Comparative Assessment of Pediocin PA-1 Fermented Whey Formulations for Microbial Reduction in Raw Cow Milk

Rohit Kumar Shukla, Usha Devi*

Division of Animal Biochemistry, National Dairy Research Institute, Karnal – 132001, India

Corresponding Author: sharmausha3112@gmail.com

Abstract

The present study was carried out to assessment of various formulations of pediocin PA-1 containing fermented cheese whey for reducing total microbial load in raw cow milk. Different formulations were prepared after fermenting cheese whey and supplemented cheese whey with Pediocin PA-1 producing *Pediococcus pentosaceous* NCDC273. Liquid cheese whey was prepared after dissolving 8 gm of dried cheese whey powder in 100 ml distilled water. Liquid cheese whey was supplemented with yeast extract (10 g/L), Tween 80 (1 mL/L), magnesium sulphate (0.2 g/L), and manganese sulphate (0.05 g/L) and pH 7.0. Feremented cheese whey were used as such and after boiling, after centrifuging & boiling supernant and after ulfiltration (through 3 kDa cutoff memebrane) of boiled supernatant obtained after centrifugation of fermented cheese whey, without and with supplementation. All the pediocin preparations were found effective in reducing total count for viable bacteria on TGYEA, lactic acid bacteria on MRS, Yeast & Mould on PDA, Coliforms on VRBA and *Staphylococcus aureus* on Baird Parker determined using plating raw cow milk with agar media.

Keywords Pediocin PA-1; *Pediococcus* pentosaceous NCDC273; Cheese whey medium; reducing total microbial load; raw cow milk

1. Introduction

Milk in its natural state is highly perishable because of rapid spoilage by naturally occurring enzymes and contaminating microbes. Good hygiene practice from the farm to dairy, effective refrigeration regimes, reduction of storage times and technologies for reduction of spoilage and pathogenic microorganisms are basic measures needed to be accomplished for preserving good quality of raw milk (Sorhaug and Stepaniak, 1997). It is very difficult to maintain the good hygienic practice from the farm to dairy as many factors are responsible for contamination like air, milking equipment, feed, soil, faeces, grass, feeding and housing strategies of milking animals (Coorevits *et al*., 2008), rinsing water for milking machine and milking equipment washing (Bramley, 1990). Raw milk microbiota is composed of lactic acid bacteria (LAB), spoilage and even pathogenic bacteria. Introduction of cold storage of milk on farms resulted in microbial shift from mesophilic aerobic bacteria towards psychrotrophic microorganisms. Latter is one of the most unwanted milk spoilage bacteria, which

have adverse effect on milk and milk products and the same is attributed to their ability of producing heat resistant proteolytic and lipolytic enzymes at refrigerating temperatures (Cousin, 1982). Moreover facing the economic crisis, where milk market is of no exception, new solutions for costs lowering are searched for Biopreservation may offer a potential for reduction of spoilage and pathogenic microorganisms in raw milk. Biopreservation is the safe and natural means of preservation, involving empirical use of micro-organisms and their natural products to preserve the food and dairy products (Cleveland et. al., 2001). Among the several microorganisms, lactic acid bacteria have the major potential to be used as a biopreservatives as it produces an array of active antimicrobial compounds like acetic acid, organic acids, hydrogen peroxide, bacteriocins, etc. These days, bacteriocins have been extensively explored for their use as biopreservatives. Bacteriocins are the ribosomaly synthesized, extracellularly released, cationic antimicrobial peptides produced by lactic acid bacteria and have wide range of antimicrobial spectrum. Till now, several bacteriocins have been characterized including nisin, pediocin, curvacin, mesenterocin, sakacin, etc. Among these bacteriocins, nisin, produced by *Lactococcus lactis*, has been granted GRAS status for applications as food biopreservative by FDA (Deegan *et al*., 2006). Nisin and pediocin are the two bacteriocins, which are now available commercially under the name, Nisaplin™ (Blackburn *et al*., 1998) and Alta 2341™, respectively. Pediocin is a class IIa bacteriocin produced by *Pediococcus spp*. and is made up of 44 amino acids, has both cationic and hydrophobic region and is non-toxic and heat-stable (Sood and Sinha, 2003). It kills bacteria by forming pores in the cell membrane. Pediocin provides an advantage over nisin as it provides site directed mutagenesis for improving its potency. It was shown to have an extra C-terminal disulfide bond which improves its potency at elevated temperatures and widens its antimicrobial spectrum (Deegan *et al*., 2006).

