Biopreservation of milk and milk-based food products using antibacterial peptide of *Bacillus licheniformis* Me1
5.1.1. Abstract

The application of antibacterial peptide as natural preservative in foods is an interesting and rapidly growing field of study. The biopreservative efficacy of *B. licheniformis* Me1 was evaluated by direct application of its ABP in milk and milk-based food products, such as cheese and paneer. Commercially available pasteurized milk samples were supplemented with partially purified ABP (ppABP) at a final concentration of 1600 AU/ml and inoculated with food-borne pathogens (*L. monocytogenes* Scott A, *M. luteus* ATCC 9341 and *Staph. aureus* FRI 722), individually. After inoculation the samples were stored at two different temperatures (4 ± 2°C and 28 ± 2°C). Milk without ppABP served as the control. During the incubation period at 4 ± 2°C, in the presence of ppABP, the count of *L. monocytogenes* Scott A decreased from $4 \log_{10}$ to $2 \log_{10}$ CFU/ml within 24 h, whereas, in the control milk samples, the count increased to $8 \log_{10}$ CFU/ml at the end of the storage period. Results indicate that the ppABP produced by *B. licheniformis* Me1 was as effective as the commercially available biopreservative, nisin. The other pathogens (*M. luteus* ATCC 9341 and *Staph. aureus* FRI 722) were completely inhibited in the presence of ppABP during the storage period. Similarly, reduced growth rate of the tested pathogens was observed ppABP in milk samples with ppABP as compared to the control, incubated at 28 ± 2°C. The shelf-life of milk samples increased to 4 days in the presence of ABP, whereas, curdling and off-odour were noticed in the control milk samples just after 24 h of storage at 28 ± 2°C. Milk samples with ppABP were sensorily acceptable and showed no significant difference in any of the parameters analyzed as compared to the control milk samples. Further studies were performed to determine the efficiency in preservation of other dairy food products, such as cheese and paneer. Samples of 1 g pieces of cheese/paneer contaminated with *L. monocytogenes* Scott A and flooded with ppABP solution also showed significant reduced viable counts of pathogen when compared with the controls, kept at 4 ± 2°C. Thus, the results of this study indicate that the ppABP from *B. licheniformis* Me1 can be utilized as biopreservatives in food systems to control the growth of spoilage and pathogenic bacteria, thereby reducing the risk of food-borne diseases.
5.1.2. Introduction

Food-borne pathogens are widely distributed in environment and occur naturally in many raw foods (Gram et al. 2002). The most common bacterial pathogens that are found in raw milk and milk products include *L. monocytogenes*, *Salm. typhimurium, M. luteus* and *B. cereus* (Faye and Loiseau 2000). Meat and meat products also provide suitable environment for proliferation of spoilage microorganisms and common food-borne pathogens, including *Staph. aureus* and *Salmonella* species (Ayulo et al. 1994; Lima and Gorlach-Lira 1999). *L. monocytogenes* being a psychrotrophic and halotolerant (Seelinger and Jones 1986) grows in many refrigerated food products with extended shelf-lives (Barakat and Harris 1999). The cross contamination of cooked ready-to-eat products, such as cheese, meat and fish, and other delicatessen products by *L. monocytogenes* is a major concern of the food industry (Adzitey and Huda 2010). The contamination of raw milk and meat or its products has been the major source of several outbreaks (Brett et al. 1998; Centers for Disease Control and Prevention [CDC], 1999, 2000, 2002; Ericsson et al. 1997; Jemmi et al. 2002; Reij et al. 2004).

Biopreservation is defined as the extension of shelf life and enhanced safety of foods by the use of natural or controlled microbiota and/or antimicrobial compounds (Schillinger et al. 1996; Stiles 1996). Nowadays, there is a strong interest in the use of natural antimicrobials for preservation of minimally processed foods (Chen and Hoover 2003; Delaquís et al. 2002). The natural and GRAS antimicrobial nisin produced by LAB have been the subject of intensive study because of their potential use as biopreservatives in the food industry (O'Sullivan et al. 2002). Nisin and other antimicrobial, like pediocin from LAB have been shown to reduce or inhibit *L. monocytogenes* in dairy and meat products, particularly in pasteurized cheese (Berry et al. 1990; Muriana 1996; Nielsen et al. 1990). Nisin has been approved by FDA for application as food additives for biopreservation (Cleveland et al. 2001; Federal Register 1988, Galvez et al. 2007).

Like LAB, some representatives of *Bacillus* spp., such as *B. subtilis, B. licheniformis* and *B. coagulans* are GRAS in food industry and agriculture (Durkee 2012; Sharp et al. 1989). However, the biopreservative application of antimicrobial
compounds produced by *Bacillus* cultures in food was rarely evaluated (Bizani et al. 2008; Martirani et al. 2002; Sorokulova et al. 1997), as compared to the extensive application of LAB bacteriocins against food pathogens and/or spoilage bacteria.

In previous sections of the thesis (Chapter 2 and 3), characterization of the culture *Bacillus licheniformis* Me1, a native isolate from milk, which produces a potent ABP exhibiting antimicrobial activity against several food-borne pathogens is documented. The bactericidal effect of this ABP was found to be apparently by disturbing the membrane function of target organisms. The ABP of this culture was also found to be stable at wide range of pH and temperature, which makes it a potential candidate for application in food system as biopreservatives. Furthermore, the culture was classified as safe for application in food system (Chapter 4). In this section, the biopreservative efficacy of the ABP produced by the culture *B. licheniformis* Me1 was evaluated. To determine the biopreservative efficacy, the effects of the ABP on the survival of food-borne pathogens in milk and milk products were studied. The sensorial acceptability of the milk added with ABP was also evaluated.

### 5.1.3. Materials and methods

#### 5.1.3.1. Bacterial strains and culture conditions

The pathogens used in this study included *M. luteus* ATCC 9341, *L. monocytogenes* Scott A and *Staph. aureus* FRI 722. The culture *B. licheniformis* Me1 and the pathogenic strains used in this study were maintained as described previously (Section 2.3.2).

#### 5.1.3.2. Preparation of antibacterial peptide

For the production of ABP, the culture *B. licheniformis* Me1 was grown in a modified media (pH 8 ± 0.2) consisting of corn steep liquor (2%), yeast extract (0.5%) and NaCl (0.25%) for 24 h at 37°C and with agitation speed of 150 cycles/min. After incubation, the CFS was subjected to a fractionated precipitation of the protein by the slow addition of ammonium sulphate to 65% saturation as described in section 3.3.7. The precipitated protein collected after centrifugation (10,000 g for 10 min), was resuspended in 0.1 mM phosphate buffered saline (100 mM PBS; pH 7) and further extracted with n-Butanol. Butanol was evaporated using Rota vaporizor (BUCHI India Pvt. Ltd.) at 14 lbs and 55°C, and the dried residue was
dissolved in 1/100th volume of sterile distilled water. The resulting solution was partially purified ABP (ppABP) and stored at -20°C, until further use. The antibacterial activity of the ppABP against the food-borne pathogens and the residual activity were determined as discussed in section 3.3.3.

