

Inhibitory effect of plantaricin peptides (Pln E/F and J/K) against *Escherichia coli*

Gargi Pal · Sheela Srivastava

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Abstract Plantaricins are small bioactive peptides produced by *Lactobacillus plantarum* strains that exhibit significant antimicrobial activity against closely-related Gram-positive bacteria, including food spoilage organisms. In comparison, bacteriocins including plantaricins, are usually less effective against Gram-negative organisms. In this study, we demonstrate that heterologously expressed and purified plantaricins, Pln E, -F, -J, and -K when tested against Gram negative model organism *Escherichia coli* K-12 were highly effective under certain conditions. The apparent tolerance of Gram-negative members to these peptides has been explained on the basis of the presence of the outer membrane (OM) that acts as a protective barrier. We have shown that agents and/or conditions that destabilize OM of *E. coli* K-12, make it susceptible to plantaricin peptides. In order to further strengthen this conclusion, an OM lipoprotein-defective *lpp* mutant strain of *E. coli* K-12 was also studied and compared. A significant loss of cell viability both in terms of CFU/ml as well as with live–dead dual staining combined with flow cytometry, could be demonstrated with the *lpp* mutant in comparison to the wild type strain. The results indicate that plantaricins can inhibit Gram-negative bacteria if the outer-membrane is weakened and it can be used in preservation of food with the help of some food-grade chelating agents.

Keywords Plantaricin · Gram-negative bacteria · Cell viability · Outer membrane · Flow cytometry

Introduction

Microorganisms produce a variety of compounds which demonstrate antibacterial properties. One group of these compounds, the bacteriocins, consists of relatively small bactericidal peptides with a relatively narrow antimicrobial spectrum (Cleveland et al. 2001; Stern et al. 2006). They are now being considered for a variety of antimicrobial uses especially in foods and medicine (Papagianni 2003; Cotter et al. 2005).

The bacteriocins produced by lactic acid bacteria (LAB) have tremendous potential for use in food safety, probiotics, and are also seriously being considered as human therapeutics. Typically, these peptides are pH- and heat-tolerant and show very little, if any, inhibitory activity toward eukaryotic cells (Chen and Hoover 2003; Cotter et al. 2005). Many bacteriocins display potent activity against food-spoilage and pathogenic bacteria often at concentrations much lower than conventional antibiotics (Martin-Visscher et al. 2011). Plantaricins produced by *Lactobacillus plantarum* strains belong to class IIb. These comprise 2-peptide bacteriocins, wherein the complementary peptides, such as, PlnE/F and PlnJ/K show synergistic effect against target bacteria (Anderssen et al. 1998; Moll et al. 1999; Pal and Srivastava 2013).

While most antibiotics have specific targets, bacteriocins kill the sensitive cells by damaging the cytoplasmic membrane or inhibition of cell wall biosynthesis, or both. Because of the complex mode of action, development of resistance against bacteriocins is generally not reported. One of the major limitations in the wide-spread usage of most of the bacteriocins from Gram-positive organisms is their inability to kill Gram-negative pathogens (Chen and Hoover 2003; Galvez et al. 2007; Gillor et al. 2008). But in the last decade, some research papers have demonstrated that bacteriocins

G. Pal · S. Srivastava (✉)
Department of Genetics, University of Delhi South Campus,
New Delhi 110021, India
e-mail: sslab222012@gmail.com

are also active against certain Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium* (Gong et al. 2010) and interestingly against *Campylobacter jejuni*, the major cause of gastroenteritis world-wide (Stern et al. 2006; Svetoch et al. 2011). Few plantaricins, or bacteriocins from *L. plantarum* strains have also been reported to exert inhibitory effects on several Gram-negative organisms, for example: plantaricin 35 days (Messi et al. 2001), plantaricin ST26MS and ST38MS, AMA-K (Todorov 2009), plantaricin LR/14 (Tiwari and Srivastava 2008), and plantaricin MG (Gong et al. 2010).

One unique feature of the cell envelope organization of Gram-negative bacteria is the presence of the outer membrane (OM) that not only protects the cell wall (peptidoglycan) and the cell membrane but also acts as a barrier to many compounds, including antibiotics, hydrophobic compounds, detergents and dyes or other agents that normally damage the cell membrane (Vaara 1992; Raetz and Whitfield 2002). OM is composed of a network of lipids and polysaccharides referred to as lipopolysaccharides (LPS), which forms a tight shield and contributes to the resistance against antibiotics and other membrane damaging agents. It is, therefore, not surprising that these bacteria appear impervious to bacteriocins as well. The anionic LPS layer is stabilized by divalent cations, particularly Mg^{2+} and Ca^{2+} . If these cations are removed, lipopolysaccharide molecules are released from the OM, exposing the underlying phospholipid bilayer and jeopardizing the integrity of the OM (Vaara 1992; Vaara and Nurminen 1999).