However, for developing pediocin for food biopreservation, it is necessary to produce it in usable form not only at large scale but also from a food grade and cost-effective medium. High production usually occurs in complete growth medium such as MRS (Vijay Simha *et al*., 2012). Therefore purification upto food grade level becomes the bottleneck due to high cost of production and purification. Whey and whey permeate powders may serve as the basis of food-grade inexpensive fermentation media and require minimum nutritional supplementation for the production of bacteriocins. Whey provides an excellent growth medium for LAB bacteria as it has a high biological oxygen demand. It has been widely used for the production of various compounds including organic acids, single-cell protein, enzymes, ethanol and bacteriocins (Garsa *et al*., 2014a). Consequently, there has been increased interest in using cheese whey as a basis of culture media (Sood and Sinha 2009; Garsa *et al*. 2014b). *Ex situ* produced bacteriocins can also be added in the form of raw concentrates obtained by cultivation of the producer strain in food-grade substrate, whey. Pediocin PA-1was found more potent against food spoilage and pathogenic bacteria viz., *S. aureus* NCDC 110, *B. cereus* NCDC 240 and *S. agalictiae* NCDC 208 as compared to nisin. On the other hand, it was found that pediocin was not effective to inhibit lactic acid bacteria (Garsa *et al*., 2014c), indicating that addition of pediocin to raw milk will not affect subsequent processing of milk by lactic acid bacteria. Therefore, the present study was carried out to evaluate efficacy of cheese whey

fermented with pediocin producing *Pediococcus pentosaceous* NCDC273 (Sood *et al*. 2013), for reducing total viable count in raw cow milk.

2. Material and Methods:

2.1. Raw milk

Raw cow milk was collected under aseptic conditions from the cattle yard of the National Dairy Research Institute (NDRI), Karnal, and Haryana, India.

2.2. Bacterial strains and growth media

Pediococcus pentosaceus NCDC 273 (producer strain) and *Enterococcus faecalis* NCDC 114 (indicator strain) were obtained from the National Collection of Dairy Cultures, NDRI, and Karnal. Growth media used included Nutrient Broth (NB), deMan Rogosa Sharpe (MRS) broth and agar, Tryptone Glucose Yeast Extract Agar (TGYEA), Violet Red Bile Agar (VRBA), Baird Parker Agar (BPA), and Potato Dextrose Agar (PDA) (HiMedia, India). All chemicals used were of analytical grade. Cheese whey and a supplemented cheese whey medium were prepared as described in Section 2.3.

2.3. Preparation of pediocin containing fermented cheese whey formulations

The overnight activated culture of *Pediococcus pentosaceus* NCDC273 was inoculated at 1% into both cheese whey (CW) and supplemented cheese whey (SCW) media, and incubated at 37°C for 24 hours to allow fermentation (Garsa et al., 2014). Liquid cheese whey was prepared by dissolving 8 g of dried cheese whey powder (procured from Venkatesh Natural Private Limited, M.P., and India) in distilled water. For the preparation of supplemented cheese whey medium, the cheese whey solution was enriched with yeast extract (10 g/l), Tween80 (1 ml/l), magnesium sulphate (0.2 g/l), and manganese sulphate (0.05 g/l). The pH was adjusted to 7.0 before sterilization by autoclaving at 121°C for 15 minutes. This supplemented medium served as a nutritionally improved substrate for the growth of *P. pentosaceus* NCDC273 to enhance pediocin production. After fermentation, various formulations were developed from both CW and SCW by applying specific post-fermentation treatments, including: Boiling in a water bath for 10 minutes, Centrifugation at 7,000 rpm for 20 minutes at 4°C, Ultrafiltration of the supernatant using a sterile 3 kDa cut-off membrane (Millipore, Bedford, MA, USA). These treatments were performed to prepare distinct formulations for evaluating the stability and activity of pediocin in different physical states and conditions. The formulations prepared are listed and described in Table 2.1. To evaluate the antimicrobial activity of pediocin in each formulation, a spot-on-lawn assay was conducted.

For this, 5 µl of serial double dilutions of each formulation were spotted on agar plates seeded with the indicator organism. The highest dilution that produced a clear zone of growth inhibition was recorded, and the activity units per milliliter (AU/ml) were calculated using the following formula:

Activity units per ml = 200 x reciprocal of highest dilution that gives a clear zone

Table 2.1. Formulation of various pediocin preparations

Sr. No.	Pediocin Preparation Abbreviation	Meaning
1.	CW	Sterile cheese whey
2.	FCW	Fermented Cheese Whey*
3.	BFCW	Fermented Cheese Whey Boiled in water bath for ten Minute
4.	CBFCW	Supernatant obtained upon Centrifugation of preparation of 3
5.	UCBFCW	Supernatant obtained upon Ultra filtration of preparation 4 through 3 KDa cutoff membrane
6.	FSCW	Fermented Supplemented Cheese Whey**
7.	BFSCW	Fermented Supplemented Cheese Whey Boiled in water bath for ten minute
8.	CBFSCW	Supernatant obtained upon Centrifugation of preparation of 7
9	UCBFSCW	Supernatant obtained upon Ultra filtration of preparation 8 through 3 KDa cutoff membrane

