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The Effect of Bacteriocin Produced by *Lactobacillus plantarum* on the Growth of *Listeria monocytogenes*

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The inhibitory effect of *Lactobacillus plantarum* (*Lb. plantarum*) which is bacteriocin-producing strain against the growth of *Listeria monocytogenes* (*L. monocytogenes*) was examined in trypticase soy broth (TSB). TSB was inoculated with 10^4 cells/mℓ *L. monocytogenes* and then with different numbers (10^6, 10^4 and 10^2 cells/mℓ) of *Lb. plantarum*. The mixed cultures were incubated at 37, 25 and 4℃. The most effective inhibition of was found at 37℃ and a less inhibition at 25℃. However, there was no significant change in the cell numbers of both *L. monocytogenes* and *Lb. plantarum* at 4℃. At same incubation temperature, the higher initial inoculum level of *Lb. plantarum*, the better inhibitory effect against *L. monocytogenes*. In addition, TSB was inoculated with *L. monocytogenes* at different initial inoculum levels of 10^6, 10^4 and 10^2 cells/mℓ and then supplemented with 0, 30, 60 and 100 AU/mℓ of bacteriocin produced by *Lb. plantarum*. The mixed cultures were incubated at 37, 25 and 4℃. *L. monocytogenes* of three different initial inoculum levels began to be inhibited in the presence of more than 60 AU/mℓ of bacteriocin at 37℃. In TSB containing more than 60 AU/mℓ of bacteriocin and incubated at 25℃, *L. monocytogenes* decreased by 2 log-units during the period of 12 hrs incubation and thereafter remained steady. At 4℃, *L. monocytogenes* decreased by 1.5 log-units in the presence of 60 AU/mℓ bacteriocin during the period of 4 days incubation and dropped to the non-detectable level in TSB with 100 AU/mℓ bacteriocin.

Key words: *Listeria monocytogenes*, *Lactobacillus plantarum*, bacteriocin

Introduction

Some lactic acid bacteria produce antimicrobial proteins known as bacteriocins which inhibited the growth of the pathogenic and spoilage bacteria in foods (Tagg et al., 1976; Hurst, 1981; Barefoot and Klaenhammer, 1983; Pucci et al., 1988). Antimicrobial activity of bacteriocin-producing bacteria has been studied with respect to the potential use for food preservation. Bacteriocin-producing *Lactobacillus* spp. and *Pediococcus* spp. inhibit the growth of *L. monocytogenes*, a well-known foodborne pathogen (Barefoot and Klaenhammer, 1984, Bhunia et al., 1988; Pucci et al., 1988).

Since *L. monocytogenes* is commonly found in a variety of food products and causes foodborne disease, the industries and regulatory agencies of food have been paid attention to its occurrence in food (Brackett, 1988; Bailcy et al., 1989; Johnson et al., 1990). The prevalence of *L. monocytogenes* ranges from 0 to 9% in raw meats and poultry and from 3 to 13% in ready-to-eat products, in which typical plate counts ranges 10 to 1000 CFU/g (Buchanan et al., 1989; Johnson et al., 1990). Morris and Ribeiro (1989), however, detected 10^4 CFU/g in a processed plate and 10^3-10^6 CFU/g in contaminated slices of prepacked meats. Thermal treatment could be effective to eliminate undesirable bacteria such as *L. monocytogenes*, but this method is unlikely to be applied to raw meats and poultry because of post-processed contamination (Johonson et al., 1990; Zaika et al., 1990). Biopreservation systems, therefore, such as organic acid, peroxides and bacteriocins have gained increasing attention as a means of natural controlling of the growth of pathogenic bacteria including *L. monocytogenes* (Luchansky and Doyle, 1991; Degnan et al., 1992).

In this study, the inhibitory effect of *Lb. plantarum* which is bacteriocin-producing strain against the growth of *L. monocytogenes* in trypticase soy

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broth was examined at 37 (optimum growth temp.), 25 (abusive temp.) and 4℃ (refrigerated temp.). In addition, inhibitory effect of different concentration of bacteriocin against *L. monocytogenes* with different initial inoculum levels was also examined.