Although Gram-negative cells are relatively resilient, they may not be necessarily insensitive to LAB bacteriocins. Numerous studies have demonstrated that certain bacteriocins could inhibit Gram-negative bacteria in the presence of chelating agents, such as the common food preservative, EDTA (Ananou et al. 2005; Lappe et al. 2009; Martin-Vischer et al. 2011), and lactoferrin (Murdock et al. 2007). Other treatments that destabilize the OM, include osmotic shock (Kordel and Sahl 1985), temperature (Bover-Cid et al. 2008), pH variation (Ananou et al. 2005), and pulsed electric fields and high hydrostatic pressure (Galvez et al. 2007).

The disruption of bacterial growth and metabolism by various treatments can be evaluated by a number of different techniques. One conventional method is to assess viability that is frequently equated with the ability to form colonies on solid growth medium or to proliferate in nutrient solutions. By these criteria, antimicrobial activity is defined as the ability to limit bacterial growth. Such a viability assay, however, is not only time consuming and labour intensive but also allows the bacterial death to be viewed only in retrospect, necessitating viability indicators that can be assessed at the single-cell level (Berney et al. 2007).

A number of fluorescence-based assays for evaluating bacterial viability have been introduced over the past few

decades. These are based mostly on fluorescent molecules, which can be detected with epifluorescence microscopy, solid state cytometry, or flow cytometry. Each indicator is based on the criteria that reflect different levels of cellular integrity or functionality (Berney et al. 2007). Over the last several years, multi-parameter flow cytometry has become a powerful tool in microbiology, particularly in biotechnological processing, food preservation, and chemical disinfection processes because it is fast and allows single-cell analysis (Berney et al. 2007). We have heterologously expressed and purified the 2-peptide plantaricins (PlnEF/PlnJK) and showed their efficacy against a number of Gram-positive bacteria (Pal and Srivastava 2013).

In the present investigation, we have evaluated these peptides against *E. coli* K-12, a model Gram-negative bacterium. The bactericidal effect was compared in the presence of certain agents, such as EDTA, SDS, sodium deoxycholate, $CaCl_2$ and $MgSO_4$, and conditions, such as low temperature. An *E. coli* mutant strain defective for major lipoprotein (*lpp*) in OM has been included to unravel the role of OM.

Materials and methods

Strains used

Two bacterial strains *E. coli* K-12 wild-type (MTCC 1302) and its *lpp* mutant (CGSC 6672) (*lpp-254*, *pps-6*, *hisG4* (O^C), *xylA5*, *mtl-1*, *argE3* (O^C), *thiE1*) were used. Both the strains were grown in LB medium at 200 rpm and 37 °C unless otherwise mentioned. The mutant strain was procured from *E. coli* Genetic Stock Centre (CGSC), Yale University.

Source of plantaricins and treatment of *E. coli* K-12

Plantaricins used in this study belonged to two peptide class IIb bacteriocins produced by *L. plantarum*. In this study, *plnE*, *-F*, *-J*, and *-K* genes were amplified from soil metagenome, cloned in pET32a (+) vector and the respective recombinant plantaricin peptide was obtained by heterologously expressing and purifying by Ni-NTA affinity chromatography from *E. coli* BL21 (DE3) strain (Pal and Srivastava 2013). Initially, *E. coli* K-12 strain was grown overnight and subcultured with an initial OD_{600} of 0.01. Then 1 ml culture per well in a 24-well microtitre plate was treated with different concentrations (1.0–10 μ g/ml) of plantaricin peptides both individually and in combination. Effect on cell viability was monitored as described later.

Treatment with outer membrane destabilizing agents

The one agent known to permeabilize OM is the chelating agent EDTA, which sequesters divalent cations that

contribute to the stability of the OM. To study its effect, a microtitre plate assay was carried out, as described above with different EDTA concentrations starting from 0.1 mM up to 20 mM for 8 h. An untreated control was also maintained. IC₅₀ concentration was determined on the basis of viable cell count. At this specific concentration of EDTA (1 mM), 10 µg/ml each of Pln peptide, singly or in appropriate combination, was added and cells were incubated for 8 h. Percent inhibition was calculated on the basis of viable cell count in comparison to the untreated control. In the next step, *E. coli* cells were first exposed to EDTA (1 mM) for 3 h followed by plantaricin (10 µg/ml) treatment. After 8 h treatment, the cell viability was determined on the basis of CFU/ml.