5.1.3.3. Efficacy of ppABP to control spoilage organisms in the milk

Commercially available pasteurized milk samples were used in this study. Samples of 10 ml were dispersed in test tubes under sterile conditions and *L. monocytogenes* Scott A at a concentration of $10^4$ CFU/ml was inoculated in to each milk sample. The tubes containing milk samples along with indicator strains were divided into two sets. To the one set of tubes, ppABP was added at a final concentration of 1600 AU/ml. Another set of tubes without any ppABP were kept as the control. After addition, the milk samples were stored at 4 ± 2°C and at higher temperature (28 ± 2°C). Nisin at a concentration of 1600 AU/ml were used as positive control. Individual tubes were removed at 2 day intervals and the count of *L. monocytogenes* Scott A was analysed by plating on *Listeria* selective (LS) medium. Similar experimental setup was made for testing the efficacy of the ppABP on the survivability of *M. luteus* ATCC 9341, *Staph. aureus* FRI 722 and normal microflora in milk samples during storage at 4 ± 2°C and 28 ± 2°C. The number of viable cells in the milk samples of these cultures was determined by plating on LB agar, Mannitol Salt agar and Plate count agar, respectively. All determinations were done using three independent samples.

5.1.3.4. Biopreservation of cheese using ppABP

Cheese purchased from local market was made in to small pieces of 1 g each and then divided into three sets. The first and the second set of cheese pieces were dipped into two different concentrations of ppABP solution 400 and 1600 AU/ml, respectively. After dipping, the cheese pieces were kept for drying for 15 min and then again dipped in a suspension of indicator strain *L. monocytogenes* Scott A ($10^2$ CFU/ml). The third set of cheese pieces were dipped only in the *L. monocytogenes* Scott A suspension and was used as a control. The cheese pieces were stored at 4 ± 2°C in sterile containers. Individual sample from each group were removed at 3 days interval and homogenized in saline (0.85% w/v NaCl) and then viable count of the indicator strain was determined by plating on LS medium.
5.1.3.5. Biopreservation of paneer using ppABP

The application of ppABP in the paneer samples was carried out in two ways; i) surface application and ii) direct incorporation of the ppABP in paneer. Paneer was prepared as described below. Milk (1000 ml) was boiled and 5 ml of lemon juice was added in the hot milk to separate the curds from the whey. Then the curds were drained and pressed out to remove the excess water in cheesecloth, and subsequently kept for moulding. After moulding, the paneer was cut in to pieces of 1 g and divided into three groups. For surface application of the ppABP, two groups of paneer pieces were dipped in two different concentrations of ppABP solutions, 400 and 1600 AU/ml, respectively. The treated paneer pieces were inoculated with indicator pathogen by dipping the paneer pieces in a suspension of \textit{L. monocytogenes} Scott A at a dilution of $10^4$ CFU/ml. Paneer pieces without ppABP and inoculated with indicator organism was used a negative control. The paneer pieces were then stored at $4 \pm 2^\circ$C for a period of 14 days in sterile containers. Individual samples were removed at 3 days interval and checked for the viability of \textit{L. monocytogenes} Scott A by plating on a LS medium.

The whole experimental procedure was same for direct incorporation of ABP in the paneer. However, the incorporation of ppABP in the paneer was done only during the preparation of paneer by adding the ppABP at a final concentration of 1600 AU/ml in the hot milk before adding lemon juice for curdling. Untreated paneer samples served as the control.

5.1.3.6. Sensory evaluation of the milk samples with ppABP

Sensory analysis was carried out for samples of milk incorporated with two different concentration of ppABP, 400 and 1600 AU/ml and compared with the control milk samples. Evaluations were conducted under white fluorescent light, with the booth area maintained at temperature $22 \pm 2^\circ$C and RH $50 \pm 5\%$. A suitable score card was developed using “free choice profiling” method by selecting suitable terminology specific to milk analysis. Qualitative Descriptive Analysis (QDA) was used to assess the quality of the milk samples.
5.1.4. Results

5.1.4.1. Inhibition of food-borne pathogens in milk

Samples of pasteurized milk inoculated with food-borne pathogens stored at different temperatures were tested to evaluate the effect of ppABP on the growth of the pathogens, which included *L. monocytogenes* Scott A, *M. luteus* ATCC 9341 and *Staph. aureus* FRI 722.

5.1.4.1.1. Storage at low temperature (4 ± 2°C)

The viable count of the pathogens in milk samples stored at 4°C was monitored at 3 days interval for a period of 13 days. During the period of storage at 4 ± 2°C, in control milk samples, the count of *L. monocytogenes* Scott A increased above 8 log_{10} CFU/ml [Fig. 5.1(a)]. In milk samples with added 400 AU/ml of ppABP, the count of *L. monocytogenes* Scott A decreased from 4 log_{10} to 2 log_{10} CFU/ml values within 24 h, and thereafter the count remained constant during the entire storage. The positive control samples, where nisin was added at a concentration of 1 mg/ml (1000 U) also showed similar results [Fig. 5.1(a)].

Other common contaminants of food industry tested include *Staph. aureus* FRI 722 and *M. luteus* ATCC 9341. Interestingly, there was complete reduction of the pathogens within 4 days in the milk samples with ppABP [Fig. 5.1(b,c)]. While, in control samples, the viable count of the indicator strain slightly increased during the incubation period. The milk samples with added ppABP were found stable during the entire incubation period. The total plate count of the pasteurized market milk samples was found to be 4.2 log_{10} CFU/ml. The count of the normal flora remained almost static with slight increase to 5 log_{10} CFU/ml in the presence of ppABP, whereas in the control milk the count got increased to 8 log_{10} CFU/ml (Fig. 5.1.2) within 6 days of the incubation.
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**Figure 5.1.1.** Effect of ppABP from *B. licheniformis* Me1 on the growth of pathogens in milk samples stored at 4 ± 2°C; (a) *L. monocytogenes* Scott A, (b) *Staph. aureus* FRI 722, and (c) *M. luteus* ATCC 9341. No ppABP (■) and 1600 AU/ml of ppABP of Me1 (●), 1600 AU/ml of nisin (●) were added to milk samples before inoculation with the tested pathogens. Each point is the mean ± SEM of three independent experiments.

**Figure 5.1.2.** Effect of ppABP on normal microflora in milk samples during 13 days incubation at 4 ± 2°C; a) Milk with ppABP, b) Control milk. Each point is the mean ± SEM of three independent experiments.
5.1.4.1.2. Storage at higher temperature (28 ± 2°C)

The milk samples kept at 28 ± 2°C were analyzed daily to determine the viable count of the pathogens for an incubation period of 4 days. In the control milk samples, the count of all the pathogens increased exponentially during the storage. In case of *L. monocytogenes* Scott A, a reduced growth rate and a lesser count of viable cells was observed as compared to that of the control samples [Fig. 5.1.3(a)]. However, the presence of ppABP in milk samples caused a drastic reduction in the number of viable cells of *M. luteus* ATCC 9341 and *Staph. aureus* FRI 722, and complete inhibition was observed by 4th day of the incubation [Fig. 5.1.3(b, c)]. Interestingly, none of the treated milk samples got spoiled even after 4 days of incubation at room temperature, while, curdling and off-odour was noticed in control milk samples just after 24 h of storage. Also, there was a reduction in the count of normal flora in milk samples treated with ppABP as compared to the control samples (Fig. 5.1.4).

