RESEARCH

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Discovery

Bacillus species isolated from cow milk samples

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ABSTRACT

In cow milk samples to isolate probiotic microorganisms, *B.subtilis(KF477278), B.thuringienesis(KF477279)* were isolated from healthy cow's milk samples *and* Psychotropic *Bacillus cereus (KF525235), B.amyloliqufaciens (KF477284),* was isolated from cow milk exhibiting 99% similarity at the 16S RNA level. Milk and fermented milk products were collected from ten different dairies of Kodaikanal and surrounding areas, Tamilnadu in India. To study the psychotropic bacteria present in them. Due to high nutritive value, milk is an excellent culture medium for a variety of microorganisms and being a perishable type of food, gets easily spoiled by the activity of microorganisms present in it. Milk and milk products are generally preserved at refrigeration temperature (4-7^{0}C). Psychotropic bacteria are able to multiply under this temperature. During the study of these psychotropic bacteria present in milk products were collected from ten different dairies of Kodaikanal surrounding areas, Tamilnadu in India, the isolates was found to be *Bacillus cereus(KF525235), B.amyloliqufaciens(KF477284),* which was identified on the basis of morphological, biochemical, physiological features as well as 16S rRNA sequencing. Probiotics colonize the intestines and exert an antibacterial effect on pathogens. Therefore, probiotics could be used as a preventive agent against lethal infections. To isolate probiotic microorganisms, *B.subtilis(KF477278), B.thuringienesis(KF477279)* were isolated from healthy cow's milk and were subjected to Gram-stain, morphology and biochemical analyses, and 16S rRNA analysis. Two of the strains identified as *Bacillus (B.) thuringinesis* and *Bacillus subtilis.*

Keywords: rRNA-ribosomal RNA, *B.subtilis-Bacillus substilis*

1. INTRODUCTION

Probiotics include *Bacillus, Enterococcus, Streptococcus, Lactobacillus* species (sp), and yeast and are commonly found in the fermentation process of dairy products, crude oil, and in the intestines of animals and humans. For example, Lactobacillus sp maintains an acidic pH in the intestines, which inhibits the growth of intestinal pathogens such as *E. coli*, Clostridium sp, or bacteria causing diarrhoea, and supports a healthy intestinal micro flora. It has been suggested that probiotics may be useful as therapeutics. Probiotics might be used to minimize the need to use antibiotics in feed. Antibiotics are commonly added into feed in the livestock industry to prevent diseases caused by pathogenic microorganisms, thus increasing the economic productivity of livestock. However, because of the emergence of antibiotic-resistant bacteria and antibiotic residuals that have recently emerged as a serious problem, the use of antibiotics tends to be regulated. Therefore, the use of antibiotics is only encouraged for the treatment of diseases, with the use of probiotics being suggested for prevention or convalescence of diseases.

2. MATERIALS AND METHODS

Collection of samples

Specimens were collected from healthy 40 Cow and milk products were collected from ten different dairies of Kodaikanal and surrounding areas, Tamilnadu in India, The samples were immediately transported to the laboratory in an ice box at 4°C for microbiological analysis within 2-3 hours. 20 ml of the cow milk were expressed and collected in sterile sampling bottles (114 ml) and immediately frozen prior to microbiological and biochemical analyses. Microbiological analysis of cow milk samples were inoculated on triptone Soya agar (Oxoid, England), a general purpose agar that can support the growth of both aerobes and anaerobes when supplemented with 1% (w)/v cysteine hydrochloride, (APHA, 1998) and Mac Conkey agar, for the isolation of coli-aerogenes-like enteric organisms (Itah and Ben, 2004), and incubated at 37°C for 24 hours. Primary isolates were repeatedly sub cultured on fresh media using streak plating techniques to obtain pure cultures.

