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Inactivation of *Listeria monocytogenes* on Raw Pork Treated with Modified Atmosphere Packaging and Nisin

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ABSTRACT

The response of *Listeria monocytogenes* Scott A was examined on exposure to a combination treatment of nisin and modified atmosphere at 4 and 20℃. Atmospheres employed were 100 and 80% CO_2/20% air; and an air control. The organisms were exposed to these atmospheres on raw pork tenderloin treated with various concentrations of nisin (0, 10^4, and 5 × 10^4IU/ml). Treated samples were packaged in PET/Al/PE bags and stored at 4 and 20℃ for 30 and 10 days, respectively. Changes in pH, microbial numbers, gaseous headspace composition, and nisin concentrations were monitored during the storage period. In the absence of nisin, the anaerobic modified atmosphere (100% CO_2) resulted in the failure of both the aerobic plate counts and *L. monocytogenes* to grow at the temperatures tested. In addition, both the *L. monocytogenes* and aerobic microorganisms grew in air at 4 and 20℃. In combination with nisin, all atmospheres were inhibitory to the growth of this pathogenic microorganism. These inhibitory effects for MAP/nisin combination system were more pronounced at 4℃ than at 20℃. The combination treatment was also increasingly effective with increasing CO_2 and nisin concentrations.

Key words : *Listeria monocytogenes,* Nisin, MAP, Pork

INTRODUCTION

Controlled and modified atmosphere packaging (MAP) are commonly used to slow the ripening process, to inhibit fungal deterioration of fresh fruits and vegetables during storage and to extend the shelf life of perishable refrigerated foods[1]. It has become widely practiced in the poultry industry for both raw and cooked products. Inhibition of Gram-negative microorganisms such as *Pseudomonas, Moraxella* and *Acinetobacter* by CO_2 has been demonstrated[2]. Carbon dioxide-modified atmosphere packaging has also been shown to be effective against some pathogenic bacteria[3]; however, MAP is not effective against *Listeria monocytogenes*[4].

Listeria monocytogenes is the pathogenic bacteria responsible for several food-related outbreaks of disease[5]. Listeriosis may cause death; the disease characteristics of *L. monocytogenes* have been described[6]. The association of *L. monocytogenes* with red meat, poultry and meat products is well-established[7,8]. Incidence rates

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of *L. monocytogenes* in raw meat vary considerably, the rates were as low as 4% and as high as 92%[9]. Survival of *L. monocytogenes* from several food processes such as sausage-making [10], cheese-making[11] and yogurt-making[12] has been reported. Since *L. monocytogenes* is psychrotrophic, this pathogenic organism not only survives, but grows, at temperatures at or below 7°C in various foods[13]. The potential growth of *L. monocytogenes* in refrigerated foods prompted many investigators to search for methods to control the pathogens in those products.

Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis,* has been tested on bacon[14] and chicken frankfurter emulsion[15] for its antibotulinal activity. It can inhibit the outgrowth of *Bacillus licheniformis* [16], and *Clostridium sporogenes* spores[17] and the growth of lactic acid bacteria from cured and fermented meat products[18]. The action of nisin against pathogenic bacteria such as *L. monocytogenes* has been tested[19,20], with nisin approved as a food additive in many countries including the United States[21].

The relationship between the growth of spoilage organisms and pathogens is a critical safety factor in MAP. There is little or no information on the effectiveness of combining MAP with nisin as a integrated antimicrobial system. The objective of this investigation was to evaluate the effect of different concentrations of nisin combined with MAP on the inhibition of *L. monocytogenes,* and to compare the growth of this pathogenic organism on raw pork stored under this combination system at abusive temperatures. The impact of nisin and/or MAP on aerobic flora was investigated as well.

MATERIALS AND METHODS

I. *Cultures*

Micrococcus luteus CCRC 10452 was obtained from Culture Collection and Research Center, Food Industry Research and Development Institute, Taiwan, R.O.C. It was maintained on brain heart infusion agar (BHIA, Difco) medium at 4°C and transferred monthly. *Listeria monocytogenes* Scott A was obtained from the culture collection of the Biochemistry Laboratory in the Department of Food Science, National Chung Hsing University. It was maintained at 4°C on Tryptic soy agar (TSA, Difco) slants and was transferred monthly. One loop of *L. monocytogenes* culture from TSA slant was streaked onto TSA plate and incubated at 30°C for 24 h. A colony of the TSA plate culture was transferred into 100 ml of sterile Tryptic soy broth (TSB, Difco) and incubated for 24 h at the same temperature. *L. monocytogenes* was subcultured twice in TSB and after the second incubation, this organism was inoculated to 200 ml TSB at 1% inoculum. A standard curve for *L. monocytogenes* was prepared by growing the cells in TSB at 30°C in a shaker incubator. Periodically, a small sample of the suspension was removed and centrifuged to concentrate the cells. The pellet was resuspended in normal saline solution (0.87% NaCl). The optical density (OD) of the diluted sample was read at 600 nm against the saline solution. This microorganism was diluted and plated on the Palcam medium as describe below to determine the number of CFU per ml. A standard curve was prepared and the regression line determined for this pathogenic organism[22].