Other permeabilizers used were the detergent SDS and sodium deoxycholate. For this, *E. coli* cells were grown as described above and treated with different concentrations, such as 0.1, 0.25, 0.5, 0.75, 1, 2, and 5 % of SDS and sodium deoxycholate. At 0.5 % SDS and 0.75 % sodium deoxycholate (IC₅₀) concentrations, respectively, 10 µg/ml each of Pln E, -F, -J, and -K peptides, both individually and in appropriate combinations were added and cell viability was monitored. Cells were also pre-treated with these concentrations of SDS and sodium deoxycholate and then treated with 10 µg/ml of these peptides. In another experiment, pre-treatment of *E. coli* cells was carried out with 10 mM each of CaCl₂ and MgSO₄, individually before treating them with plantaricins. Percent inhibition of cell viability was calculated in terms of CFU/ml by comparing it with untreated control, in all the cases.

Temperature

To study the effect of low temperature, *E. coli* K-12 cells were grown at 18 °C, and the cells were treated with 5 and 10 µg/ml concentration of PlnE, -F, -J, and -K peptides. An untreated control was also maintained at same temperature.

Antimicrobial assay against *E. coli* K-12 *lpp* mutant strain

To check whether OM acts as a barrier for plantaricin activity, a lipoprotein mutant strain of *E. coli* K-12 was employed as indicator organism and effect of all the four plantaricins (at 2.5 and 5 µg/ml concentration) was observed against it and compared with the wild type strain.

Assay for antimicrobial peptides

The concentration of purified plantaricins was determined by UV absorption at 280 nm and with the help of BCA method using Bicinchoninic Acid Kit for Protein Determination (BCA1-1KT, Sigma-Aldrich, USA). The activity

of pantaricins was quantified by percent inhibition method with the help of microtitre plate assay and by flow cytometry.

Percent inhibition of cell viability

The activity of purified peptides was quantified in a 24-well microtiter plate using *E. coli* K-12 wild type and its *lpp* mutant as indicator strains. Cells were derived from a fresh, overnight culture in LB and subcultured in the same medium at an initial OD₆₀₀ of 0.01. One millilitre of culture in each well was grown with different concentrations (2.5, 5, and 10 µg/ml) of the peptides singly and in combination (PlnE–PlnF and PlnJ–PlnK) for 8 h at 37 °C. When the cells were grown at 18 °C, the bacteriocin treatment was given for 16 h. The viable cell count was estimated by plating an appropriately diluted suspension on LB agar, and represented as CFU/ml.

Checkerboard assay

The combined effect of plantaricins was tested via checkerboard assay in sterile microtitre plate. The inoculum was prepared using above described percent inhibition method and EDTA permeabilized (3 h) *E. coli* K-12 cells were treated with different concentrations (2.5, 5, and 10 µg/ml) of plantaricin EF/JK mixed at different ratios. The plates were then incubated at 37 °C and cell-viability inhibition was calculated at OD₆₀₀ after 8 h.

Flow cytometry

The standard plate counting method was compared with the help of flow cytometric enumeration. For flow cytometry, bacteriocin treatment (2.5 µg/ml) was given for 8 h at 37 °C, with initial OD₆₀₀ set at 0.05. Overnight grown control and bacteriocin-treated cells were harvested by centrifugation (8,000g for 5 min), washed once in phosphate-buffered saline (PBS [pH 7.2]), and resuspended in 200 µl of PBS. Dead cells were prepared by heat-killing a cell suspension at 90 °C for 30 min. While untreated and heat-killed washed *E. coli* K-12 cells were used as positive control for SYTO9 and PI staining, respectively, unstained cells were used as negative control. SYTO9 was added to a final concentration of 5 µM from a 5 mM stock solution in dimethyl sulfoxide, and the cells were incubated for 30 min at 30 °C in dark. PI was added to a final concentration of 30 µM. Ten thousand cells were acquired to observe the staining efficiency for analysis. The control samples were run simultaneously.

Flow cytometry was carried out with a FACSLSR II instrument (BD Biosciences, USA) equipped with an argon ion laser providing 488 nm (Blue laser) and 561 nm