*Culture obtained after fermentation of cheese whey with *Pediococcus pentosaceus* NCDC 273 with 1% inoculation, incubated at 37°C for 22 hours

** Culture obtained after fermentation of supplemented cheese whey with *Pediococcus pentosaceus* NCDC 273 with 1% inoculation, incubated at 37°C for 22 hours

2.4. Spot on Lawn assay

The efficacy of pediocin produced by *Pediococcus pentosaceus* NCDC 273 was evaluated against *Enterococcus faecalis* and various microorganisms isolated from raw cow milk using the spot-on-lawn assay. A base layer of hard agar was first prepared using the appropriate culture medium. It was overlaid with 6–8 mL of soft agar (0.75%) inoculated with 5 µL of an overnight-activated indicator culture. After allowing the overlay to solidify for 15–30 minutes, the agar surface was marked into 1.5 cm × 1.5 cm grids. Then, 5 µL of cell-free supernatant (CFS) from each pediocin-containing formulation was spotted in serial double dilutions onto the lawn of indicator organisms. A negative control spot (containing only sterile broth) was also included. The plates were left at room temperature for 1–2 hours to allow diffusion of the test solutions. Subsequently, they were incubated at 37°C for 16–20 hours. Growth inhibition was assessed by observing clear, growth-free zones around the spots, indicating antimicrobial activity of the formulations.

2.5. Counting of microbes

Standard plate count (SPC) was enumerated by plating one ml of appropriate sample dilution in petridish, pouring melted & cooled (42°C) Tryptone Glucose Yeast Extract Agar (TGYE), Coliform count, *Staphylococcus*

aureus count, MRS count while mixing. The agar media was allowed to solidify and inverted plates were incubated at 37^{o}C for 42 to 72 hours respectively. Similarly, Yeasts and moulds count was enumerated plating one ml of appropriate sample dilution in petridish, pouring melted and cooled (42^{o}C) PDA while mixing. The agar media was allowed to solidify and inverted plates were incubated at 20^{o}C for 3 to 5 days. The total microbial count, *S. aureus* count, *Lactobacillus* count, coliform count and yeasts and moulds count were represented as colony forming units per milli-liter (cfu/ml) of raw cow milk. The cfu/ml values were trans-formed into log cfu/ml through taking log of all colonies forming unit per milliliter grown on various agar medium plates.

2.6 Efficacy of pediocin containing fermented cheese whey formulation

0.5 millimeter of each preparations of pediocin containing fermented cheese whey formulations (Table 2.1) was added to 4.5 ml of raw milk and appropriate microbial load was enumerated using plating method immediately (0 hr) and kept on the shelf at room temperature for six hour after adding pediocin containing fermented cheese whey formulations to raw cow milk.

3. Result & Discussion

3.1. Efficacy of pediocin containing fermented cheese whey formulation

Total microbes present in raw cow milk at 0 and 6 hrs were enumerated by plating Raw milk on different types of media viz. TGYEA, VRBA, BPA, MRS and PDA Total count was represented as cfu/ml in (Figure 3.1) Various pediocin containing formulations were prepared as described in Table 2.1 and these formulations were found to contain pediocin (AU/ml) as shown in Table 3.1. In view of the wide spectrum of the pediocin various formulations were added in raw cow milk @10% to evaluate its efficacy in reducing total microbial load. The efficacy was assessed by following microbiological criteria (Total Viable Count, MRS Count, *Staphylococcus aureus* Count, Coliform Count, Yeast and Mould Count) as shown in Figures 3.2 to 3.6. All the preparations were effective in reducing total microbial load. Fermented cheese whey was found to be more effective after boiling. Centrifuged preparation of the fermented cheese whey resulted in loss of pediocin activity which may be due to binding of pediocin molecules to the producer cells. In addition ultra-filtration through 3 KDa cut off membrane resulted in more effective preparation as compared to boiled supernatant of centrifuged fermented cheese whey. Bali *et al.*, 2013 reported reduction in plate count of aerobic bacteria upon addition of bacteriocin BS-13 in paneer and khoya. Shashikumar and Puranik, 2011 reported the use of optimum level of lactoferrin present in whey for extending the shelf life of paneer. In the present study which used fermented cheese whey preparations therefore the contribution of lactoferrin in reducing total microbial load cannot be ruled out therefore reduction in yeast and mold count (Fig. 3.4) and coliform count (Fig. 3.5) may be due to lactoferrin. Therefore the efiicacy of *Pediococcus pentosaceus* NCDC 273 fermented cheese whey shows the potential for reducing total microbial load in raw cow milk. In addition, Pediocin is less detrimental to lactic acid culture showing that Pediocin containing preparations

can be added to raw cow milk without compromising subsequent processing of raw cow milk with lactic acid bacteria. (Garsa *et al.*, 2014b).