Materials and Methods

1. Bacterial strains

The baceriocin-producing strain, the bacteriocin sensitive strain and the indicator strain for antimicrobial test were *Lactobacillus plantarum* (*Lb. plantarum*) LMG7945, *Listeria monocytogenes* (*L. monocytogenes*) ATCC15313 and *Proteus mirabilis* (*P. mirabilis*) NCTC5887, respectively. All strains were obtained from BCCM (Belgium Coordinated Collections of Microorganisms), maintained in trypticase soy agar (TSA, Difco) slant at 4℃ and transferred monthly. Cells of each strain were propagated twice before experiments.

2. Crude bacteriocin preparation and bacteriocin assay

Bacteriocin was prepared as described by Kim et al. (1995). The bacteriocin producing organism, *Lb. plantarum*, with the initial level of 10^5 cells/㎖ was inoculated to MRS (Oxoid) broth and cultured at 37℃ for 24 hrs. The cells were harvested by centrifugation (4,000×g for 30 min at 4℃) and the cell-free supernatant was concentrated by rotary evaporation (55℃, 100 rpm). This was prepared for the crude bacteriocin.

Antimicrobial activity of bacteriocin was tested by the disk diffusion method as described by Sobrino et al. (1992) and Jepperson and Huss (1993). The MRS agar test plates (1.5% agar) were overlaid with about 2.5×10^5 cells/㎖ *P. mirabilis*, the bacteriocin indicator organism, in 4 ㎖ of soft MRS agar (0.7% agar). Sterile paper discs (0.8 mm, TOYO) containing 25 ㎕ of bacteriocin were placed on the agar plates and the plates were incubated at 37℃ for 18 hrs. Antimicrobial activity of bacteriocin was measured by the diameter of the clearing zones formed around the discs, which is the inhibition zone of *L. monocytogenes*, the bacteriocin sensitive organism. The inhibition zone with 0.5 mm diameter was determined as positive and the activity was defined as the reciprocal of the last serial dilution demonstrating inhibition activity presented as activity unit (AU) per milliliter.

3. Enumeration of the strains

To enumerate *L. monocytogenes* and *Lb. plantarum*, both organisms were pour-plated and duplicated on Listeria enrichment agar (Difco) and MRS agar respectively and (Oxoid), incubated at 35℃ for 48 hrs.

4. Preparation of mixed culture of *L. monocytogenes* with *Lb. plantarum*

100 ㎖ TSB was inoculated with about 1% of precultured *L. monocytogenes* and incubated at 37℃ for 24 hrs. In order to obtain about 10^4 cells/㎖ of the mixed cultures, cells were harvested by centrifugation (5,500×g for 10 min at 4℃) and washed in 100 ㎖ of sterile physiological saline solution (PSS) and resuspended in TSB.

MRS broth was inoculated with about 1% of precultured *Lb. plantarum* and incubated at 37℃ for 24 hrs. Cells were sedimented, washed in 100 ㎖ of PSS and resuspended in TSB containing 10^4 cells/㎖ of *L. monocytogenes*. The initial inoculum levels of *Lb. plantarum* were about 10^2, 10^4 and 10^6 cells/㎖ and then each mixed culture was incubated at 37, 25 and 4℃.

5. The inhibitory effect of bacteriocin against growth of *L. monocytogenes*

TSB was inoculated with *L. monocytogenes* of the initial level of about 10^2, 10^4 and 10^6 cells/㎖ and then 0, 30, 60 and 100 AU/㎖ the crude bacteriocin was added to the culture. Each culture was incubated at 37, 25 and 4℃.

Results

1. Behavior of *L. monocytogenes* in the presence of *Lb. plantarum*.

About 10^6, 10^4 and 10^2 cells/㎖ *Lb. plantarum* were added to *L. monocytogenes* culture with about 10^4 cells/㎖. The three mixed cultures were incubated at 37, 25 and 4℃.