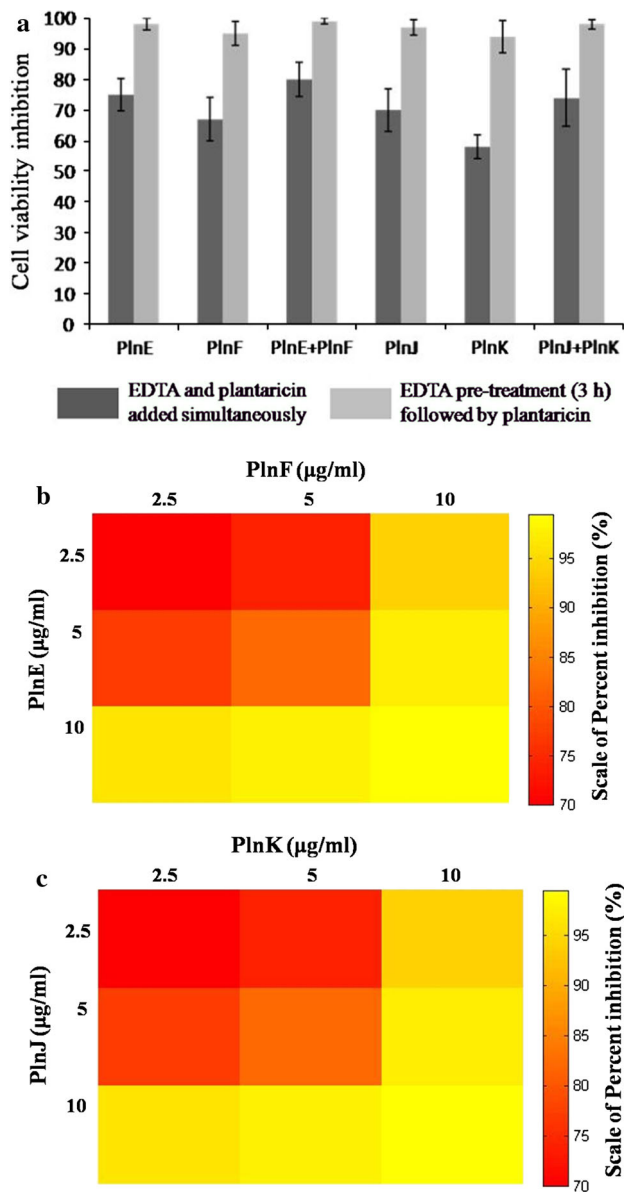


Fig. 1 **a** Cell viability inhibition by plantaricins and EDTA treatment in *E. coli* K12: dark grey bar EDTA and plantaricin added simultaneously, and light grey bar EDTA pre-treatment (3 h) followed by plantaricin. The experiments were done in triplicates and the results are expressed as percent of mean values of cell viability inhibition ($n = 3$) \pm standard deviation. **b** Heat map representing combined effect plantaricins (PlnE/F) against EDTA pre-treated *E. coli* K-12 cell. **c** Heat map representing combined effect plantaricins (PlnJ/K) against EDTA pre-treated *E. coli* K-12 cell

(Yellow green laser) with the standard filter setup. All parameters were collected as logarithmic signals. Fluorescence emission was detected in blue laser (530 nm with 505LP, 530/30BP) for SYTO 9 and at the yellow green laser (561 nm with 570LP, 575/26BP) for PI. The fluorescence emissions from live and treated *E. coli* cells were compared with the background fluorescence of each stain alone at the optimal excitation wavelength.

Results

Effect of plantaricins on *E. coli*

We have earlier observed that heterologously expressed and purified plantaricins (PlnE, -F, -J, and -K) are active against several Gram-positive organisms, in micro to nanomolar range, both singly and in combination. However, no effect was observed at these concentrations, when Gram-negative, *E. coli* K-12, was used as indicator organism. Even at 10 $\mu\text{g/ml}$ concentration, no inhibition was observed. As a result, membrane-permeabilizing agents such as chelating agent EDTA and detergents (SDS and sodium deoxycholate) were used in conjunction with plantaricins.

EDTA

When combined with 1 mM of EDTA (IC_{50} concentration), PlnE was found to be most effective ($\sim 75\%$), and PlnK the least ($\sim 58\%$) among the four peptides tested against *E. coli* K-12. Complementary peptide combinations were highly effective as the synergistic effect was observed with PlnE–PlnF showing $\sim 80\%$ and PlnJ–PlnK $\sim 75\%$ viable cell inhibition, respectively. Cells pre-treated with same concentration of EDTA showed a significant increase in inhibitory effect, as all the peptides showed almost 95–100% inhibition of cell viability under this condition (Fig. 1a).

Through checkerboard assay it was observed that the antimicrobial effect of plantaricins along with EDTA increases in a dose dependent manner. Different ratios of plantaricin concentration was used and maximum inhibition was observed when PlnE/F and PlnJ/K were used at a concentration of 10 $\mu\text{g/ml}$ of each (Fig. 1b, c).

SDS/sodium deoxycholate

A similar pattern of inhibition was observed, when the cells were treated with 0.5% SDS. Further an increase in inhibition by 10–25% was observed when plantaricin peptides were combined with SDS, singly as well as in combination. PlnE, -F, -J, and -K peptides showed $\sim 69, 60, 67, 57\%$ cell viability inhibition, respectively. In combination, synergistic effect was very apparent as PlnE–PlnF and PlnJ–PlnK showed ~ 75 and $\sim 70\%$ loss of viability. However, when the cells were first exposed to SDS, for 3 h prior to bacteriocin treatment, more than 90% inhibition was observed in all the cases (Fig. 2).

Similar effect was observed when plantaricins were applied along with IC_{50} concentration of sodium deoxycholate (0.75%). However, when the cells were pre-treated with 10 mM each of CaCl_2 or MgSO_4 , no significant inhibition was observed (data not shown).

