DISCUSSION
**Discussion**

*Lactobacillus plantarum* strain LR/14 is a Gram-positive, facultative anaerobic bacterium isolated from the rhizosphere. Two previous studies carried out on strain LR/14 demonstrated its bacteriocinogenic potential, the purification and characterization of two peptide plantaricin LR14 (Tiwari and Srivastava, 2008c) and the promising probiotic properties (Ghosh et al., 2008) as well as identification of *pln* locus from strain LR/14 (Ghosh, unpublished results). The present investigation is an extension of the previous studies in terms of enhancing the yield of antimicrobial peptides (AMPs LR14), and studying the mode of action and expanding their inhibition spectrum, so as to suggest their possible application/(s). Different tests were carried out to demonstrate the biosafety aspects, of these peptides. All this has culminated in suggesting a few applications of AMPs LR14 in a food system.

During its growth *Lb. plantarum* LR/14 synthesizes a number of compounds such as organic acids, including lactic acid, and antimicrobial peptides such as bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial peptides secreted extracellularly by the cell. Plantaricin LR14, a bacteriocin produced by *Lb. plantarum* LR/14 has been purified and characterized (Tiwari and Srivastava, 2008 a, b, c). Plantaricin LR14 showed remarkable thermo-stability and stability in the presence of organic solvents, detergents, and surfactants, with maximum activity in the acidic pH regime. Moreover, the protein was stable under storage. The crude and the purified plantaricin LR14 not only inhibited related strains but also some Gram-positive and Gram-negative pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Yersinia enterocolitica* and urogenic *E. coli* (Tiwari and Srivastava, 2008a, b). Media optimization led to an eight-fold increase in bacteriocin production (Tiwari and Srivastava, 2008c). Purified plantaricin LR14 consisted of two peptides designated as plantaricin LR14α and -β with molecular masses of 3 and 5.6 kDa, respectively and showed synergistic action. Upon amino acid sequencing, the N-terminus of both the peptides was found to be blocked; the partial sequence obtained showed no homology.
with known bacteriocins, thus indicating it to be a novel compound (Tiwari and Srivastava, 2008b).

The one major constraint of the previous work was the yield of the plantaricin. As the present investigation was planned to work out its mode of action and inhibition spectrum, the first aim was to enhance the yield.

Since bacteriocins are proteinaceous in nature and are secreted into the culture medium, most strategies started with a step to concentrate them from the culture supernatant. Different methods, such as, pH-based cell surface adsorption and later desorption (Yang et al., 1992), acid extraction followed by RP-HPLC (Daba et al., 1994), and using different resins have been tried (Ross et al., 1993; McAuliffe et al., 1998; Zendo et al., 2003; Aso et al., 2008). However, the yield was reported to be low and inappropriate for large-scale purification (Nissen-Meyer et al., 1992; Andersson et al., 1998).

Taking advantage of the cationic and hydrophobic nature of bacteriocins, different types of column chromatography have also been used (Liu and Hansen 1990; Floriano et al., 1998; Palacios et al., 1999; Berjeaud and Cenatiempo 2004; Saavedra et al., 2004). This general method of purification is the most widely used, however, it is time consuming and ultimately leads to lesser recovery of peptides. To circumvent this problem, the isolation of peptides directly from the crude culture supernatant was carried out through expanded bed-absorption using a strong cation-exchanger (Callewaert and de Vuyst, 1999). Precipitation of protein from the culture supernatant though provided an easy method to recover and concentrate them, the degree of purification may still not be optimized (Guyonnet et al., 2000).

In the present study, therefore, the method of three phase partitioning (TPP) was applied for the separation of proteins from the culture supernatant. TPP is a much simpler process that consists of the addition of tertiary butanol (t-butanol) in presence of ammonium sulfate that pushes the protein out of solution in the form of an
interfacial precipitate layer between lower aqueous and upper organic phase (Dennison and Lovrien, 1997).

In this process, inorganic sulfate in molar concentrations pushes and tightens the conformation of expanded protein molecules, via kosmotropicity, exclusion and crowding, and t-butanol reinforces the behavior of sulfate ions. Moreover, t-butanol because of its size and bushy structure, unlike methanol and ethanol, does not easily penetrate folded proteins and thus helps in maintaining the native conformation of proteins (Baker, 1994). Thus, on the whole, TPP addition enhances salting out and kosmotropicity to precipitate proteins (Dennison and Lovrien, 1997). In the present study, antimicrobial peptides labeled as AMPs LR14, could be achieved at an overall yield of 20%. The antimicrobial function was assigned to four peptides as resolved by ultra performance liquid chromatography (UPLC).

Purification by butanol extraction with increased yield has been reported by many workers. In many of these studies, ammonium sulfate was combined with butanol. Some examples consist of edentomocin 110 from Bacillus thuringiensis entomocidus HD110 (Cherif et al., 2008), plantaricin AA135 from the culture supernatant of Lb. plantarum AA135 (Abo-Amer, 2007), and pediocin A, from P. pentosaceus FBBGl (Piva’ and Headon, 1994). In the present study, TPP was followed by size exclusion chromatography to get purified AMPs LR14. Purification of peptide with butanol extraction followed by cation-exchange chromatography and reverse-phase chromatography (Pantev et al., 2002) is also available in the literature.

Our results indicated that the antimicrobial activity could be assigned to four distinct peptides, that are individually active against the indicator strain, M. luteus. As demonstrated by UPLC all the peptides could be purified to homogeneity. The total antimicrobial activity recovered was ~15-times higher than that present in the initial supernatant and ~20% active protein could be recovered. The purification of AMPs/bacteriocins is always a challenging process in terms of the percentage yield and because of the smaller size (from ~2kDa to 5kDa) (Gonzalez’ et al., 1994; Jack et
al., 1995; Eijsink et al., 1998; Remiger et al., 1999; Parada et al., 2007; Tiwari and Srivastava 2008b).

Though the TPP method employed in the present study gave better results in terms of yield, however, the HPLC profile of purified peptides was different from plantaricin LR14α and LR14β peptides purified by the conventional method (Tiwari and Srivastava 2008b, c). It was therefore, important to know whether this method has identified a new class of AMPs produced by strain LR/14.

The MALDI (Matrix assisted laser desorption/ionization) MS/MS analysis of the peptides revealed peptide fingerprint sequences that did not show homology with any of the known bacteriocins/peptides in database. MALDI-MS/MS have been used to identify such (small) peptides showing antimicrobial activity (Lu et al. 2009; Kaur et al., 2013).

N-terminal sequencing by Edman degradation gave a partial 10 amino acid sequence, that also did not reveal similarity with any of the known peptides in the database. As described earlier, Lb. plantarum strain LR/14 possesses full pln regulon consisting of 5 plantaricin structural genes, pln E, -F, -J, -K, and –N as well as pln A (Nitika Ghosh, unpublished results).

