6.1 General consideration:

The fermented milk peptides play a natural role in many biochemical and immunological mechanisms in human body and they can be formulated as various oral supplements to bring out positive health effect for all age groups. Many reports are available for various Immunomodulatory activities, antihypertensive potential to lower the blood pressure and to be used as a sports medicine CPP [16]. Further work in the area can be performed to understand the mechanism of CPP’s role as an Immunomodulatory agent.

6.2 Initial standardization:

During the fermentation of milk by various lactic acid producing microorganisms, the pH reduced and becomes acidic in all the samples but not much difference in the ranges of pH was observed among the 4 fermenting agents, i.e. Aavin curd, Dodla curd, Lactobacillus acidophilus and Lactobacillus bulgaricus. Compared to control the titratable acidity was in uniform range for all the fermented milk taken up for study. The test samples intra comparison also proved that titratable acidity did not vary much among them. The lowest pH observed was in the case of milk fermented using Lactobacillus bulgaricus (Table 5.4).
Titratable acidity shown in table 5.6 acts as an indicator of acid volume produced in the fermented milk due to fermentation process and low level variation shows that the volume of acid formed in all the test samples of fermented milk is almost the same. The lowest titratable acidity was observed in the case of milk fermented by *Lactobacillus bulgaricus* and highest titratable acidity was observed in the case of milk fermented by commercial Dodla. As observed in table 5.7, not a substantial variation in viscosity was observed between the four fermented milk samples and the control, the non-fermented milk. Viscosity is directly related to the physical appearance and palatability of the fermented milk samples. If viscosity is high, it affects the shear stress of the fermented milk, thus making the flow a bit less smoother. The lowest viscosity was observed in the case of milk fermented by *Lactobacillus acidophilus* and highest viscosity was observed in the case of milk fermented by commercial Dodla (Figure 5.3). Excess increase of viscosity in fermented milk drastically affects the palatability of the fermented milk. All the test samples had their viscosity in the range which favoured high palatability of the fermented milk samples ensuring a good shear stress balance in accordance with Funian *et al.*, 2004 [116].

6.3 Microbiological and Scanning Electronic Microscopic (SEM) analysis:

The presence of *Lactobacillus sp.* was confirmed by gram staining and SEM. Ness *et al.*, 2000 [67] and Drago *et al.*, 1997 [183] have stated that commercially produced fermented milks always contains mixed cultures including more than one bacterial culture and it corresponded with our findings. This mixed culture model
ensures speedy and effective fermentation of milk which is commercially economical for them. The test samples of milk fermented by *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* contained a single strain. Although there is a variation in the cultures used and the texture of all the samples remained the same when examined physically indicating that mixed cultures produce fermentation in a quicker way than single culture samples but has no significant impact on texture and related physico-chemical properties. The presence of *Lactobacillus* sp. as rod shaped *bacillus* in the fermented milk was demonstrated in photo 5.3 by SEM analysis.

### 6.4 Anti-microbial activity:

The anti-microbial activity (photo 5.4) of our CPP isolate against common clinical pathogens such as *Escherichia coli* and *Pseudomonas sp.* corresponds to the mucosal secretions associated with milk peptides and fermented milk peptides and our findings correspond with Sun *et al.*, 2002 and Pihlanto *et al.*, 1999 [51, 37]. There was a slightly higher zone of inhibition formed by CPP against *Pseudomonas sp.* when compared with *Escherichia coli*. Pihlanto *et al.*, 1999 [37] have reported that Lactoferrin is a milk peptide in that front towards which relatively higher time and studies had been devoted. This fact can be ascertained by the literature review of the milk and fermented milk peptides [134,151, 172, 174, 187]. Production of anti-microbial agents from fermented milk samples especially employing CPP will mark a new beginning in nutraceuticals. CPP will be a value addition to the existing food based nutrients available in the market.
6.5 High Pressure Liquid Chromatography (HPLC) and Fourier Transform Infra Red (FTIR) spectroscopy analysis of CPP:

HPLC analysis of the fermented milk samples of our study produced exclusive peaks which were characteristic to fermented milk peptides [55, 154] and the peaks which corresponded to components produced during fermentation is seen in Figures 5.5 – 5.9. The HPLC analysis of the control sample, i.e. non-fermented milk did not produce the peaks which were produced by fermented milk test samples indicating the formation of new components in milk due to fermentation. FTIR analysis of the same test samples yielded similar results. The fermented milk samples exhibited peaks which are characteristic of the bioactive component which was absent in control sample. Both the HPLC, FTIR results confirm the above mentioned sentence of new bioactive peptides formation.

6.6 Molecular weight determination by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE):

Molecular weight determination was ascertained using SDS-PAGE and the value was found to be 3.5 – 4 kilo Daltons (Photo 5.5). Molecular weight is always of critical importance to bioactive components as it indirectly determines their mode of delivery in to human body as a nutraceutical and therefore efficacy as stated by Antonius et al., 2000 [106]. The bioactive components present in fermented milk peptide of CPP could be broken in to smaller components for an improved formulation and effective delivery. Production of these peptides at nano scale could be pondered upon in the future which will open new vistas of fast acting nutraceutical.
6.7 Animal studies:

CPP’s effect on the weight gain of albino mice was demonstrated by animal studies. The Dodla CPP isolated from milk fermented by Dodla culture produced the highest increase in weight in mice was 2.59 grams as seen in table 5.10. The least weight increase by a test batch was 2 grams by Aavin CPP, i.e. CPP which was isolated from milk fermented by commercial Aavin. When compared with the control batch (non-fermented milk) weight increase of 1.28 grams, all the four test batch of fermented milk CPP’s produced substantial increase in the weight of albino mice in a injection period of 10 days (Figure 5.15), proving the point that continuous intake of fermented milk products contribute to uniform increase in body weight.

As far as the weight increase over the 15 days injection period is concerned, the Dodla CPP produced the highest weight increase in mice which was 4.33 grams. The least weight increase by a test batch was 3.17 grams by Aavin CPP, i.e. CPP which was isolated from milk fermented by Lactobacillus bulgaricus. When compared with the control batch (non-fermented milk) weight increase was 1.56 grams (table 5.19), all the four test batch of fermented milk CPP’s produced substantial increase in the weight of albino mice in a injection period of 15 days indicating that injection period was directly proportional to the weight increase as seen in the Figure 5.16. These results were in accordance with Mogensen et al., 1977 [137].
6.7.1 Gastroprotective action against *E. coli*

The mice mortality during post infection state indicated the gastroprotective effect of CPP. Gastroprotective nature of the fermented milk peptides has been already shown by Antonio *et al.*, 2001 [97]. The highest gastroprotective effect against *Escherichia coli* infection was observed in the case of Dodla CPP and *L. bulg.* CPP which had the lowest percentage of mice mortality at 17%. Control mice had 100% mortality due to lack of CPP injection while only 33% mortality rate was observed in the 10 days CPP fed groups indicated their gastroprotective effect. In case of 15 days injection, the *L. acid.* CPP fed group showed the highest gastroprotective effect against *Escherichia coli* and *L. bulg.* CPP fed group showed the lowest percentage mortality as 17%. Due to the lack of CPP injection the control group mice showed 83% mortality.

6.7.2 Gastroprotective action against *Salmonella sp.*

The highest gastroprotective effect against *Salmonella sp.* infection was observed in the case of Dodla CPP which showed 0% mortality. Control mice had 100% mortality due to lack of CPP injection. 17 - 33% mortality rate by other CPP’s indicated their gastroprotective ability during the injection period of 10 days. In case of 15 days injection, the highest gastroprotective effect against *Salmonella sp.* infection was observed in Dodla CPP which was confirmed by 17% mortality where as in Control 100% mortality was observed due to lack of CPP injection. Rearrangements of cytoskeleton with the formation of membrane ruffles of the
intestinal tissues is the possible mechanism by which gastroprotection was enabled and this has already been dealt by Benkerroum et al., 2002 [167]