In case of mixed culture with 10^4 cells/㎖ *L. monocytogenes* and 10^6 cells/㎖ *Lb. plantarum* (Fig. 1), the inhibitory effect against *L. monocytogenes* at 37℃ was higher than at 25℃. At 37℃, inhibition against *L. monocytogenes* begun between 9 and 12 hrs incubation and dropped to the non-detectable level after 27 hrs. The concentration of produced bacteriocin was 53 AU/㎖ and 126 AU/㎖ after 9 and 12 hrs incubation, respectively. At 25℃, the cell numbers of *L. monocytogenes* began to decrease

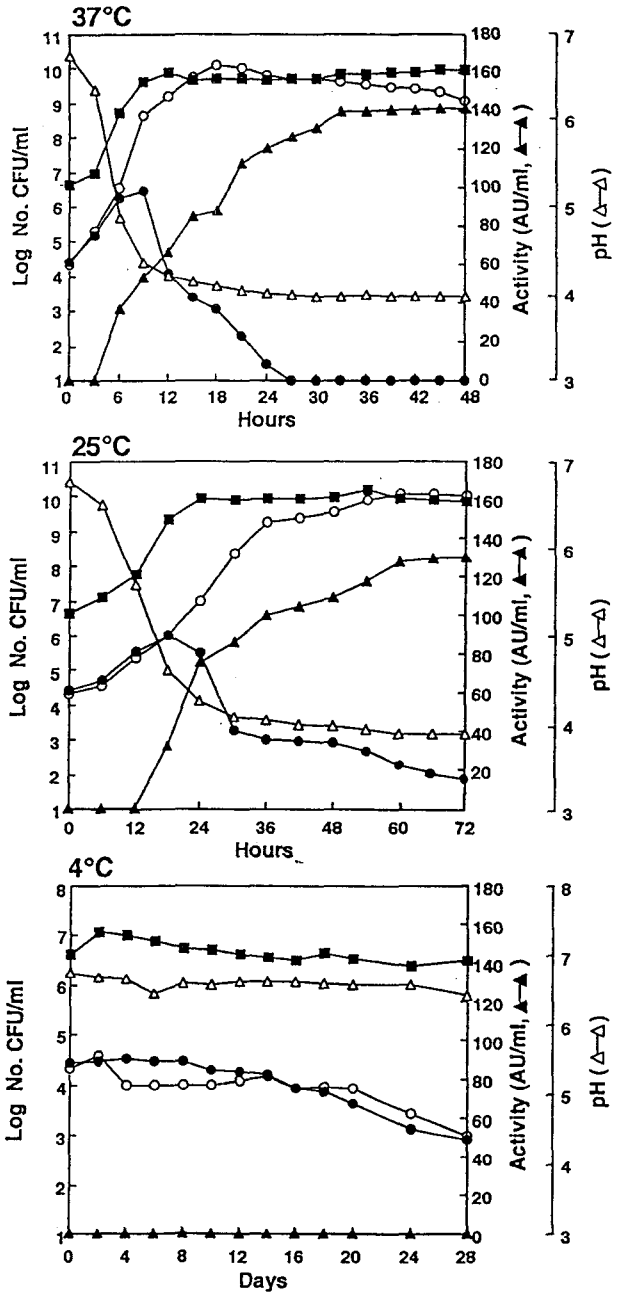

Fig. 1. Effect of *Lb. plantarum* (10^6/mℓ) on growth of *L. monocytogenes* (10^4/mℓ) in trypticase soy broth cultured at 37, 25, 4℃.
○—○ ; *L. monocytogenes* in control.
●—● ; *L. monocytogenes* in mixed culture.
■—■ ; *Lb. plantarum* in mixed culture.

after 20 hrs incubation, rapidly decreased between 20 and 35 hrs, and then remained relatively steady until 72 hrs. The bacteriocin concentration was 50 AU/mℓ by 20 hrs incubation and 130 AU/mℓ by 72 hrs. At 4℃, there was no change in the cell numbers of *L. monocytogenes* and *Lb. plantarum* up to 28 days. The number of *L. monocytogenes* decreased by 1 log unit, while there was no reduction in the cell numbers of *Lb. plantarum*. Similarly, there was no change in the pH of the culture over 28 days and no detectable bacteriocin activity in the mixed cultures at 4℃. The growth of *L. monocytogenes* was not inhibited at 4℃ because lactic acid and bacteriocin were not produced.

