CHAPTER 5
SUMMARY AND CONCLUSION

'Peptic ulcer' refers to an ulcer of the lower oesophagus, stomach or duodenum which forms when lining of the digestive system is corroded by acidic digestive juices. Peptic ulcers are produced by an imbalance between the gastro-duodenal mucosal defense mechanisms and damaging forces of gastric acid and pepsin, combined with superimposed injury from an environmental or immunologic agent or caused due to bacterial infection. It is estimated that about 5% -10% of adults globally are affected by peptic ulcers at least once in their lifetimes. A major causative factor (60% of gastric and up to 90% of duodenal ulcers) is chronic inflammation due to *H. pylori* that colonizes the antral mucosa. But some ulcers are caused by chronic use of NSAIDs (aspirin) that cause suppression of mucosal prostaglandin and have direct irritative topical effect. Repeated use of corticosteroids in high dose, alcoholic cirrhosis, personality disorder, psychological stress, ischemia and cigarette smoking impair healing of peptic ulcers and favor their recurrence.

Situation becomes worse due to emergence of drug resistance in *H. pylori.* According to Mukhopadhyay and coworkers, 85% of the Calcutta strains of *H. pylori* have shown resistance to metronidazole and 7.5% strains to tetracycline, which is quite high as compared to other reports in India. Pathogen sometimes develops antibiotic resistance due to spontaneous mutations and decreased binding of antibiotics to the ribosomes. Hence, alternative therapeutics are required to inhibit growth of *H. pylori* to control peptic ulcer disease.

Probiotic lactic acid bacteria (LAB) have been suggested to increase efficacy of *H. pylori* eradication therapy by preventing antibiotic-associated side effects and thus increasing compliance. Lacticin A164 of *Lactococcus lactis* subsp. *lactis* A164 and

lacticin BH5 of *L. lactis* BH5 are two bacteriocins of *Lactococcal* origin with anti-*Helicobacter* activity that kill pathogen in a dose-dependent manner. Two more anti-*Helicobacter pylori* bacteriocins namely bulgaricin BB18 produced by *L. bulgaricus* BB18 and enterocin MH3 produced by *E. faecium* MH3 have recently been identified. LAB constitute a phylogenetically related group of anerobic Gram-positive bacteria, including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus, Streptococcus* and *Bifidobacterium* species that share the ability to ferment sugars primarily into lactic acid. They are generally recognized as safe (GRAS) grade organisms due to their ubiquitous appearance in food and their contribution to the healthy micro flora of human mucosal surfaces. Many probiotic strains exhibit their antimicrobial property by synthesizing proteinaceous toxins or bacteriocins that inhibit growth of similar or closely related bacterial strain(s).

Only a few studies have actually focused on role of bacteriocins in treating *H. pylori* infections *in vivo*. Keeping in view the ever growing demand of natural therapeutics to combat recurrence and control of *H. pylori* induced peptic ulcer disease, present research work was undertaken which aimed at **" Characterization and therapeutic potential of anti-*Helicobacter pylori* bacteriocin of a lactic acid bacterial isolate ".**

1. **Screening, isolation and identification of Anti-*Helicobacter pylori* bacteriocin producing lactic acid bacterial strain from faecal samples of healthy individuals.**

Study employed more than 100 faecal samples which were collected from healthy individuals with informed consent and 153 LAB strains were isolated using pure culture

techniques. Samples were collected in sterilized NaCl (0.85%) and transferred to the MRS broth, an enrichment culture media selective for isolation of LABs. Further screening was carried out on the basis of bacteriocin production activity which was assayed using standard methodologies like spot-on-lawn, well-diffusion and disc-diffusion assays. Out of total tested isolates, bacteriocin production trait was observed only in fourteen LAB isolates designated as GI54, GI69, GI90, GI75, GI76, GI59, GI19, GI28, GI40, GI16, GI80, BA28, BA30 and BA31. Isolates namely BA28, BA31 and GI76 possessed extremely attractive antimicrobial spectrum which was highest among all the tested indicators, especially against *H. pylori* DMSZ 10242 and therefore, these three strains were selected for further study.

The selected LAB isolates were identified biochemically as per Bergey's Manual of Determinative Bacteriology 9th Edition. The strains consisted of Gram-positive cocci showing tetrad arrangement of cells with convex and smooth surface and were found to be catalase negative. Isolates were mesophillic in nature and were able to grow under alkaline environment (pH 9.0) from 10 to 42 ^{o}C. They successfully fermented trehalose, xylose and mannose but could not utilize sorbitol, lactose and galactose. They were unable to hydrolyze gelatin, starch, esculin, casein, urea and Tween 20, Tween 40, Tween 60 and Tween 80. Strains neither have oxidase activity nor have indole and gas production. Isolate BA28 is salt (12% *w/v* NaCl) as well as bile tolerant (30% *w/v*) that gave positive methyl red test, whereas a negative Voges-Proskaeur reaction. They were capable of producing ammonia from arginine that reflects presence of arginine deaminase activity. Based upon carbohydrate fermentation profile and other physiological and biochemical tests, strains were classified as *Pediococcus acidilactici*.

