), *L. planetarium* (22 isolates), and *L. lactis* subsp. *lactis* (22 isolates) in MRS broth separately, incubated at 37°C for different time intervals

Table 2: Morphological characteristics of isolated bacteria

Bacteria Investigated	Motility test	Gram staining	Flagella staining	Endospore staining
Aeromonas hydrophila	Motile	Gram-negative Rod	Polar flagella	No endospore
Aeromonas sobria	Motile	Gram-negative	Single polar flagella	No endospore
Bacillus cereus	Motile	Gram-positive Rod	Peritrichous flagella	Central
Bacillus subtilis	Motile	Gram-positive Rod	Peritrichous flagella	Subterminal
Clostridium perfringens	Non Motile	Gram-positive Rod	No flagella	Subterminal
Enterobacter aerogenes	Motile	Gram-negative Rod	Peritrichous flagella	No endospore
Enterococcus faecalis	Non Motile	Gram-positive Cocci	No flagella	No endospore
Escherichia coli	Motile	Gram-negative Rod	Peritrichous flagella	No endospore
Klebsiella oxytoca	Non Motile	Gram-negative Rod	No flagella	No endospore
Klebsiella pneumonia	Non Motile	Gram-negative Rod	No flagella	No endospore
Lactobacillus acidophilus	Non Motile	Gram-positive Rod	No flagella	No endospore
Lactobacillus plantarum	Non Motile	Gram-positive Rod	No flagella	No endospore
Lactococcus lactis subsp. lactis	Non Motile	Gram-positive Rod	No flagella	No endospore
Listeria monocytogenes	Motile	Gram-positive Cocci	Peritrichous flagella	No endospore
Salmonella enterica serovar Paratyphoid	Motile	Gram-negative Rod	Peritrichous flagella	No endospore
Salmonella enterica serovar Typhi	Motile	Gram-negative Rod	Peritrichous flagella	No endospore
Salmonella enterica serovar Typhimurium	Motile	Gram-negative Rod	Peritrichous flagella	No endospore
Salmonella enterica serovar Enteritidis	Motile	Gram-negative Rod	Peritrichous flagella	No endospore
Staphylococcus aureus	Non Motile	Gram-positive Cocci	No flagella	No endospore
Staphylococcus chromogenes	Non Motile	Gram-positive Cocci	No flagella	No endospore
Staphylococcus epidermidis	Non Motile	Gram-positive Cocci	No flagella	No endospore
Staphylococcus haemolyticus	Non Motile	Gram-positive Cocci	No flagella	No endospore
Staphylococcus sciuri	Non Motile	Gram-positive Cocci	No flagella	No endospore
Staphylococcus simulans	Non Motile	Gram-positive Cocci	No flagella	No endospore
Staphylococcus xylosus	Non Motile	Gram-positive Cocci	No flagella	No endospore
Streptococcus agalactiae	Non Motile	Gram-positive Cocci	No flagella	No endospore

such as 48 h, 72 h, 96 h, and 120 h anaerobically (5% CO_2 incubation). A total of six different indicator bacteria were investigated to test the antagonistic activity initially. The ability of the isolates to produce a high amount of bacteriocin was tested by performing a well diffusion assay method.[15] The culture was centrifuged at 10,000 rpm for 5 min, and the supernatant was collected. A volume of 1 mL inoculum of each indicator bacteria (A = 1.0 at 540 nm) were swabbed on pre-poured sterilized NA agar plates using pre-sterilized cotton bud. Wells of 7 mm in diameter and 5 mm deep were cut in each plate and 30 µL of culture supernatant was poured. The sizes of clear zones were recorded for different indicator bacteria to select the best strains. From the result obtained four isolates of *L. acidophilus*, three isolates of LP, and two isolates of *L. lactis* subsp *lactis* showed bacteriocin activity. These isolates were further tested for antagonistic activity to find out a novel bacteriocin which is very effective.[16] The conditions for the production of bacteriocin by LAB were also optimized based on its effective antimicrobial activity against indicator organisms.

Scanning Electron Microscopy

Bacteriocin-producing bacterial isolates were grown on NA agar plates and were fixed with Karnovsky's fixative (pH 7.3) and incubated at 4°C for 4 h. Samples were washed twice with 0.1 M Sodium Cacodylate buffer (pH 7.4) (Sigma, USA) and incubated at 4°C for 15 min for each wash, postfixed with the same mix for 12 h at 4°C and dehydrated in a series of acetone from 30% to 100%, twice in each dehydrating solution for 15 min at 4°C. The samples were dried using the drying reagent tetramethylsilane (Sigma, USA) for 15 min at 4°C and air dry in air hood for 15 min. The samples were mounted on aluminum stubs, with adhesives taps and sputter coated with carbon for 5 min using a polaron energy beam and examined under the scanning electron microscope (SEM) (Jeol-Jem, Japan).

