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Biopreservation of meat by probiotic bacteria isolated from dairy products

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ABSTRACT

Biopreservation of meat is one of the recent trends in food technology. This increases the shelf life and safety of food by the use of naturally occurring organisms and their metabolic products. Our study was aimed at the isolation of probiotic organisms from two sources of milk samples, viz. donkey and cow, also from butter, which could be used for biopreservation of meat. The dairy samples were collected from Vellore district, Tamil Nadu. Isolation of the probiotic bacteria was carried out on MRS Agar media (de Man, Rogosa and Sharpe medium). A total of 7 isolates were selected which included DM1 and DM2 from donkey milk, CM1 from cow milk and BT1, BT2, BT3 and BT4 from butter sample. Meat spoilage organisms were isolated on Nutrient agar medium from meat samples collected from Vellore district, Tamil Nadu. Antagonist activity of the probiotic organisms was assessed by Agar Well diffusion technique. The duration of meat preservation was observed for 2 weeks.

Key words: Meat spoilage, Biopreservation, Probiotics, MRS agar, Agar well diffusion

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INTRODUCTION

The concept of Probiotics evolved around 1900 AD when Ellie Metchnikoff proposed that the long life of Bulgarian peasants is due to their intake of fermented milk and milk products. Probiotics are a group of microorganisms that positively affect the health of the host. The term “probiotics” is a composite word from Latin and Greek that literally means ‘for life’. They play various assisting roles like preservation of milk and meat products by production of lactic acid and other antimicrobial compounds, production of flavour compounds and improving the nutritional quality of food and control of serum cholesterol levels. It was reported that people who had a drink of yoghurt with *Lactobacillus* spp. have low levels of cholesterol. The commonly used antibiotics are considered useless as the organisms become resistant to them and thus Probiotics are regarded as a ray of hope. The use of probiotics in antibiotic resistance is known as microbial interference therapy [1&2]. Probiotic bacteria are generally delivered via food. Therefore they should have the ability to resist the acidic conditions in the stomach and intestine. Thus probiotic strains should be salt tolerant, bile tolerant, acid tolerant and should possess the adherence property [3]. They should be able to grow in the lower intestinal tracts before they can start providing any health benefits. Probiotic bacteria exert their effects by adhering to the gut mucosa thereafter diffusing the antimicrobial compounds. The production of bile salt hydrolase (BSH) is also reported which is known to reduce the serum cholesterol level [4].

Preservation of food efficiently is a major hurdle that has to be still crossed by the modern day food technology. To present consumers with food that is ready to eat, highly nutritious, fresh, minimally processed and preserved and at the same time avoiding contamination with food-borne pathogens and lowering the food processing costs are the major challenges of the food industry [5]. Use of microorganisms and their natural by-products for biopreservation has been commonly practiced throughout the history of mankind [6]. Lactic acid bacteria (LAB) are known to produce many antimicrobial compounds like organic acids, anti-fungal peptides, hydrogen peroxide and bacteriocins [7&8]. They are being used in many dairy milk products. They are non-pathogenic, bile tolerant and salt tolerant. Lactobacillus is the most studied bacteria among LAB as they are used in food fermentation and can be ingested as

probiotics [3]. LAB as bio preservative agents is an alternative to chemical food preservatives as they increase the shelf-life of the food and increases its safety using natural micro flora [5].

Meat spoilage is a major challenge in countries where there are poor storage facilities. Meat is highly proteinaceous but perishable with low shelf-life [8]. It undergoes deterioration in its quality from the time that it is slaughtered till consumption. The micro flora that is prevalent in the meat ecosystem leads to its spoilage. The bacteria that spoil meat include *Salmonella* spp., *Shigella* spp., *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus proteus, Bacillus cereus* and faecal streptococci. These organisms colonize the meat, break it down and releases toxins that causes food poisoning when ingested [8].

Among the major food spoiling microorganisms, *Staphylococcus aureus* and many other Gram-positive bacteria are frequent contaminants. Contamination by *S. aureus* can be from the raw material used, from the processing unit or due to human handling. *S. aureus* food poisoning mostly affects cooked foods and fermented foods [9].

For better probiotic products, we need to grow and isolate new strains of lactic acid bacteria that have the probiotics traits and that have favourable health effects on humans and animals. Natural unexploited sources can be searched to get these isolates. In the present study, we have used donkey milk, cow milk and butter as the probiotic microbial sources.

MATERIALS AND METHODS

Chemicals

All the chemicals and media used in the study were from Merck & Co. and Himedia Chemicals, Mumbai, India, respectively.