II. *Sample preparation and Experimental protocol*

Lean pork tenderloins were obtained from Shinung Co., Taiwan, and stored at 20°C for two days. Before the experiments were performed, samples were thawed at room temperature for 2 h and cut into pieces 5.0 by 4.0 by 0.5 cm (about 12 g per piece) with a sterile knife. The experimental protocol followed in this study was illustrated in Figure 1. Each piece of pork was immersed in various concentrations of nisin solution (0, 10^4, and 5 x 10^4 IU/ml) for 10 min and was then removed and allowed to drain for 1 min. Each of the pork samples was then transferred to a sterile Petridish. The working cul-

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tures of *L. monocytogenes* was prepared by incubating this pathogenic bacterium in 100 ml of TSB for 24 h at 30°C. After centrifugation, the pellet was resuspended in sterile physiological saline containing 0.1% peptone (SP). The pork tenderloins were surface-inoculated with 1.0 ml of diluted culture to give an initial populations of approximately 10^3 CFU/g for this pathogen. All sample Petridishes, with lids removed, were then placed in 17 cm × 20 cm plastic barrier bags (PE $T_{20\mu}/Al_{30\mu}/PE_{25\mu}$; permeability for air: 0-1 cc/m^2/24h; permeability for moisture: 0-1 cc/m^2/24h; Sun A Enterprises, Corp., Taiwan). Bags containing samples for air-storage were heat-sealed and stored at 4 and 20°C. Bags containing MA-storage samples were filled with gas mixtures (100% CO_2; and 80% CO_2+20% air; Tong-Yang Gas Co., Taiwan) by creating a vacuum followed by flushing with gas mixtures at one bar pressure and heat-sealed (Super Vac GK166 REGM, Busch Co., Austria). These bags then were stored at 4 and 20°C. At sampling time, two bags were sampled and 10 g of the pork was transferred aseptically to sterile Stomacher bags (Seward Medical Co., England). Then 90 ml of sterile SP solution was added to the package, and the contents were stomached for 2 min (Model 400 BA7221, Seward Medical Co., England). The resultant slurry was diluted with SP or plated directly as appropriate onto Palcam medium (Oxide)[23] for *L. monocytogenes* enumeration, or onto PCA (Difco) for enumeration of total aerobic bacteria[24], using a spiral plater (Spiral system, Model DU2, Cincinnati, OH)[25]. Selected colonies of *L. monocytogenes* were identified and biochemically confirmed using the FDA procedure[26]. The pH of the blended meat slurry was determined using a Hanna pH meter (model 8417).

III. *Nisin preparaton and determination of remaining activity of nisin on meat*

Purified nisin (Aplin & Barrett Ltd., Dorset, England) was stored at 4°C in a desiccator. Nisin working solution was prepared by dissolving the nisin in double distilled water and the nisin solution was sterilized by filtration (0.2 μm Nylon membrane filter). For the nisin treatments, fresh-prepared nisin solution was used. The plate diffusion assay method developed by Tramer and Fowler[27] was used to dtermine the remaining nisin concentration on meat. Each piece of meat was placed in 120 ml of 0.02 N HCl (pH 2.0), the mixture was then boiled for 5 min to release nisin and centrifuged. The supernatant was used for the assay. *M. luteus* CCRC 10452 was used as the test organism, and brain heart infusion agar (soft agar, Difco) was used as the assay medium[27].

IV. *Analysis of headspace composition*

Gas samples were drawn with a gas-tight syringe through the silicone sampling port. Composition of the gaseous headspace was evaluated using a model GC 6-AM gas chromatography (Shimadzu, Japan) equipped with a thermal conductivity detector (TCD). Nitrogen and oxygen were separated using a Porapak Q (2 m, stainless steel, 80-100 mesh) column while a Molecular sieve 5A (2 m, stainless steel, 60-80 mesh) column was used to elute nitrogen and carbon dioxide. The critical temperatures for injector, detector, and oven were 80, 60, and 30°C, respectively. Hydrogen at a flow rate of 30 ml/min was used as the carrier gas[28].