Effect of Concentration of NaCl on Growth

Previous studies reported that NaCl tolerance is the important physiological parameter for the growth of a cell as the

Table 3: Percentage of isolates positive for biochemical tests

Bacteria	No of isolates	Percentage of isolates positive for biochemical tests																								
		NR	SH	VP	CT	H_2S	MR	CAMP	IN	NOB-R	HE	CA	OX	CO	ALD	AP	DN	GE	UR	HI	ES	LE	BG	ARD	LDC	ODC
Aeromonas sobria	34	98	-	65	98	78	-	-	89	-	-	-	85	-	-	-	0	-	0	-	0	88	-	-	-	0
Bacillus cereus	22	95	90	92	100	-	-	-	0	-	80	85	0	-	-	-	-	78	55	-	89	-	0	-	0	0
Bacillus subtilis	31	92	85	98	98	-	-	-	-	-	86	88	65	-	-	-	-	-	-	0	82	-	84	-	0	0
Clostridium perfringens	70	-	-	-	-	92	-	-	-	-	100	0	0	-	-	-	92	92	-	-	-	88	-	-	-	-
Enterobacter aerogenes	43	98	-	-	95	0	-	-	0	-	-	85	0	-	-	-	0	0	-	-	-	0	-	-	92	95
Enterococcus faecalis	30	-	-	88	93	-	-	-	-	-	0	0	-	-	-	0	-	-	-	89	98	-	0	-	-	-
Escherichia coli	86	100	-	0	0	0	-	-	98	-	-	86	0	-	-	-	0	0	0	-	0	0	-	-	85	72
Klebsiella oxytoca	12	94	-	98	98	0	-	-	89	-	0	78	0	-	-	-	0	0	86	-	89	0	-	-	85	-
Klebsiella pneumonia	21	92	-	98	90	0	-	-	0	-	0	80	0	-	-	-	0	0	85	-	78	0	-	-	84	-
Lactobacillus acidophilus	18	0	92	94	-	88	85	-	0	-	35	0	0	0	88	-	-	78	-	-	92	-	-	-	-	0
Lactobacillus plantarum	22	-	-	-	-	-	-	-	-	-	-	0	0	-	-	-	-	98	-	-	92	-	-	-	-	-
Lactococcus lactis subsp. *lactis*	22	0	0	89	0	0	-	-	0	-	-	-	0	0	-	-	-	-	0	-	-	-	-	-	-	0
Listeria monocytogenes	82	-	-	92	-	-	98	-	-	-	100	98	0	-	-	-	-	-	0	88	85	92	98	-	-	-
Salmonella enterica serovar Paratyphoid	10	100	-	0	-	-	98	-	0	-	-	80	0	-	-	-	0	0	0	-	0	0	-	-	0	88
Salmonella enterica serovar Typhi	8	95	-	0	-	92	98	-	0	-	-	92	0	-	-	-	0	0	0	-	0	0	-	-	88	0
Salmonella enterica serova Typhimurium	12	92	-	0	-	-	88	-	0	-	-	85	0	-	-	-	0	0	0	-	0	0	-	-	-	-
Salmonella enterica serovar Enteritidis	9	98	-	0	88	92	98	-	0	-	-	88	0	-	-	-	0	0	0	-	0	0	-	-	88	85
Staphylococcus aureus	11	100	-	98	-	-	-	-	-	0	92	90	0	88	-	80	75	-	30	-	-	-	0	55	-	-
Staphylococcus chromogenic	9	98	-	0	-	-	-	-	-	0	0	92	0	0	-	88	92	-	85	-	-	-	0	80	-	-

(*Contd...*)

Table 3: (*Continued*)

Bacteria	No of isolates	Percentage of isolates positive for biochemical tests																						
		NR	SH	VP	CT	H_2S	MR	CAMP	IN	NOB-R	HE	CA	OX	CO	ALD	AP	DN	GE	UR	HI	ES	LE	BG	ARD	LDC	ODC
Staphylococcus epidermidis	12	60	-	100	-	98	-	-	-	0	78	92	0	0	-	88	0	-	85	-	-	-	0	78	-	-
Staphylococcus haemolyticus	7	45	-	50	-	-	-	-	-	0	100	100	0	0	-	0	65	-	0	-	-	92	0	78	-	-
Staphylococcus sciuri	9	95	-	0	-	-	-	-	-	98	0	85	88	0	-	75	88	-	0	-	-	-	0	0	-	-
Staphylococcus simulans	8	92	-	80	-	-	-	-	-	0	75	95	0	0	-	98	0	-	92	-	-	-	85	75	-	-
Staphylococcus xylosus	8	68	-	25	-	-	-	-	-	92	78	92	0	0	-	58	60	-	80	-	-	-	90	0	-	-
Streptococcus agalactiae	54	-	0	95	-	-	-	92	-	-	92	0	0	0	88	85	-	0	0	92	0	-	-	-	-	-