To investigate whether MS/MS fingerprints or partial amino acid sequence conform to any of the known plantaricins, PlnJ, K, E, F and A were in-silico digested with trypsin and the sequences were analyzed and compared with MS/MS fingerprints. However, no similarity or homology with these peptides was revealed. This type of analysis is a helping tool to detect the peptides after trypsin digestion (Lu et al., 2009). Moreover, the UPLC profile of these peptides was very different from the known plantaricins.

All these results strongly suggested that Lb. plantarum strain LR/14 produces more antimicrobial peptides other than plantaricins LR14α, LR14β, PlnJ, PlnK, PlnE or
Many reports suggest the production of different antimicrobial peptides by LAB (Ehrmann et al., 2000; Eijsink et al., 2002; Lu et al., 2009; O’Shea et al., 2011). Thus, it is not an unusual phenomena for a strain to produce many types of AMPs to widen its inhibitory spectrum and for successfully competing with diverse bacteria in the same environment. Therefore, the peptides identified during present study were labeled as AMPs LR14, and were taken together for further studies.

The characterization of AMPs LR14 was an important parameter keeping in mind its importance for various applications. The bacteriocinogenic property of AMPs LR14 like many other bacteriocins remained unaffected in the range of pH 2-6, with marginal loss at neutral and increasing loss of activity at alkaline pH. This feature is shared with many others, such as antimicrobial activity of *Lb. gasseri* (Charteris et al., 2001), bacteriocin from *Lb. delbrueckii* ssp. *Bulgaricus* (Balasubramanyam and Varadraj, 1998) and plantaricin LR14 from *Lb. plantarum* LR/14 (Tiwari and Srivastava, 2008b, c). Contrastingly, a bacteriocin-like substance produced by *L. plantarum* TF711 and bacteriocin produced by *L. plantarum* F1 showed activity between pH 2.0-11.0 (Hernandez et al., 2005).

Similarly, extreme temperatures ranging from (-)20°C to boiling and autoclaving did not affect the activity of AMPs LR14. The peptides also remained stable at different storage temperatures for one year. Reports on several other bacteriocins show a variable response to temperature. Generally speaking, high temperatures such as boiling and autoclaving lead to partial loss of activity. Some such examples are the bacteriocin produced by *Lb. plantarum* F1 (Ogunbanwo et al., 2003), bacthuricin F4 (Kamoun et al., 2005), plantaricin LC74 (Rekhif et al., 1994), plantaricin UG1 (Enan et al., 1996), bacteriocin produced by *Lb. plantarum* TF711 (Hernandez et al., 2005), and, plantaricin 423 (Van Reenen et al., 1998). Plantaricin LR14 from *Lb. plantarum* LR/14 showed high thermal and pH stability, and long term storage ability at variable temperatures (Tiwari and Srivastava, 2008b). Reports on bacteriocin produced by *P. pentosaceus* suggest that the bacteriocin was stable at room temperature for 1 month (Wu et al., 2004).
Though most of the bacteriocins are proteinaceous in nature, some may contain additional carbohydrate or lipid moiety. The antimicrobial property of AMPs LR14 was sensitive to different proteolytic enzymes, reiterating its proteinaceous nature. Moreover, the ineffectivity of α-amylase and lipase on the same suggested that AMPs LR14 is neither glycosylated nor contains lipid moiety. Similar results have been reported for the bacteriocins from different groups of LAB (Park et al., 2003; De Kwaadsteniet et al., 2005; Todorov and Dicks, 2005; Cocolin et al., 2007).

The main emphasis of the present study was to understand the mode of action of AMPs LR14 and its inhibition spectrum. When tested against Gram-positive and Gram-negative bacteria, though its effect was variable, it was noteworthy that Gram-positive strains *M. luteus* and *L. monocytogenes* were more sensitive than Gram-negative strains, *E. coli* and *Y. enterocolitica*. Significant loss in viable cell counts indicated the bactericidal mode of action in all the cases. AMPs LR14 also showed inhibition against various important clinical pathogens.

Previous studies conducted on other bacteriocins indicate that most of them show antimicrobial effect only against Gram-positive bacteria. Gram-negative bacteria are either very feebly inhibited or they require a membrane disrupting agent (Belfiore et al., 2007). While pediocin PD-1 showed similar mode of action (Bauer et al., 2005), the antimicrobial substance produced by *Lb. plantarum* TF711 showed bacteriostatic action (Hernandez et al., 2005). Bacteriocin produced by *P. acidilactici* HA-6111-2 acted bactericidal to stationary-phase cells of *E. faecium* HKLHS, and bacteriostatic to stationary-phase cells of *L. innocua* N27 (Albano et al., 2007). Thus, the mode of action of bacteriocins can be bactericidal or bacteriostatic resulting in death or extension of lag phase, respectively.

Antimicrobial peptides produced by LAB are generally not active against Gram-negative bacteria. The outer membrane of such bacteria acts as a permeability barrier to the entry of antimicrobial compounds (Lappe et al., 2009). However, studies done on few bacteriocins report their activity against Gram-negative bacteria. Examples are bacteriocin ST151BR (Todorov and Dicks, 2004), thermophylin, and bacteriocins
ST28MS and ST26MS (Todorov and Dicks, 2005), plantaricin LR14 (Tiwari and Srivastava, 2008b), enterocin LR/6 (Kumar and Srivastava, 2011) and bacteriocin-like substances produced from LAB (Ponce et al., 2008) which were active against *E. coli, Yersinia pseudotuberculosis* and *Y. enterocolitica, Salmonella typhimurium* and *Acinetobacter baumanii*, besides some Gram-positive bacteria.

Antimicrobial activity against Gram-negative bacteria is an unusual property and has so far been reported only for few bacteriocins (Ivanova et al., 1998; Tiwari and Srivastava, 2008a; Gong et al., 2010; Kumar et al., 2011). Cerein 8A, an antimicrobial peptide produced by *B. cereus* 8A showed inhibitory activity against *Salmonella enteritidis* when combined with EDTA and sodium lactate (Lappe et al., 2009). In contrast, AMPs LR14 could target the Gram-negative bacteria even in the absence of any membrane disrupting agent.