RESULTS AND DISCUSSION

Tables 1 and 2 show the evolution of the pH of the pork tenderloins treated with various concentrations of nisin combined with MAP during storage at 4 and 20°C, respectively. The pH values of meats at day 0 were similar; even different treatments including MAP were applied. Although a rapid pH drop in the tissue, caused by the application of carbon dioxide atmospheres, has been reported[29], no such pattern was found in this investigation when samples were stored at 4 or 20°C. A possible reason for this phenomenon is that high pH muscle

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Table 1. Effect of nisin concentration combined with MAP on pH of the pork tenderloin artificially contaminated with *L. monocytogenes* and stored at 4°C.

Treatment	pH at n days of storage					
	0	6	12	18	24	30
Air/No nisin	6.09	6.73	6.74	6.93	7.02	7.22
Air/10^4IU/ml nisin sol'n	5.99	6.71	6.73	6.89	7.00	7.10
Air/5 × 10^4IU/ml nisin sol'n	6.11	6.74	6.80	7.00	7.10	7.05
100%CO_2/No nisin	6.08	6.27	6.29	6.39	6.41	6.47
100%CO_2/10^4IU/ml nisin sol'n	6.00	6.23	6.26	6.48	6.52	6.54
100%CO_2/5 × 10^4IU/ml nisin sol'n	6.05	6.32	6.41	6.51	6.61	6.60
80%CO_2/No nisin	6.11	6.33	6.00	6.13	6.29	6.47
80%CO_2/10^4IU/ml nisin sol'n	6.01	6.29	6.18	6.55	6.59	6.64
80%CO_2/5 × 10^4IU/ml nisin sol'n	6.07	6.20	6.38	6.49	6.53	6.57

Table 2. Effect of nisin concentration combined with MAP on pH of the pork tenderloin artificially contaminated with *L. monocytogenes* and stored at 20°C.

Treatment	pH at n days of storage					
	0	1	2	4	7	10
Air/No nisin	6.09	6.56	6.77	6.83	6.99	7.03
Air/10^4IU/ml nisin sol'n	5.99	6.58	6.89	6.94	6.95	7.22
Air/5 × 10^4IU/ml nisin sol'n	6.11	6.53	6.78	6.83	6.96	7.17
100%CO_2/No nisin	6.08	6.17	6.31	6.33	6.80	6.42
100%CO_2/10^4IU/ml nisin sol'n	6.00	6.20	6.18	6.17	6.35	6.43
100%CO_2/5 × 10^4IU/ml nisin sol'n	6.05	6.25	6.34	6.34	6.42	6.53
80%CO_2/No nisin	6.11	6.28	6.18	6.39	6.48	6.63
80%CO_2/10^4IU/ml nisin sol'n	6.01	6.03	6.42	6.18	6.33	6.43
80%CO_2/5 × 10^4IU/ml nisin sol'n	6.07	6.24	6.37	6.34	6.46	6.59

spoils more rapidly than normal muscle because the carbohydrate available for microbial metabolism is limited. When growing on muscle with a high pH, compared with muscle at normal pH 5.3, bacteria use protein as an energy source and produce organoleptically obnoxious compounds much earlier during storage[30]. Papageorgious and Marth[31] indicated that pH values remained unchanged in whey and skim milk inoculated with *L. monocytogenes* and incubated at 4°C. Fey and Regenstein[32] also reported that the pH of salmon was not affected by the CO_2 treatment. During the 30 days storage period at 4°C or 10 days storage period at 20°C in this investigation, those samples stored in the air control atmosphere exhibited a steadily increasing pH from near pH 6.0 to excess of pH 7.0.

The effect of the treatment with various concentrations of nisin combined with MAP on *L. monocytogenes* stored at 4 and 20°C is given in Figures 2 and 3, respectively. *L. monocytogenes* grew under MAP (100% CO_2 and 80% CO_2+20% air) at the temperatures tested in the absence of nisin and the growth of this pathogen was more slow at 4°C than at 20°C. The fact that these gas mixtures in the absence