Collection of Samples

The milk samples were collected from different sources including cow and donkey milk and the butter sample from a dairy farm of Vellore District,12.9202° N, 79.1333° E Tamil Nadu. The samples were collected in sterile bottles which were then transferred to the Molecular and Microbiology Research Laboratory in VIT University, Vellore. Prior to processing, the samples were stored at 4°C in refrigerator for further use.

Isolation of Probiotic Microorganisms

Simple spread plate technique was employed for the isolation of probiotic bacteria. The samples were serially diluted up to 10^{-7} and dilutions 10^{-3} to 10^{-5} was used for inoculation. 1µl of the dilutions was poured onto MRS agar plates and the plates were incubated at 37°C for 48 hours [10]. Morphologically distinct colonies were separated and preserved as slant cultures at 4°C.

Morphological identification

The morphology of the colonies on the MRS agar plates were observed for the shape, nature, and colour. Characterization of the organism was performed by Gram staining technique followed by motility test, endospore test and catalase and oxidase tests [11].

Assessment of Probiotic Properties

Acid Tolerance Test

All the isolates from different samples were inoculated in MRS broth and kept for incubation at 37°C for 48 hours. They were centrifuged in 7000 rpm for 5mins and the supernatant was discarded. The pellet was then diluted in 1X Phosphate Buffered Saline (PBS) of pH 7.2. Centrifugation was repeated and the supernatant was drained out, the pellet was diluted in 1X PBS of pH 1 and 3. Streak plate method was followed to check the acid tolerating capability of the isolates after 30 mins and 24 hrs of incubation [10].

Adherence Test

Adherence test was performed as a preliminary screening method on all the isolates from the different samples by inoculating them in beakers containing 1X PBS and cover-slips in them. The cover-slips were then stained with crystal violet and checked for adherence under microscope after 5mins, 10mins and 1hr of incubation [12].

Bile Tolerance Test

The isolates were inoculated in MRS broth containing different concentrations of bile salt like 0.1%, 0.3%, 0.5% and 1% and kept for incubation at 37°C for 48 hours. Centrifugation was performed at 7000 rpm for 5 mins at 4°C and the supernatant was drained out. The pellet was then streaked on MRS agar plates and kept for incubation at 37°C for 24 hours [13].

Hemolytic Test
For testing the hemolytic activity, freshly prepared isolates were streaked on Blood agar plates 5% (w/v). The plates were incubated at 37°C for 48 hours. They were examined for any sign of hemolysis [14].

Salt Tolerance Test
The isolates were inoculated in MRS broth containing different concentrations of salt like 6.5%, 10%, and 15% and kept for incubation at 37°C for 48 hours. Centrifugation was performed at 7000 rpm for 5 mins at 4°C and the supernatant was drained out. The pellet is then streaked on MRS agar plates and kept for incubation at 37°C for 24 hours [15].

Antibiotic Sensitivity Test
The isolates were streaked on MRS agar plates and the antibiotic discs of 6 different kinds of antibiotics were arranged on the plates. The plates were incubated at 37°C for 48 hours, and then observed for zone of inhibition around the discs.

Screening of antimicrobial activity of the probiotic microorganism
The antimicrobial activity of the isolates was determined by using Agar Well Diffusion technique. The isolates were inoculated in MRS broth and incubated at 37°C for 48 hours. Human clinical isolates like *Salmonella typhi, Listeria monocytogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus* and the meat pathogen *Staphylococcus aureus* were inoculated in nutrient broth and were kept for incubation at 37°C for 24 hours. These test pathogens were then swabbed onto MHA plates using sterile cotton swabs. The agar well diffusion method was performed by cutting out wells on the MHA plates using a sterile gel borer. Then 100µl of each of the supernatant obtained after centrifugation of the isolates in MRS broth was poured into each well. The plates were incubated at 37°C for 48 hours, and then observed for zone of inhibition [16].

Preservation of Meat
To check the biopreservation potency of the isolates, each isolate was inoculated in MRS broth and kept for incubation at 37°C for 48 hours. They were subjected to centrifugation at 7000 rpm for 5 mins at 4°C. 50 µl of the supernatant obtained was added to evenly dissected small pieces of meat and kept in air tight conditions. Observation was noted down every day. The meat samples were found to be preserved for two weeks.

RESULTS AND DISCUSSION

The isolation was successful with plating on MRS agar media. Seven different colonies showing different morphological characteristics were obtained. The isolated strains were designated as DM1, DM2, CM1, BT1, BT2, BT3 and BT4. Gram staining of all the strains revealed gram positive rods. Oxidase and catalase tests were also found to be positive for all the strains. The morphological characteristics are given in the table1.