Gram Staining

The gram reaction of the isolates was determined by light microscopy after gram staining. LABS are known to be gram positive. It means that they give blue-purple color by gram staining. Cultures were grown in appropriate mediums at 37 °C for 24 h under anaerobic conditions. Cells from fresh cultures were used for gram staining. After incubation cultures were transferred aseptically into 1.5 ml eppendorf tubes and centrifuged for 5 min at 6000 rpm. Then, supernatant was removed and cells were resuspended in sterile water. Gram staining procedure was applied. Then, under light microscopy gram Positives and purified isolates were determined.

Inoculation into Broth

In the laboratory the milk sample was inoculated into tryptose soy broth under sterile condition. After inoculation, the test tube was incubated at 37oC for overnight (Cruickshank et al., 1975).

Inoculation into Tryptose Soy Agar

A loopful of culture from the tryptose soy broth was streaked onto tryptose soy agar plates. After inoculation the plates were incubated at 37oC overnight (Cruickshank et al., 1975).

Inoculation into Agar Plates

Well isolated colonies from the TSA plates were streaked onto the following agar plates,

-Mac Conkey Agar

-Eosin Methylene Blue Agar (Cruickshank et al., 1975).

Biochemical Tests

Indole Test

A loop full of culture from 24h growth medium was inoculated in Tryptone medium and incubated at 37° C for 24 h. After incubation 0.5ml of Kovac's reagent was added to it and observed for the ring formation (Cruickshank et al., 1975).

Methyl Red Test

Sterile MR broth was inoculated with the isolate and incubated at 37° C for 24-48 hours after incubation, methyl red solution was added and shaken well (Cruickshank et al., 1975).

Voges Proskauer Test

VP broth was inoculated with cultures and tubes were incubated at 37° C for 24-48 hours. Baritt's reagent was added in all test tubes and the result was observed (Cruickshank et al., 1975).

Citrate Utilization

The test culture was inoculated into Simmons citrate medium and inoculated at 37° C for 24 hours and observed for colour change (Cruickshank et al., 1975).

Eijkman Test

1.Durham's tube was placed inside the test tubes containing Mac conkey broth. Care should be taken that there should be no air bubbles.
2.Inoculate the isolate into the sterilized test tubes.
3.Incubated for 24 – 48 hours at 44° C Formation of air bubbles inside the Durhams tube indicated the positive results (Cruickshank et al., 1975).

Sugar Fermentation Test

The cultures were incubated in the respective sugar medium (glucose, sucrose. Lactose, maltose, mannitol, mannose, raffinos, xylose, sorbitiol, trehalose, fructose) and incubated at 37 C over night. After incubation, Andrads indicator was added to the culture tubes. Red colour was formed as a result of the production of acid and gas confirms the positive reaction (Cruickshank et al., 1975).

The protocol of sequencing service

1. Sequencing Kit: ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits
2. Sequencer: ABI PRISM® 3730XL Analyzer (96 capillary type) (Over 20)
3. PCR machine: MJ Research PTC-225 Peltier Thermal Cycler
4. Sequencing protocol

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using [Universial or what you selected] primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Primer information

S. no	Primer Name	Type	Type2	Sequence (5 to 3)
1	518F	Universal	Forward	CCAgCAgCCgCggTAATACg
2	800R	Universal	Reverse	TACCAgggTATCTAATCC
3	27F	Universal	Forward	AgAgTTTgATCMTGGCTCAg
4	1492R	Universal	Reverse	TACggYTACCTTgTTACgACTT

Note:
Primer 1 & 2 for Sequencing Reference
Primer 3 & 4 for PCR Amplification

Analysis Procedure

1. Preparation of template DNA

It is important to use a pure cultivated bacterium for identification. Colonies are picked up with a sterilized toothpick, and suspended in 0.5 ㎖ of sterilizes saline in a 1.5 ㎖ centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5 ㎖ of InstaGene Matrix (Bio-Rad, USA). Incubated 56℃ for 30 min and then heated 100℃ for 10 min. After heating, supernatant can be use for PCR.