Preliminary biochemical investigation was confirmed and validated by 16S rRNA gene sequencing of isolate BA28. Sequence was deposited with NCBI's Genbank database vide Accession no. JX431046. Phylogeneic analysis by BLASTn scoring of 1462 bp 16S rRNA gene sequence of *P. acidilactici* BA28 against non-redundant nucleotide database revealed its 100% sequence similarity with *Pediococcus acidilactici* strain UL5, and a close homology with other strains of *Pediococcus* species.

2. Production and purification of the bacteriocin at flask level.

Bacteriocin produced by *P. acidilactici* BA28 was designated as pediocin BA28 and it was purified by standard adsorption-desorption method. There was 2.69 fold increases in specific activity of the partially purified bacteriocin than crude bacteriocin preparation. Further, its purity was analyzed by reverse phase HPLC where a single peak was observed in the chromatogram at retention time of 3.367 min. Purified pediocin BA28 has a molecular weight of 6.4 KDa as observed in SDS-PAGE and by MALDI-TOF analysis (without trypsinization).

RSM has been successfully applied for optimization of media constituents for enhancing bacteriocin production in *P. acidilactici* BA28. It reduced the number of media components from 10 to 5 as compared to MRS media for supporting optimal bacterial growth and bacteriocin production. The statistically optimized media is much more cost effective than MRS media which is generally used for selective enumeration of heterofermentive *Lactobacilli*. A similar pattern of *P. acidilactici* BA28 growth was observed in both MRS as well as RSM optimized media, however, the specific activity in RSM optimized medium symbolizes a magnificent increase from 30,000 AU/ml to 55,000 AU/ml during late log period from 16 - 24 h.

3. *In vitro* characterization of bacteriocin produced

(a) Biochemical characterization

Pediocin BA28 has a great commercial importance owing to its remarkable heat stability (121^{o}C for 20 min), activity over a wide pH range (2.0 to 9.0), higher specificity and effectiveness even at very low concentrations. Complete or partial reduction in bacteriocin activity was observed after proteolytic treatment with α-chymotrypsin, papain, pepsin and proteinase K, while it is not affected following treatment with organic solvents such as isoamyl alcohol and formaldehyde. Partial loss in pediocin BA28 activity was observed with SDS, Tween-20, Tween-80 and Triton X-100 whereas; it was completely lost upon EDTA and urea treatment. Purified pediocin BA28 is stable upto 30 days at refrigeration temperature (4 °C) and it is highly encouraging to report its usefulness as a food biopreservative at refrigeration temperature.

(b) Antimicrobial spectrum

Antimicrobial activities of isolated LAB strains were comparatively investigated against a panel of microorganisms and highly significant results were obtained in the study. LAB produce a variety of antibacterial factors and their inhibitory spectrum varies between narrow and broad range depending upon the LAB species. A number of pathogenic and non-pathogenic tested Gram-positive bacterial strains were inhibited by the bacteriocin producing LAB isolates including *B. fragilis, B. ovatus, B. vulgatus, C. albicans, C. sporogenes*, *E. coli, E. faecalis, G. vaginalis, H. pylori, K. pneumoniae, L. mesenteroides, L. monocytogenes, M. flavus, N. gonorroeae, N. mucosa, P. aeruginosa, P. mirabilis, Staphylococci, Streptococci, S. typhi* and *V. cholerae.* Among non-

pathogenic indicators, *L. brevis, L. bulgaricus, L. helveticus* and *P. acidilactici* LB42 were strongly inhibited by isolate GI69 and GI76 respectively. *H. pylori* causing peptic ulcer in humans was strongly inhibited by all the 14 bacteriocin producing LAB isolates but maximum growth inhibition was observed in case of pediocin BA28. As these bacteriocins are potential antimicrobial agents and in conjunction with their producers, they may have use in applications to contribute a positive effect on the balance of intestinal microflora.

There has been a great interest in the application of bacteriocins and bacteriocin-producing strains (especially those produced by LAB) in food industry. Biopreservative potential of pediocin BA28 was tested in different model food systems (including pasteurized milk, vegetable fresh cuts, moong sprouts, channa sprouts and minced meat) to prove their effectiveness in killing of food spoilage organism and promising results were observed when used at higher concentrations. Because of broad spectrum of inhibition and remarkable molecular stability of pediocin BA28, it can be a sound candidate for decontamination of vegetable foods containing *L. monocytogenes* and other foodborne bacteria sensitive to this bacteriocin.