In the present study, inhibition was observed when AMPs LR14 were targeted to various pathogens, important for causing food-spoilage and health-related problems. AMPs LR14 led to inhibition of both Gram-positive as well as Gram-negative bacterial strains, suggesting its wider inhibitory spectrum. This suggests the suitability of AMPs LR14 as potential antimicrobial agent in both food and clinical sector. In the food industry, *Listeria monocytogenes* and *Y. enterocolitica* have been identified as important food-borne pathogens causing microbial spoilage of food (Hartmann et al., 2011). Class IIa bacteriocins are known to have strong anti-listerial properties (Jack et al., 1995). According to Tagg et al. (1976), bacteriocins are bactericidal against closely-related bacteria. However, bacteriocins have increasingly been shown to elicit bactericidal activity against various important pathogenic bacteria as well (Ogunshe et al., 2007; Tiwari and Srivastava, 2008a; Carroll et al., 2010; Gong et al., 2010), as also shown by the present investigation.

A number of reports are available that highlight the importance of AMPs in inhibiting the growth of various pathogens. Some examples include cell-free culture filtrates of different lactic acid bacteria (Mantovani and Russell, 2003; Tiwari and Srivastava,
Nisin was identified to be anti-listerial (Scannell et al., 2000). Lacticin 3147 and nisin acted against Mycobacterium (Chung, 2000, Caroll et al., 2010) and bacteriocin AMA-K (Todorov, 2008) against Enterococcus spp., E. coli, Klebsiella pneumoniae and Listeria spp., Acinetobacter spp., Alcaligenes spp., Enterobacter aerogenes, Proteus mirabilis, Pseudomonas aeruginosa and Shigella flexneri (Ogunshe et al., 2007).

Our results are fully in accordance with bactericidal mode of action of AMPs LR14 since the viability of the treated bacteria is strongly compromised as also reported for many bacteriocins. Such an action is reported to be mediated through formation of pores in the cell membrane, leading to the leakage of various intracellular ions and molecules resulting in cell death (Yoneyama et al., 2011). Nisin, is known to disturb the membrane barrier function by pore formation as well as the inhibition of cell wall biosynthesis (Christ et al., 2008).

Bacteriocins generally have a cationic character and easily interact with Gram-positive bacteria that have a high content of anionic lipids in the membrane determining the formation of pores (Chung et al., 2000; Cleveland et al., 2001; Chen and Hoover, 2003). Pores in the cytoplasmic membrane clearly affect the energy status of the cell, i.e. dissipation of proton motive force that causes an arrest of ΔpH- and Δψ (transmembrane electrical potential) -dependent (e.g. transport) processes while certain bacteriocins cause ATP efflux (Moll et al., 1999).

Electrostatic interactions with negatively-charged phosphate groups on target cell membranes contribute to the initial binding of many bacteriocins to the sensitive cell’s membrane (Chen et al., 1997; Lins et al. 1999). Other factors, such as the phospholipid composition of the target bacterial membrane and the environmental pH, influences the bacteriocin’s activity (Chen et al., 1997).

The results of the present study have shown that AMPs LR14 could dissipate the transmembrane proton potential of both the Gram-positive and Gram-negative target cells and its action is quite comparable to that of valinomycin, a known ionophore.
Dissipation of $\Delta \psi$ is a typical characteristic of the pore-forming bacteriocin, such as nisin, and enterocin P (Montville and Bruno, 1994; Herranz et al., 2001) and indicates the disturbance of the membrane’s integrity by the bacteriocin molecules. Our studies have also shown that treatment of *M. luteus* and *E. coli* with AMPs LR14 led to the release of intracellular K$^+$. Earlier studies in our lab showed that treatment of *M. luteus* and *E. coli* with enterocin LR/6, from strain *Enterococcus faecium* LR/6 also caused leakage of intracellular K$^+$ (Kumar and Srivastava, 2011). This action of AMPs LR14 is in agreement with many other bacteriocins (Herranz et al., 2001; Suzuki et al., 2005; Naghmouchi et al., 2008).

ATP release from both *M. luteus* and *E. coli* cells treated with AMPs LR14 also suggested its membrane destabilization effect. Similar observations have been reported for pediocin-like bacteriocins (Cleveland et al., 2001; Herranz et al., 2001). Bacteriocins are generally known to cause the release of small ions and molecules, however, there are reports where the pores formed in the cell membrane may also be big enough to cause the leakage of bigger molecules, such as ATP (Moll et al., 1999; Zhou et al., 2008).

The other effect observed was the increase in $P_i$ levels corresponding to increased ATP hydrolysis (ATPase activity), by the AMPs LR14-treated cells in comparison to the untreated controls. Both efflux and intracellular ATP hydrolysis are known to contribute to the depletion of intracellular ATP pool. ATP hydrolysis may result from a shift in ATP equilibrium due to inorganic phosphate efflux or due to a futile attempt to regenerate PMF (Li et al., 2005; Zhou et al., 2008). Piscicocin CS526 is known to bring about rapid depletion of intracellular ATP without its leakage from the cells. These results suggest that the pores formed only allow passage to small ions (e.g. K$^+$) but not to larger (e.g. ATP) molecules (Suzuki et al., 2005). Release of UV-absorbing materials detected after treatment of cells with AMPs LR14 also suggested membrane perturbations as reported by other authors as well (Motta et al., 2008; Gong et al., 2010).
While the viability assay established bactericidal mode of action of AMPs LR14 it could be further corroborated by live-dead dual staining, microscopic observations, and TEM studies. These studies clearly indicated the dose-dependent killing of target cells by AMPs LR14.

In the TEM observations, the membrane deformity, blebbing and notches observed both in *M. luteus* and *E. coli* could be explained due to some disturbance in the osmotic balance of the cell leading to shrinkage of the protoplasm as is reported for nisin (Hyde *et al*., 2006). The cell disruption reflecting the leakage of intracellular contents and cell lysis at higher concentrations of AMPs LR14, as shown by the present studies has also been reported to be induced by many bacteriocins (Montville and Bruno, 1994; Bauer *et al*., 2005; Khalil *et al*., 2009; Lappe *et al*., 2009). The membrane-active compound cerein 8A, a pore-forming peptide damages the cell membrane and causes cell lysis (Bizani *et al*., 2005). The cytoplasmic membrane appeared to be retracted from the outer membrane and disorganized unlike the control where an intact cell envelope was present (Motta *et al*., 2008). Cell elongation due to treatment with AMPs LR14 was also evident in both the strains. Though this is in accordance with previous reports on lactococcin 972 and mersacidin (Brotz *et al*., 1995; Martinez *et al*., 2000), whether it is due to inhibition of septum formation, is yet to be seen. It has been reported that treatment of *B. subtilis* with nisin resulted in elongated cells or minicells, due to improper functioning of cytoskeletal and cell division proteins. This leads to delocalization of morphogenetic proteins and thus dissipation of the membrane potential (Strahl and Hamoen, 2010).