It was found that the inhibitory effect against *L. monocytogenes* depended on the incubation temperature of the mixed culture with 10^4 cells/mℓ *L. monocytogenes* and 10^4 cells/mℓ *Lb. plantarum* (Fig. 2). The inhibitory effect against *L. monocytogenes* was also higher at 37℃ than at 25℃. At 37℃, 120 AU/mℓ of bacteriocin was produced by the end of 48 hrs incubation and the population of *L. monocytogenes* dropped to the non-detectable level at 40 hrs. At 25℃, bacteriocin concentration was 78 AU/mℓ and initial *L. monocytogenes* population (10^4 cells/mℓ) decreased to 3.2×10^3 cells/mℓ after 48 hrs.

Fig. 3 shows that the inhibition rate against *L. monocytogenes* was higher in TSB with 10^4 and 10^6 cells/mℓ *Lb. plantarum* than that of 10^2 cells/mℓ.

2. Behavior of *L. monocytogenes* in the presence of bacteriocin

The crude bacteriocin of 0, 30, 60 and 100 AU/mℓ was added to TSB containing about 2.9×10^6 cells/mℓ *L. monocytogenes* and incubated at 37, 25 and 4℃ (Fig. 4).

At 37℃, the population of *L. monocytogenes* in the absence of bacteriocin increased from the initial level of 2.9×10^6 cells/mℓ to 3.2×10^9 cells/mℓ after 30 hrs incubation. The growth of *L. monocytogenes* in the presence of 30 AU/mℓ of the crude bacteriocin delayed about 10 hrs. In the presence of 60 and 100 AU/mℓ of bacteriocin, the growth of *L. monocytogenes* began to be inhibited as soon as the bacteriocin was added. The *L. monocytogenes* population decreased to the non-detectable level after 33 and 24 hrs incubation at 60 and 100 AU/mℓ of bacteriocin, respectively. At 25℃, the cell numbers of *L. monocytogenes* decreased progressively until 12 hrs of incubation in the presence of 60 and 100 AU/mℓ of bacteriocin and thereafter did not change by 72 hrs incubation. At 4℃, the population of *L. monocytogenes* decreased slightly from the initial level of 2.9×10^6 cells/mℓ to 2.2×10^4 cells/mℓ in the presence of 60 and 100 AU/mℓ of bacteriocin during the incubation period. *L. monocytogenes*, however,