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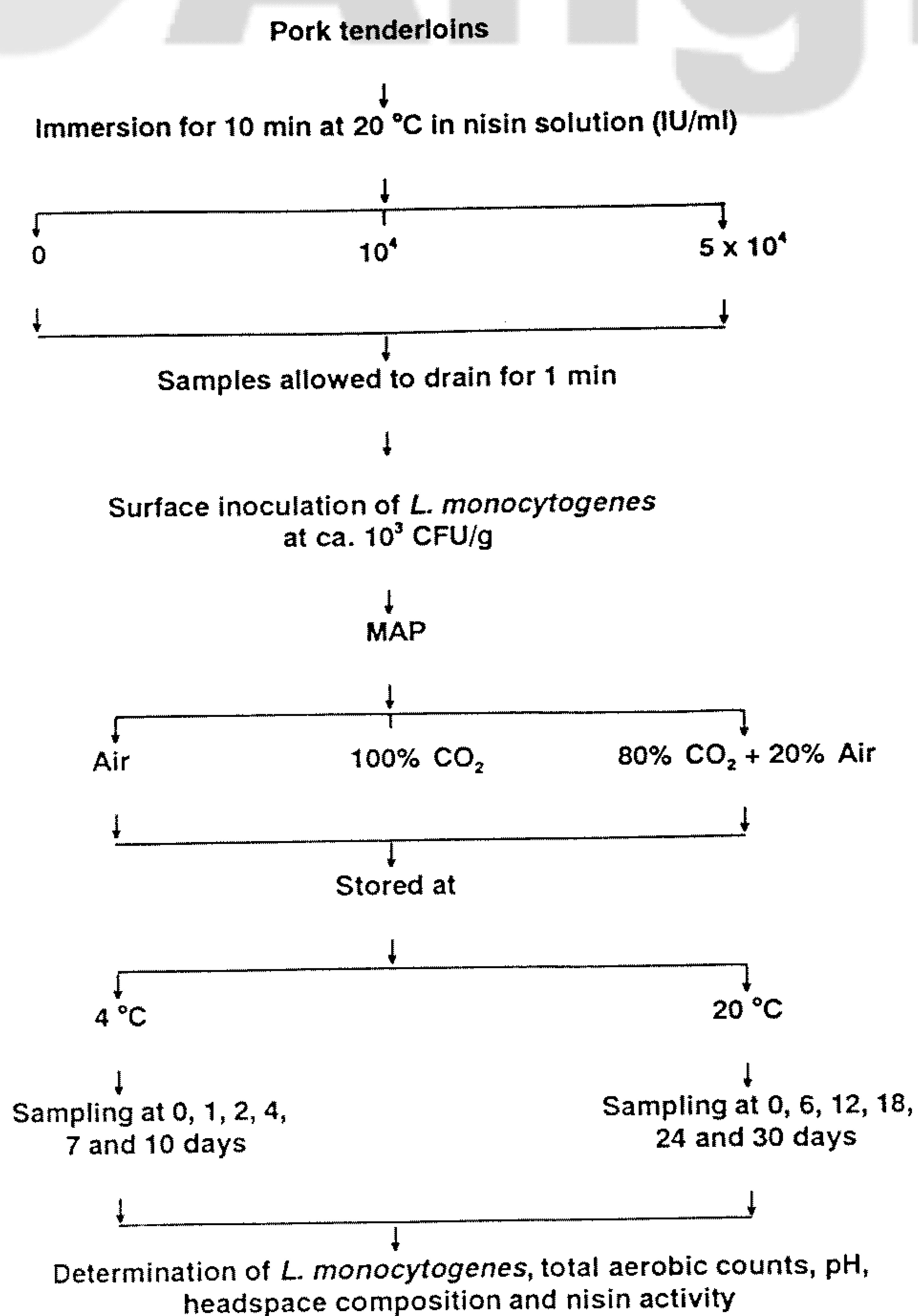

Figure 1. The experimental procedure.