NR: Nitrate reduction test, SH: Starch hydrolysis test, VP: Voges–Proskauer test, CT: Citrate test, H2S: Hydrogen Sulfide, MR: Methyl Red test, CAMP- CAMP Test, IN: Indole test, NOV-R: Novobiocin resistance test, HE: Hemolysis test, CA: Catalase test, OX: Oxidase test, CO: Coagulase test, ALD: Alkaline dehydrogenase test, PA: Alkaline phosphatase test, GE: Gelatinase test, ES: Esculinase test, LE: Lecithinase test, BG: Beta-galactosidase test, ARD: Arginine dehydrogenase test, LYD: Lysine decarboxylase test, ORD: Ornithine decarboxylase test, UR: Urease test, HI: Hippuricase test

physiological saline could prevent the cell from osmotic shock.[17] The MRS broth with varying concentration of NaCl from 2% to 8% was prepared and sterilized. They were inoculated with the single isolated colony and incubated overnight at 37°C. The growth was observed using Ultraviolet spectrophotometer at 660 nm for all concentration.

Effect of pH Over Growth

The MRS broth medium with different pH (4, 5, 6, 7) using NaOH (1.0 M) or HCl (1.0 M) was prepared and sterilized. The isolated single colony of bacteria was inoculated in all the tubes and incubated at 37°C for 24 h to observe the ability of the growth of *Lactobacillus* under different pH values.[18] After overnight incubation, the growth of bacteria was measured at 560 nm using a spectrophotometer.

RESULTS AND DISCUSSION

Isolation and Identification of Food Borne Pathogens and LAB

Major food spoilage pathogens, bovine mastitis pathogens, and bacteriocin-producing bacteria were identified which were isolated from various dairy, food, and fermented food products. From 389 samples, a total of 688 isolates of bacterial species, namely *Aeromonas, Bacillus, Clostridium, Escherichia, Klebsiella, Listeria, Salmonella, Streptococcus, Staphylococcus, Enterobacter, Enterococcus,* and *Lactobacillus* were isolated [Table 3]. From milk and dairy products, the maximum number of *Listeria, Streptococcus, Staphylococcus, Enterobacter, Enterococcus,* and *Lactobacillus* species was isolated. Meat was found to be a good source for the isolation of *Clostridium, Escherichia,* and *Salmonella* species. Maximum number of *Klebsiella* and *Bacillus* species was isolated from raw vegetables and products [Figure 1]. The suspected colonies were picked up and purified by repeated streaking and restreaking on respective agar media plates until the pure cultures were obtained and were observed for morphological [Table 4] and biochemical [Tables 5 and 6] characteristics [Figure 2]. Similar isolation and identification of food spoilage pathogens, bovine mastitis pathogens, and bacteriocin-producing bacteria from various dairy, food, and fermented food products have been reported.[19] On serial dilution and continuous streaking, various species have been isolated. Milk products showed an average of 28% of organisms isolated from it, becoming the food item with the most organisms in it. From this, *Enterobacter* and *Enterococcus* species became the most isolated one with a percentage of 46 and 40 each and further *Staphylococcus, Listeria, Lactobacillus, Streptococcus,* and *Bacillus* species also showed a percentage of 37, 35, 35, 30, and 30, respectively. *Salmonella* sp. showed the least percentage of 15% from the organism isolated from milk products. The second food item is the meat which showed an average of 20% of organisms isolated from it and among