Antibiotic resistance is increasing at a rapid pace in infection-causing bacteria which is a serious threat to mankind all over the world. Although bacteriocins are not antibiotics, there is growing concern that repeated exposure to bacteriocins will render sensitive cells more resistant to them, a fact that has not been very well substantiated. In the present study, no genetic resistance against AMPs LR14 was observed in *M. luteus* and *E. coli* at least under short-term multiple exposure. In this context, infact, antimicrobial peptides (such as, AMPs LR14), particularly
bacteriocins have provided an alternative to kill infectious pathogens (Hurdle et al., 2011; Hassan et al., 2012). In fact, the narrow inhibition spectrum of AMPs can be exploited to the utmost advantage because their off target effect would be minimal in contrast to known antibiotics.

In laboratory settings, nisin-resistant Gram-positive and Gram-negative bacteria have been reported (Crandall and Montville, 1998). The molecular mechanisms leading to non-susceptibility have been shown to involve changes in the bacterial cell membrane or cell wall, although the precise nature of the factors involved in resistance development remains elusive, and bacteria may employ several strategies simultaneously to acquire nisin resistance (Crandall and Montville, 1998; Macwana and Muriana 2012). Most researches show that a change in cell envelope (e.g. membrane fluidity or cell surface charge) can mask or interfere with the bacteriocin–receptor interaction thus leading to resistance of the cells to bacteriocins (Kjos et al., 2011). Our study suggested as also reported by Macwana and Muriana (2012) that there are multiple targets that need to be mutated independently of each other allowing different mechanisms of acquiring resistance. Resistance has not been reported in S. salivarius K12 when tested against clinical pathogens for 10 passages (Masdea et al., 2012).

The antibacterial effect of AMPs/bacteriocins produced by lactic acid bacteria has been reported for various related and unrelated bacteria. However, keeping in mind the general applicability of AMPs LR14, the present studies were devoted to see their effect on other organisms as well. The suggested application of bacteriocin (AMPs) in food biopreservation and safety led us to look at some food spoilage pathogens. The AMPs LR14 were able to inhibit various Gram-positive and Gram-negative bacteria which are important food-spoilage deteriogens as discussed above. However, food-spoilage by various fungi is another major concern of the food industry. Many fungi are known to spoil foods, not only the raw materials but also foods at different stages of preparation (Rouse et al., 2008; Gerez et al., 2009; Wang et al., 2012). In a study, it was estimated that there is a loss between 5 and 10% of the world’s food production
due to fungal deterioration. In addition mould spoilage of bread alone is estimated to cause major economic losses. *Penicillium* and *Aspergillus* species are reported to be spoilage organisms for a wide range of food and feeds (Schnürer and Magnusson, 2005).

In the present investigation, the antifungal effect of AMPs LR14 was assessed against four fungal deteriorogens *viz.*, *Aspergillus niger*, *Rhizopus nigricans*, *Mucor racemosus*, and *Penicillium chrysogenum*. Interestingly, all the four moulds were found to be inhibited, suggesting that these peptides possess antifungal property as well. The bioactive peptides from LAB have generally been assessed for their antibacterial effects and there are relatively fewer reports on specific antifungal compounds from LAB (Magnusson and Schnürer, 2001; Schnürer and Magnusson, 2005; Dalié et al., 2010). In this context, AMPs LR14 appear to possess a broader inhibition spectrum.

Qualitative and quantitative validation of antifungal activity of AMPs LR14 on different growth phases concluded that spore germination was the most susceptible of all the stages. It has been shown that the antifungal ability is not only dependent upon LAB strain and fungal species but the susceptibility is also stage-dependent. While majority are able to inhibit spore germination only some inhibit mycelial growth (Gerez et al., 2009). Whether a fungicide inhibits germination and/or acts after germination is determined by its mechanism of action and the role the affected cellular process plays in fungal biology (Slawecki et al., 2002). Hydration and subsequent metabolic activation are important components of germination. Our results on spore germination and also those discussed later tempted us to conclude that AMPs LR14 are perhaps interfering with the imbibition process as reported by other authors (Rouse et al., 2008; Gerez et al., 2009; Wang et al., 2012). The effect of AMPs LR14 on hyphal and mycelial growth may suggest its action on the cell wall as well. Viability test of treated spores confirmed the fungicidal activity of AMPs LR14. The results are in agreement with the studies conducted on various *Lb. plantarum* strains isolated from Nigerian foods, where considerable inhibition is reported on spore germination and fungal growth (Adebayo and Aderiye, 2010).
Though spore germination was found to be more vulnerable, the hyphal extensions were also inhibited which is in accordance with the observation of Coda et al. (2011). Hyphal growth is associated with the cytoskeletal structures (actin, microtubules and associated molecular motors) which organize at the growing tip, nevertheless, turgor pressure is also a very important parameter for cellular expansion and growth (Lew, 2011). AMPs LR14 inhibited the hyphal growth, suggesting the hinderance in the normal mechanism of turgor induced elongation of mycelium as also the germination as discussed above. That these peptides may also affect the cytoskeletal structure, as discussed later, seem to be another possibility.

The antifungal effect by the culture supernatant of various lactobacilli strains against few food-spoilage fungi has been worked out by various other workers (Lavermicocca et al., 2000; Magnusson and Schnürer, 2001; De Muynck et al., 2004; Yang and Clausen, 2005; Sathe et al., 2007; Smaoui et al., 2009). The fungistatic activity was also observed by pentocin TV35b and BacTN635 against C. albicans (Okkers et al., 1999), and against Fusarium (isolated from cereals) by two lactobacilli strains (Laitila et al., 2002). The ability of AMPs LR14 to kill some food-spoilage fungi should make them a potential candidate for their use to prevent the growth of contaminating fungi. The strong inhibition of spore germination by these peptides make them suitable for these applications, as these fungi are predominantly propagated by their spores.

With both antibacterial and antifungal effects exhibited by AMPs LR14, it provided the impetus to test them against important pathogens, two of which were selected: Plasmodium falciparum, causing cerebral malaria, and Mycobacterium tuberculosis, the causal agent of tuberculosis. Interestingly, AMPs LR14 also exerted the antiplasmodial effect. This assessment was based on the fact that these parasites lack the ability to synthesize the purine ring de novo and thus are reliant upon salvage of purines from the host. The incorporation of these purines is therefore a reliable indicator of their viability (Downie et al., 2008). The experiments showed that the viability of the plasmodial parasites was compromised in a dose-dependent manner in
the microgram concentration range. That this effect was specific to the parasite could also be demonstrated as only a low hemolytic activity was obtained in the parasitized RBCs in contrast to the substantial loss in viability of the parasite. Conclusively, the data suggest the anti-plasmodial action of AMPs LR14 with almost negligible hemolytic activity. Also, uninfected cells did not show any lysis. Several natural antimicrobials, especially the membrane active peptides have been recommended as of potential biomedical value. However, only a limited number of such peptides are known to be active against eukaryotic pathogen (Gelhaus et al., 2008). These authors have shown the selective activity of NK-2, representing the cationic core region of lymphocytic effector protein causing hemolysis of parasitized RBCs, destroying the integrity of parasite plasma membrane and inhibiting P. falciparum. The uninfected cells were not harmed. Kaushik et al. (2012) have shown that the inhibition of the growth of malaria parasite by synthetic peptides is accompanied with lysis of infected red cell. Studies done on ultra short peptides, report that the nature of the N-terminal residue is very important for anti-malarial activity. Moreover, presence of non polar amino acid residues (providing lipophilic anchors) dramatically increases the biological activity, because those residues might be helping in their penetration (disruption) through plasma membrane (Liu et al., 2007; Mason et al., 2009; Pérez-Picaso et al., 2009).