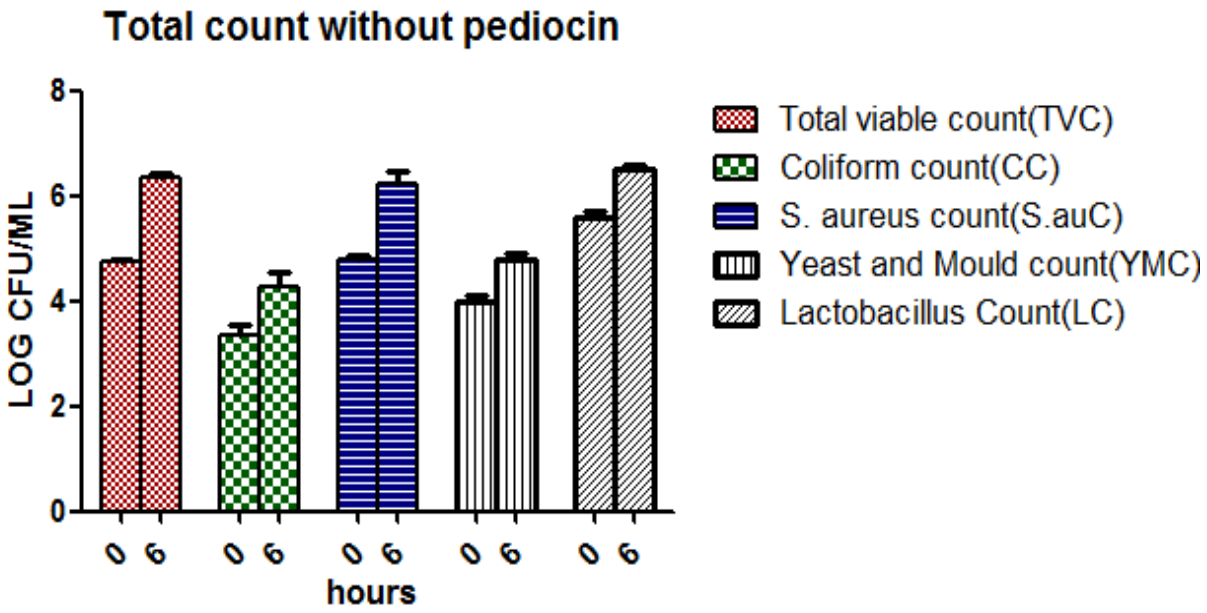

Fig: 3.1. logcfu count of different microbes isolated on different media

Table 3.1: Pediocin (AU/ml) in various formulations shown in Table 2.1.

Pediocin Preparation Abbreviation	**AU/ML**
CW	0
FCW	25600
BFCW	25600
CBFCW	409600
UCBFCW	409600
FSCW	25600
BFSCW	25600
CBFSCW	409600
UCBFSCW	409600