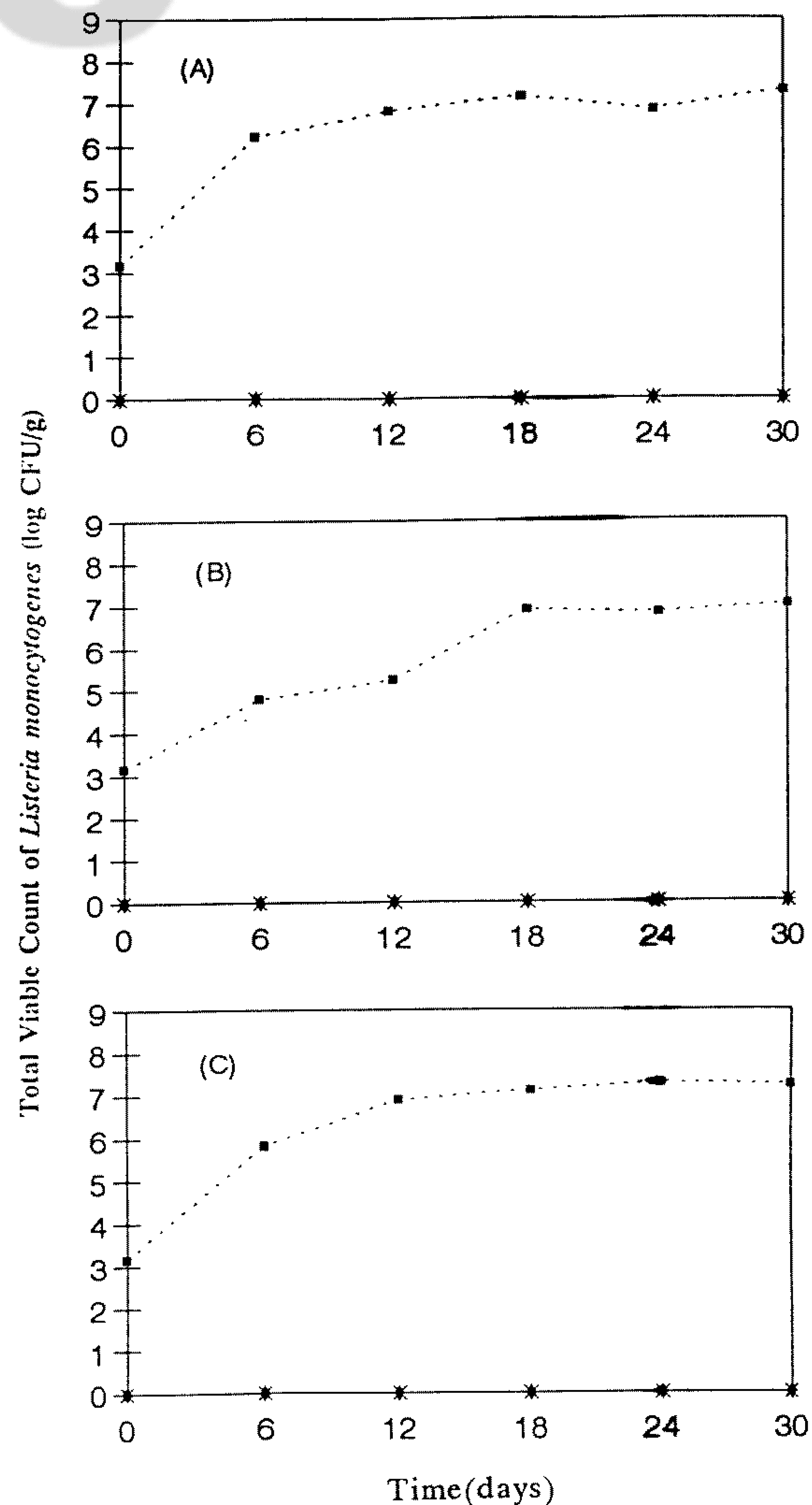

Figure 2. Effect of nisin and packaging atmosphere on the growth of *L. monocytogenes* when stored in air (A), 100% CO_2 (B), and 80% CO_2+20% air (C). Nisin concentration: 0 IU/ml (-■-), 10^4 IU/ml (◆), and 5 × 10^4 IU/ml (✱) at 4°C.

of nisin was unable to inhibit the growth of *L. monocytogenes* under nearly all conditions tested suggests that MAP alone, as tested in this investigation, may not be appropriate for use with raw pork products to inhibit the growth of this pathogen. Ingham et al.[4] used MA gas mixes containing 50% CO_2+10% O_2 and 80% CO_2 and no O_2 to investigate the growth rate of *L. monocytogenes* on cooked chicken loaf stored under these MAPs. They indicated that neither MAPs appeared to be effective at inhibiting growth of *L. monocytogenes* on such a loaf[4]. Similar result was obtained by Fang and Lin[22] as well when cooked pork samples were sotred under 100% CO_2, air, and 80% CO_2/20% air. The population of *L. monocytogenes* on fresh vegetables was also found to increase when these vegetables were stored under controlled atmosphere[33]. Growth of *L. monocytogenes* Scott A in raw chicken packaged under modified atmospheres and in air was investigated by Wimpfheimer et al.[8] who found that this pathogenic microorganism grew in air at 4, 10, and 27 °C. The modified atmosphere used in their investigation was unable to inhibit the growth of *L. monocytogenes*, and the numbers of this organism increased by nearly 6 log_{10} CFU/g at 4°C in 21 days.