The present investigation is a preliminary study but identifies an important lead that could be pursued further, more so in terms of administration of these peptides during infection. Moreover, the significance of the present study increases because of the fact that the anti-plasmodial peptides are derived from a GRAS organism. The mechanism for this anti-plasmodial activity is though unclear, however, important parallel could be drawn with differences in the membrane composition of Plasmodium spp. and the cells that they infect which are predicted to enhance the activity of anti-plasmodial peptides. These modifications include a marked increase in erythrocyte membrane fluidity, alteration in host-cell lipid fatty acid composition and phospholipid distribution, and increased membrane permeability through erythrocyte membrane pores. In contrast, healthy erythrocyte membranes retain asymmetry, and
phosphatidylserine is not presented at the external surface prior to a pathological stimulus (Mohandas and Gallagher, 2008; Gelhaus et al., 2008; Mason et al., 2009). Antimicrobial peptides may also have an indirect effect on malaria parasite survival. For example, some synthetic peptides have been shown to kill intracellular blood-stage forms of the malaria parasite (Ghosh et al., 1997), whereas some studies have shown that antimicrobial peptides can induce cells to undergo apoptosis (Risso et al., 1998). Generally speaking, the positively-charged peptides are expected to interact electrostatically with the altered and negatively-charged plasma membrane of the infected cells, traversing the membrane of host and then that of parasite to reach its target (Gelhaus et al., 2008). Selective toxicity towards *P. falciparum*, low level lysis of infected RBC and practically no hemolysis of uninfected RBC (as also discussed later) are the characteristic properties of AMPs LR14.

The studies conducted on inhibition of *Mycobacterium smegmatis* and *M. tuberculosis* showed that AMPs LR14 is not effective against Mycobacteria. Nisin is reported to be bactericidal against *M. tuberculosis* H37Rv (Sosunov et al., 2007) and *M. smegmatis* (Montville et al., 1999), and lacticin 3147 was found to be active against *M. tuberculosis* H37Ra (Carroll et al., 2010). These results clearly suggest that inhibition by AMPs is strongly dependent upon the type of target cells. It could be hypothesized that this may be due to differential penetration of these peptides through the cell wall as has been shown in the case of Gram-negative bacteria (Lappe et al., 2009).

The broad inhibition spectrum strongly suggested the application of AMPs LR14 as a food bioprotectant and a therapeutic molecule. However, for any such application, it becomes mandatory to test and provide information on toxicity/ill-effects of the compound under consideration. While humans are the endpoint beneficiary for all such applications, it is not possible due to ethical reasons, to conduct toxicity testing directly on human beings. The problem is further multiplied by the large number of such compound to be tested. Therefore, a set of tests have been identified that should
provide the preliminary data on the toxicity before even animal experiments are conducted.

The measurement of toxicity is also complex. Toxicity may be acute or chronic, and may vary from one organ to another as well as with age, genetics, gender, diet, physiological condition, or the health status of the organism. As opposed to experimental animals, which are highly inbred, genetic variation is a most important factor in human toxicity since the human population is highly outbred and shows extensive genetic variation. Even the simplest measure of toxicity, the LD$_{50}$ (the dose required to kill 50% of a population under stated conditions) is highly dependent on the extent to which the above variables are controlled. Selective toxic agents have been developed to protect crops, animals of economic importance, and humans from the vagaries of pests, parasites, and pathogens (Hodgson, 2004). These tests are broadly classified into two categories: the in-vitro and in-vivo toxicity testing, with the former followed by the latter.

*In vitro* cytotoxicity assays are useful in identifying the intrinsic ability of a compound to cause cell death or loss of viability, damages to different cellular functions, and the required doses to be tested. In one such tests, the viability and proliferation of mammalian cells was evaluated using Chinese hamster ovary (CHO) cells. CHO cells are often used in biological and medical research, that includes genetics, toxicity screening, nutrition, and gene expression, particularly of recombinant proteins. The study was important to monitor the dose-dependent response of AMPs LR14 against mammalian cells. MTT assay is a standard test employed to check the cytotoxic effects of an antimicrobial peptide of both prokaryotic and eukaryotic origin (Maher and McClean, 2006). In case of AMPs LR14, lower doses (upto 200 µg/ml) were safe as loss of viability was not more than ~20% but higher concentrations showed strong inhibition. It may be reiterated, that this dose has a good antibacterial potential. Similar dose-response effect has been reported for different antimicrobial peptides (Paiva *et al*., 2012). However, in these studies, the IC$_{50}$ concentration and the cell lines used were different (Murinda *et al*.,
Some AMPs have shown a cell-line specific cytotoxic effect that has been explained on the basis of membrane lipid composition and the presence of cholesterol (Paiva et al., 2012).

Studies done by many workers report that bacteriocins/antimicrobial peptides present a low cytotoxicity to various mammalian cell lines. Carnobacteriocins displayed significantly no cytotoxicity towards gastro-intestinal cell lines, Caco-2 and HT-29 (Jasniewski et al., 2009). Studies done by Murinda et al. (2003) reported nisin, pediocin, and Col E6 to be very cytotoxic bacteriocins; SV40-HC cells demonstrated greater sensitivity than Vero cells (Murinda et al., 2003). Differential toxicity was observed in VERO cells treated with peptide P34 and nisin by MTT assay (Vaucher et al., 2010, 2011). Nisin A, and Magainin I and II exhibited significant loss of cellular viability than gallidermin in both cells, HT29 and Caco-2, at concentrations at which vancomycin was not toxic (Cruz-Chamorro et al., 2006; Maher and McClean, 2006). In general, prokaryotic antimicrobial peptides are less inhibitory than those derived from eukaryotic sources (Maher and McClean, 2006). Overall, the antimicrobial activity demonstrated by such peptides against indicator bacteria and pathogens is significantly lower than the cytotoxicity in some cell lines (Maher and McClean, 2006).