![Figure 5.1.3](image-url)

*Figure 5.1.3.* Effect of ppABP to control indicator organisms in milk samples stored at 28 ± 2°C; (a) *L. monocytogenes* Scott A, (b) *Staph. aureus* FRI 722 and (c) *M. luteus* ATCC 9341 No ABP (■) and 1600 AU/ml of ppABP (●) were added to milk samples before inoculation with the indicator organisms. Each point is the mean ± SEM of three independent experiments.
5.1.4.2. Effect of ppABP on the inhibition of *L. monocytogenes* Scott A in dairy products

5.1.4.2.1. In cheese

The ABP of *B. licheniformis* Me1 was applied to commercially available cheese by dipping pieces of cheese (1 g) in two different concentrations of ppABP solution and then the development of inoculated *L. monocytogenes* Scott A was monitored at 4 ± 2°C. In the control samples, the count of the pathogen increased during the incubation period and reached 4 log\(_{10}\) CFU/ml within 6 days of incubation (Fig. 5.1.5). However, a decrease in the number of viable cells of *L. monocytogenes* Scott A was observed in the cheese samples coated with ppABP solution. Both the dilution of ppABP was found to be effective. As the concentration increased to 1600 AU/ml, the degree of inhibition increased indicating the complete inhibition of the pathogen is possible if higher concentration of ppABP concentration is used.

5.1.4.2.2. In paneer

The efficacy of the ABP of *B. licheniformis* Me1 to inhibit the development of pathogens in paneer was determined in two conditions. The number of viable cells of *L. monocytogenes* Scott A in paneer samples incorporated with 1600 AU/ml of ppABP during the manufacture of paneer reduced to ~1 log\(_{10}\) CFU/ml within 24 h of incubation (Fig. 5.1.6). The paneer samples coated with ppABP by dipping in ppABP solutions of different concentrations (400 and 1600 AU/ml) showed significant lower viable counts and a delay of 4 days in the development of *L. monocytogenes* Scott A (Fig. 5.1.6). However, in the control samples, the count of *L. monocytogenes* Scott A increased during the incubation period.
5.1.4.3. Sensory analysis of milk samples

Results of the sensory analysis indicated that the milk samples had typical white colour, optimum body, milky and creamy aroma, slightly sweet taste. There was no significant difference between the control and the pABP added milk samples (Fig. 5.1.7). The milk samples with both the effective concentration of ppABP were acceptable.
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5.1.5. Discussion

The shelf-life of pasteurized milk is expected to be 10-14 days in some countries, while in some 4-5 days or even less, depending on the state of art of processing, handling and storage conditions (Rysstad and Kolstad 2006). Cooling reduces the bacterial growth but does not eliminate the microorganisms already present in the milk. Usually in a milk sample, the initial acceptable bacterial load (10⁴ CFU/ml) will not reach the limit of 10⁶ CFU/ml for at least 4 days, if the storage temperature is 4°C. Conclusively, the addition of ppABP to pasteurized milk stored at 4°C seems to have less importance in practical applications. However, refrigeration temperature are not always constant during food handling and in case of cross-contamination, milk at low temperatures favours the predominance of psychrotrophic bacteria, leading to the degradation in the nutritive property of the milk and food-poisoning outbreaks. This explains that the shelf-life of food products stored at lower temperatures is largely determined by the growth of psychrotrophic bacteria. Thus, the control of spoilage and pathogenic microorganisms in food products with antimicrobial compounds, especially with those ABPs obtained from a bacterial strain isolated from natural ecological niche can be a novel approach in order to increase the shelf life of the processed foods. Furthermore, milk can serve as a model system to evaluate the effect of components of milk on the activity of the ABP against food-borne pathogens, as reported elsewhere (Bizani et al. 2008; Maisnier-Patin et al. 1995).

![Sensory evaluation of milk added with ppABP; control (O), 400 AU/ml (■), 1600 AU/ml (Δ).](image)

**Figure 5.1.7** Sensory evaluation of milk added with ppABP; control (O), 400 AU/ml (■), 1600 AU/ml (Δ).
Several reports are available on the preservation of milk and milk-based foods with added antimicrobial compounds, such as nisin and pediocin from LAB in the last few years (Davies et al. 1997; Harris et al. 1991; Kim et al. 2008; Pinto et al. 2011; Zapico et al. 1999). However, strains of *L. monocytogenes* demonstrating increased tolerance or resistance to nisin have also been reported (Martinez et al. 2005; Mazzotta et al. 2000). Furthermore, the efficacy of nisin to inhibit food-borne pathogens in milk largely depends upon the fat content. For instance, nisin activity against *L. monocytogenes* dropped by 33% in skim milk, by 50% in milk with 1.2% fat, and by 88% in milk with 12.9% fat (Jung et al. 1992). Thus, research for new compounds from food-grade microorganisms showing inhibitory activity against wide range of food-borne pathogens is an interesting and important field. Among ABPs-producing Gram-positive bacteria, *Bacillus* is an emerging organism for application as biopreservatives (Abriouel et al. 2010; Baruzzi et al. 2011).

In the present study, the addition of ABP of *B. licheniformis* Me1 to milk samples inoculated with pathogens resulted in either reduced growth of *L. monocytogenes* Scott A or complete lysis of the cells in case of *M. luteus* ATCC 9341 and *Staph. aureus* FRI 722. The growth inhibition of pathogens suggests that the ABP has bacteriolytic effect; this had been already demonstrated by monitoring the effect of the ABP on the growth pattern of the pathogens in BHI broth (section 3.4.11/12/13). The bacteriostatic nature of the ABP can also be concluded on terms that the viable counts of *L. monocytogenes* Scott A remained constant during the entire storage after a decrease in the counts. Similar observation was reported for cerein 8A treated milk samples inoculated with *L. monocytogenes* by Bizani et al. (2008). He observed a concentration of 160 AU/ml was effective in control of *L. monocytogenes* in UHT and pasteurized milk. Zapico et al. (1999) also achieved a reduction of 3.7-3.8 log units in whole milk and 3.6 log units in fat-in-water emulsion by the addition of 100 IU/ml of nisin. Addition of pediocin 5 showed a bactericidal effect on three *L. monocytogenes* strains inoculated in partially skimmed milk containing 1% and 3.25% milk-fat at 4°C (Huang et al. 1994). Gallo et al. (2007) reported that the reduction of *L. innocua* in liquid cheese whey with a combination of low pH and nisin (pH = 5.5, 300 IU/ml of nisin) was more at 7°C than at 20°C. Likewise, in the present study, a drop in storage temperature along with ABP resulted in high degree of reduction in growth of *L. monocytogenes* in the milk samples. There are no reports on the studies of the effect of ABP from *Bacillus* in preservation of
milk sample against pathogens, *Staph. aureus* and *M. luteus*. In this study, complete inhibition of *Staph. aureus* FRI 722 and *M. luteus* ATCC 9341 in the milk samples with added ABP kept at high and low temperatures was observed within 4 days as compared to the control. Pinto et al. (2011) also observed a higher growth of *Staph. aureus* in skim milk samples as compared to all other nisin-treated samples (100 - 500 IU/ml). Moreover, the ABP was found to have a considerable inhibitory effect on normal flora present in the milk, and count has been controlled at both tested temperature, for a period of time as compared to control sample. These findings suggest the possible application of the ABP from *B. licheniformis* Me1 for control of wide range of food-borne pathogens/spoilage microorganisms in milk.