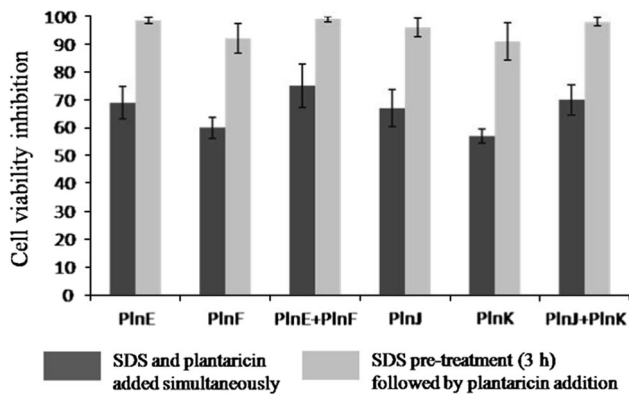


Fig. 2 Percent inhibition of cell viability by plantaricin and SDS treatment in *E. coli* K-12: dark grey bar SDS and plantaricin added simultaneously, and light grey bar SDS pre-treatment (3 h) followed by plantaricin addition. The experiments were done in triplicates and the results are expressed as percent of mean values of cell viability inhibition ($n = 3$) \pm standard deviation

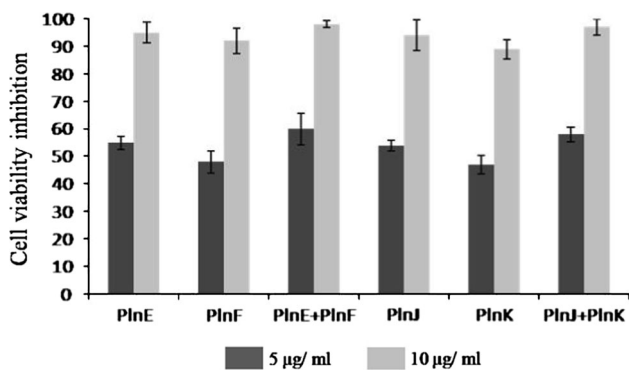


Fig. 3 Percent inhibition of viable cells by plantaricins at low temperature in *E. coli* K12 at dark grey bar 5 µg/ml concentration, and 10 µg/ml concentration. The experiments were done in triplicates and the results are expressed as percent of mean values of cell viability inhibition ($n = 3$) \pm standard deviation

Low temperature

Plantaricin treatment at low temperature was highly effective. When the cells were grown at 18 °C with 10 µg/ml of plantaricins, complete loss of viability (90–100 %) was observed. When the concentration of plantaricin was lowered to 5 µg/ml each of the individual peptides, ~50 % inhibition was recorded. A combination of PlnE–PlnF once again was more potent (65 % inhibition) in comparison to PlnJ–PlnK (60 % loss of viability) (Fig. 3). However, at 37 °C, no significant inhibition was observed at any of these concentrations of plantaricins.

Effect on *lpp* mutant strain

Keeping in line with the effect of membrane permeabilizing agents, the effect of plantaricins was much more

pronounced against *E. coli* K-12 *lpp* mutant strain. The effect was found to be dose-dependent, as at 5 µg/ml of plantaricin, complete inhibition was observed and even at 2.5 µg/ml of individual plantaricin, a significant decrease in viability (45–60 %) was observed in terms of CFU/ml. In the wild-type cells, these concentrations of peptide(s) would lead to no significant inhibition (data not shown).

To have a comparative analysis, and to find the correlation between cell counts determined flow cytometrically and by CFU assays, this concentration (2.5 µg/ml) was used to perform flow-cytometric experiment.

Flow cytometric analysis

The analysis was based not only on the differences in excitation and emission spectrum of the dyes, but also could be correlated with the percent distributions of viable and dead bacteria due to the smooth transition of the viability states, if any.

Figure 4, represents the results of two controlled populations from both the strains subjected to dual staining, based on the differential response of SYTO9 and PI, with former staining both live and dead cells, and the latter only the dead and damaged cells. The results clearly showed a shift of cell population in Q2 quadrant representing the heat-killed population (a1–a2), whereas a scattered pattern was observed in case of live cell population (a3–a4). This pattern was common for the control cells of both wild type and the mutant strain. When compared with the viability status, heat-killed population showed just about 5 % viable cells in comparison to control live cell population.

In the next step, plantaricin-treated (2.5 µg/ml) wild type and mutant cells were stained and analysed, which resulted in a distinctive and reproducible fluorescence pattern, indicating different cellular states. As shown in Fig. 5, plantaricin-treated wild type cells (a1–a3; c1–c3) showed a similar fluorescence distribution as that of untreated cell population, though in peptide combinations (a3 and c3), there appeared to be a slightly higher differentiation of damaged cells. In comparison, treated mutant cells exhibited distinctly higher population of damaged/dead cells. It clearly demonstrated that the cells of mutant strain (*lpp*) are much more sensitive to plantaricins compared to the wild type strain (Fig. 5b1–b3, d1–d3).