The other test used was the hemolytic assay. With AMPs LR14 no hemolytic activity was detected even in the milligram concentration range. The assessment of hemolytic activity is used as a measure of cytotoxicity, as well as to estimate the therapeutic index of antimicrobial peptides (Maher and McClean, 2006). Several studies have used hemolysis as a parameter to assess toxicity and have shown that it varies with bacteriocins and their doses (Maher and McClean, 2006; Cruz-Chamorro et al., 2006; Vaucher et al., 2010; Paiva et al., 2012). No hemolysis has been reported for cerein 8A, a bacteriocin like peptide from B. cereus against human and sheep erythrocytes (Bizani et al., 2005). The peptide P34 revealed higher hemolytic activity (5.8%) when compared to nisin at the highest concentration (2.5 mg/ml) tested (Vaucher et al., 2010). Dose-dependent hemolysis was observed in rabbits and mice treated with
antimicrobial peptides (Cruz-Chamorro et al., 2006; Maher and McClean, 2006). A study done by Sandiford and Upton (2012) on epidermicin NI01 has shown that the peptide did not show hemolysis at 100 times the minimum inhibitory concentration (MIC) tested and nisin may be haemolysing at a concentration, 1000-fold higher than its antimicrobial dose (Maher and McClean, 2006).

Again, the level of hemolysis though variable is low (Maher and McClean, 2006; Cruz-Chamorro et al., 2006; Vaucher et al., 2010; Paiva et al., 2012). As discussed earlier, this could be due to specific composition of erythrocyte membrane (Mohandas and Gallagher, 2008). All the studies including the present one have clearly demonstrated that toxic effects depend upon the type of antimicrobial peptide, its concentration and exposure time, the composition of the target cell membrane, the metabolic activity of the sensitive cells tested and the assay system used (Paiva et al., 2012). What appears to be more important is to demonstrate the relationship between the concentration which is antimicrobial and the one which is hemolytic. This is an important parameter and a low level of hemolysis, if caused, should not cause concern due to the massive selective toxicity associated with these peptides (Maher and McClean, 2006).

Since the use of animals in toxicology research and testing is a concern for both science and ethics, it is important to consider other alternative systems before the studies are conducted on mammals. Plants can be valid alternatives because chemicals which cause chromosomal aberrations in plant cells also produce chromosomal aberrations in cultured animal cells and most frequently the aberrations are identical (Grant, 1978). An efficient test organism for the assessment of introduction of chromosomal aberrations should have chromosomes which are easy to analyze in terms of size, morphology and number. Allium cepa (onion) root tip has served as an ideal test system for the study of mutagenesis by a test chemical (Asita and Matobole, 2010).
In the present study, onion-root tip mitosis was employed. One striking observation was that root formation did not occur when onion bulbs were exposed to different concentrations of AMPs LR14. Thus, when normally formed roots were exposed to AMPs LR14, no gross chromosomal abnormality or effect on mitotic index was observed. The inhibition of root formation was also reported by Aksoy et al. (2011) in onion bulbs treated with industrial waste water. Exposure to nano silver particles (Babu et al., 2008), benomyl, a systemic fungicide (Dane and Dalgic, 2005), wastewater samples from different industries (Matsumoto et al., 2006; Aksoy et al., 2011), and pesticides (Asita and Makhalemele, 2009) have all been reported to induce chromosomal aberrations in onion root meristem cells.

A substance to be deemed a useful chemotherapeutic agent, it must have selective toxicity for the target; this means that at an effective concentration in the tissues, the substance must have low toxicity to the host cells and high toxicity to the invading pathogenic organisms. Antimicrobial peptides present new opportunities for the inhibition of pathogens (Maher and McClean, 2006; Cruz-Chamorro et al., 2006; Vaucher et al., 2010; Paiva et al., 2012). Bacteriocins and antimicrobial peptides produced by bacteria have found applications in food industries as an alternative to chemical preservatives, as they can help to reduce the proliferation of microbes (Cotter et al., 2005; Galvez et al., 2007; Settani and Corsetti, 2008). AMPs LR14 is an antimicrobial peptide produced by a lactic acid bacterium, so to substantiate its role in food applications, it was necessary to check its toxicity on in vivo systems. Moreover, the information obtained from in-vitro tests cannot be used directly as they usually do not exhibit all the desirable characteristics, such as absorption, distribution, metabolism, excretion, and toxicity (Pandey and Nichols, 2011). Once again various authors have recommended an alternative model system. Traditional animal models, such as rodents, are a poor choice for a whole-animal primary screening platform, because of the prohibitive costs and the time required to conduct the experiments and validate the results (Pandey and Nichols, 2011).
The fruit-fly, *Drosophila melanogaster*, a eukaryotic organism with its interrelated organ systems and activation mechanisms is considered one such alternative in the drug discovery process, mainly because the key physiological processes are well conserved from fly to humans. Moreover, because of a short-life cycle, distinct developmental stages, easy cultivation, low cost maintenance, numerous offspring, and its strong cytogenetic/genetic background, *Drosophila* has served as a useful model system to study several biological processes including toxicity (Schneider, 2000; Pandey and Nichols, 2011; Panacek et al., 2011). Toxicity studies have been based mainly on different pesticides, insecticides, nanomaterials, and other toxic compounds (Mukhopadhyay et al., 2002; Nadda et al., 2005; Tsukamoto et al., 2005; Gupta et al., 2008; Karatas et al., 2009; Akmoutsou et al., 2011; Panacek et al., 2011).

However, the toxicity of an AMP of bacterial origin, has not been demonstrated, to the best of our knowledge, on *D. melanogaster*. Our study has clearly shown that AMPs LR14 provided through the food adversely affects the reproductive fitness and developmental program of *D. melanogaster*. Though the dose required was much higher than the antimicrobial and antiplasmodial effect. Moreover, as reported with other toxic compounds, dose-dependent adverse effects, developmental delays and snags, the compromised reproductive capacity and larvicidal and adulticidal effects were also observed. The number and development of larvae, pupation ability, number and development of pupae, overall developmental time, the number of hatched adult individuals, and also the fecundity of adult flies were all affected as reported by other authors, with different toxicants, as well (Akmoutsou et al., 2011; Memmi and Atli, 2011; Panacek et al., 2011).