The results of this investigation demonstrated that MAP alone was insufficient to in-

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hibit the growth of *L. monocytogenes*. However, the growth of this pathogen under MA storage can be prevented by the addition of nisin. Figures 2 and 3 show the effect on *L. monocytogenes* of treatment with various concentrations of nisin combined with MAP. The population of *L. monocytogenes* on the tenderloins treated with nisin (10^4 and 5×10^4 IU/ml) and stored under air, 100% CO_2, and 80% CO_2+ 20% air at 4°C was also effective when the samples were stored at 20°C (Figure 3); however, nisin at a concentration of 10^4 IU/ml was unable to inhibit the growth of the pathogen at this temperature after four days of storage. This bacteriostatic effect on *L. monocytogenes* increased with increasing concentration of nisin in pork tenderloin and with decreasing storage temperature.

The effect of the treatments on the total aerobic counts during storage at 4 and 20°C are

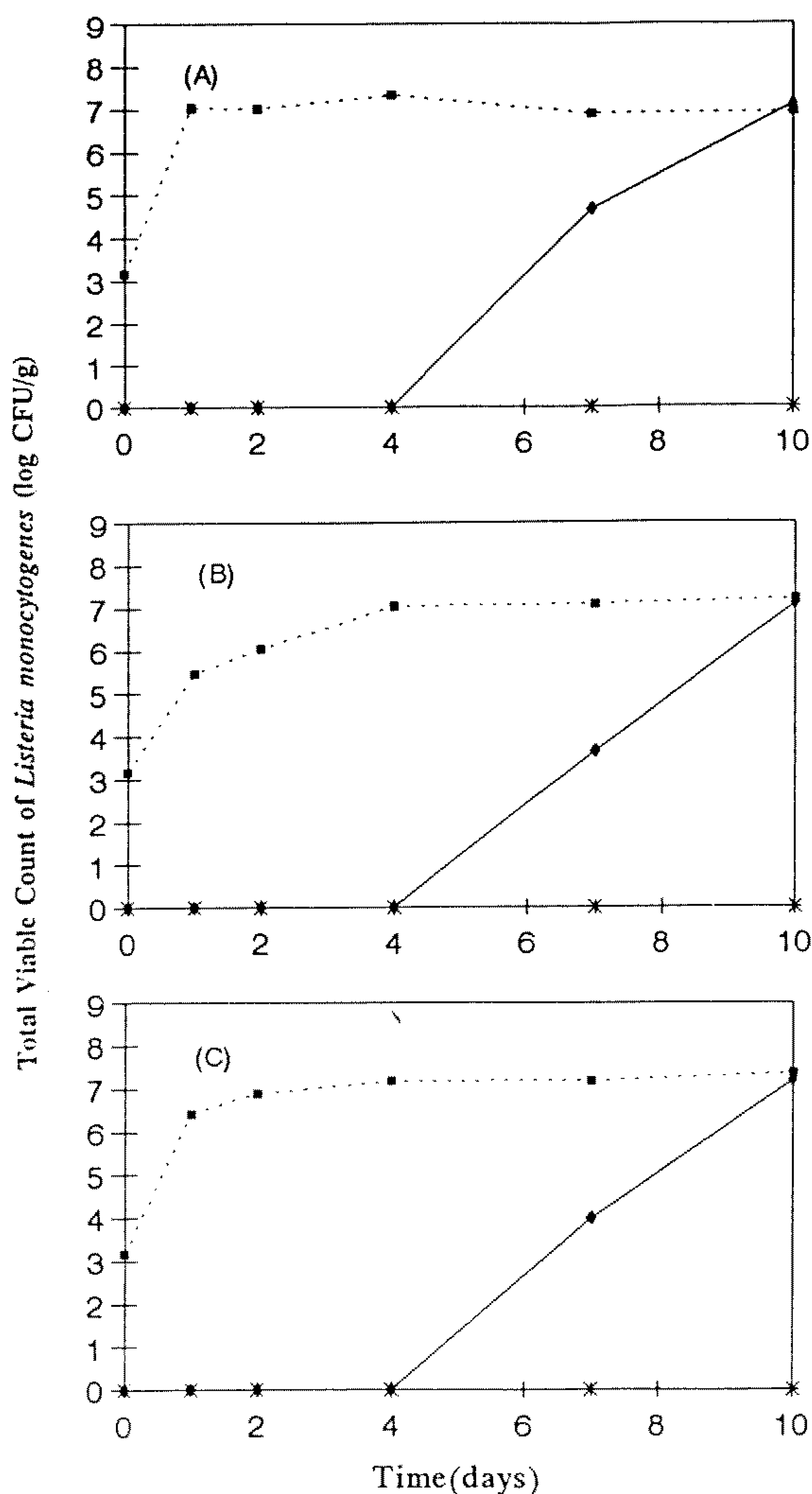

Figure 3. Effect of nisin and packaging atmosphere on the growth of *L. monocytogenes* when stored in air (A), 100% CO_2 (B), and 80% CO_2+20% air (C). Nisin concentration: 0 IU/ml (-■-), 10^4 IU/ml (◆), and 5×10^4 IU/ml (✳) at 20°C.