Further studies were also carried out to determine the biopreservative efficacy of the ABP of *B. licheniformis* Me1 in milk-based food products, such as cheese and paneer. Significantly, lower viable counts of *L. monocytogenes* Scott A were observed in cheese and paneer samples flooded with ABP solution when compared with the controls. There are reports on the cheeses made with sufficient nisin to provide protection against growth of *Staph. aureus*, *L. monocytogenes* and *Clostridium* spp. (Davies et al. 1997; Pinto et al. 2011; Zottola et al. 1994). Davies et al. (1997) observed inhibition of *L. monocytogenes* and increase in self-life of ricotta-type cheese up to 8 weeks in the presence of 2.5 mg/ml of nisin. Bizani et al. (2008) observed that the addition of 400 AU/ml of cerein 8A during the manufacture of Minas-type cheese increased the time lag to reach exponential growth for *L. monocytogenes* as compared to cheese without bacteriocin. They also reported when cheese flooded with bacteriocin (400 AU/ml), the count of *L. monocytogenes* was below 2 log\(_{10}\) CFU/ml up to 10\(^{th}\) day of incubation at 4°C.

The effect of ppABP to inhibit pathogens in cheese and paneer samples was highly dependent on the concentration of the ABP. The number of *L. monocytogenes* Scott A in case of surface applied paneer samples with ppABP solution of 1600 AU/ml was significantly lower than 400 AU/ml treated samples, indicating that higher concentration may lead to the complete inhibition of the pathogen. The direct incorporation of ppABP (1600 AU/ml) in boiled milk during the manufacture of paneer prior to the start of milk coagulation was found to be more effective in control of *L. monocytogenes* Scott A development as compared to surface application of the ppABP on paneer. The reason for this difference in case of ppABP incorporated
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Paneer samples might be due to the binding of the ppABP to milk fat/protein or would have entrapped to the floating coagulated proteins in the milk. Similar results were found by the addition of nisin to Anthotyros cheese (Samelis et al. 2003). The addition of 500 mg of nisin to the whey prior to cheese making was found to maintain *L. monocytogenes* count below the initial population level up to 30 to 40 days of incubation at 4°C as compared nisin added to cheese after post processing. However, Bizani et al. (2008) reported the less effective activity of incorporated ABP in comparison with its application in cheese surface.

The less efficiency of antilisterial effect of ppABP in paneer as compared to cheese may be due to high average moisture content of paneer prepared in the laboratory. Furthermore, the increase in the number of *L. monocytogenes* Scott A in the ppABP-treated samples after four days of incubation might be due to one of the several other factors, including recovery of *L. monocytogenes* Scott A or development of ABP resistant sub-population of the pathogen.

### 5.1.6. Conclusion

The ABP from *B. licheniformis* Me1 proved to be an efficient antimicrobial agent against food-borne pathogens in milk and milk-based food products. The particular properties of this ABP (pH and temperature tolerance, proteolytic inactivation, wide range of inhibitory activity, stability during storage) including control of pathogens in food systems and sensorily acceptable when incorporated to milk, makes convincing evidence for the potential application of this bacteriocin as biopreservatives in food items. These observations indicates that the ABP from *B. licheniformis* Me1 can be used as an alternative to the commonly used chemical preservatives (e.g., Nitrate, NaCl) and biological antimicrobial agents, such as nisin and pediocin for food preservation. The application of this ABP in food system can be an efficient way of extending shelf life and food safety through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of raw materials and food products.
Chapter – 5, Section – 2

Development and application of active films for food packaging using antibacterial peptide of *Bacillus licheniformis* Me1
5.2.1. Abstract

In this study, an attempt was made to evaluate the effectiveness of ppABP produced by *B. licheniformis* Me1 for food preservation by means of active packaging (packaging film coated with ppABP). The ppABP of the culture *B. licheniformis* Me1 was used for the development of active packaging films using two different packing materials [low density polyethylene (LDPE) and cellulose films]. Two different methods for the preparation of active films with ABP were used; soaking and spread coating. The activated film showed inhibitory activity against tested pathogens, such as *M. luteus* ATCC 9341, *L. monocytogenes* Scott A, *Staph. aureus* FRI 722, *B. cereus* F 4433 and *Salm. typhimurium* MTCC 1251, which are major contaminants of dairy industry. The release study of ppABP from coated film showed that the LDPE films liberated ABP as soon as it comes in contact with water, while gradual release of coated ppABP was observed in case of cellulose films. This indicates that the ABP got adsorb to the LDPE film, while cellulose film have the ability to absorb the ppABP, which makes it to have a controlled release of ABP over a period of time. Release of the ABP from films under simulating food storage conditions was also evaluated. The activated LDPE films demonstrated a loss in activity at both the temperatures tested (4 and 37°C) and with increased pH (7 and 9), while the cellulose film retained its activity at the above conditions. However, a regain in activity was observed in the LDPE films incubated at 4°C after 8 h. The biopreservative efficacy of the activated films was studied in two dairy products; cheese and paneer. The experiment revealed the bacteriolytic and bacteriostatic effect of ppABP on the dairy pathogen, *L. monocytogenes* Scott A. The ppABP from active films got diffused into the food matrix and reduced the growth rate and maximum growth population of the target microorganism. Overall, both the types of active films were found to be effective carrier of the ABP and can be used as a packaging material to control spoilage and pathogenic organisms in food, thereby extending the shelf-life of foods.
5.2.2. Introduction

In response to the changes in market trends and increasing demands of consumers for high quality, safe and extended shelf-life of food products, active packaging is creating a niche in the market and is becoming increasingly significant. Active packing has been defined as “a type of packaging in which the package, the product and the environment interact to extend shelf-life or improve safety or improve convenience or sensory properties while maintaining the quality and freshness of the product (European FAIR-project CT 98-4170).

Antimicrobial packaging is a promising and innovative form of active packaging. One of the major concerns of the food industry is the spoilage of food by microbial contamination. Most of the contamination of the foods occurs mainly on the surface due to post processing and handling (Perez-Perez et al. 2006). The delay or prevention of spoilage of foods has been done either by dipping and spraying foods with antimicrobials or by packing the foods with antimicrobial packages. The former approach is less efficient as these compounds may get neutralized on contact or get diffused rapidly into the food matrix or may get diluted to below effective concentration (Appendini and Hotchkiss 2002; Hoffman et al. 2001; Quintavalla and Vicini 2002). Whereas, antimicrobial packaging is an efficient technology and helps in reducing the risk of pathogen development, as well as extending shelf-life and maintaining food quality and safety (Han 2000; Mauriello 2005). Furthermore, the packaging films with antimicrobial agents confer residual activity during transport, storage and distribution (Cutter 2001; Quintavalla and Vicini 2002).