Table 1
Mean total bacterial counts for cow milk

S.No	No of samples treated in cow milk	Total bacterial count(cfu/m×l0¯4)	Identified bacterial species
1.	15	5.0×l0¯4	***Bacillus***
2.	15	4×l0¯5	***Bacillus***
3.	10	5.0×l0¯6 3.0×l0¯6	***Bacillus***
Total	40		

2. PCR

Add 1 ㎕ of template DNA in 20 ㎕ of PCR reaction solution. Use 27F/1492R primers for bacteria, and then perform 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. DNA fragments are amplified about 1,400 bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

3. Purification of PCR products

Remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

4. Sequencing

The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers as described (Primer Name File). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

3. RESULTS AND DISCUSSION

In cow milk samples to isolate probiotic microorganisms, *B.subtilis(KF477278), B.thuringienesis(KF477279)* were isolated from healthy cow's milk *and* Psychotropic *Bacillus cereus (KF525235)* ,*B.amyloliqufaciens (KF477284),* was isolated from cow milk exhibiting 99% similarity at the 16S RNA level. Milk and fermented milk products were collected from ten different dairies of Kodaikanal surrounding areas, Tamilnadu in India to study the psychotropic bacteria present in them. Due to high nutritive value, milk is an excellent culture medium for a variety of microorganisms and being a perishable type of food, gets easily spoiled by the activity of microorganisms present in it. Milk and milk products are generally preserved at refrigeration temperature (4-7^{0}C). Psychotropic bacteria are able to multiply under this temperature. During the study of these psychotropic bacteria present in milk products were collected from ten different dairies of Kodaikanal surrounding areas, Tamilnadu in India (Table 1). Probiotics colonize the intestines and exert an antibacterial effect on pathogens. Therefore, probiotics could be used as a preventive agent against lethal infections. To isolate probiotic microorganisms, *B.subtilis(KF477278), B.thuringienesis(KF477279)* were isolated from healthy cow's milk and were subjected to Gram-stain, morphology and biochemical analyses, and 16S rRNA analysis (Plate 1). Two of the strains identified as *Bacillus (B.) thuringinesis* and *Bacillus subtilis*. *Bacillus subtilis*, as with many in the Bacillus genus, is an extremely common bacterium. It is found in soil, water, air, and decomposing plant matter. Bacteria in the Bacillus genus are spore-forming, which means that they create a thick wall which surrounds their DNA and other internal cell structures. In this way, they are very hardy and impervious to extreme temperatures, chemicals, environmental factors, even some types of radiation. This makes them excellent for use in industrial processes. Bacillus subtilis is widely used for laboratory studies, but more for genetic research as opposed to health research. The bacteria are highly responsive to genetic mutation, giving it a many experimental uses in a laboratory setting (Table 2).

Though Bacillus subtilis presents some risk to humans, the instances of this are incredibly rare. Part of the problem with its sometimes shady reputation can actually be attributed to other members of its genus. The *Bacillus* genus encompasses a large number of species. At one time all aerobic, spore-forming bacilli were named part of the subtilis species. Many of the species are closely related, making it very difficult to tell them apart. However, the disease-causing Bacillus species are now easily distinguishable from the helpful strains such as *Bacillus subtilis*. The subtilis species is not to be confused with *Bacillus cereus* (KF525235), which is a common cause of food poisoning, and *Bacillus anthracis,* which is pathogenic to humans and other animals

(Figure 1). *Bacillus subtilis* is beneficial is many ways, including industrial applications. It is used to produce a variety of enzymes, including amylase, which is helpful in the de-sizing of textiles and starch modification for the sizing of paper. *Bacillus subtilis* also produces the enzyme protease, including subtilisin, which is used in detergents and the leather industry. Perhaps more notably, *Bacillus subtilis* is used to produce many antibiotics, such as difficidin, oxydifficidin, bacilli, bacillomyin B, and Bacitracin, which is helpful in treating bacterial skin infections and preventing infection in minor cuts and burns. Bacillus subtilis is also used as a fungicide. The bacteria colonize the root system, leaving no room for fungal disease organisms. It is used on agricultural seeds of vegetables, soybeans, cotton, and peanuts and on flower and ornamental seeds. It is also being used to produce insect toxins, including one to kill malarial mosquito larvae.