TABLE1. COLONY MORPHOLOGY

COLONY	MARGIN	ELEVATION	COLOUR
DM1	Irregular	Flat	Cream
DM2	Irregular	Flat	Cream
BT1	Irregular	Flat	Yellow
BT2	Irregular	Flat	White
BT3	Irregular	Flat	Yellow
BT4	Irregular	Flat	White
CM1	Irregular	Flat	Yellow

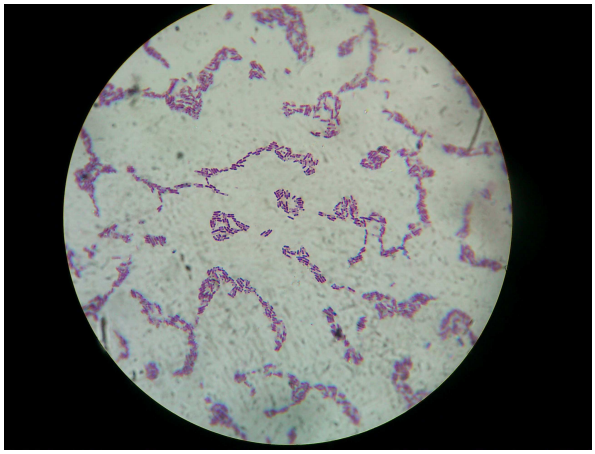

Fig1. Gram staining

The tests of probiotic characterization gave positive results. The results are tabulated below in Table2.

TABLE2. PROBIOTIC CHARACTERIZATION

Tests	DM1	DM2	BT1	BT2	BT3	BT4	CM1
Acid tolerance							
pH 1							
pH 3	-	-	-	-	-	-	-
pH 7	+	-	-	+	+	+	+
	+	+	+	+	+	+	+
Adherence							
5 min	+	+	+	+	+	+	+
10 min	+	+	+	+	+	+	+
30 min	+	+	+	+	+	+	+
Bile tolerance							
0.1%							
0.3%	+	+	+	+	+	+	+
0.5%	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+
	+	+	+	+	+	+	+
Salt tolerance							
6.5%							
10%							
15%	+	+	-	+	+	+	+
	+	+	-	+	+	-	+
Hemolytic test	+	+	-	+	+	+	+
	α		α	α	γ	α	γ

'+' = Positive, '- '= Negative, % = Percentage, α – Alpha, γ –Gamma

The antibiotic sensitivity test results are as in Table 3.

TABLE 3 – ANTIBIOTIC SENSITIVITY TEST

Antibiotics	DM1	DM2	BT1	BT2	BT3	BT4	CM
Gentamicin	S	S	S	S	S	S	R
Ampicillin	S	S	S	S	S	S	R
Cholramphenicol	S	S	S	S	S	S	I
Vancomycin	S	S	S	S	S	S	S
Rifampicin	S	S	S	S	S	S	S
Nitofurantoin	S	S	S	S	S	S	S

S = Sensitive, R = Resistant, I = Intermediate.

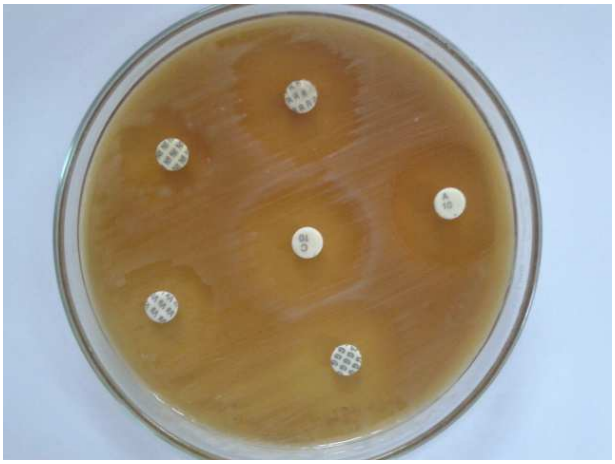

Fig2. Antibiotic sensitivity test

The meat spoilage organism when plated on nutrient agar showed yellow coloured colonies. Gram staining resulted in identification of purple coloured cocci in clusters. The colony when cultured on MSA (Mannitol Salt Agar) produced golden yellow colonies. It showed catalase positive result. The organism was found to be *Staphylococcus aureus* from the above tests.

On performing agar well diffusion against human and meat pathogens, the highest inhibition was seen against the meat pathogen. Three of the human pathogens, *Salmonella typhi*, *Pseudomonas aeruginosa*, and S*taphylococcus aureus* were also inhibited. The results are shown in table 4 and graphically represented using figure 3.