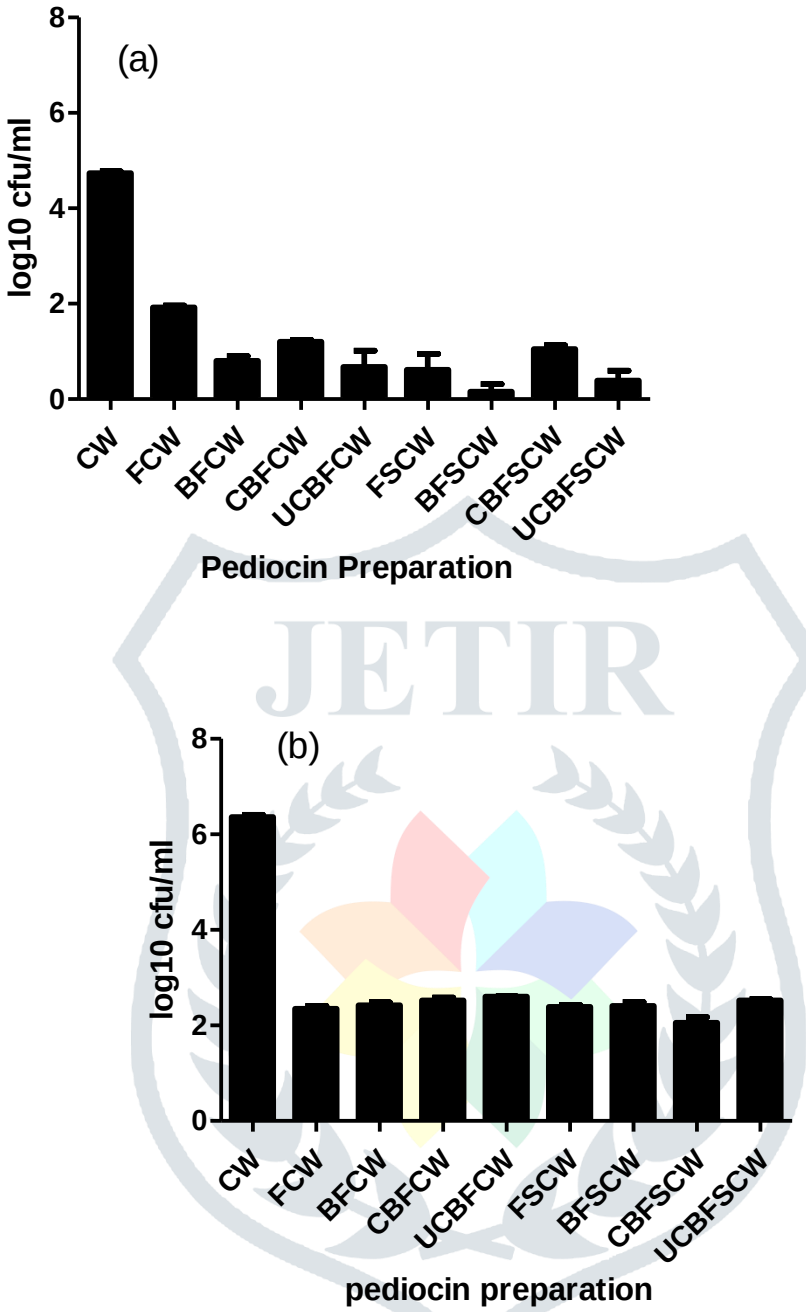

Fig: 3.2 Total viable count (TVC) at (a) 0 hour and (b) 6 hours without and with added pediocin preparations as defined in Table 2.1

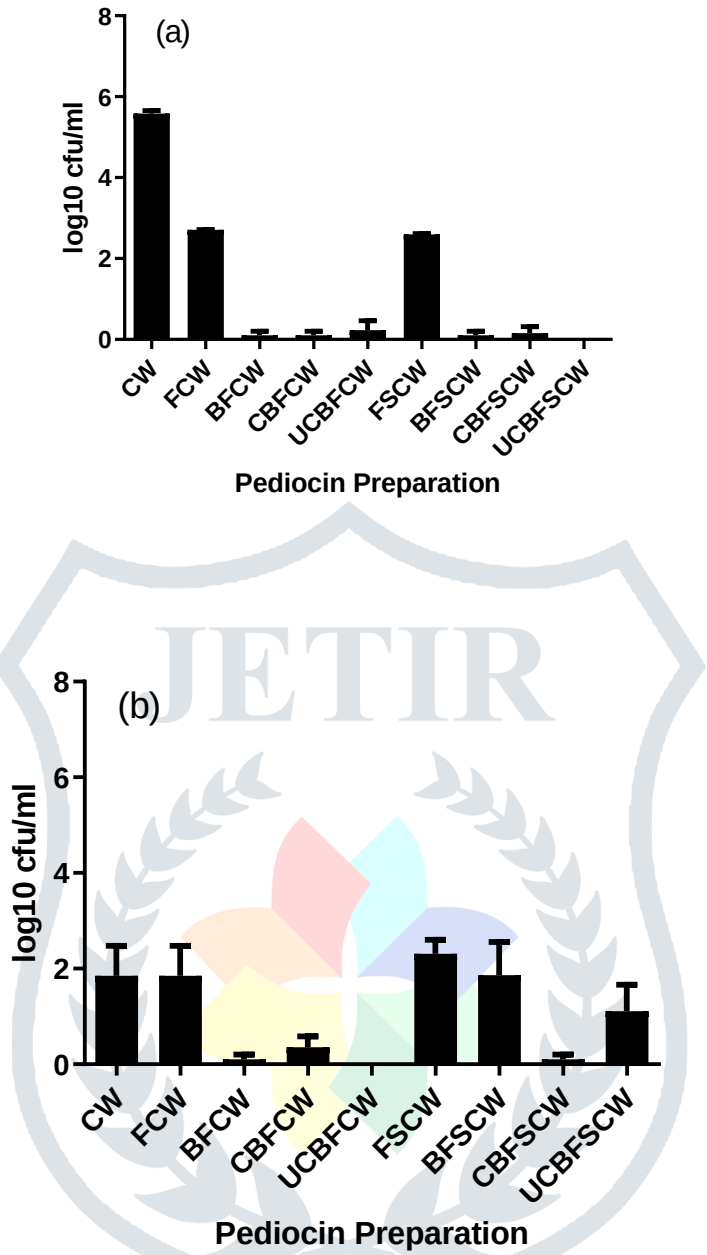

Fig: 3.3 MRS at (a) 0 hour and (b) 6 hours without and with added pediocin preparations as defined in Table 2.1