Table 2

The Biochemical Test Used For Identification of the Isolated Bacillus Species

Test	**Species and result of test**			
	1	2	3	4
Gram Stains	+	+	+	+
Motility	+	+	+	+
Spore Position	VX	VX	VTX	VX
Swelling of the cell	_	+	+	+
Carbohydrates acid from Ass:				
Glucose	+	+	+	+
Cellobiose	+	+	+	+
Galactose	_	+	+	_
Mannose	_	+	+	_
Raffinose	_	_	+	_
Salicin	+	_	+	+
xylose	_	_	+	_
Citrate	+	_	_	+
Urease	+	+	_	+
Indole	_	_	_	_
V.P	+	_	+	+
Nitrate	+	_	_	+
Casein hydrolysis	+	_	+	+
Oxidase	_	+	_	_

V: Sub: terminal, X: Spore Oval, T: Spore Terminal,

1. *Bacillus cereus(KF525235)*
2. *B.Subtilis(KF477278)*
3. *B.amyloliqufaciens(KF477284)*
4. *B.thuringienesis(KF477279)*

Table 3

Antibiotic resistances of *Bacillus (B.) thuringinesis* and other Bacillus species

Antibiotic resistance growth inhibition diameter (mm)											
B.Species	N	S	K	TE	P	E	ENR	VA	GM	OX	AM
B. thuringinesis	21	21	24	24	28	18	30	28	22	22	19
B.subtilis	25	20	28	22	13	12	ND	25	32	11	16

Table 4

Genera of bacteria isolated from the Cow milk sample in 16srna sequencing

S.NO	IDENTIFIED ISOLATES	MAX IDENT	GEN BANK ACCESSION NO
1.	*Bacillus cereus(KF525235)*	**99%**	**KF525235**
2.	*B.Subtilis(KF477278)*	**99%**	**KF477278**
3.	*B.amyloliqufaciens(KF477284)*	**99%**	**KF477284**
4.	*B.thuringienesis(KF477279)*	**99%**	**KF477279**