The effect of AMPs LR14 was so pronounced on the larval growth that it suggested some gross changes in the general cellular architecture. Larvae reared on AMPs LR14 supplemented food showed a highly reduced size and the sluggish movement as compared to the control larvae. Movement and developmental defects due to ingestion of toxic nanomaterials and microbial infection has been reported by Liu *et
al. (2009) and Olcott et al. (2010). DAPI and phalloidin-TRITC staining of salivary gland from the larvae and ovary from the adult were examined to see the effect. The confocal images obtained with sub-lethal concentration showed that AMPs LR14 treatment has led to the disruption of the cell’s cytoskeletal architecture. The absence of phalloidin uptake suggested the degeneration of actin filament network in both the organs in the larvae and the adults resulting in massive tissue disorganization. Moreover, defects in ovarian development, shrinkage of cytoplasm, and nuclear fragmentation also explained the drastic effect of the peptide. Apoptosis, though a normal physiological process, can also be induced by a number of exogenous factors, such as chemicals, oxidative stress, anoxia, and radiation (Ziegler and Groscurth, 2004). Leventis et al. (2011) have shown that phalloidin-TRITC and DAPI staining could be used to visualize uniform arrangement of nurse cells and nuclei in the normal development and could also reveal any defect occurring in the ovarian development. The present study suggests that AMPs LR14 can also be developed as an insecticidal agent.

All these tests are only primary indicators of toxicity, however, whether the compound is truly toxic can only be confirmed in a mammalian system. Animal tests for toxicity are conducted prior to human clinical investigations as part of the non-clinical laboratory tests of pharmaceuticals (Hodgson, 2004). There is no single species of animal that can be used for all toxicity tests. In some cases, it may not be possible to use the most desirable animal for testing because of animal welfare or cost considerations. For example, use of monkeys and dogs is restricted to special cases and that too in the final stages of drug testing, even though they represent the species that may react closest to humans. Rodents and rabbits are the most commonly used laboratory species due to their availability, low costs in breeding and housing, and past history in producing reliable results.

The results of acute oral toxicity tests of AMPs LR14 in wistar rats determined the LD$_{50}$ of the peptides in between 1000 and 2000 mg/kg body weight. This value was far beyond the one that is required for its antimicrobial, antiplasmodial, and anti-
Drosophila action. Also when compared with other AMPs, AMPs LR14 appear to be very safe. Nisin, the commercially available bacteriocin, is currently permitted in the European Union in a concentration range of 3 mg to ~15 mg/kg.

US Food and Drug Administration (FDA, 1988) has recommended a dose of 4.9 mg/kg bw, with a safety factor of 100, and an Acceptable Daily Intake (ADI) of 0.049 mg/kg bw/day. This corresponds to 2.9 mg/person/day. The SCF (Scientific Committee on Food, 1992) allocated an ADI of 0.13 mg pure nisin/kg bw. The oral LD\textsubscript{50} of nisin in rats was reported to be >25 mg/kg bw (Frazer et al, 1962), whereas in the mouse it was determined to be 174 mg/kg bw (Hara et al., 1962; EFSA Journal, 2006). Another study on peptide p34 stated that the LD\textsubscript{50} in BALB/c mice was higher than 332.3±0.76 mg/kg of bw (Vaucher et al., 2011).

Moreover, AMPs LR14 failed to elicit any immunogenic response and thus no antibodies were generated when injected in rabbit. These results are in accordance with other bacteriocins/antimicrobial peptides that have been reported to lack immunogenic response in mice or rabbit when these animals were immunized with such peptides. The antibodies produced only when these peptides were conjugated with carrier proteins. Examples include generation of polyclonal antibodies against pediocin PA-1 conjugated to carrier protein keyhole limpet hemocyanin (KLH) and Freund’s adjuvant (Martínez et al., 1998, 1999). Nisin was conjugated to KLH for generation of antibodies (Suárez et al., 1996; Bouksaim, 1998; Gupta et al., 2008). Monoclonal antibodies (MAb) against pediocin RS2 and AcH were generated in mice conjugated to a polyacrylamide gel (Bhunia, 1994). Peptide P34 was conjugated to Freund’s complete adjuvant and the antibodies could be raised against it (Vaucher et al., 2011). The evaluation of an antimicrobial peptide through in-vitro and in-vivo toxicity is considered to be an essential step before it could be considered for use in food.

The studies on applications of bacteriocins have gained much attention due to consumer demands of ready-to-eat foods and concern over minimally-processed
foods. The demand for safe foods with a long shelf-life without the addition of chemical preservatives is on the rise. Bacteriocins are promising candidates as they are produced by food-grade GRAS organisms (LAB), usually heat stable and can inhibit many of the primary pathogenic and spoilage organisms (Cotter et al., 2005; Parada et al., 2007). Bacteriocins are also known to inhibit or retard the growth of microorganisms in foods without changing the nutritional content, thus satisfying the consumer demands to combat undesired bacterial growth in foods and beverages (Santiago-Silva et al., 2009). Till date, nisin and pediocin AcH are the only bacteriocins permitted as biopreservative in the food industries (Gálvez et al., 2007; Settanni and Corsetti, 2008).

Two most commonly used chemicals in processed/preserved foods are sugar and salt. Thus, it became important to study the efficacy of AMPs LR14 in the presence of different concentrations of salt and sugar. These compounds tend to tie up moisture and thus exert a drying effect on both food and microorganisms and thus prevent the activity of food spoilage and pathogenic organisms. Salts are added in brine and curing solutions or applied directly to foods to preserve pickles, fish and other meats (Galvez et al., 2007). Sugar plays a very important role in the preservation of fruit syrups, candies, chocolates, condensed milk, cakes and pies etc. The shelf stability of these products is largely due to the preserving effect of high concentrations of sugar. The activity of AMPs LR14 was not affected even in the presence of high concentrations (10%) of sugar or salt thus suggesting that the peptide could be used even in the processed foods. Reports to date suggest that salt (NaCl) resulted in an increased cell growth and thus bacteriocin production (Neysens et al., 2003).

Bacteriocins/antimicrobial peptides produced by lactic acid bacteria have been reported to be involved in preservation of apple juices and meat (Udhayashree et al., 2012) fermented vegetables (olives, sourdough, sauerkrauts, kimchi, cider), non-fermented vegetables such as pickles, mungbean sprouts, mashed potatoes, soymilk, fruit and vegetable juices, canned vegetables, fresh-cut products, salads, and puree (Settanni and Corsetti, 2008; Bellei et al., 2011).
The other aspect of food bioprotection is to prevent their spoilage during storage. The damage to agricultural produce during storage occurs mainly due to rodents, insects, and microorganisms. Fungi, are the major cause of deterioration during storage and are most undesirable because of the byproducts they generate could pose a danger to the public health (Sinha and Sinha, 1990). Grains when stored in improper and unhygienic damp conditions in godowns and store-houses, lead to infection with fungi and other microbes. In that situation, the precocious germination of seeds can occur and spoilage of nutritional quality and production of toxins by deteriogens make the grains unfit for human consumption. Since the antifungal activity of AMPs LR14 was already explored, the preservation of wheat grains was also tested.