When FCM analysis was compared with the percent distribution of damaged/dead cells (Q2 quadrant) of both the wild-type and mutant strain, the results described above could be confirmed. In case of wild-type *E. coli* K-12, percent cell death was <10 % in all the cases, when treated with PlnE, -F, -J, and -K individually. The number was slightly higher when a combination of PlnE/PlnF and PlnJ/PlnK was used (~12 %). In comparison, PlnE, PlnF, PlnJ,

and PlnK treated mutant strain led to 55, 50, 57, 40 percent cell death, respectively and their combination led to 66 and 68 % inhibition, respectively, indicating synergistic action.

The FCM analysis, therefore, confirmed the results obtained with the viability assessment under different conditions. As described earlier, the plantaricin's concentration used did not show any effect on the cell viability of wild type *E. coli* K-12. Though FCM analysis indicated low levels of damage, it was not different from the control cells. It is thus clear that low concentrations of peptides could not inflict any damage. That the apparent tolerance of the wild-type *E. coli* cells to these plantaricins is indeed due to the intact OM acting as a barrier became apparent, as the mutant strain (*lpp*) possessing a weaker OM was ~ fivefold to sixfold more sensitive at the same concentration of plantaricin.

Discussion

The plantaricins belong to class IIb of bacteriocin or peptide antibiotics produced by different strains of *L. plantarum*.

Fig. 5 Flow cytometric analysis of *E. coli* K-12 wild-type and *lpp* mutant cells treated with plantaricins and stained with SYTO 9 and PI: PlnE, PlnF, PlnE + PlnF against *E. coli* K-12 wild-type (**a1–a3**), and *lpp* mutant (**b1–b3**); PlnJ, PlnK, PlnJ + PlnK against *E. coli* K-12 wild type (**c1–c3**), and *lpp* mutant (**d1–d3**)

These 2-peptide bacteriocins act synergistically, and a strain may produce multiple of such peptides (Anderssen et al. 1998; Moll et al. 1999). Bacteriocins produced by LAB have received considerable attention as food preservatives and as potential replacement of antibiotics (Todorov et al. 2007; Todorov 2009). However, there are major considerations in their usage because of their narrow antimicrobial spectrum and low level or unstable production. It is, therefore, important that these peptides are available in large quantities in purified form. Though chemical synthesis is one opinion, we have demonstrated that heterologous expression can also be employed to enhance their yield (Pal and Srivastava 2013). It is also clear that Gram-negative bacteria may be highly tolerant to these peptides owing to their OM acting as a protective barrier (Lappe et al. 2009; Martin-Visscher et al. 2011). Our results have confirmed this, as *E. coli* cells