The use of bacteriocins or other natural antimicrobials in packaging films to control food spoilage and pathogenic organisms has increased significantly as it involves lesser risk for the consumers (Nicholson 1998; Perez-Perez et al. 2006; Suppakul 2002). Among bacteriocins, nisin has been the subject of extensive study and use, either as direct application in food or indirect use in antimicrobial packages for food biopreservation (An et al. 2000; Hoffman et al. 2001; Kim et al. 2002b; Ko et al. 2001). Similarly, Natrajan and Sheldon (2000) studied the effectiveness of using nisin-coated polymeric films such as PVC, linear low density polyethylene (LLDPE) and nylon on fresh broiler drumstick skin for inhibition of Salm. typhimurium. Nisin coated into a LDPE film was used to inhibit M. luteus ATCC 10240 and the microflora of raw milk during storage (Mauriello et al. 2005). The growth of aerobic bacteria reduced significantly in chopped meat, where active packing using cellophane coated with nisin was utilized (Guerra 2005).
Studies of new food-grade bacteriocins as preservatives and development of suitable systems for bacteriocin treatment of plastic films for food packaging are important issues in food biotechnology, both for implementing and improving effective hurdle technologies for better preservation of food products. Although, some of the *Bacillus* spp. are found safe for use in food and agricultural industry (Sharp et al. 1989; PR Newswire 2009), and are known to produce several antimicrobial compounds that show inhibitory activity against broad range of food-borne pathogens (Stein 2005; Abriouel et al. 2010), the use of *Bacillus* bacteriocins in packaging materials is so far unknown. Study on new food-grade bacteriocins as preservatives and development of packages with such bacteriocins may also offer an alternative to nisin. In the previous section of this Chapter, the biopreservative efficacy of ABP of the culture *B. licheniformis* Me1 by direct application in milk and milk-based products was investigated. The ABP was found to be suitable for application in such food systems to control the growth of food-borne and spoilage microorganisms during storage.

Thus, keeping in view the potential application of antimicrobial compounds for development of antimicrobial packages that would prevent the contamination and growth of spoilage microorganisms in food systems, the indirect use of ABP through active films was evaluated. As an attempt to determine this, active packaging films incorporated with the partially purified ABP of the culture *B. licheniformis* Me1 was developed. Further, the effectiveness of such active films in inhibiting the growth of common food-borne pathogens was evaluated. The release of ABP from the activated film and the efficacy of the developed films in inhibiting the growth of *L. monocytogenes* Scott A during the storage of dairy products were also verified.

**5.2.3. Materials and methods**

**5.2.3.1. Packaging material and bacteriological media**

The packaging films (LDPE and cellulose) used for developing active films with ABP of *B. licheniformis* Me1 was generous gift from the Department of Food Packaging, CFTRI, Mysore, India. The media, such as LB and BHI used for the growth of the indicator organisms were procured from Himedia, India.
5.2.3.2. Bacterial strain and culture conditions

The pathogens used in this study included *M. luteus* ATCC 9341, *L. monocytogenes* Scott A, *Staph. aureus* FRI 722, *Salm. typhimurium* MTCC 1251 and *B. cereus* F 4433. The culture *B. licheniformis* and the pathogenic strains used in this study were maintained as discussed in section 2.3.2.

5.2.3.3. Preparation of antimicrobial agent

The ppABP of the culture *B. licheniformis* Me1 to be used for making active films was prepared as discussed in section 5.1.3.2. The antibacterial activity of the ppABP was also evaluated as described in section 3.3.8.

5.2.3.4. Preparation of antimicrobial packaging films

5.2.3.4.1. Soaking

A solution of ppABP was prepared at a final concentration of 800 and 6400 AU/ml. Samples of low-density polyethylene (LDPE) and cellulose films of size 2 x 2 cm were soaked in ppABP solution of the above concentrations for different incubation period (1 and 5 h for LDPE, and 1, 3 and 5 h for cellulose films). After soaking, the films were air-dried and then their antibacterial activity was assayed against the indicator organisms by bioactive assay.

5.2.3.4.2. Coating by spreading

The ppABP was prepared at a concentration of 6400 AU/ml in sterile distilled water. LDPE films and cellulose films (size 30 x 3 cm) were spread-coated with ppABP uniformly with the help of a spreader dipped in bacteriocin solution. After spreading, the films were exposed to warm air in order to dry the ppABP solution and promote a homogenous distribution of the ppABP onto the surface of the films. Once dried, the treated films were assayed for antibacterial activity against the indicator organisms by bioactive assay.

5.2.3.5. Bioactive assay

The above treated LDPE and cellulose films were assayed for antimicrobial activity against food-borne pathogens, such as *M. luteus* ATCC 9341, *L. monocytogenes* Scott A, *Staph. aureus* FRI 722, *B. cereus* F 4433 and *Salm. typhimurium* MTCC 1251 as described elsewhere (Mauriello et al. 2004). Briefly, samples (2 x 2 cm) of the treated films were placed onto the surface of BHI soft
(0.8%) agar plates seeded with $10^4$ CFU/ml of 16 ± 2 h grown indicator organism. The treated face of the film was in contact with the agar. The untreated films were also assayed and served as negative controls. After incubating the plates at 37°C for 18 ± 2 h, the antagonistic activity was evaluated by observing a clear zone of growth inhibition in correspondence with the active film.

5.2.3.6. Adsorption and release of ABP from spread-coated active films

For checking the adsorption rate of ppABP by the films, drops of 20 µl of 6400 AU/ml ppABP were spotted on the surface of untreated LDPE and cellulose films, and then removed after 1, 5, 10, 15, 30, 45, 60 min (Mauriello et al. 2004). The film was then placed on BHI agar plates seeded with *M. luteus* ATCC 9341 for checking the antibacterial activity, as described above (section 5.2.3.5).

The study of release of ppABP from active LDPE and cellulose films was performed as described previously (Mauriello et al. 2004). Briefly, 20 µl of sterile deionised water was spotted onto the surface of active films with ppABP and then incubated in a humid chamber. The water was removed at 1 h interval for 5 h. The collected water samples were then checked for antibacterial activity against *M. luteus* ATCC 9341 by well diffusion assay as described previously in section 3.3.4.

In order to check the residual activity of the films after release of ABP, the release experiment was also conducted by another method. Briefly, the active films with ppABP (2 x 2 cm) were placed in 2 ml of sterile deionised water and kept under gentle stirring (100 cycles/min) at 28 ± 2°C. The antibacterial activity of the solution in which film was dipped was checked against *M. luteus* ATCC 9341 at 1, 2, 4, 8, 24 and 48 h of incubation using agar well diffusion assay (section 3.3.4). The residual activity of the treated films was checked by placing the film after treatment, on the agar plates seeded with indicator organism, as described previously (section 5.2.3.5).

Similar experimental procedure was followed to determine the release of ppABP from active films under stimulating conditions such as temperature and pH. The activated films were placed in 2 ml of sterile distilled water in different test tubes and then incubated at different temperatures (4 and 37°C). At different incubation times (1, 3, 8, 12 and 24 h), samples of 50 µl of water from each incubation temperature were taken and assayed for antimicrobial activity against *M. luteus*
ATCC 9341, by well diffusion assay (section 3.3.4). Simultaneously, the activity of the temperature treated activated films removed at different incubation period were also evaluated as described above (section 5.2.3.5).

For determining the effect of pH on the release of ppABP from the activated films, the films were placed in 5 ml of PBS solutions of pH 3, 7 and 9 and incubated at room temperature (25 ± 2°C) for 6 h. After incubation, the pH of the PBS solutions with pH 3 and 9 were neutralized to pH 7 before testing its activity. Also, the films were dried and assayed for antimicrobial activity against the indicator strain, *M. luteus* ATCC 9341.