(c) Gene localization

Genetic analysis of *P. acidilactici* BA28 indicated plasmid linkage of bacteriocin production trait which was associated with a cryptic plasmid of 10.8 kb size. Curing experiments were performed using ethidium bromide to correlate linkage of bacteriocin production trait with 10.8 kb plasmid DNA in *P. acidilactici* BA28. Ethidium bromide caused concentration dependent viability loss in *P. acidilactici* BA28 and loss of cryptic plasmids from native strains of *P. acidilactici* BA28, which was also

confirmed by bacteriocin assays. Further to confirm plasmid curing, antibiogram of the *P. acidilactici* BA28 strain was determined using OCTA-discs of Himedia, India. Both the *Pediococcus* isolates (with and without curing) were found to be resistant to fosfomycin and vancomycin, while wild type strain was resistant to amikacin, cephalexin, erythromycin, norfloxacine that became sensitive to these antibiotics after curing of plasmid DNA using ethidium bromide. Results also indicated that four antibiotic resistance factors are associated with genes located on to the 10.8 kb plasmid DNA of *P. acidilactici* BA28. This strain was sensitive to the rest of the tested antibiotics including Amoxycillin, Amoxyclav, Ampicillin, Cefazolin, Cefixime, Ciprofloxacin, Co-trimoxazole, Erythromycin, Gentamicin, Gentamycin, Levofloxacin, Linezolid, Norfloxacin, Penicillin-G, Pristhiomycin, Roxithromycin, Streptomycin, Tetracyclin, Tigecycline which favours its categorization on the WHO recommended GRAS list of microorganism.

A pair of sequence specific primers against pediocin operons was used to amplify 618 bp segment of *P. acidilactici* BA28 plasmid DNA. The nucleotide sequencing of the gel extracted 618 bp amplified product from plasmid was carried out and has been deposited with Genbank database under the accession number KC693734. The sequence analysis revealed the presence of a *pediococcal* promoter having 5' TTTTAAAAT 3' consensus at -10 and 5' TTACCA 3' conserved stretch at -35 positions. Pediocin structural gene i.e *ped A* was located between nucleotide 63-246 of the amplified segment. *Ped A* gene has its own ribosomal binding sites at position 48 having 5' AAGGAG 3' sequence and at position 275 having 5' AAGGGG 3' sequence. BLASTp analysis of the longest ORF detected in 618 bp amplicon against non-

redundant protein database revealed a 100% sequence similarity with prepediocin CP2 produced by *Pediococcus acidilactici* MTCC 5101, Pap A protein produced by *Pediococcus acidilactici* and Pre-pediocin produced by *Enterococcus faecium.* Pediocin BA28 contains an extra stretch of 15 amino acid on C-terminal as compared to already reported pediocin sequences when analysed using Clustal omega program. Due to presence of extra residues at the C-terminal end, a wide spectral inhibition was attributed to bacteriocin produced by *P. acidilactici* BA28.

4. Mechanism of bacteriocin action on *Helicobacter pylori*.

Mechanism of bacteriocin action on *H. pylori* was studied *in vitro* by treatment of *H. pylori* cell suspensions with different concentrations of purified pediocin BA28. A 1.78 log cycles reduction in *H. pylori* counts was observed upon treatment with 100 µg/ml bacteriocin but by over 4.26 log cycles when treated with 400 µg/ml bacteriocin, which indicates that a decrease in viability of the sensitive cells is dependent upon concentration of the bacteriocin used. Bactericidal mode of action of pediocin BA28 against *H. pylori* was confirmed by SEM analysis which shows changes in cell morphology and topography upon bacteriocin treatment. Disruption of the *H. pylori* cell wall and cell membrane occurred due to formation of a large number of pores (of small to moderate size) causing extensive damage to the cells and thus inducing viability loss in the sensitive cells. Pediocin BA28 is therefore reported as a highly potent anti *H. pylori* agent with numerous therapeutic applications.

5. Study of *in vivo* therapeutic potential of Anti-*Helicobacter pylori* bacteriocin producing lactic acid bacterial isolate in mice model.

In vivo therapeutic potential of *P. acidilactici* BA28 was studied in C57BL/6 mice models as per IAEC guidelines. Mice receiving continuous feed of probiotic *P. acidilactici,* showed a proliferation in their LAB counts, whereas a decrease in *H. pylori* counts was observed in the stool samples of the test group as compared to the control group. Results were further validated by histopathological evaluation of gastric biopsy specimens of the infected mice. Results clearly demonstrate a growth retardation as well as gastric colonization by *H. pylori* upon antagonistic probiotic feeding with *P. acidilactici* BA28. More interestingly, probiotic BA28 strain could reverse the infection if administrated for a longer duration and could check reoccurrence of *H. pylori* infection also. Evidence provided in the current study may pave the way to propose the use of probiotic *P. acidilactici* BA28 as a co-adjunct of standard antibiotic treatment.