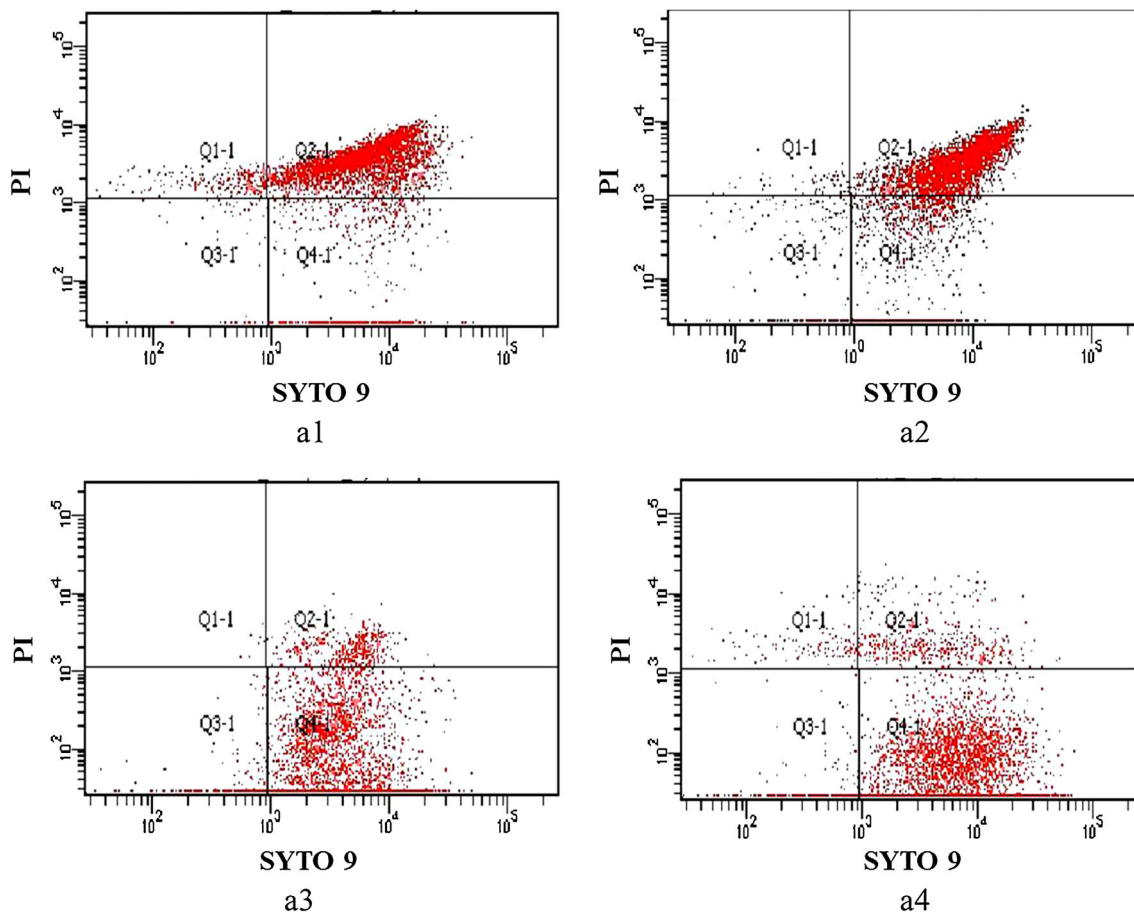
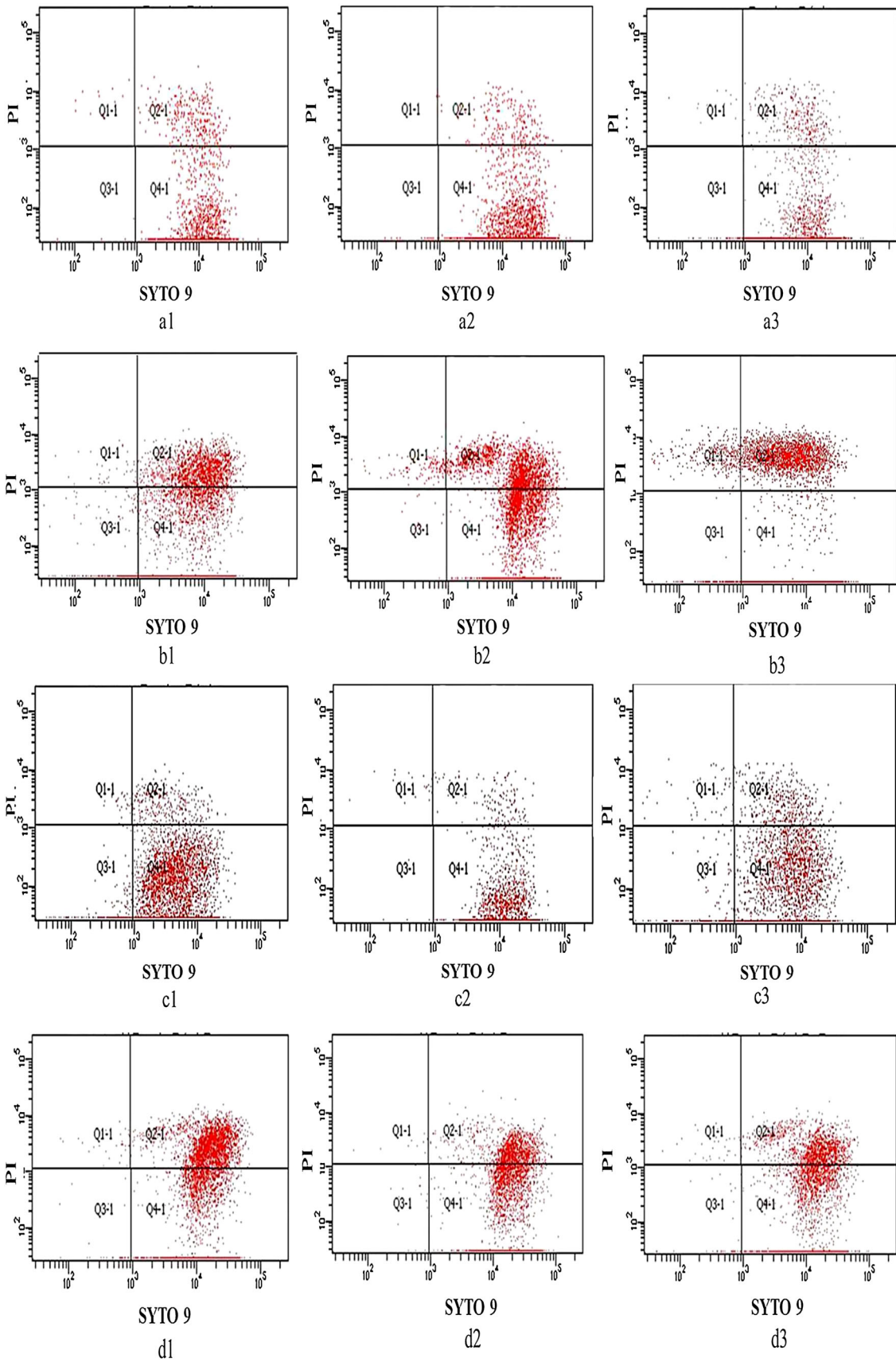


Fig. 4 Flow cytometric analysis of *E. coli* K-12 wild type and *lpp* mutant strain stained with SYTO 9 and PI: heat killed cell stained with SYTO 9 and PI (**a1, a2**), live untreated cell stained with SYTO 9 and PI (**a3, a4**)



otherwise impervious to plantaricins became sensitive only when treated with membrane destabilizing agents.