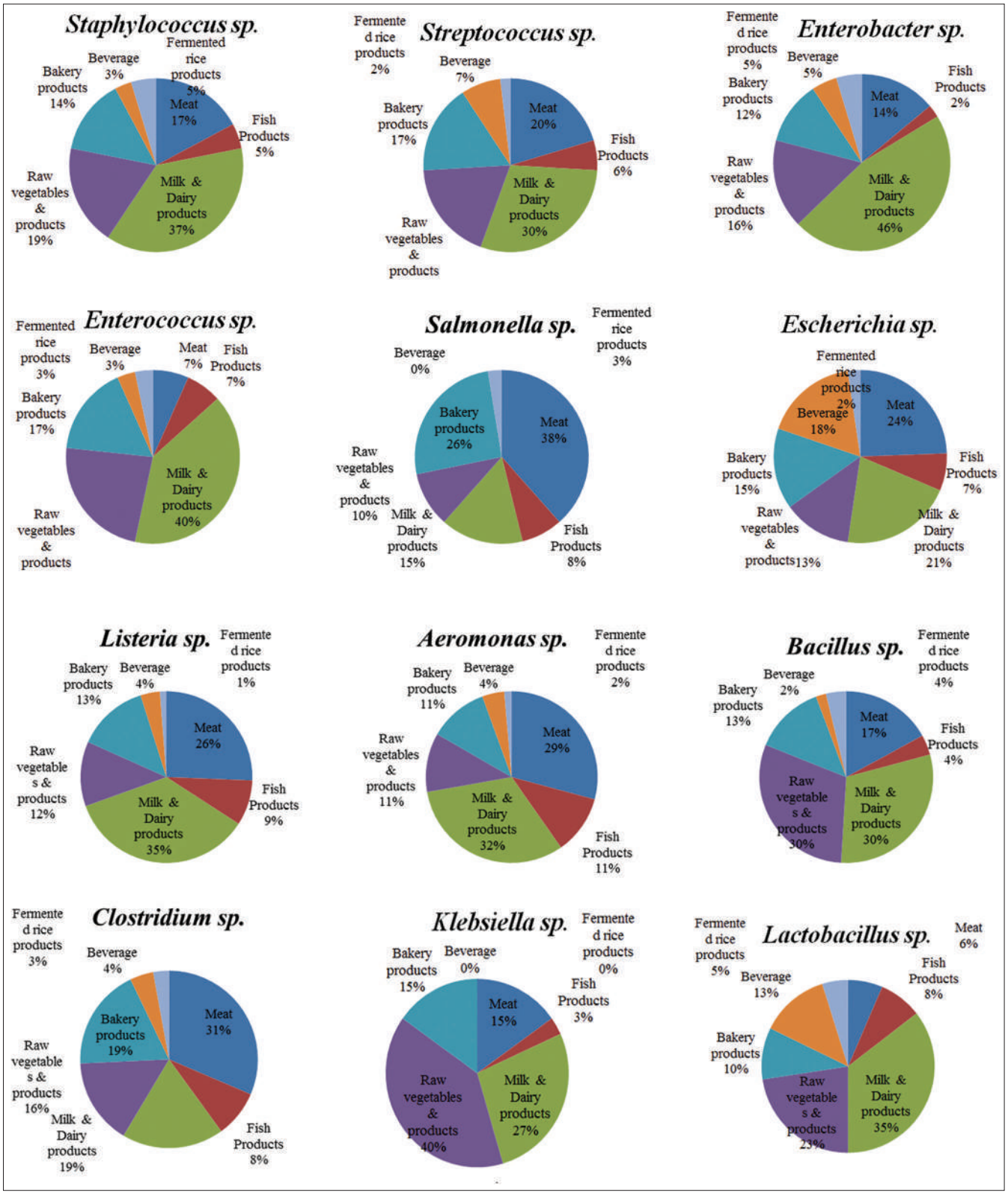

Figure 1: Distribution of source for different bacteria isolated

its *Salmonella* and *Clostridium* species became the most isolated one with a percentage of 38 and 31. *Lactobacillus* and *Enterococcus* species showed the percentage of 6 and 7 from the isolated organisms. Raw vegetables are known to get spoiled easily if not kept in refrigerator so from this we were able to get an average of 19% of organism from it and among it the most isolated one is *Klebsiella* species with 40% of all the isolated organism and the least one isolated is *Aeromonas* and *Listeria* species with 11% and 12%. In case of business, bakery products play a pivotal role so in this the

Table 4: Percentage of isolates positive for sugar fermentation tests

Bacteria	No of isolates	Percentage of isolates positive for sugar fermentation tests															
		Arabinose	Cellobiose	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Raffinose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
Aeromonas sobria	34	0	88	-	-	92	0	88	82	85	0	-	0	0	98	82	0
Bacillus cereus	22	0	45	88	0	98	0	89	0	0	0	78	-	0	60	80	0
Bacillus subtilis	31	0	90	98	75	85	35	-	75	88	92	95	84	90	80	78	82
Clostridium perfringens	70	-	-	92	82	-	100	78	-	85	-	86	-	-	92	-	-
Enterobacter aerogenes	43	90	68	-	-	88	98	98	92	88	86	-	78	-	92	92	85
Enterococcus faecalis	30	0	78	100	88	92	82	-	78	72	-	85	-	-	98	78	0
Escherichia coli	86	88	-	-	-	-	-	-	74	68	-	-	-	-	-	74	-
Klebsiella oxytoca	12	88	82	-	-	92	-	94	85	75	78	-	78	-	-	88	82
Klebsiella pneumonia	21	85	85	-	-	95	-	98	78	85	80	-	75	-	-	80	75
Lactobacillus acidophilus	18	-	88	-	82	92	98	85	-	-	-	-	76	-	92	58	-
Lactobacillus plantarum	22	55	80	-	-	-	-	-	78	-	80	85	-	76	88	75	50
Lactococcus lactis subsp. *lactis*	22	-	-	-	-	100	100	-	-	78	88	-	-	-	82	0	-
Listeria monocytogenes	82	-	-	-	60	85	88	-	-	-	-	0	-	45	90	78	0
Salmonella enterica serovar Paratyphi	10	-	0	-	-	92	-	-	98	85	0	-	-	-	0	-	-
Salmonella enterica serovar Typhi	8	-	0	-	-	92	-	-	85	78	0	-	-	-	0	-	84
Salmonella enterica serovar Typhimurium	12	-	0	-	-	75	-	-	78	92	0	-	-	-	0	-	-
Salmonella enterica serovar Enteritidis	9	-	0	-	-	95	-	-	80	75	0	-	-	-	0	-	88
Staphylococcus aureus	11	0	0	95	98	-	85	88	76	74	-	80	0	-	78	85	0
Staphylococcus chromogenic	9	0	0	68	78	-	82	25	50	92	-	88	0	-	92	75	0

(*Contd...*)

Table 4: (*Continued*)