5.2.3.7. Application of active films as packing material

The cottage cheese (paneer) was prepared as described previously (section 5.1.3.5). After preparation, the paneer was cut in to pieces of 1 g and divided into three sets. The paneer pieces were surface inoculated by dipping paneer pieces in a suspension of *L. monocytogenes* Scott A at a concentration of 10⁶ CFU/ml. The paneer pieces from first and second set were wrapped individually with spread coated LDPE and cellulose films with ABP solution of concentration, 1600 AU/ml. The third set of paneer pieces were wrapped individually by untreated film of both types separately and served as the negative control. All the wrapped paneer pieces were stored in sterile containers at 4 ± 2°C. Individual samples were removed at an interval of 3 days for a period of 12-15 days, homogenised in 9 ml of saline and then appropriate dilution were placed on LO agar. The plates were then incubated at 37°C for 24 h and CFU/ml of the sample was determined.

For the preservation studies of cheese using active packages, the cheese was purchased from local market and made into 1 g pieces. The procedure for inoculation of cheese with indicator strain, packaging and detecting the effect of active packages on the viability of the inoculated pathogen was done as described for paneer. The results of both paneer and cheese biopreservation studies are based on three independent experiments.
5.2.4. Results

5.2.4.1. Antibacterial activity of activated films

The activated LDPE and cellulose films with ABP of *B. licheniformis* Me1 were prepared by two different methods; soaking and spread-coating. Both the types of activated films showed antibacterial activity against indicator organisms, *M. luteus* ATCC9341, *L. monocytogenes* Scott A, *Staph. aureus* FRI 722, *B. cereus* F4433 and *Salm. typhimurium* MTCC 1251. Some of plate images showing inhibitory activity of the activated LDPE and cellulose films against tested pathogens are shown in Figure 5.2.1 and Figure 5.2.2, respectively. The zone of inhibition was not confined to the film area of both the types of film. The soaked activated films showed regular zone of inhibition along the periphery of the film as compared to spread-coated films, suggesting even diffusion of the ppABP from the film into the agar. The activated films prepared with two different concentrations of ppABP (800 and 6400 AU/ml) exhibited difference in the intensity of the inhibitory activity against the indicator strains. There was no marked difference in the intensity of the antimicrobial activity among the activated LDPE films against *L. monocytogenes* Scott A prepared by soaking at different incubation times (1 and 5 h) [Fig. 5.2.3(i)]. However, the activated cellulose films showed difference in inhibition intensity when treated for 1, 2 and 5 h [Fig. 5.2.3(ii)]. In all the cases, the untreated films did not show any activity against the indicator strains.

Interestingly, the coated films displayed a clear and stable antilisterial activity after 3 months of coating which was kept under at 4°C and at room temperature. Moreover, the activated film maintained its activity even after rubbing.

5.2.4.2. Adsorption of ppABP to films with increasing time

In order to assess whether the binding of ppABP was affected by the time of incubation with bacteriocin, aliquots (20 μl) of ppABP solution (6400 AU/ml) were spotted on the surface of the LDPE and cellulose films for different contact times (1, 5, 10, 15, 30, 45, 60 min) and the antimicrobial activity of the film was then tested.
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Figure 5.2.1. Antibacterial activity of ppABP activated LDPE films against food-borne pathogens. Inhibition zone of 1) soaked activated and 2) spread activated films with 6400 AU/ml of ppABP (a), 800 AU/ml of ppABP (b) and Untreated films (c) against pathogens (i) L. monocytogenes Scott A, (ii) Staph. aureus FRI 722 and (iii) B. cereus F 4433.

Figure 5.2.2. Antibacterial activity of ppABP activated cellulose films against food-borne pathogens. Inhibition zone of (1) Soaked activated films and (2) Spread activated films, with 6400 AU/ml of ppABP (a) or 800 AU/ml of ppABP (b) and Untreated films (c), against tested pathogens, such as (i) M. luteus ATCC 9341, (ii) L. monocytogenes Scott A, (iii) Salm. typhimurium MTCC 1251 (iv) Staph. aureus FRI 722.
Figure 5.2.3. Antibacterial activity of packaging films soaked in ppABP solution for different incubation time. The inhibition zone of (i) LDPE and (ii) cellulose films soaked in ppABP solution (1600 AU/ml) for (a) 1 h, (b) 2 h, (c) 5 h and (d) untreated.

As shown in Figure 5.2.4(a), in LDPE film, the observed antibacterial activity, in correspondence to the spot area of ppABP solution, was almost the same for all of the contact time except a slight increase at 1 h. It may be probably due to drying of ppABP by slight evaporation of water and thereby an increase in concentration of ppABP in the spot. While, in case of cellulose film an increasing trend in the zone of inhibition diameter against the indicator organism was observed in correspondence to the ppABP spotted region as the contact time of the ppABP solution with the film increases [Fig. 5.2.4(b)].

Figure 5.2.4. Antibacterial activity of LDPE and cellulose film spotted with ppABP for different contact time. Inhibition zone corresponding to 1, 5, 10, 15, 30, 45, and 60 min of contact time with ppABP (6400 AU/ml) in case of LDPE (a), and Cellulose (b) film.
5.2.4.3. Release of ppABP from the activated film

The activated LDPE films were subjected to ppABP release in water at different incubation times (every 1 h till 5 h). The water spots of 20 µl collected after different incubation times showed the same intensity of antibacterial activity against *M. luteus* ATCC 9341 in agar well diffusion assay which indicates the release of the ppABP from activated LDPE film. There was no back-absorption of the ppABP from the water to the film until 5 h, as there was no marked difference in zone of inhibition of the collected water samples. Although, the release of the ppABP was confined to the area where the water drops were placed, the activated films, after being assayed for the ppABP release, showed inhibitory activity. This may be attributed to the fact that the ppABP present in the outer circumference of the water drop placed on the film might be getting diffused and inhibiting the pathogens in the area of the spot of released ppABP. To confirm this further (release of ppABP from LDPE), the film was soaked in water under stirring condition and the antibacterial activity of 20 µl of water collected at every 1 h interval for 5 h was checked. There was no marked difference in the zone of inhibition for any of the water samples collected till 5 h [Fig. 5.2.5(a) (ii)]. After the release assay, when the film was checked for antibacterial activity, it failed to show any zone of inhibition against *M. luteus* ATCC 9341 [Fig. 5.2.5(a)(i)], indicating complete release of ppABP into water.

The activated cellulose film showed contradictory results as compared to LDPE films in the ppABP release assay. Initially, the water drops did not show any zone of inhibition against *M. luteus* ATCC 9341 until 2 h. After 2 h of incubation, a zone of inhibition of very small diameter was observed which slightly increased with further increase in incubation time of water drops [Fig. 5.2.5b (ii)]. The activated film, after being assayed for ppABP release, displayed antibacterial activity, indicating that the ppABP is still retained in the film [Fig. 5.2.5b (i)].

5.2.4.4. Release of ppABP from the activated films under simulated conditions

5.2.4.4.1. Release of ppABP at different temperature

The activated LDPE film showed a loss in activity within one hour of incubation at both the temperatures (4 and 37ºC) [Fig. 5.2.6(1)]. However, a regain in activity was observed in the films which were incubated at 4ºC, from 8 h onwards.
In the case of activated cellulose film, there was no marked difference in the release of ppABP in both the temperature conditions [Fig. 5.2.6 (2)]. Even after 24 h of incubation in sterile distilled water, the activated cellulose film retained antibacterial activity, indicating its potential use in long term storage of food products. Furthermore, there was a gradual increase in the inhibitory activity of the water, indicating controlled release of the ppABP from the film and not dependant on the temperature difference.