Effect of EDTA, one such agent, has been reported to be differential, not only depending upon the type of bacteriocin under consideration but also the target bacterial species (Martin-visscher et al. 2011). It has also been reported that even closely related species perhaps possessing similar OM permeabilities, may actually have different cell-envelope properties. It is, therefore, very important that the conditions needed to inhibit target bacterial species are optimized. In other words, it must be recognized that Gram-negatives are not created all equal (Schweizer 2012). Chelators sequester divalent cations that contribute to the stability of the OM by providing electrostatic interactions with proteins and LPS. Sensitization of Gram-negative cells with the help of detergents such as SDS, and sodium deoxycholate has also been reported (Alakomi et al. 2006). Thus, food-grade permeabilizers in combination with other antimicrobials would be ideal in inhibiting Gram-negative spoilage bacteria and pathogens in food materials (Alakomi et al. 2006).

Our results have also demonstrated that perturbation of OM results in the marked efficiency of inhibitory activity of plantaricins. Hence by pre-treatment with these agents, plantaricins may have had a better access to the target cell membrane leading to enhanced inhibition.

Similarly, many changes in bacterial fatty acid composition and membrane fluidity occur in response to temperature fluctuations (Mansilla et al. 2004). We have shown that at low temperature, plantaricins were significantly more effective in inhibiting *E. coli*, in comparison to optimum growth temperature of 37 °C. The reason might be that greater proportion of unsaturated or branched fatty acids allows the phase transition to occur at a lower temperature, whereas a greater proportion of saturated fatty acids allows the transition temperature to be elevated (Marr and Ingraham 1962). This property of plantaricins can be highly beneficial during food preservation as low temperature storage of food is a common practise.

The role of OM in plantaricin activity was further confirmed as an *lpp* mutant of *E. coli* K-12, lacking a specific OM lipoprotein, both the free and the bound forms, showed much higher sensitivity to plantaricins. This mutant has been reported to be defective in producing mRNA for lipoprotein synthesis, and is hyper-sensitive to EDTA, cationic dyes, and detergents (Hirota et al. 1977). The cell viability of the mutant strain was remarkably affected by plantaricin treatment at a concentration which was absolutely ineffective against wild-type K-12 strain. Flow cytometric analysis using SYTO9–PI dual staining further confirmed these results. Thus, it could be stated that the OM does play a key role and acts as a protective barrier against plantaricin activity, as with many other bacteriocins.

Treatments of plantaricins in combination with selected agents affecting outer-membrane (OM) permeability, would increase their effectiveness against Gram-negative cells, which are generally resistant. The combined effect of various concentrations of PlnE/F and PlnJ/K in different ratios was checked against *E. coli* K-12 through the checkerboard microtitre plate assay. However, synergistic activity of the complementary peptides reported in literature was not very remarkable in this case (Anderssen et al. 1998; Moll et al. 1999; Opegård et al. 2007). This difference could be explained on the basis of use of different host strains which are phylogenetically distant from each other. In this study, Gram-negative model organism *E. coli* was used as indicator organism, which has a totally different machinery as well as cell-wall organization. Due to the presence of LPS molecules in the outer leaflet of the membrane, most of the Gram-negative bacteria acquire an inherent resistance to several antimicrobial compounds and therefore are not inhibited even at a concentration 10–100 fold higher than the one that inhibited some Gram-positive and other LAB members (Gao et al. 1999). Even it is also reported that individual plantaricin peptides could exert strong anti-*Candida* effect at a higher concentration, but did not exhibit any synergistic effect (Sharma and Srivastva 2014). Thus it can be possible that the mode of action of plantaricins is strictly dose- and host-dependent.

Moreover, the effect of antimicrobial compound depends on several other things, such as, initial cell density of target population, the growth rate of the bacteria, and byproduct resources generated from dead bacteria, which can also make difference in the inhibition pattern (Hartmann et al. 2010).

Finally, it can be concluded that the PlnE, -F, -J, -K have strong antimicrobial activity, and its narrow antibacterial spectrum can be extended by manipulating the treatment conditions. It should thus become possible to use them not only in preservation of food, but also as a therapeutic molecule.

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