Bacteria	No of isolates	Percentage of isolates positive for sugar fermentation tests															
		Arabinose	Cellobiose	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Raffinose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
Staphylococcus epidermidis	12	0	0	78	92	-	68	88	0	82	-	45	0	-	92	0	0
Staphylococcus haemolyticus	7	0	0	39	46	-	35	85	56	0	-	50	0	-	92	98	0
Staphylococcus sciuri	9	35	85	92	95	-	55	62	80	60	-	78	82	-	94	84	0
Staphylococcus simulans	8	0	0	89	40	-	92	70	98	48	-	25	0	-	80	65	0
Staphylococcus xylosus	8	95	0	94	55	-	65	92	46	98	-	33	48	-	90	93	84
Streptococcus agalactiae	54	0	-	-	-	94	-	90	0	-	0	70	-	0	-	-	0

Table 5: Production of bacteriocin at by different LAB isolates

Indicator bacterial isolates	*Lactobacillus acidophilus*				*Lactobacillus plantarum*			*Lactobacillus lactis*		
	LA-BP-14	LA-DP-7	LA-DP-11	LA- FP 12	LP 8	LP 19	ADP 67	LL-DP-12	LL-FP-18	LL-FP-19
	Zone of inhibition of growth (mm)									
Aeromonas hydrophila (MTCC 3613)	5	4	4	5	6	5	7	4	4	3
Aeromonas sobria (MTCC 1608)	6	6	5	5	7	7	8	6	5	5
Escherichia coli(MTCC 723)	7	6	6	6	8	7	8	5	6	5
Klebsiella oxytoca (MTCC 2275)	5	3	4	5	4	5	6	4	5	4
Listeria monocytogenes(MTCC 1143)	7	6	6	7	7	8	8	5	5	6
Salmonella enterica serovar Typhimurium (NCIM 2501)	2	0	2	2	2	2	3	3	2	0
Staphylococcus aureus (NCIM 2079)	6	7	7	6	7	7	8	6	5	4
Staphylococcus chromogenes (MTCC 3545)	2	1	1	2	2	2	5	1	2	1
Staphylococcus epidermidis (NCIM 2493)	9	7	8	8	9	10	12	7	8	8
Streptococcus agalactiae (NCIM 2401)	5	4	5	4	6	7	8	6	4	3

MTCC: Microbial Type Culture Collection, LAB: Lactic acid bacteria

Table 6: Occurrence of bacteria in various food samples

Nature of sample	Source and number of samples	No of *Staphylococcus* isolated (%)	No of *Streptococcus* isolated (%)	No of *Enterobacter* isolated (%)	No of *Enterococcus* isolated (%)	No of *Salmonella* isolated (%)	No of *Escherichia* isolated (%)	No of isolate *Listeria* (%)	No of *Aeromonas* isolated (%)	No of *Klebsiella* isolated (%)	No of *Clostridium* isolated (%)	No of *Bacillus* isolated (%)	No of *Lactobacillus* isolated (%)
Meat	Pork Meat (22)	3 (14)	3 (14)	2 (9)	1 (5)	4 (18)	7 (32)	6 (27)	8 (36)	2 (9)	8 (36)	3 (14)	2 (9)
	Beef Meat (17)	3 (18)	3 (18)	2 (12)	0	5 (29)	6 (35)	8 (47)	7 (41)	1 (6)	6 (35)	2 (12)	1 (6)
	Poultry Meat (20)	5 (25)	5 (25)	2 (10)	1 (5)	6 (30)	8 (40)	7 (35)	6 (30)	2 (10)	8 (40)	4 (20)	1 (5)
Fish Products	Fish pickle (19)	3 (16)	3 (16)	1 (5)	2 (11)	3 (16)	6 (32)	7 (37)	8 (42)	1 (5)	6 (32)	2 (11)	5 (26)
Milk and Dairy products	Cow milk (18)	6 (33)	4 (22)	3 (17)	3 (17)	2 (11)	4 (22)	8 (44)	5 (28)	2 (11)	3 (17)	3 (17)	5 (28)
	Buffalo milk (20)	6 (30)	3 (15)	3 (15)	2 (10)	1 (5)	3 (15)	7 (35)	6 (30)	2 (10)	3 (15)	3 (15)	4 (20)
	Curd (23)	4 (17)	3 (13)	3 (13)	2 (9)	1 (4)	3 (13)	3 (13)	3 (13)	1 (4)	3 (13)	3 (13)	5 (22)
	Paneer (16)	3 (19)	3 (19)	5 (31)	2 (13)	1 (6)	4 (25)	5 (31)	4 (25)	2 (13)	2 (13)	3 (19)	4 (25)
	Cheese (18)	5 (28)	3 (17)	6 (33)	3 (17)	1 (6)	4 (22)	6 (33)	5 (28)	2 (11)	2 (11)	4 (22)	4 (22)
Raw vegetables and products	Potato (16)	3 (19)	2 (13)	2 (16)	2 (13)	0	1 (6)	2 (13)	1 (6)	3 (19)	2 (13)	5 (31)	1 (6)
	Radish (15)	2 (13)	3 (20)	0	2 (13)	1 (7)	2 (13)	2 (13)	1 (7)	3 (20)	2 (13)	4 (27)	1 (7)
	Pickle (25)	3 (12)	2 (8)	2 (8)	1 (4)	1 (4)	3 (12)	2 (8)	2 (8)	3 (12)	2 (8)	2 (8)	5 (20)
	Soybean (20)	2 (10)	1 (5)	2 (10)	1 (5)	1 (5)	2 (10)	2 (10)	3 (15)	2 (10)	3 (15)	3 (15)	4 (20)
	Sauerkraut (18)	2 (11)	2 (11)	1 (6)	1 (6)	1 (6)	3 (17)	2 (11)	1 (6)	2 (11)	2 (11)	2 (11)	3 (17)
Bakery products	Sandwich (18)	2 (11)	2 (11)	0	1 (6)	2 (11)	3 (17)	1 (6)	1 (6)	3 (17)	2 (11)	3 (17)	1 (6)
	Pudding (20)	4 (20)	4 (20)	3 (15)	2 (10)	3 (15)	4 (20)	5 (25)	3 (15)	1 (5)	6 (30)	2 (10)	3 (15)
	Pastries (22)	3 (14)	3 (14)	2 (9)	2 (9)	5 (23)	6 (27)	5 (23)	4 (18)ti	1 (5)	5 (23)	2 (9)	2 (9)
Beverage	Beer (20)	1 (5)	2 (10)	1 (5)	1 (5)	0	7 (35)	2 (10)	1 (5)	0	1 (5)	1 (5)	4 (20)
	Wine (22)	1 (5)	2 (9)	1 (5)	0	0	8 (36)	1 (5)	2 (9)	0	2 (9)	0	4 (18)
Fermented rice products	Idly (20)	3 (15)	1 (5)	2 (10)	1 (5)	1(5)	2 (10)	1 (5)	1 (5)	0	2 (10)	2 (10)	3 (15)
Total (%)	389	64 (16)	54 (14)	43 (11)	30 (8)	39 (10)	86 (22)	82 (21)	72 (19)	33 (8)	70 (18)	53 (14)	62 (16)