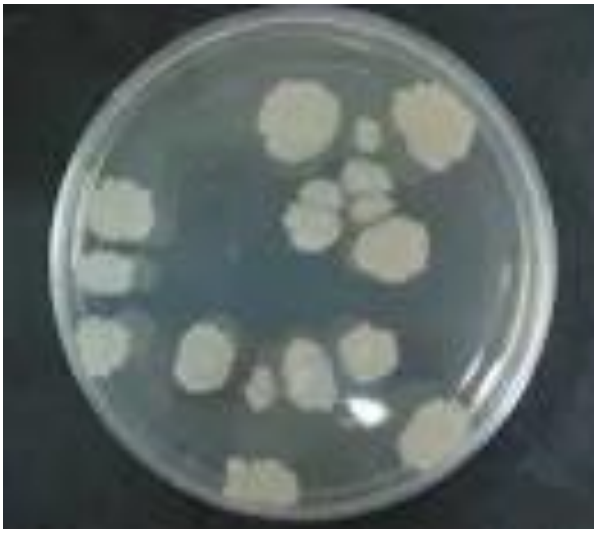

Plate 1
Bacillus subtilis isolated from cow milk samples

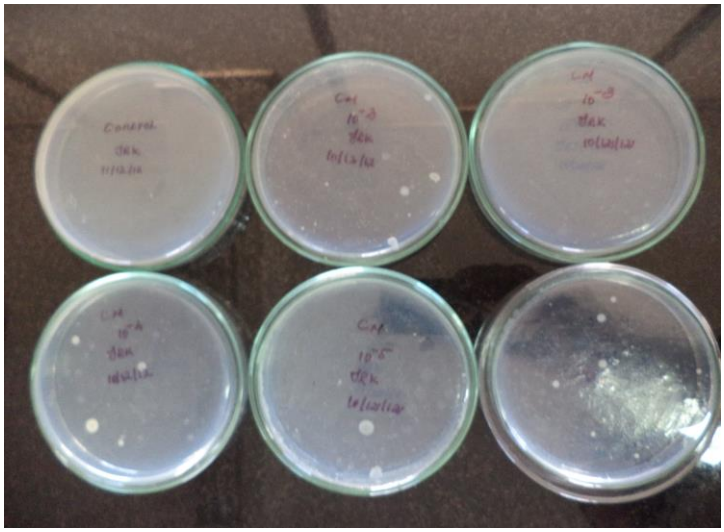

Plate 2
Cow milk in serial dilution

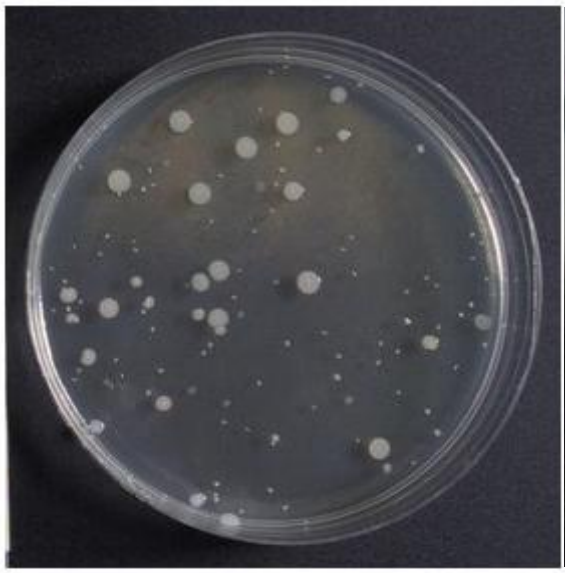

Plate 3
Colony morphology of *Bacillus thuringesis*

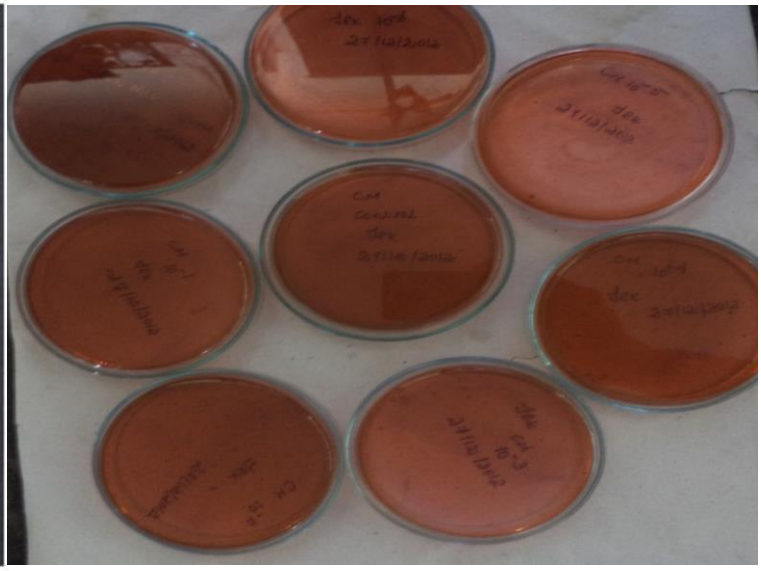

Plate 4
Cow milk in EMB agar in serial dilutions

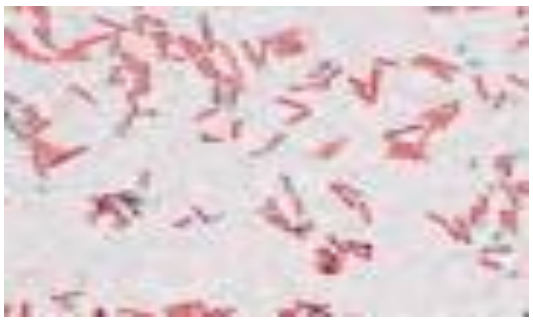

Figure 1
Bacillus subtilis in gram staining from the cow milk samples

Plate 5
Bacillus cereus (KF525235) isolated from cow milk samples

Table 5

Morphological, Biochemical, Physiological and Molecular Characteristics of *B amyloliquefaciens* in cow milk