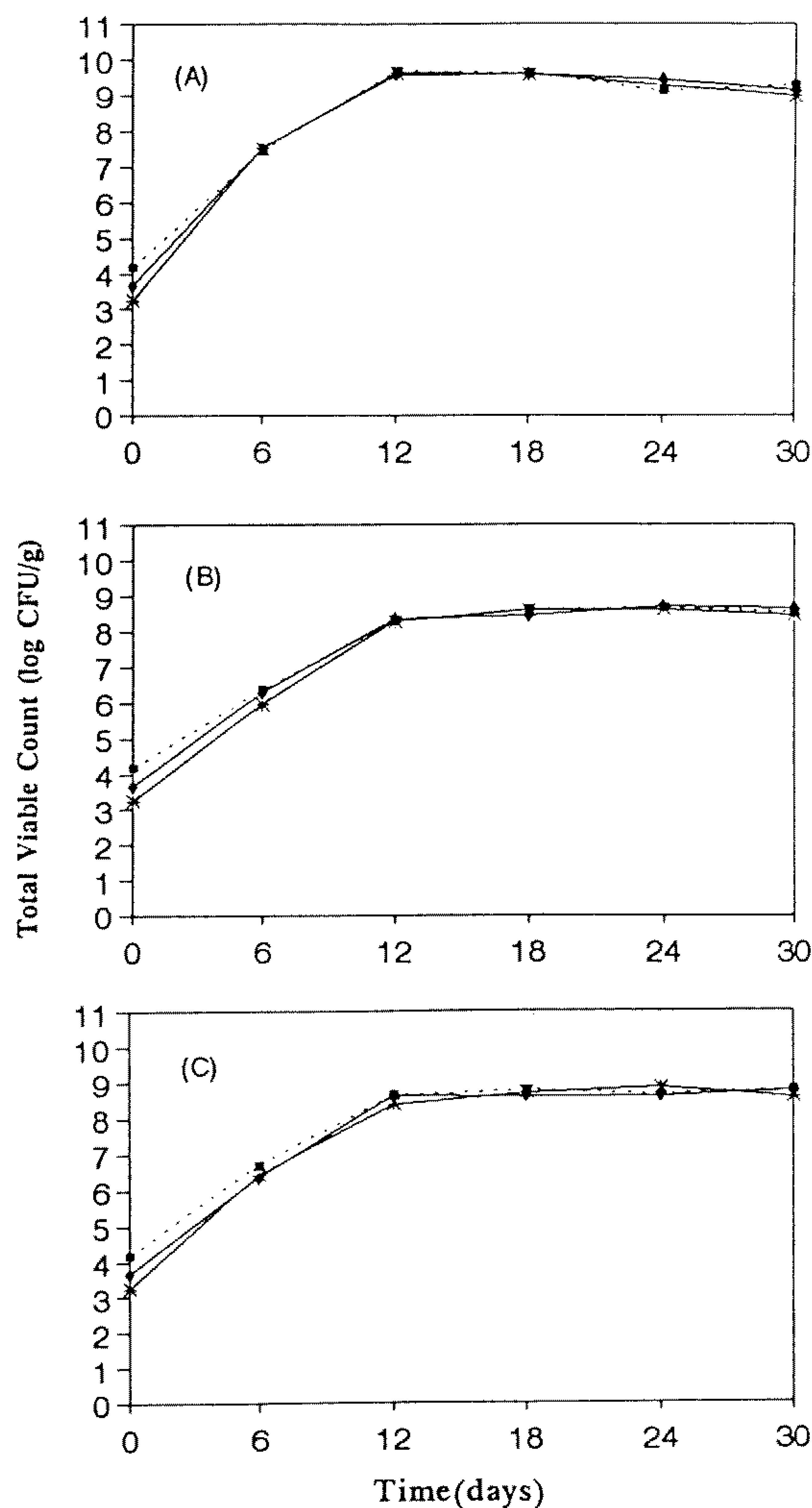

Figure 4. Effect of nisin concentration and packaging atmosphere on the growth of total aerobic counts when stored in air (A), 100% CO_2 (B), and 80% CO_2+20% air (C). Nisin concentration: 0 IU/ml (-■-), 10^4 IU/ml (◆), and 5×10^4 IU/ml (✳) at 4°C.