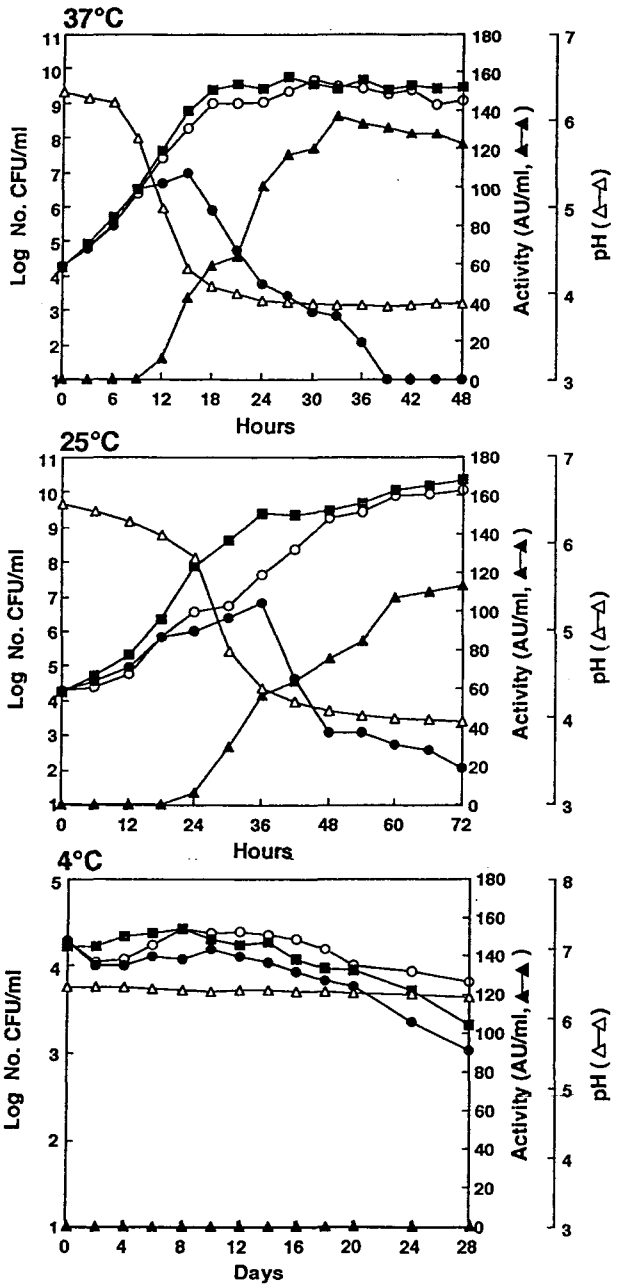

Fig. 2. Effect of *Lb. plantarum* (10^4/mℓ) on growth of *L. monocytogenes* (10^4/mℓ) in trypticase soy broth cultured at 37, 25, 4℃.
Symbol definition is as described for Fig. 1.

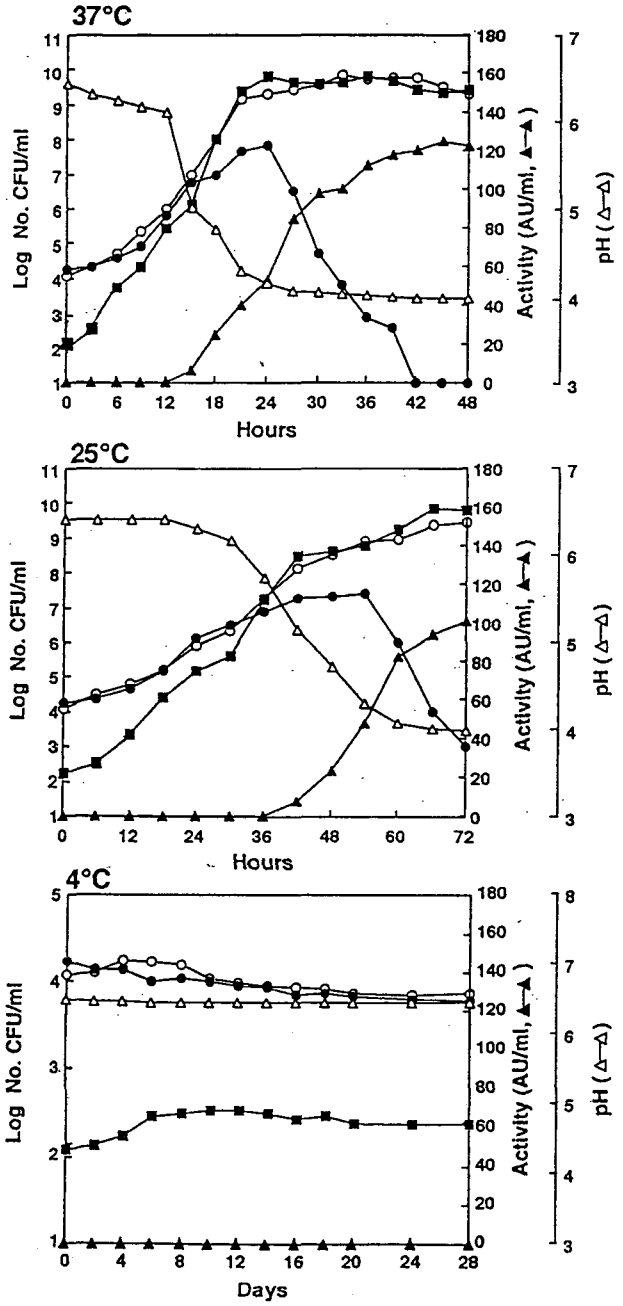

Fig. 3. Effect of *Lb. plantarum* (10^2/mℓ) on growth of *L. monocytogenes* (10^4/mℓ) in trypticase soy broth cultured at 37, 25, 4℃.
Symbol definition is as described for Fig. 1.

was not detected in the presence of 100 AU/mℓ bacteriocin after 24 hrs.

When the initial inoculum levels of *L. monocytogenes* were 10^4 cells/mℓ (Fig. 5) and 10^2 cells/mℓ (Fig. 6), the overall pattern of the inhibition of *L. monocytogenes* was similar to the previous result of inhibition pattern (Fig. 4). However, the inhibition rate decreased in proportion to the lower concentration of bacteriocin and initial levels of *L. monocytogenes*.