The treatment of wheat grains with AMPs LR14 prevented the infection by fungi as confirmed by the seed health test. Moreover, the antifungal effect was retained even when the seeds were stored for a long time under laboratory conditions. The majority of the nutritional content in the seed comprising carbohydrates and proteins, were not affected. However, the seed viability was affected to a large extent. Therefore, such a treatment can be recommended only for storage but not for agricultural purposes.

Similarly, mungbean sprouts, a rich source of nutrition, are consumed all over. Seed sprouts are usually eaten raw in salads or in sandwiches, and concerns for the safety of these raw foods have, therefore, increased. In addition, with increasing complexity in lifestyles, they are available as ready-to-eat packaged foods. Thus, the choice of preservatives is a matter of concern. The sprouts are prone to bacterial infection, including Salmonella and E. coli (Kumar et al., 2006; Mohle-Boetani et al., 2009). AMPs LR14 were able to retard the bacterial/fungal growth on the mungbean sprouts as seen visually. Bacteriocinogenic strains of LAB (Enterococcus mundtii ATO6 and Pediococcus parvulus ATO34 and ATO77) were investigated for their ability to inhibit L. monocytogenes on mungbean sprouts (Bennik et al., 1999).

Antioxidants are compounds which play a role in the scavenging of free radicals. The antioxidant effect of lactic acid bacteria has been reported since a long time (Kaizu et
Although oxidation is essential to living organisms for the production of energy, so as to fuel biological processes, oxidative stress can damage biological molecules. It is well established that oxygen-centred free radicals and other reactive oxygen species are continuously produced in vivo (Halliwell, 1994). A wide variety of reactive oxygen species can be formed in the human body and in food systems and they are implicated in several medical problems (Halliwell and Gutteridge, 1999). Investigations on AMPs LR14 indicated that it possesses a good antioxidative effect. The antioxidative effects have been reported by a lactic acid bacterium that releases peptides with antioxidant activity through the proteolysis of native cereal proteins (Coda et al., 2012).

With respect to medical applications, antimicrobials produced by probiotic LAB might play a role during in vivo interactions occurring in the human gastrointestinal tract, hence contributing to gut health. Further research is needed to unravel the precise role of LAB bacteriocins in this process. For the various applications, AMPs will have to move through the gastro-intestinal tract. Their effectivity through this passage will be based on their stability. The results of the present study obtained after evaluation of the activity of AMPs LR14 through the entire passage simulating the gastro-intestinal tract suggested that pepsin could lead to a significant reduction in the activity of AMPs LR14. Thus, if the AMPs LR14 is present in food, its consumption by humans should not lead to any toxic side-effect. Also, since the peptide is broken down it should not lead to their accumulation in the GI tract and thus the chances of developing resistance against them will also be ruled out.

Ingested nisin is inactivated by trypsin and pancreatin and has no effect on the gut microflora (EFSA Journal, 2006). Similar studies done earlier (Gardiner et al., 2007; Lohans and Vederas, 2012) have shown that the peptides, lacticin 3147 and pediocin PA-1 are inactivated during intestinal transit and therefore the probability of causing an adverse effect can be ruled out. However, these studies including the present investigation, do point out that this limits the application of AMPs as a therapeutic molecule for direct human consumption.
Another aspect, that the present investigation looked into was the establishment of *Lb. plantarum* strain LR/14, the producer of AMPs LR14 in the gut of a host organism. For this also, we selected *D. melanogaster* as a host. The choice was based on the fact that *Drosophila* midgut has been reported to harbor various bacterial species *viz.*, *Commensalibacter intestini*, *Acetobacter pomorum*, *Gluconobacter morbifer*, *Lactobacillus plantarum*, and *Lactobacillus brevis* (Shin et al., 2011). Similarly, several species of LAB have been shown to be present in the gut of *D. simulans*. These comprised, *Lb. plantarum*, *Lb. paracasei*, *Lb. sanfranciscensis*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Enterococcus faecalis* and *Pediococcus pentosaceus* (Dillon and Dillon, 2004). One of the most favorite niche of *Drosophila* species, the rotten fruit, can also be a principal source of LAB and yeasts (Dillon and Dillon, 2004; Stamps et al., 2012), and their association, therefore, appears to be natural.

Moreover, recent studies done on fly intestinal pathology suggest that this model is suited to study intestinal stem cell physiology during aging, stress and infection. Though vertebrates and insects share a diverse physiology, *Drosophila* can be forwarded as a model system for studying intestinal diseases in humans because of the high degree of conservation between *Drosophila* and mammals with respect to the signaling pathways that control intestinal development, regeneration, and disease. The well-studied intestinal stem cell lineage, as well as the tools available for its manipulation *in vivo*, provide a promising framework that has been used to elucidate many aspects of human intestinal pathology (Apidianakis and Rahme 2011). *Lb. plantarum* LR/14, strain under study, shows features of a probiotic strain (Ghosh et al., 2008). Keeping all these facts in mind, gut association of probiotic strain LR/14 was studied using a *Drosophila* system. In this investigation, axenic *D. melanogaster* was found to maintain *Lb. plantarum* strain LR/14, when ingested through the food. However, it must be acknowledged that some non-bacterial, microbes such as archaea, yeasts, and other commensal bacteria, especially anaerobes, may remain to be associated with the host. Inspite of this, strain LR/14 could still be traced out as plantaricin-gene specific PCR amplification was positive. Thus, the present study has
demonstrated the usefulness of *D. melanogaster* as a system, not only to study the host response to microbial infection (Olcott *et al.*, 2010, Loper *et al.*, 2012), but also to study interaction of commensal and introduced bacteria with that of a pathogen. Moreover, since the AMPs LR14 may be digested in the intestinal transit, their in-situ production in the host may be more advantageous. The strain establishment and persistence in the gut may also be tested for further applications (O’ Shea *et al.*, 2011).

The present study has contributed towards revealing additional paradigms of the environmental strain, *Lactobacillus plantarum* strain LR/14. Previous studies have identified a 2-peptide bacteriocin, plantaricin LR14 with broad inhibition spectrum, the identification of a *pln* regulon, and the probiotic potential of this strain. Present study has shown that the strain LR/14 produces in addition small antimicrobial peptides (AMPs LR14) that have strong antibacterial, antifungal, antiplasmodium, and a possible insecticidal property. These peptides appear to be safe and fairly non-toxic and non-immunogenic. Therefore, they could be applied for food biopreservation and bioprotection of seeds. Bacteria, for their successful existence, have faced a variety of selection pressure of both abiotic and biotic in nature. It is, therefore, not intriguing that a strain may possess an armoury of defense mechanisms so as to establish itself in an environmental niche. *Lb. plantarum* strain LR/14, being an environmental isolate, could serve as a model system to understand some basic ecological phenomena and its usefulness could be exploited for various applications.