6.7.3 Gastroprotective action against *Shigella sp.*

The highest gastroprotective effect against *Shigella sp.* infection was observed in the case of *L. acido*. CPP which had the 0% percentage of mice mortality. Control mice had 83% mortality due to lack of CPP injection. 17 - 33% mortality rate by other CPP’s indicated their gastroprotective ability during the injection period of 10 days. In case of 15 days injection period mice, the highest gastroprotective effect against *Shigella sp.* infection was observed in the case of *L. acido*. CPP which had 17% percentage mortality as seen in table 5.30. Control mice had 100% mortality due to lack of CPP feed. Increase in the injection period was found to be directly proportional to the enhancement of gastroprotective effect on albino mice, drastically reducing the mortality percentage as indicated in figure 5.34.

6.8 Pathogen count determination Visceral Organs:

The pathogen count present in visceral organs of albino mice after post infection by GIT pathogens such as *Escherichia coli*, *Salmonella sp.* and *Shigella sp.* was determined. The visceral organs in which pathogen count was determined were liver, spleen, kidney and small intestine. As observed in table 5.34, the lowest pathogen count in case of *Escherichia coli* infection was observed in the case of *L. acido*. CPP fed mice liver. The highest pathogen count in case of *Escherichia coli* infection was observed in the case of Aavin CPP whereas control mice in comparison
had $51 \times 10^3$ pathogens due to lack of CPP injection (Figure 5.35). Similarly the test batch of mice had lower pathogen count in other visceral organs such as kidney, spleen and small intestine when compared with control batch, indicating the role of CPP in reducing the pathogen count in visceral organs during post infective period.

The lowest pathogen count in *Salmonella sp.* infected mice was observed in the *L. acido.* CPP fed mice liver. The highest pathogen count was observed in Aavin CPP fed mice whereas control mice had $79 \times 10^3$ pathogens because there was no injection of CPP. Similarly the test batch of mice had lower pathogen count in other visceral organs such as kidney, spleen and small intestine when compared with control batch, indicating the role of CPP in reducing the pathogen count in visceral organs during post infective period. The lowest pathogen count was observed in *Shigella sp.* infected mice liver fed with *L. bulg.* CPP. The highest pathogen count was observed in Shigella *sp.* infected mice fed with *L. acido.* CPP whereas the control mice had $93 \times 10^3$ pathogens due to lack of CPP injection. Similarly the test batch of mice had lower pathogen count in other visceral organs such as kidney, spleen and small intestine when compared with control batch, indicating the role of CPP in reducing the pathogen count in visceral organs during post infective period. Ours is the first study to show the effect of CPP on the lowering of pathogen count in GI tract infected mice. CPP injection was able to effectively reduce the pathogen count in the infected mice when compared with control unfed mice.
6.9 Histopathological studies:

Histopathological studies of the small intestines from the albino mice infected with GIT pathogens like *Escherichia coli*, *Salmonella sp.* and *Shigella sp.* showed the disruption in cell organization when compared with the uniformly organized cell organization present in test batch mice fed with CPP for 10 and 15 days. The difference in cell structure was in line with the findings of Warenjo *et al.*, 2004 [69]. The level of disorientation in cells was quite low in test mice batches when compared with control mice batches where the level of disorientation was relatively higher. Histopathology of albino mice spleen, kidney and liver cells infected with *Escherichia coli*, *Salmonella sp.* and *Shigella sp.* showed similar disruption in cell organization of control batch mice when compared with the uniformly organized cell organization present in test batch mice fed with CPP for 10 and 15 days. Histopathological studies proved the gastroprotective potential of CPP at the cellular and molecular level. CPP was able to bring down the cell attrition rate and preserve the cellular integration which was evident by its impact on test batches (Photos 5.6-5.9).