Discussion

The growth of *L. monocytogenes* in the presence of *Lb. plantarum* began to be inhibited at the middle or late logarithmic phase of *Lb. plantarum* where the concentration of bacteriocin was about 50 ~60 AU/mℓ. The inhibitory effect against *L. monocytogenes* was affected by incubation temperature. In the case of 37℃ incubation, *L.*

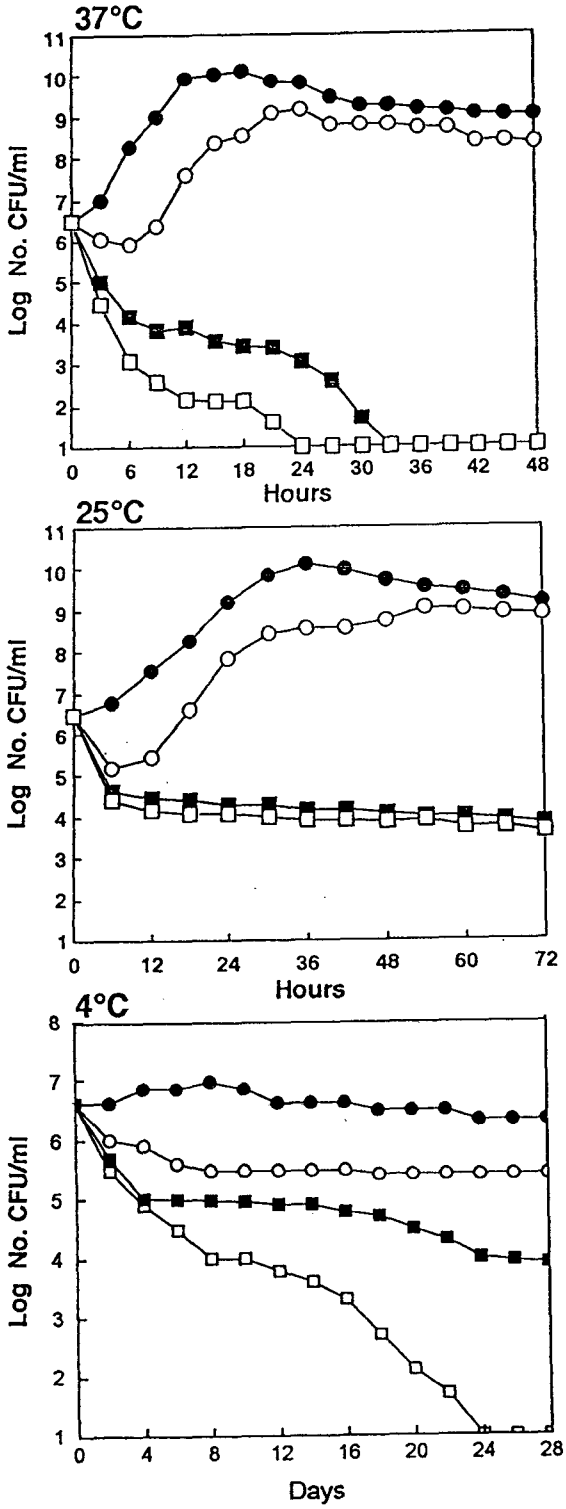

Fig. 4. Effects of different concentration of bacteriocin on the growth *L. monocytogenes* (10^6 /mℓ) in trypticase soy broth at 37, 25, 4℃. ●−−● ; control, ○−−○ ; 30 AU/mℓ, ■−−■ ; 60 AU/mℓ, □−−□ ; 100 AU/mℓ

