2.2 MATERIALS AND METHODS

2.2.1 Microorganisms

The indicator organisms namely *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis Escherichia coli* and *Pseudomonas aeruginosa* were taken for the inhibitory activity which were isolated from Bovine mastitis infected udder milk and maintained in the Department of Biotechnology and Microbiology, Karnataka University, Dharwad.

2.2.2 Inhibitory spectrum of Bacteriocin produced by *Lactococcus garvieae*.

The indicator organisms *E. coli*, *B. subtilis*, *B. cereus*, *S. aureus*, *P. aeruginosa* were cultured in BHI medium. The inhibitory activity of bacteriocin is expressed in Arbitrary Unit (AU/ml). (Sri Usmiati et al., 2009).

Bacteriocin activity (mm²/ml) = \[ \frac{L_z - L_s}{V} \]

\[ L_z \] = Clear zone area (mm²)

\[ L_s \] = Well area (mm²).

Isolated *L. garvieae* was inoculated in 100 ml of BHI broth and incubated at 37°C for 24 h. The broth was subjected for 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 this has been designated as BLIS. For inhibitory activity BLIS was filter sterilized by 0.22 μm membrane filterpaper (Millipore, India) to carry out the anti-microbial activity by well diffusion assay (VijVijai Pal et al., 2005).
2.2.3 Effect of salts and detergents on the antimicrobial activity of bacteriocin.

To test the sensitivity to various salts and detergents the bacteriocin sample was treated with 1% w/v, v/v of SDS, Tween 20, Tween 80, Urea, EDTA and NaCl and control (no treatment).

2.2.4 Effect of enzymes on antimicrobial activity of bacteriocin.

To test effect of enzymes the bacteriocin sample was treated with Amylase, proteinase K, lysozyme treatments and no treatment (control). The enzymes were filter-sterilized by passing through 0.45 μm membrane filterpaper (Millipore, India), and treated the selected bacteriocins by adding to a final concentration 1 mg/ml. The mixtures of enzyme and bacteriocins were incubated at 37°C for 2 h. Then, the residue bacteriocin activity was determined.

2.2.5 Effect of incubation period on antimicrobial activity of bacteriocin

To determine the effect of incubation period on the activity of bacteriocin the 5 ml culture extracts of L. garvieae was taken at period of 10, 20, 30, 40 and 50 h of incubation and at temperature of 37 °C and then the antimicrobial activity was determined.

2.2.6 Effect of temperature on antimicrobial activity of bacteriocin

To test of temperature sensitivity bacteriocin sample was treated under different conditions as following; heating at 30, 40, 50, 60, 80 °C, for 15 min for each temperature then, assayed for bacteriocin activity.

2.2.7 Effect of pH on antimicrobial activity of bacteriocin

To determine the effect of pH on bacteriocin activity, the bacteriocin was adjusted, using 6 N Hcl or 6 N NaoH, to pH between pH, 2.0, 4.0, 6.0, 8.0, 10.0, and
12.0. The samples were incubated at 4°C for 2 h. The antimicrobial activity was determined.

2.2.8 Determination of minimum inhibitory concentration of bacteriocin from *Lactococcus garvieae*.

Nine dilutions of bacteriocin have to be done with BHI for MIC. In the initial tube 20 microliter of drug was added into the 380 microliter of BHI broth. For dilutions 200 microliter of BHI broth was added into the next 9 tubes separately. Then from the initial tube 200 microliter was transferred to the first tube containing 200 microliter of BHI broth. This was considered as $10^{-1}$ dilution. From $10^{-1}$ diluted tube 200 microliter was transferred to second tube to make $10^{-2}$ dilution. The serial dilution was repeated up to $10^{-9}$ dilution for each drug. From the maintained stock cultures of required organisms, 5 microliter was taken and added into 2ml of BHI (brain heart infusion) broth. In each serially diluted tube 200 µl of above culture suspension was added. The tubes were incubated for 24 h and observed for turbidity (Schwalve, Moore and Goodwin, 2007).

2.2.9 Evaluation of effect of bacteriocin on various indicator organisms by Fourier transform infrared spectroscopy (FTIR).

The bacteriocin treated and untreated cells of indicator bacteria such as *Staphylococcus aureus, Salmonella typhi, Bacillus subtilis, Bacillus cereus and Escherichia coli* were subjected to FTIR analysis. For this, the cells were pelleted and treated with 4600 AU ml$^{-1}$ of bacteriocin preparation of *Lactococcus garvieae*. The treated and untreated cells of the test organism were washed thrice with distilled water. The washed cells were dried to remove moisture. The dried cells were mixed with finely grounded potassium bromide (KBr) and IR spectra were recorder in the
Drift mode in absorbance between 4000 and 400 cm$^{-1}$ using a Thermo Nicolet 6700 spectrometer. (Kemp et al., 1991).

### 2.2.10 Evaluation of effect of bacteriocin on various indicator organisms by raman spectroscopy.

The FT-Raman spectra were obtained by Thermo Nicolet 6700 spectrometer NXR FT-RAMAN module. The test conditions were as follows, the laser line of 1064 nm with the power of 500 mW was used as excitation energy; samples were scanned from 200 to 3500 cm$^{-1}$, with temperature maintained at 25 °C. The data analysis software (Origin 8, 2007/10) was used to analyze the spectra. Before examining the interaction between the bacterial cells and bacteriocin, the samples were equilibrated for 24 h (Lu et al., 2011).