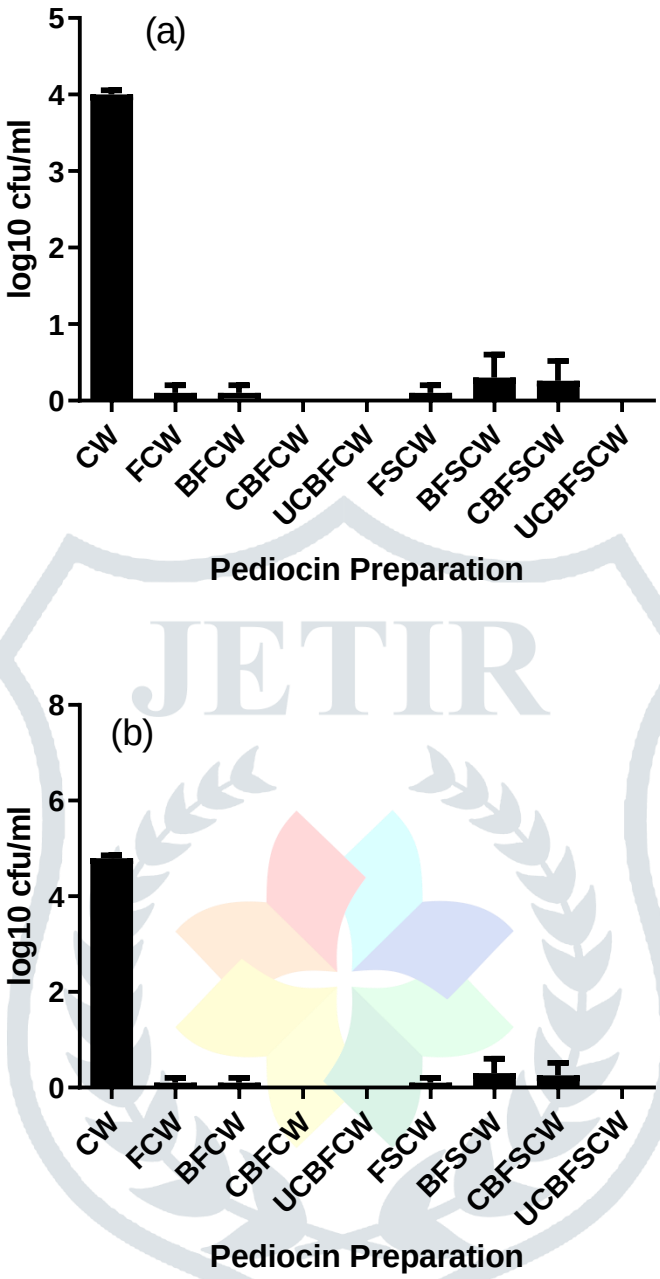

Fig: 3.4 Yeast & Mould count (YMC) at (a) 0 hour and (b) 6 hours without and with added pediocin preparations as defined in Table 2.1