6.10 Immunomodulatory activity:

The immunomodulatory roles of CPP on the GIT pathogen infection were determined by immunofluorescence assay [189] using mice small intestine tissue samples. In *E. coli* infected mice the highest number of secretary IgA cells was produced in *L. acido*. CPP 10 days fed mice intestine and least was observed in Dodla CPP 10 days fed mice. Control batch had produced just 8 IgA cells (table 5.37). In case of 15 days fed group, the highest number of secretary IgA cells was produced by
L. bulg. CPP and least secretary IgA cells being produced by L. acido. CPP. Control batch had produced only 6 IgA cells. As seen in Figure 53, the highest number of secretary IgA cells was produced by L. bulg. CPP in case of mice fed with CPP for 10 days and then subsequently infected using Salmonella sp. with the least secretary IgA cells being produced by Aavin CPP. Control batch had produced just 10 IgA cells. In case of 15 days (Figure 5.41) injection period test batches, highest number of secretary IgA cells was produced by L. bulg. CPP and least secretary IgA cells being produced by Dodla CPP. Control batch had produced 14 IgA cells. The results were in accordance with Rojas et al., 2002 [180].

In Shigella infected mice the secretary IgA cells produced was highest (table 5.39) by L. bulg. CPP 10 days fed mice and it was low in Dodla CPP 10 days fed mice. Control batch had produced just 5 IgA cells. In case of 15 days injection period batches, highest number of secretary IgA cells was produced by L. bulg. CPP and least secretary IgA cells being produced by Dodla CPP. Control batch had produced 11 IgA cells. All the fermented milk batches seem to induce a considerable immune enhancement in albino mice which is evident from the substantial increase in the number of IgA secretary cells by CPP. The immune enhancement potential of CPP had been proved beyond a doubt by the concurrent checking with all the four batches of CPP isolated from milk sources fermented by four different fermenting agents. The possible mechanism by which immune enhancement observed is shown by the increase in secretary IgA cells production as stated by various other studies [112,141,97]. This mechanism also explains the lower mortality rate, lesser percentage
of weight loss in experimental mice group fed with CPP compared to control group which was not fed with CPP. Another possible mechanism for immune enhancement effect observed due to CPP enhances the production of IgA secreting cells.

6.11 Anti-Genotoxic role of CPP:

Few works have been carried out to study the anti-genotoxic role of milk and fermented milk peptides [191,192]. The deleterious effect of gamma radiation on the albino mice and fish were determined using the number of micronuclei formed, number of binucleated cells seen and the number of multinucleated cells observed. Our experimental results (tables 5.40-5.45) proved the presence of anti-genotoxic component in all the four CPPs isolated from fermented milk. The number of micronuclei formed, number of binucleated cells seen and the number of multinucleated cells observed were all relatively lower in the CPP fed mice and CPP treated fish compared to control after exposed to radiation at different periods of time.(Photos 5.13-5.24).

Lourens-Hattingh et al., 2001 [185] have stated that the possible mechanism by which fermented milk products could act as anti-genotoxic agents and the CPP, a class of fermented milk peptide bringing about anti-genotoxicity is in accordance with the same possible mechanism [125]. A number of radioprotectants of chemical nature are present today but a nutraceutical based anti-genotoxic agent could be of immense benefit. One possible mechanism of anti-genotoxicity was due to stimulation of enzymatic secretion in the small intestine cells by CPP which was confirmed by the positive oxidase and catalase enzyme tests carried out for test batches of albino mice,
test batch fish, control batch of albino mice and control batch of fish. The presence of these enzymes could be the probable reason for the anti-genotoxic nature of CPP [170].

6.12. Application to human model:

The concentration of CPP in 0.5 ml of crude sample isolated from fermented milk was found to be 65 mg. Assuming that an adult human consumes 100 ml of fermented milk through his/her regular diet on a daily basis, approximately 13000 mg or 13 grams of CPP would be intaken. This amount should be enough for the body to carry out all the necessary functions that the CPP seems to be effecting.