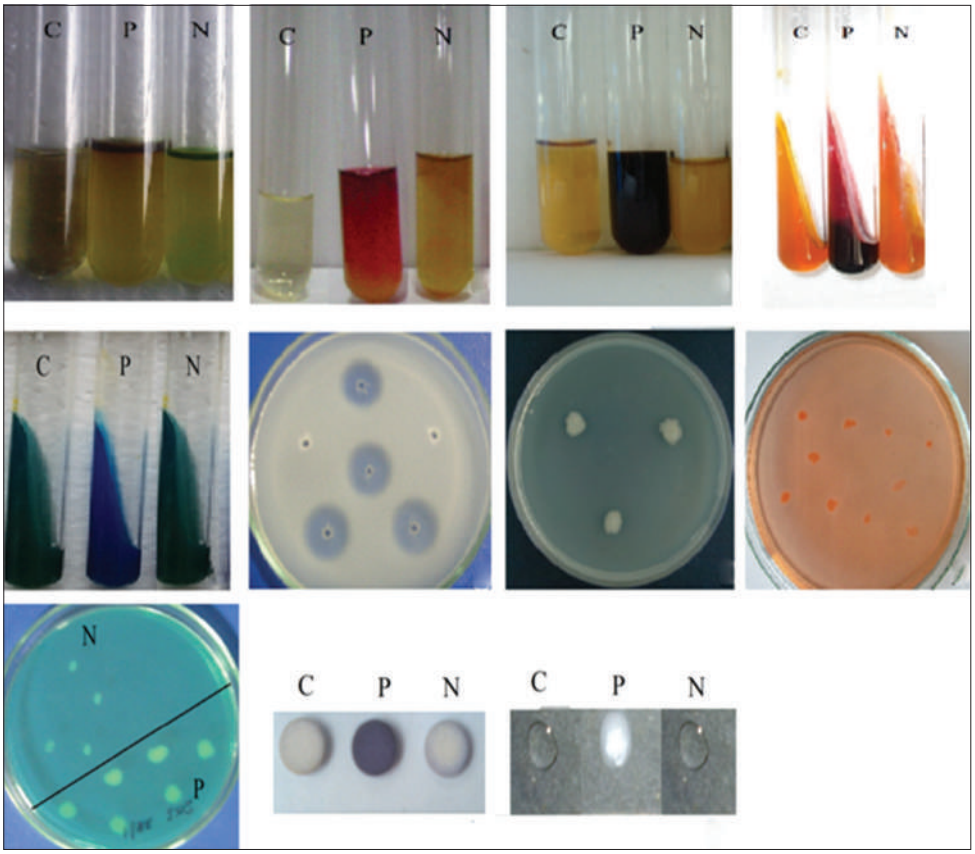

Figure 2: Biochemical and phenotypic characterization. A: Indole Test; B: Voges–Proskauer test; C: Esculin Hydrolysis test; D: TSI test; E: Citrate utilization; F: Gelatinase activity; G: Lipolytic activity; H: Congo red dye uptake; I: DNase activity; J: Oxidase test; K: Catalase test. Symbols - C: Media control; P: Positive control; N: Negative control