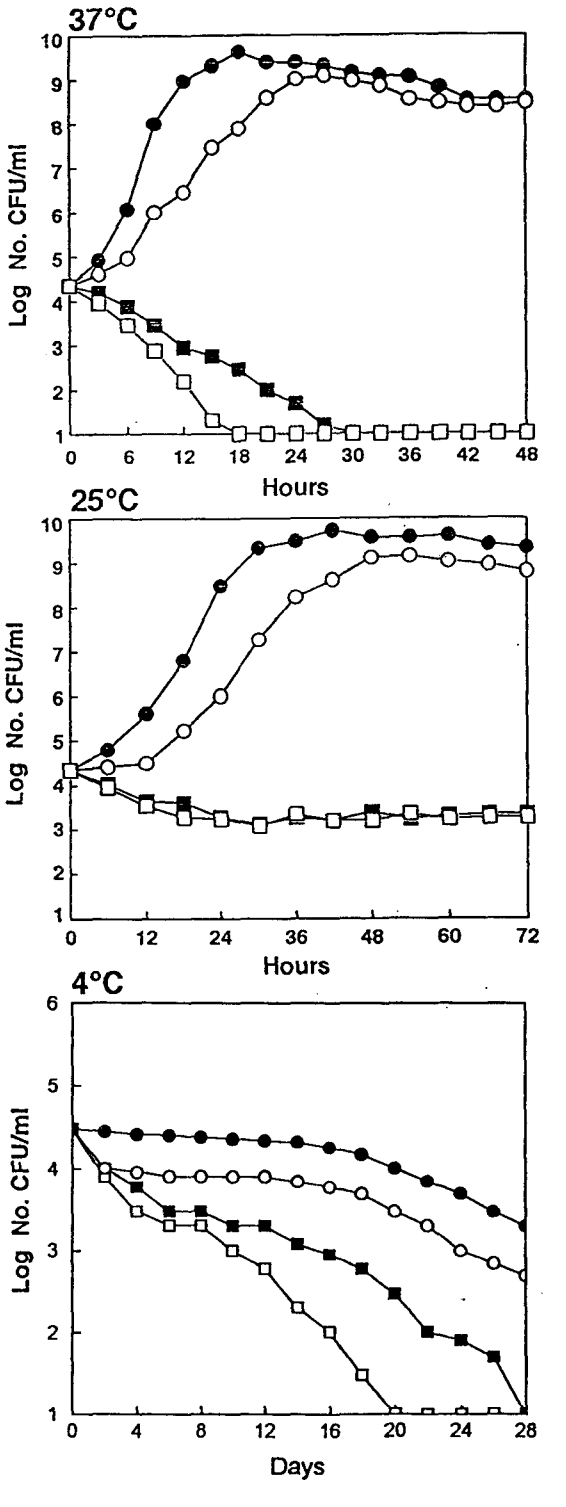

Fig. 5. Effects of different concentration of bacteriocin on the growth *L. monocytogenes* (10^4 /mℓ) in trypticase soy broth at 37, 25, 4℃. Symbol definition is as described for Fig. 4