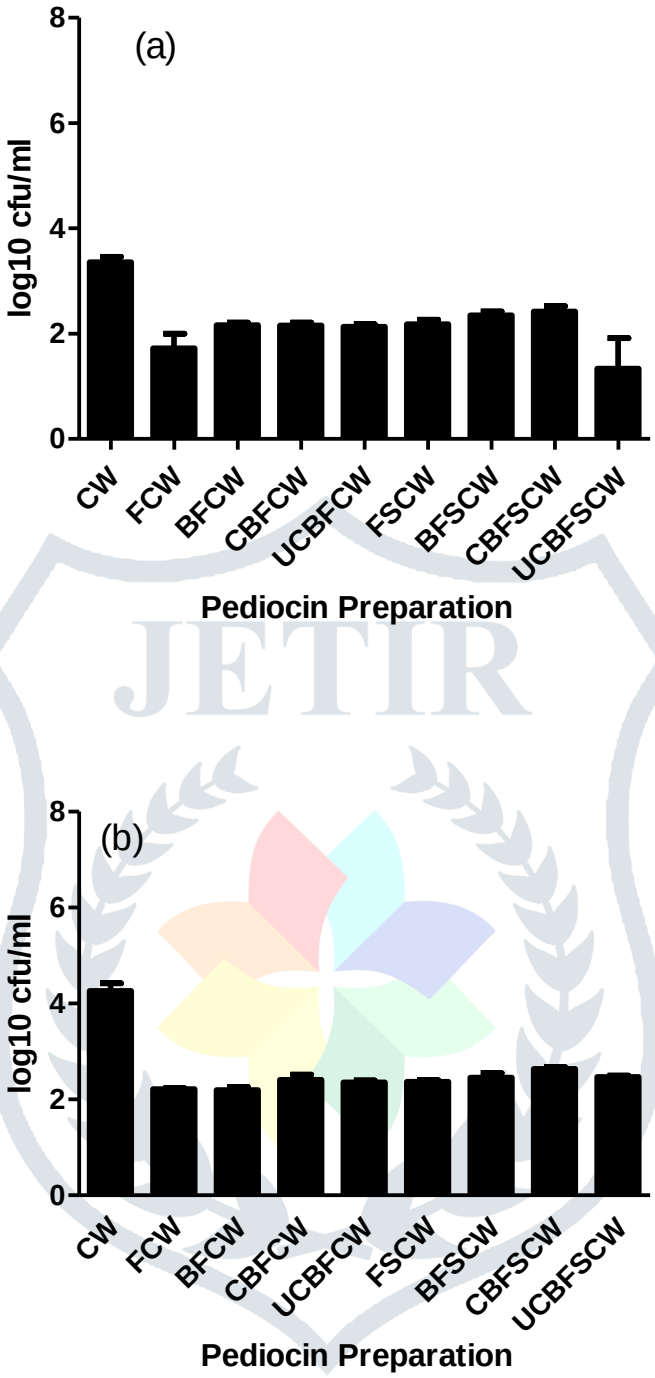

Fig: 3.5 Coliform count (CFC) at (a) 0 hour and (b) 6 hours without and with added pediocin preparations as defined in Table 2.1

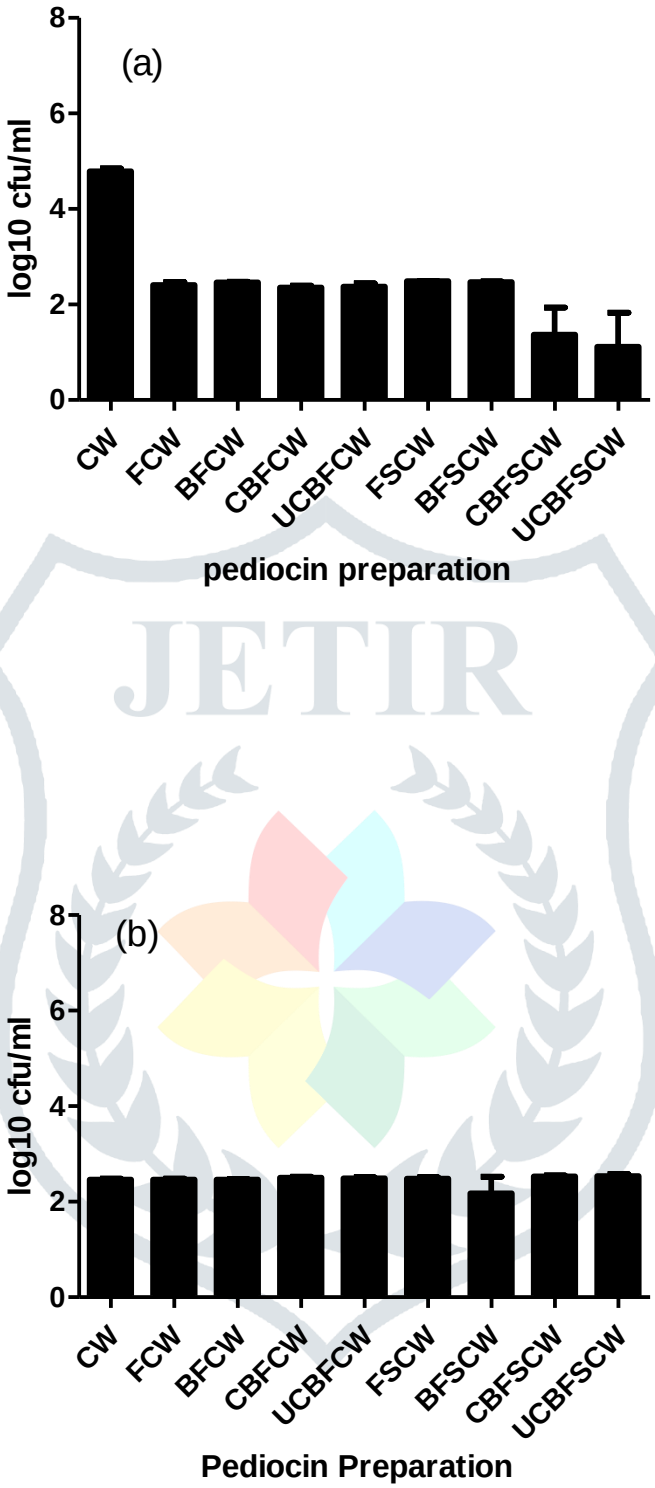

Fig: 3.6 *Staphylococcus aureus* count (SAC) at (a) 0 hour and (b) 6 hours without and with added pediocin preparations as defined in Table 2.1