Characteristics		***B. amyloliquefiaciens* in cow milk samples**
Morphological	Appearance	Wrinkle, dull, dry
	Pigmentation	White
	Size	2 mm
	Form	Irregular
	Margin	Lobate
	Elevation	Flat
Gram's reaction		Gram positive
Endospore formation		+
Growth at pH 6.0		+
Catalase		+
Oxidase		+
Nitrate reduced to nitrite		+
Arginine dihydrolase		_
Voges Proskauer		+
Citrate utilization		+
Methyl red		_
Starch hydrolysis		+
Casein hydrolysis		+
Acid production from	Glucose	+
	Lactose	+
	Galactose	_
	D-Arabinose	_
	Starch	+
	Casein	+
	Inulin	_
	L-xylose	_
	Rhamnose	_
	Sorbitol	+
	Glycogen	+
	Trehlose	_
	L-arabinose	+
	N-acetylglocosamine	_
Identification of bacterium on partial 16S rDNA sequencing (Identity %: 99%)		*Bacillus amyloliquefaciens in cow milk* (Accession No *(KF477284).*)

+: positive; -: negative

According to a Toxic Substances Control Act report from the Environmental Protection Agency, *Bacillus subtilis* "is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low. A 2009 report published in the Journal of Hepatology referenced a report by Swiss researchers and showed a possible different aspect of *Bacillus subtilis*. Liver injury occurred to two patients after taking a Herbalife product "contaminated" with *Bacillus subtilis.* They concluded that because liver damage resulted after use of the product, *Bacillus subtilis* possesses "potential hepatoxicity.Though the incidence of distress related to *Bacillus subtilis* is quite low, perhaps the best advice for its use comes from Gary Huffnagle, a Ph.D. and author of The Probiotics Revolution. Because of certain probiotic species' similarity to disease-causing strains, Huffnagle recommends consulting a healthcare professional before using supplements containing strains of *E. coli,* Enterococcus faecium, and Bacillus subtilis. Probiotics are live microorganisms that exhibit beneficial effects on a host when ingested in sufficient amounts. Generally, probiotics improve symptoms caused by intestinal bacterial fermentation. They influence micro flora congestion on the intestinal wall and produce

various products that aid in the absorption and utilization of ingested nutrients. They also generate substances that neutralize toxins produced by bacteria and inhibit pathogen growth and proliferation. *B. thuringiensis* showed the best probiotic characteristics, as demonstrated by acid- and bile salt resistances and a protective effect. These features imply for the first time that *B. turingiensis* could be a feasible probiotic of livestock through the elicitation of nonspecific immunity for the prevention of animal diseases.

Antimicrobial resistance testing Antimicrobial resistance was determined by disc diffusion method Antibiotic discs were placed onto a lawn of freshly plated bacteria on the Muller-Hinton agar and antimicrobial resistance was determined by measuring the diameter of the inhibition zone after incubation of the plate at 37°C for 16 h (Plates 2-4).

The antibiotics in each disc included ampicillin (10 µg), erythromycin (15 µg), enrofloxacin (30 µg), gentamycin (10 µg), kanamycin (30 µg), lincomycin (2 µg), neomycin (30 µg), oxacillin (1 µg), penicillin G (10 U), streptomycin (10 µg), tetracycline (30 µg), or vancomycin (30 µg) (Table 3). AM: ampicillin (10 µg), E: erythromycin (15 µg), ENR: enrofloxacin (30 µg), GM: gentamycin (10 µg), K: kanamycin (30 µg), L: lincomycin (2 µg), N: neomycin (30 µg), OX: oxacillin (1 µg), P: penicillin G (10 U), S: streptomycin (10 µg), TE: tetracycline (30 µg), VA: vancomycin (30 µg). *ND: Not determined. *Bacillus cereus (KF525235)* is an endemic, soil-dwelling, Gram-positive, rod-shaped, *Staphylococcus aureus* is a type of bacteria (Table 4). It stains Gram positive and is non-moving small round shaped or non-motile cocci. It is found in grape-like (staphylo-) clusters. This is why it is called Staphylococcus (Plate 5). Most strains of Gram positive spore forming bacteria can cause bovine mastitis like *Bacillus* spp (Reva et al., 2004; Hong et al., 2005). Some species of genus *Bacillus* are cause mastitis like *B. alvei*, *B. subtilis*, *B. megaterium* and *B. cereus* Elgadasi, (2003). In based on the literature Bacillus spp. should be considered as one of the causes of mastitis in dairy farms.