average of an isolated organism is 15% and the most isolated one is *Salmonella* species with 26% of all the isolated and others all showed a minimum percentage of their presence in the bakery products. Fish similar to meat has a high NA content but the average isolation of organism was about 6.5 % and the least one isolated is *Bacillus* species with 2% occurrence and others all showed a relative occurrence in the fish. Beverage products too similar to fish showed a lesser percentage of isolate of about 5.25% and in this *Escherichia* species was the most isolated one with a calculated percentage of 18 and others showed a minimum average existence, but both *Klebsiella* and *Salmonella* species were not isolated from it with a 0% occurrence in the beverage product. The least one is the fermented rice products with an average percentage of 2.9 isolates isolated and all the other organisms are showing a minimum level occurrence except *Klebsiella* species which showed a 0% existence in fermented rice as per the isolation done. On collaborating with it, we found the following total concentration of organism and they are *E. coli* (86, 22.1%), *Listeria* sp. (82, 21.1%), *Aeromonas* sp. (72, 18.5%), *Clostridium* sp. (70, 17.9%), *Staphylococcus* sp. (64, 16.5%), *Lactobacillus* sp. (62, 15.9%), *Streptococcus* sp. (54, 13.9%), *Bacillus* sp. (53, 13.6%), *Enterobacter* sp. (43, 11.1%), *Salmonella enterica* (39, 10%), *Klebsiella* sp. (33, 8.5%), and *Enterococcus* sp. (30, 7.7%) from the analyzed sample.

Identification of Species by PCR

In single step PCR out of 40 isolates of *Lactobacillus* sp., recA gene of 318 bp was detected by 22 isolates which is positive for LP and 23 s rRNA gene of 575 bp was detected by 18 isolates which is positive for *L. acidophilus*. The agarose gel electrophoresis images of amplified PCR products are shown in Figure 3a-c. Out of 22 isolates of *Lactococcus* sp. gad B of 602 bp were detected by all 22 isolates and were positive for *L. lactis* sub sp. *lactis*.

Screening of Isolates for Bacteriocin-production

Initial screening with six different indicator organisms of all the *L. acidophilus* (18) isolates, LP (22) isolates, and *L. lactis* (22) isolates for bacteriocin-production showed only 4 (22%), 3 (14%), and 3 (14%) were inhibiting the growth of test organisms. A varied zone of inhibition was observed for different indicator organisms. From the results observed, the hyper bacteriocin-producing LP isolate was selected and grown on NA agar plates. In this study, the formation of wide zones of clearance ranging up to 8–12 mm, was observed on the Petri dishes containing *Staphylococcus epidermis* and *L. monocytogenes* isolates. The inhibition of *S. aureus* by bacteriocin was found higher, as already reported by Jack *et al.,* 2005.

