1.2 MATERIALS AND METHODS

1.2.1 Isolation of Lactic acid bacteria (LAB)

Raw unpasteurized milk samples of Cow were collected from the local Dairy (Vinay Dairy) of Dharwad, Karnataka, India. During the lactation period under aseptic conditions in sterile tarsons tubes and brought to the lab, processed within one hour and utilized for further studies. The milk sample was serially diluted in distilled water and plated on Man Rogosa Sharpe Agar and incubated at 37 °C for 48-72 h. The colonies with typical characteristics namely pure white, small of 2-3 mm diameter with entire margins were hand picked from each petri plate. The cultures were identified according to their morphological, physiological, biochemical and molecular characteristics up to the genetic level (Harrigan et al., 1970; Sneath et al., 1986; Holzapfel et al., 1991).

1.2.2 Physiological and biochemical characterization

Bacteriocin producing strains were Gram stained and examined microscopically for cellular morphology and Gram-stain phenotype. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide. Growth was assayed in MRS broth at 4, 25, 37 and 45 °C as well as at pH of 4, 5, 7 and 9 incubated at 37 °C. Salt tolerance was tested with 5, 15 and 25 % (w/v) NaCl in MRS broth. The production of acid and CO₂ from glucose was tested in MRS broth containing Durhams tube, with citrate omitted (Schillinger et al., 1987). An assay for gelatin hydrolysis was performed in accordance with (Harrigan et al., 1970). Ability to ferment various carbohydrates was evaluated using MRS broth (Schillinger et al., 1987).
1.2.2.1 Sample preparation for Atomic force microscopy (AFM)

For imaging of dried samples a 5 ml droplet of bacteria suspension was applied to a freshly Cleaved mica surface and left to dry. For AFM in situ, mica was pre-treated with polylysine. A 10 ml drop of $10^{-2}$ M polylysine solution was applied to a freshly cleaved mica surface and left to dry. A 5-10 ml drop of bacteria suspension in distillated water was applied onto treated mica. After drying distilled water (pH 5.5) or bacteria culture medium was injected into the syringe. AFM experiments in air modes of operation were carried out using Nanosurf Easy scan 2.

1.2.3 Isolation of genomic Deoxyribonucleic acid (DNA)

Extraction of DNA from culture broth (~10-20 mg). The first step is to add Lysis solution containing SDS and Proteinase K. The lysate is then incubated at high temperature to digest proteins and release nucleic acid, followed by removal of proteins and cellular debris by precipitation and centrifugation. The nucleic acid is then recovered from the clarified lysate by isopropanol precipitation. Yields are dependent on the type and amount of sample, but are typically between 10-100 µg per preparation. The purity of the nucleic acid as determined by UV absorbance ratio (Abs 260/Abs 280) is typically between1.8-2.0.

1.2.4 Identification of bacteria by sequencing of the 16S rRNA

The genomic DNA was extracted from the isolate. Amplification of the 16s rRNA was performed using the universal primers. Sequence analysis was carried out using NCBI online tools.

16S BACTERIAL PRIMER SEQUENCE

63F-5’ CAG GCC TAA CAC ATG CAA GTC 3’
1387R-5’ GGG CGG AGT GTA CAA GCA GGC 3’
Maintenance of microorganisms

All the lactic acid bacterial cultures were maintained at 4°C in MRS broth. Pathogenic microorganisms were maintained at 4°C in Brain Heart Infusion broth. All the bacterial cultures were sub-cultured at 10 days interval.

1.2.5 Production of bacteriocin like inhibitory substances (BLIS)

Isolated bacterial cultures were inoculated in 100 ml of MRS broth and incubated at 37°C for 24 h. The broth was subjected to centrifugation at 12,000 rpm for 20 mins, the resulting cell residue was discarded giving rise to a cell free supernatant (CFS). The pH of supernatant was adjusted to 5.0 with 1N NaOH and then subjected for rotary flash evaporator; resulting solution thus obtained has been designated as BLIS. For inhibitory activity BLIS was filter sterilized by 0.22 μm membrane filter paper (Millipore, India) to carry out the anti-microbial activity by well diffusion assay (VijVijai Pal et al., 2005).

1.2.6 Antimicrobial susceptibility testing

Agar Well Diffusion Assay (AWDA)

100 μl of the 24 h old test culture was inoculated onto the nutrient agar plates by the spread plate method. 3 wells of diameter 6 mm were made in each of the plates. These wells were filled with 15 μl, 30 μl and 45 μl of BLIS and the plates were incubated at 37° for 24 hrs (Schillinger et al., 1987). The inhibition zone was measured in millimeter using zone interpretation scale (HiMedia, Mumbai).

1.2.7 Antibiotic susceptibility test of Lactococcus garvieae.

Colonies of Lactococcus garvieae were inoculated in MRS broth at 37 °C for 24 h, with micropipette an inoculum was added on to the media and the bacterial
suspension was spread evenly on the surface of the MRS agar plate. The inoculated plate was allowed to dry before placing the diffusion discs containing antibiotics. The antibiotics used for the test are Ceftriaxone (30 mcg), Penicillin (10 mcg), methicillin (5 mcg), Cephalothin (30 mcg), cloxacillin (30 mcg), Tetracycline (30 mcg), Ampicillin (10 mcg), Amoxyclav (30 mcg), Erythromycin (15 mcg), Rifampicin (30 mcg), Ciprofloxacin (5 mcg), Cefpodoxime (10 mcg), Carbenillicin (100 mcg), Gentamicin (10 mcg), which are procured from Hi-Media Laboratories Pvt, Ltd Mumbai. The zone of inhibition is measured by a zone interpretation scale from Hi-Media.