Probiotics colonize the intestines and exert an antibacterial effect on pathogens. Therefore, probiotics could be used as a preventive agent against lethal infections. To isolate probiotic microorganisms, *B.subtilis(KF477278), B.thuringienesis(KF477279)* were isolated from healthy cow's milk and were subjected to Gram-stain, morphology and biochemical analyses, and 16S rRNA analysis. Two of the strains identified as *Bacillus (B.) thuringinesis* and *Bacillus subtilis*. *B.amyloliquefaciens* is Gram-positive, catalase positive, aerobic, rod-shaped and motile. This particular organism is found in soil samples in nature. As with other members of the family Bacillaceae, it forms a strong endospore for use when conditions are not favorable and can be dispersed in this form into dust which then also gets into water supplies for plants and animals. *Bacillus amyloliquefaciens* is further classified as a low G+C organism.*B. Amyloliquefaciens* is known for its ability to degrade proteins extracellularly, which was found to be useful. It was isolated for an enzyme that it excretes to digest the proteins that it encounters. The enzyme, subtilisin, has been put to use in the newer technologies of items such as laundry detergents and contact lens cleansers (Table 5).

4. CONCLUSION

In cow milk samples isolated the bacteria in 16srna sequencing in *Bacillus cereus (KF525235), B.subtilis (KF477278), B.amyloliqufaciens (KF477284), and B.thuringienesis (KF477279).* Psychotropic *Bacillus cereus (KF525235)* was isolated from cow milk exhibiting 99% similarity at the 16S RNA level. Milk and fermented milk products were collected from ten different dairies of Kodaikanal surrounding areas, Tamilnadu in India to study the psychotropic bacteria present in them. Due to high nutritive value, milk is an excellent culture medium for a variety of microorganisms and being a perishable type of food, gets easily spoiled by the activity of microorganisms present in it. Milk and milk products are generally preserved at refrigeration temperature (4-7^{0}C). Psychotropic bacteria are able to multiply under this temperature. During the study of these psychotropic bacteria present in milk and fermented milk products, the isolates was found to be *Bacillus cereus(KF525235), B.amyloliqufaciens(KF477284),* which was identified on the basis of morphological, biochemical, physiological features as well as 16S rRNA sequencing. Most strains of Gram positive spore forming bacteria can cause bovine mastitis like *Bacillus* spp (Reva et al., 2004; Hong et al., 2005). Some species of genus *Bacillus* are cause mastitis like *B. alvei*, *B. subtilis*, *B. megaterium* and *B. cereus* Elgadasi, (2003). In based on the literature Bacillus spp. should be considered as one of the causes of mastitis in dairy farms. Probiotics colonize the intestines and exert an antibacterial effect on pathogens. Therefore, probiotics could be used as a preventive agent against lethal infections. To isolate probiotic microorganisms, *B.subtilis(KF477278), B.thuringienesis(KF477279)* were isolated from healthy cow's milk and were subjected to Gram-stain, morphology and biochemical analyses, and 16S rRNA analysis. Two of the strains identified as *Bacillus (B.) thuringinesis* and *Bacillus subtilis*.

SUMMARY OF RESEARCH

To isolate probiotic microorganisms, *B.subtilis, B.thuringienesis* were isolated from healthy cow's milk and were subjected to Gram-stain, morphology and biochemical analyses, and 16S rRNA analysis. Two of the strains identified as *Bacillus (B.) thuringinesis* and *Bacillus subtilis*.

DISCLOSURE STATEMENT

There is no financial support for this research work from the funding agency.

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