TABLE 4: ANTAGONISTIC ACTIVITY OF THE ISOLATES

Pathogens	DM1	DM2	BT1	BT2	BT3	BT4	CM
S.aureus (meat)	12mm	10mm	15mm	-	18mm	-	-
S.aureus(human)	12mm	14mm	-	-	12mm	12mm	-
Salmonella typhi	9mm	8mm	12mm	12mm	12mm	12mm	-
P. aeruginosa	-	-	17mm	-	-	-	-
L.monocytogenes	-	-	12mm	-	-	-	-

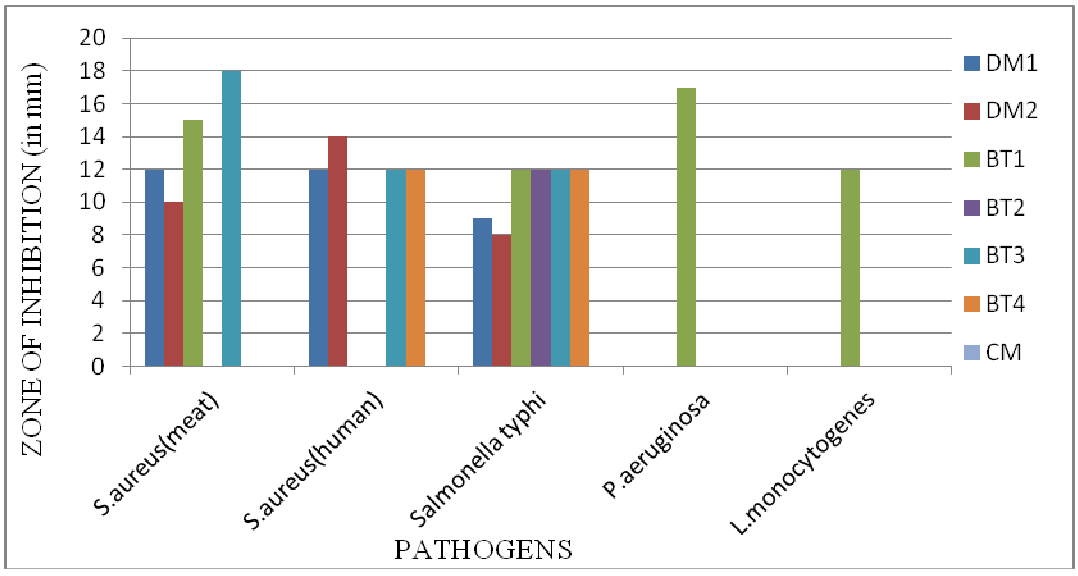

Figure 3: Potency of the isolated strains

From all the above tests the potent strain from the seven was found to be BT3 showing the best inhibition against three of the pathogens. BT1 showed the second maximum inhibition.

The meat samples kept for preservation showed no spoilage which implies that the strains are potent enough for bringing about preservation.

Milk was reported as a potential source of Lactic acid bacteria in a previous study on fermented milk [17]. As cow milk was known to cause severe allergic reactions in some children, donkey milk is now being considered as an alternative [18]. In previous studies 23 strains of LAB were obtained from 8 samples of raw cow milk [19]. In another study, around 107 isolates were obtained from 32 samples of cow milk [20]. In our experiment, only one isolation media (MRS) was used and the sampling of milk was done once from a single location. These might be the reasons for the less number of isolates that we obtained. From our study it was found that the probiotic strains isolated from milk and butter has efficiency to inhibit the common food pathogens. Out of *Staphylococcus aureus*, *Salmonella* species, *Listeria monocytogenes*, *Pseudomonas aeruginosa* species which are considered to be common meat spoilage organisms and human pathogens (URL), the growth of *Staphylococcus aureus* from meat source was found to be inhibited the most. In the test 28% (two out of seven) of the isolated strains were showing activity against the *Staphylococcus aureus* from the meat source whereas 57% (four out of seven) showed inhibition against *Salmonella typhi*. The probiotic strains must meet the characteristics like acid, salt and bile tolerance along with adherence properties [21]. The strains under our study were proven to satisfy these conditions by growth under all the stress conditions mentioned above. This is paving way towards the utilization of the strains potency. Previous studies have suggested that use of naturally occurring metabolic products by probiotic bacteria inhibit growth of spoilage microbes [22].

CONCLUSION

In the new era, people are aware of living a healthy life. Diet is playing a major role in determining the health of individuals. Hence there is an increasing trend for food preserved by probiotics. Biopreservation adds to the extension of shelf life and improvement of food quality Using microbes or their metabolites. Our study is showing the efficiency of the probiotic strain against the meat spoilage organism's growth. This gives a result that can be looked upon in future for biopreservation.

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