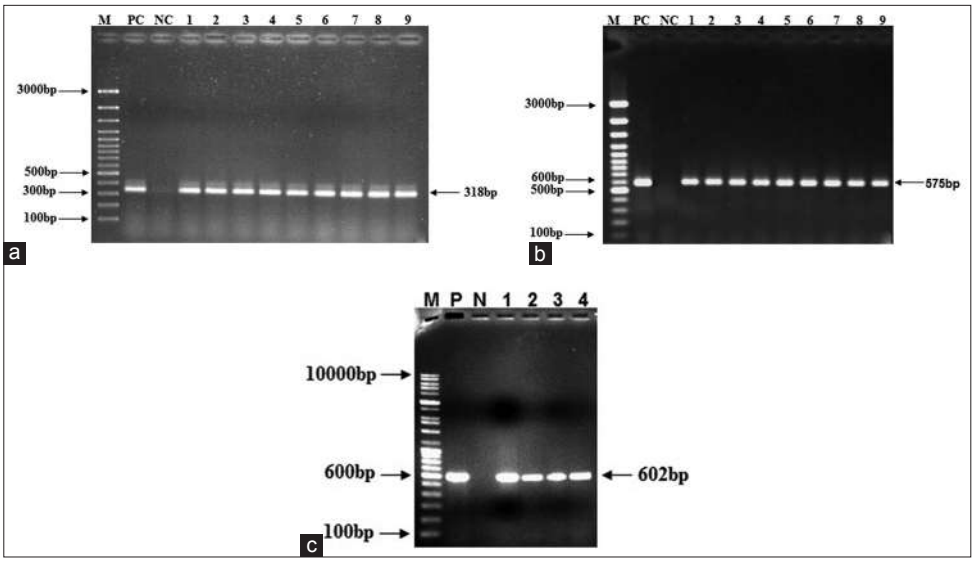

Figure 3: (a) Detection of recA gene (318bp) of *Lactobacillus plantarum* (LP) by polymerase chain reaction. Lane M: 100bp DNA ladder, lane PC: Positive control (LP NCIM 2374), lane NC: Negative control (*Escherichia coli* Microbial Type Culture Collection 723), Lanes 1 to 9: LP field isolates (b) Detection of 23SrRNA gene (574 bp) of *Lactobacillus acidophilus* by a polymerase chain reaction. Lane M: 100bp DNA ladder, lane PC: Positive control (*L. acidophilus* NCIM 2902), lane NC: Negative control (*Escherichia coli* Microbial Type Culture Collection 723), Lanes 1–9: *L. acidophilus* field isolates (c) Detection of gadB gene (602bp) of *Lactobacillus lactis* by a polymerase chain reaction. Lane M: 100bp DNA ladder, lane PC: Positive control (*L. lactis* NCIM 5635), lane NC: Negative control (*Escherichia coli* Microbial Type Culture Collection 723), Lanes 1 and 2: *L. lactis* field isolates

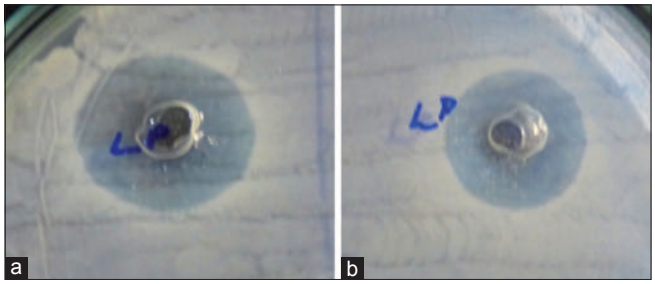

Figure 4: (a and b) Antagonistic activity of bacteriocin from Lactic acid bacteria against *Staphylococcus aureus and Staphylococcus agalactiae*

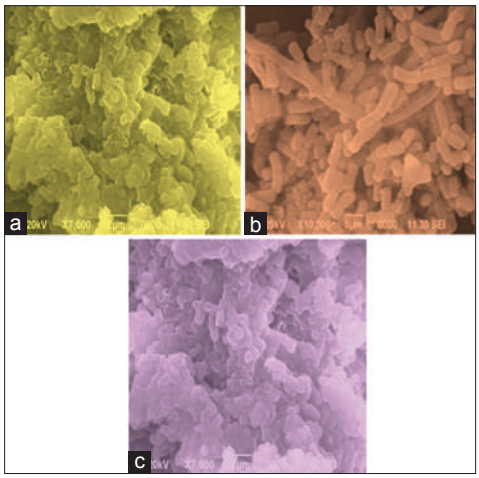

Figure 5: Ultra-structure study of *Lactobacillus* bacteria under a scanning electron microscope. (a) *Lactobacillus plantarum*, (b) *Lactobacillus acidophilus* and (c) *Lactobacillus casei*

The further test for antagonistic activity on another four different indicator organisms which excluding the six organisms chosen for initial screening with the isolates that shown bacteriocin activity revealed that the bacteriocin produced by isolates LP at the 96th h at 30 µl volume was found to be very effective [Figure 4]. Among the three isolates of LP, LP- ADP67 was found to be more effective in producing bacteriocin with high antibacterial property.

Effect of pH and NaCl on Growth

The pH test was done to reveal the environmental condition at which the bacterial species grow. LP showed optimum growth at pH 4.5–6.5 indicated that the bacteria grow well in acidic medium. *L. acidophilus* and *L. casei* have the ability to grow at pH of 5 and 7.5, respectively. The optimum temperature was found to be 37°C for the maximum growth of the above organisms. LP has the ability to grow at NaCl concentration of 6% while *L. acidophilus* and *L. casei* have the ability to grow at pH of 5 and 7.5, respectively. The ultra-structure study of LP, *L. acidophilus,* and *L. casei* in SEM was observed to be in clusters of rods of a variable in length; sometimes occurred either single or in pairs and occasionally in short chains [Figure 5].

CONCLUSION

The present research investigation confirmed the occurrences of *E. coli* (86, 22.1%), *Listeria* sp. (82, 21.1%), *Aeromonas* sp.

(72, 18.5%), *Clostridium* sp. (70, 17.9%), *Staphylococcus* sp. (64, 16.5%), *Lactobacillus* sp. (62, 15.9%), *Streptococcus* sp. (54, 13.9%), *Bacillus* sp. (53, 13.6%), *Enterobacter*. (43, 11.1%), *S. enterica* (39, 10%), *Klebsiella* sp. (33, 8.5%), and *Enterococcus* sp. (30, 7.7%) from various commercial food products. Among the bacteria isolated, *Lactobacillus* sp. can be useful in various food industries as they are known to produce bacteriocin, an antimicrobial peptide useful as biopreservative and control of many disease-causing pathogens. Therefore, these isolates can further be studied for industrial purpose. However, the prevalence of other Gram-negative and Gram-positive bacteria from the commercial food samples is an impending danger for transfer of foodborne infections to human and animals.

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