4. Summary and conclusions

a) Different formulations were prepared after fermenting cheese whey and supplemented cheese whey with *Pediococcus pentosaceous* NCDC 273. Liquid cheese whey was prepared after dissolving 8 gm. of dried

cheese whey powder. Liquid cheese whey was supplemented with yeast extract (10 g/L), Tween 80 (1 mL/L), magnesium sulphate (0.2 g/L), and manganese sulphate (0.05 g/L) and pH 7.0.

b) Fermented cheese whey were used as such and after boiling, after centrifuging & boiling supernatant and after ultrafiltration of boiled supernatant obtained after centrifugation of fermented cheese whey, without and with supplementation.

c) All the pediocin preparations were found effective in reducing total count for viable bacteria on TGYEA, lactic acid bacteria on MRS, Yeast & Mould on PDA, Coliforms on VRBA and *Staphylococcus aureus* on Baird Parker determined using plating raw cow milk with agar media.

References

1. Bali, V., Panesar, P. S., & Bera, M. B. (2013). Effect of bacteriocin extracted from *Enterococcus faecium* BS 13 on shelf life of paneer and khoya. *International Journal of Food and Nutritional Sciences*, *2*(1), 1–11.
2. Blackburn P, Polak J, Gusik S and Rubino S. (**1998).** Nisin Compositions for Use as Enhanced, Broad Range Bactericides. AMBI, Tarrytown, NY, USA, 470929 5,753,614.
3. Bramley AJ and McKinnon CH. (**1990)**. The Microbiology of Raw Milk. In: *Dairy Microbiology*, *I*, (Ed.: Robinson, R.K.). London, New York, Elsevier Applied Science 171.
4. Cleveland J, Montville TJ, Nes IF and Chikindas ML. (**2001)**. Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology* **71**: 1-20.
5. Coorevits A, De Jonghe V, roemme J, Reekmans R, Heyrman J, Messens W, De Vos P and Heyndrickx M. (**2008)**. Comparative analysis of the diversity of aerobic-spore-forming bacteria in raw milk from organic and conventional dairy farms. *Systematic and Applied Microbiology* **31(2)**: 126-140.
6. Cousin M A. (**1982)** . Presence & activity of psychrotrophic micro-organisms in milk & dairy product. A review. *Journal of Food protection* 45: 172-207.
7. Deegan LH, Cotter PD, Hill C and Ross P. (**2006)**. Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International Dairy Journal*, **16**: 1058-1071.
8. Garsa AK, Kumariya R, Kumar A, Lather P, Kapila S and Sood SK. (2014b). Industrial cheese whey utilization for enhanced production of purified pediocin PA-1. *LWT- Food Science and Technology.* DOI: 10.1016/j.lwt.2014.07.008.
9. Garsa AK, Kumariya R, Kumar A, Lather P, Kapila S, Sood SK and Kapasiya M.(2014c). In vitro evaluation of the probiotic attributes of two pediococci strains producing pediocin PA-1 with selective potency as compared to nisin. *European Food Research and Technology*. DOI: **10.1007/s00217-014-2243-7.**

10. Garsa AK, Kumariya R, Sood SK, Kumar A and Kapila S. (2014a). Bacteriocin Production and Different Strategies for Their Recovery and Purification. *Probiotics and Antimicrobial Proteins* 6: 47-58.

11. Shashikumar C.S.S. and Puranik D.B. (2011). Study on Use of Lactoferrin for the Biopreservation of Paneer. *Tropical Agricultural Research*, 23(1): 70–76.

12. Sood SK and Sinha PR. (**2003)**. Analysis of structure of YGNGV motif containing bacteriocins: A model for pore formation. *Indian Journal of Biotechnology* **2**: 227-235.

13. Sood SK and Sinha PR. (2009). Acidocin S2 containing powder obtained upon freeze-drying of fermented paneer whey reduces total viable count during storage of processed cheese. *Indian Journal of Dairy Science* **64**: 486-490.

14. Sood SK, Vijay Simha B, Kumariya R, Garsa AK, Mehla J, Meena S and Lather P. (2013). Highly specific culture-independent detection of YGNGV motif-containing pediocin-producing strains. *Probiotics and Antimicrobial Proteins* **5**: 37-42.

15. Sørhaug T and Stepaniak L. (**1997)**. Psychroptophs and their enzymes in milk and dairy products: quality aspects. *Trends in Food Science and Technology* **8**: 35-40.

16. Vijay Simha B, Sood SK, Kumariya R and Garsa AK. (**2012)**. Simple and rapid purification of pediocin PA-1 from *Pediococcus pentosaceus* NCDC 273 suitable for industrial application. *Microbiological Research* **167**: 544-549.