Figure 5.2.5. Antibacterial activity of ppABP activated films placed in sterile water under stirring conditions. Release of ppABP from LDPE film (a) and Cellulose film (b); i) the activity of the film after treatment, ii) the inhibitory activity of sterile water in which treated film were soaked for different incubation times.

Figure 5.2.6. Antibacterial activity of ppABP activated films placed in sterile water which incubated at different temperatures for different time. Release of ppABP from activated (1) LDPE and (2) Cellulose films incubated in different temperatures, 4°C [a (i,ii)] and 37°C [b (i,ii)] for different times (1, 3, 5, 8, 12 and 24 h).
5.2.4.4.2. Release of ppABP at different pH

The activated film of LDPE and cellulose was kept under different pH (3, 7 and 9) for 6 h and the film was then checked for antibacterial activity. In the case of LDPE, the film kept at the low pH (3) showed activity with less intensity, whereas, at pH 9, the film did not exhibit any antibacterial activity (Fig. 5.2.7). On the other hand the cellulose film retained its activity in all the pH conditions, however, a decrease in intensity of activity of the cellulose film kept at pH 9 was observed as compared to pH 3 and 7. No activity for the neutralised PBS solution was detected and this may be due to dilution of bacteriocin in higher volume of water (5 ml).

![Figure 5.2.7. Antibacterial activity of ppABP activated films soaked in 100 mM PBS of different pH values. Inhibitory activity of the activated films, LDPE (1) and cellulose (2) kept in different pH solutions; pH 3(a), 7(b) and 9(c) for 8 h.](image)

5.2.4.5. Inhibition of L. monocytogenes Scott A in dairy products

After preparation, samples of paneer pieces inoculated with L. monocytogenes Scott A were packed with active films (cellulose and LDPE) coated with ppABP and stored in sterile container at 4 ± 2°C. The effect of the activated films on the reduction of Listeria population compared with the control was observed just within 24 h of incubation of paneer samples. The number of viable cells of L. monocytogenes Scott A in paneer samples packed with activated LDPE films showed a reduction of around 1 log_{10} CFU/ml (Fig. 5.2.8). On the other hand, until 8 days, a slow and steady decline and a bacteriostatic effect in the count of Listeria was observed in paneer samples packed with activated cellulose film (Fig. 5.2.9) and further incubation resulted in no decrease of the cells. However, after 12^{th} day there was an increase in the number of viable cells. This can be attributed to slow rate of release of ABP from the cellulose film as compared to the LDPE. The reduction of the Listeria population indicates that the activated film exhibited bacteriostatic and bactericidal effect. Moreover, the paneer samples covered with LDPE started spoiling by 8^{th} day of incubation; while the cellulose packed paneer samples were stable up to 12 days.
A similar observation was noted for cheese samples preserved with packed activated films. However, the effect of the ppABP was higher as compared to that in paneer samples. In the treated samples, a reduction of $2 \log_{10}$ CFU/ml of the inoculated pathogen was observed in both the types of packages (LDPE and cellulose packed cheese samples) and in the case LDPE packed cheese, a bacteriostatic effect on pathogen was observed (Fig. 5.2.10). Whereas, in cellulose packed cheese, the reduction was continuous and a bactericidal action observed (Fig. 5.2.11). However, a continuous increase in the number of viable cells of *L. monocytogenes* Scott A was noticed in the cheese samples packed with untreated films in both cases.

![Figure 5.2.8](image1.png)

**Figure 5.2.8.** The viable count of *L. monocytogenes* Scott A in paneer samples packed with ppABP activated LDPE films during incubation for 12 days at 4°C; Treated package (1600 AU/ml) (●) and untreated package (●). Each point is the mean ± SEM of three independent experiments.

![Figure 5.2.9](image2.png)

**Figure 5.2.9.** The viable count of *L. monocytogenes* Scott A in paneer samples packed with ppABP activated cellulose films during incubation for 12 days at 4°C; Treated package (1600 AU/ml) (●) and untreated package (●). Each point is the mean ± SEM of three independent experiments.
5.2.5. Discussion

Foods are complex ecosystems with a range of microbial compositions, which may vary from (commercially) sterile foods to raw or fermented foods. In commercially sterile foods, post-process contaminants may easily proliferate. Increasing interest of consumers for use of bio-preserved foods has sparked the use of...
bacteriocins, especially from food-grade microorganisms because of their better adoptability in food systems (Galvez et al. 2007). The use of bacteriocins to assure microbial food safety is a novel approach and an alternative to chemical preservatives. The efficacy of the bacteriocin to control spoilage microorganisms in food largely depends on the microbial load of the contaminant, bacteriocin concentration and bacteriocin distribution (Galvez et al. 2007). One of the practical problems associated with the use of bacteriocins as food preservatives is the heterogenic distribution within the food system. In order to maximize the biopreservative potential of these antimicrobials, it is important to develop a reliable system in order to attain the objective of biopreservation of food products (McMullen and Stiles 1996).

Apart from direct application of bacteriocins by spraying and dipping, \textit{ex situ} produced bacteriocins can also be applied in the form of immobilized preparations, in which the partially-purified bacteriocin or the concentrated cultured broth is bound to a carrier (Chen and Hoover 2003; Galvez et al. 2007). In the last few years, this method of application of bacteriocins have received considerable interest, since the carrier acts as a reservoir and the concentrated bacteriocin molecules diffuses slowly into the food matrix ensuring a gradient-dependent continuous supply of bacteriocin. Furthermore, the carrier also protects the bacteriocin from inactivation by interaction with food components and enzymatic inactivation. Moreover, the localized application of bacteriocin molecules on the food surface requires much lower amounts of bacteriocin (compared to application in the whole food volume), decreasing the processing costs.

The activation procedure describes about the effectives of the activated films and thus it is necessary to apply an adequate procedure of activation in order to assure that the antimicrobial substance is linked to the film and is able to retain the antimicrobial activity during the shelf-life of film. Moreover, the activated film has to exert its preservative antimicrobial potential in food systems during storage of packed food. In the present study, the activated films (LDPE and cellulose) with ABP from \textit{B. licheniformis} Me1 showed a zone of inhibition that did not confine to the film area indicating that the ABP diffused from the films into the medium. Furthermore, the ABP retained its activity in both methods of activation (soaking and spread coating),
confirming the results of similar studies (Daeschel et al. 1992; Dawson et al. 2003; Mauriello et al. 2005). The soaking procedure proved to be more effective. Presumably, the regular inhibition zone along the periphery of the soaked activated film might be due to the homogeneous distribution of the bacteriocin on the surface of the films. However, Mauriello et al. (2004) observed irregular inhibition zone and heterogeneous distribution of the bacteriocin for soaked PE-OPA films.

The interactions of the antimicrobial agents with the film matrix have a crucial effect on the antimicrobial activity of the active films (Han 2000; Papadokostaki et al. 1997). The molecular weight, ionic charge and solubility of different additives affect their rates of diffusion in the polymer (Cooksey 2000). During incorporation of additives into a polymer, the polarity and molecular weight of the additive have to be taken into consideration. Lakamraju et al. (1996) reported that a hydrophilic surface adsorbs a higher amount of nisin than hydrophobic one. The LDPE film shows hydrophobic properties and thus rejects the hydrophilic antimicrobial formulations to a greater extent than other films (Natrajan and Sheldon 2000). On the other hand the cellulose film being hydrophilic polymer matrices absorbs higher amounts of ABP with incubation time. Similarly, in our experiments, the cellulose film exhibited a marked difference in the adsorption kinetics than LDPE indicating a higher binding ability for ABP. Adsorption studies done by spotting the ABP on the surface of the films demonstrated that even a quick contact of the bacteriocin with the surface of the film conferred activation, proving the observation of Mauriello et al. (2004). This observation leads to the conclusion that the ABP was actually adsorbed or absorbed by the surface of the films and not migrated from the cut margins into the film in the activity assay.