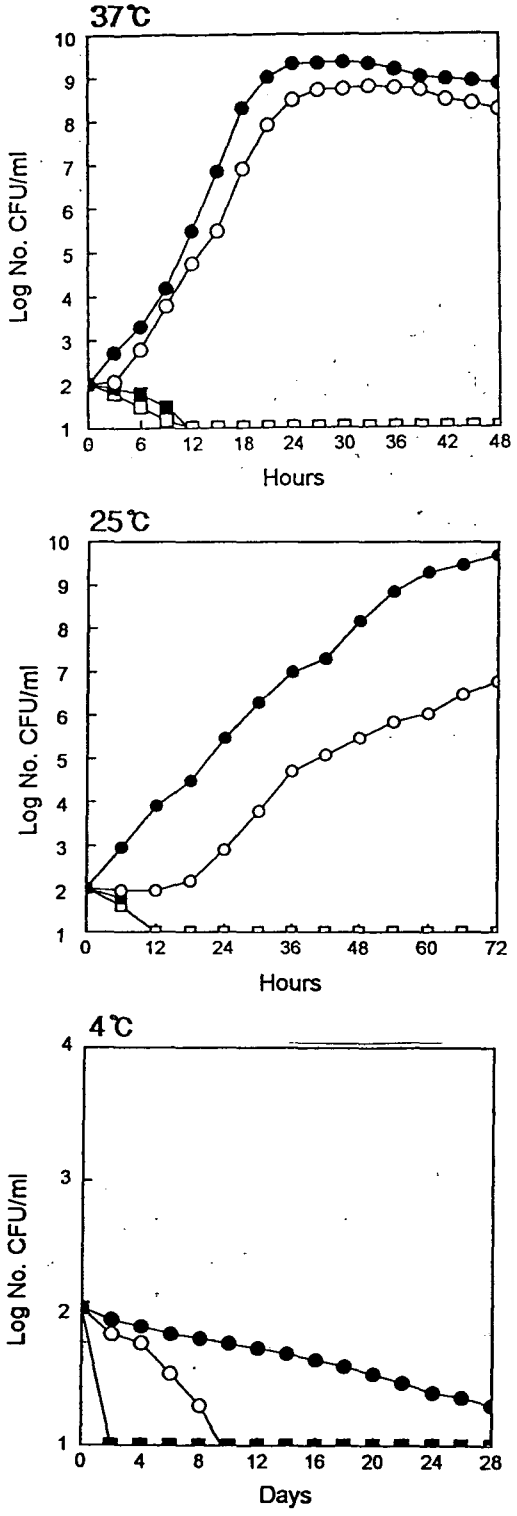

Fig. 6. Effects of different concentration of bacteriocin on the growth *L. monocytogenes* (10^2 /mℓ) in trypticase soy broth at 37, 25, 4℃. Symbol definition is as described for Fig. 4

monocytogenes was inhibited completely at the late stationary phase of *Lb. plantarum* where the concentration of bacteriocin was about 120 AU/mℓ. However, the inhibitory effect of *Lb. plantarum* against *L. monocytogenes* at 25℃ was lower than that of 37℃ in spite of same *Lb. plantarum* phase (Fig. 1-3). These results indicate that inhibitory effect depends on bacteriocin activity, and *Lb. plantarum* produce much more bacteriocin at 37℃ than at 25℃.

At 37℃, *L. monocytogenes* began to be inhibited in the presence of 60 AU/mℓ of bacteriocin and was inhibited completely at 100 AU/mℓ. However, the cell number of *L. monocytogenes* did not change throughout the incubation period at 25℃ in the presence of same concentration of bacteriocin, although it was inhibited until 10 hrs after incubation (Fig. 4). These results suggest that incubation temperature is one of the important factors for the inhibition of *L. monocytogenes* by *Lb. plantarum*.

Degnan et al. (1992) found that *L. monocytogenes* decreased by 3 log-units in 8 days at 25℃, but was not inhibited at 4℃ when meat products inoculated with *Pediococcus acidilactis* were incubated at 25 and 4℃. Schaack and Marth (1988a, b) inoculated 5.0, 1.0 and 0.5% of lactic culture with *L. monocytogenes* on yogurt, and found that higher initial inoculum level showed faster inhibition. Liao et al. (1993) found that *L. monocytogenes* was not inhibited by pediocin PO2 at 25℃ because pediocin PO2 combined irreversibly with susceptible cells and had lost its activity. Bhunia et al. (1991) provided evidence for the presence of specific receptors for pediocin AcH on the cell walls of susceptible bacteria. The elucidation of the mechanisms of bacteriocin activities against susceptible bacterial strains is important for their effective use in food preservation. The primary target of nisin was reported to be the cytoplasmic membrane (Sahl and Brandis, 1983; Ruhr and Sahl, 1985). Zajdel et al., (1985) observed that lactocin 27 obtained from *Lactobacillus helveticus* LP27 inhibited protein synthesis, caused cytoplasmic membrane damage and was bacteriostatic. Bhunia et al., (1991) found that Pediocin PA-1 bound to sensitive strains was bactericidal and caused lysis of a strain of *L. monocytogenes*. Yousef et al. (1991) reported that pH, temperature and anion salts were effective for the adsorption of bacteriocin to sensitive strains and

suggested that many factors might be responsible for the inhibition mode.

Food is a complex media in which various components, alone or in combination for bacterial growth. It is difficult to directly correlate this effect to any single component. Natural preservation systems such as bacteriocinogenic lactic acid bacteria and/or associated bacteriocin may find finally for controlling the occurrence and dissemination of *L. monocytogenes* in food. In order to use bacteriocins as natural biopreservation, further studies on the mode of action in association with many inhibition factors including temperature are necessary.

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