The diffusion rate of the antimicrobial agent and its concentration in the film must be sufficient to remain effective throughout the shelf-life of the product (Cooksey 2000). Polymer structure affects the release of active compounds (Papadokostaki et al. 1997). Hydrophilic nature of the cellulose film creates greater retention of the ABP by binding. As a consequence of this, results obtained in the release study also showed that the release rate of the active compounds from the cellulose films was lower and inhibition zones smaller in the beginning of the incubation period as compared to activated LDPE films. These results indicate that the release of ABP from the activated cellulose film was time dependent and implies
that the activated cellulose films are suitable for packing of solid foods as the coated ABP will release slowly onto the food surface and inhibit the growth of surface spoilage and pathogenic bacteria. The controlled release of ABP in food packaging applications is important since if an antimicrobial is released from the packaging during an extended period, the activity can also be extended into the transport and storage phase of food distribution (Perez-Perez et al. 2006).

Processed foods have different pH values and are exposed to different temperature profiles during handling storage and distribution. The pH of a product affects the growth rate of target microorganisms and changes the degree of ionization of the most active chemicals, as well as the activity of the antimicrobial agents (Han 2000). Several researchers have found that the increased storage temperature can accelerate the migration of the active agents in the film and deteriorate the protective action of antimicrobial films, due to high diffusion rates in the polymer (Vojdani and Torres 1989a,b; Wong et al. 1996). Furthermore, the storage temperature may also affect the activity of antimicrobial packages. Thus, release of the ABP from films in simulating food conditions should be evaluated to determine the efficacy of the films in controlling pathogens in food systems. The result of the release studies at different temperatures indicates that lower temperatures allow the back adsorption of the ABP to the LDPE films, and thus the film showed activity after 8 h of incubation at 4°C. The reason for this back-absorption behaviour remains unexplained as the mechanism for ABP binding to the plastic film is not known. Some authors have also demonstrated a loss in activity of the antimicrobial LDPE packages at 25°C (Dawson et al. 2003; Mauriello et al. 2005). The cellulose film retained its activity at both the incubation temperatures and pH treatments. This might be probably due to the chemical nature of the cellulose films, where the hydrophilic nature of the film allows higher binding of the hydrophilic antimicrobial compounds (Papadokostaki et al. 1997). This suggests the potential use of cellulose films as a packaging material for the control of spoilage microbes in acidic to alkaline foods, incubated at either lower or higher temperatures, when long-term storage is desired. The possible reason for detection of no activity in the pH solution may be due to the lower concentration of the bacteriocin released in the solution which is probably below the detection limit for agar well diffusion assay, used to detect the antagonistic activity.
To have an effective application of antimicrobial packages to food products, it is important to consider and ascertain the shelf-life of the bioactive films (Bower et al. 1995; Daeschel et al. 1992; Ming et al. 1997). Experiments used to qualitatively monitor the activity and stability of the bacteriocin coated films developed in this study demonstrated that the antilisterial activity was still stable after 3 months of film storage at 4°C and even at room temperature. Therefore, the developed active LDPE and cellulose films were assayed for their antimicrobial activity against *L. monocytogenes* Scott A in challenge tests involving the storage of dairy products at refrigeration temperatures.

Most of the research work in antimicrobial packaging has been focused primarily on the development of various methods and model systems, whereas little attention has been paid to their preservation efficacy in actual foods (Han 2000). With increasing use of minimally processed food with no chemical preservatives and development of active films with bacteriocins as packaging material, research is essential to identify the types of food that can benefit most from antimicrobial packaging materials. Furthermore, many authors have stated that future research into a combination of naturally-derived antimicrobial agents, biopreservatives and biodegradable packaging materials will highlight a range of the merits of antimicrobial packaging in terms of food safety, shelf-life and environmental friendliness (Coma et al. 2001; Nicholson 1998; Rodrigues and Han 2000). Reports are available demonstrating the biopreservation of meat samples using food packaging materials containing bacteriocins (Dawson et al. 2002; Lee et al. 2004; Mauriello et al. 2004; Ming et al. 1997; Scannell et al. 2000). However, reports on the preservation of dairy products using antimicrobial packaging materials are rare (Scannell et al. 2000), especially the application of active packages with bacteriocins from *Bacillus*. The active packaging films (LDPE and cellulose) which were used to coat dairy products, such as paneer and cheese demonstrated the control of inoculated pathogen *Listeria* by reducing the growth rate and maximum growth population and extending the lag period of the target microorganism. Similar observations were reported in raw milk, pasteurized milk and ultrahigh temperature milk (Lee et al. 2004; Mauriello et al. 2005). Scannell et al. (2000) reported that nisin-adsorbed antimicrobial packages reduced the level of *L. innocua* and *Staph. aureus* by ≥2 log and ~1.5 log units respectively, in cheese samples stored in modified atmosphere packaging at 4°C.
The control of the growth of *L. monocytogenes* Scott A by activated films was better in cheese than paneer storage. This may be due to, either the higher superficially concentrated contamination of *L. monocytogenes* Scott A on the paneer pieces, which was more difficult to control, or the nature of the paneer product itself and its possible effect on bacteriocin release and action. Furthermore, the water available (a_w) in case of paneer samples is higher than cheese, which may allow higher proliferation of *Listeria*. An increase in *L. monocytogenes* Scott A viable counts was noted after certain days of storage in all the samples, which may be due to the particular mechanism of action of bacteriocins that can inhibit as many cells as molecules available in the medium (Moll et al. 1999). Increasing the concentration of the bacteriocin in the coating solution may be also experimented with the aim of improving the preservative performance of the bacteriocin-coated films in storage of dairy products, as well as other food products. Furthermore, addition of hurdle molecules such as EDTA, lysozyme, citric acid, lactic acid, lauric acid into the coating solution may improve the antimicrobial performance of bacteriocin-activated films as reported in other studies (Natrajan and Sheldon 2000).

### 5.2.6. Conclusion

Antimicrobial packaging can play an important role in reducing the risk of pathogen development, as well as extending the shelf-life of foods. The cellulose film was found to be more efficient as a carrier of the ppABP produced by *B. licheniformis* Me1 and thus can be exploited for use in food packaging industries. However, further studies with respect to physiochemical properties of the film material after incorporation of ABP are required. The activated films showed residual activity in different simulating conditions, such as pH of food and storage temperatures. Also, the films retained their activity during long term storage at different temperatures. Moreover, both the type of active films (LDPE and cellulose) with ppABP showed potential reduction in the population of tested bacteria in dairy products (cheese and paneer), which signifies the use of the ABP from *B. licheniformis* Me1 in packaging material to control spoilage and pathogenic organisms in food. All these desirable properties of the activated film with ABP of *B. licheniformis* Me1 make them practical for food industrial applications and prove that antimicrobial substances from *Bacillus* can also be used for developing antimicrobial food packaging materials.