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illustrated in Figures 4 and 5, respectively. After 30 days of storage at 4°C, the number of aerobic mesophilic bacteria of the tenderloin treated with MAP was lower than the sample stored in the air. The same pattern was found when the tenderloins were stored at 20°C for 10 days. The aerobic mesophilic bacteria were lower in tenderloins with higher CO_2 content, and the effect of limited oxygen was more pronounced at lower temperature. The inhibitory effect of CO_2 on bacterial growth has been demonstrated by Layrisse and Matches[29]. Wimpfheimer et al.[8] also

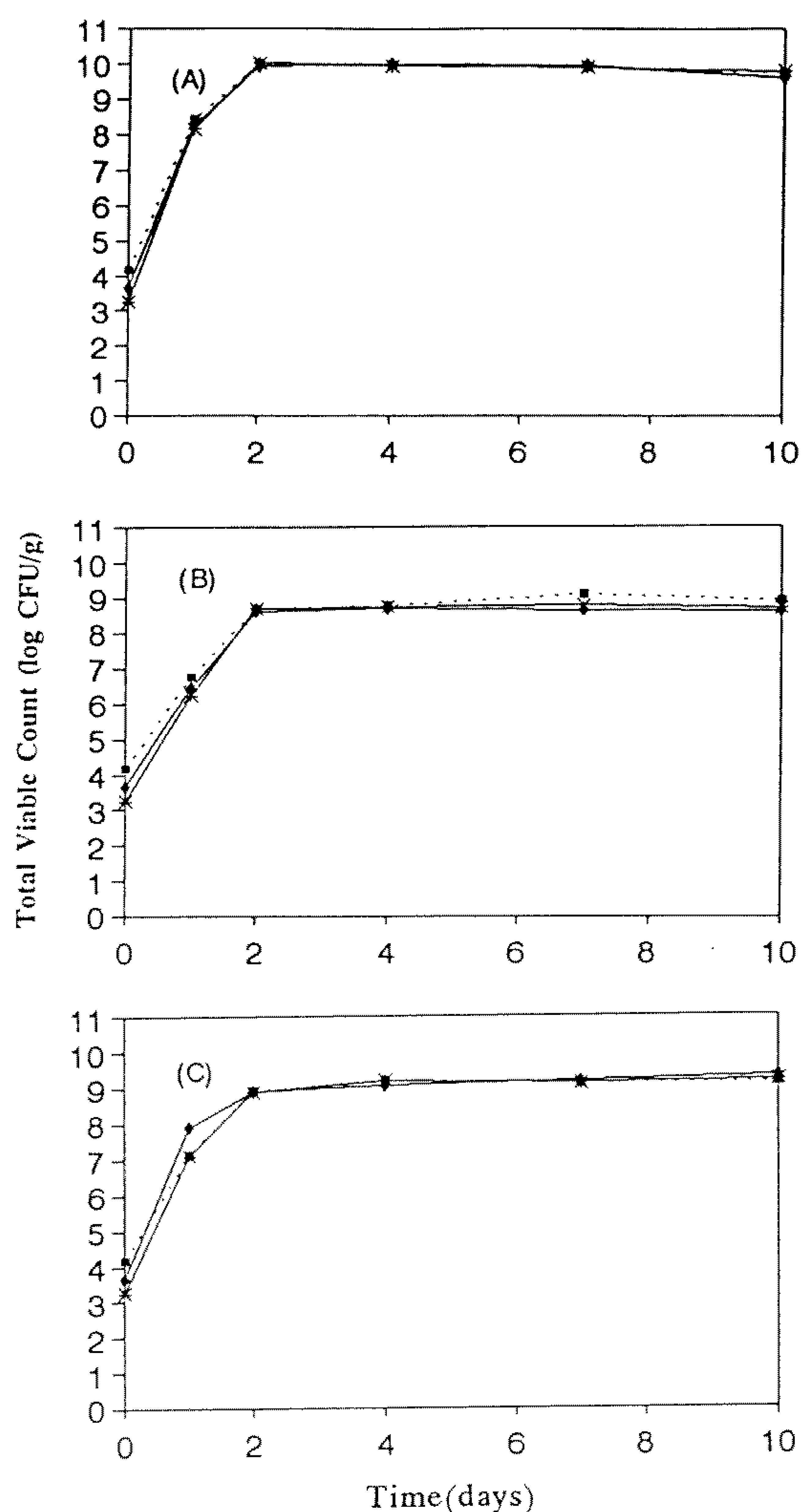

Figure 5. Effect of nisin concentration and packaging atmosphere on the growth of total aerobic counts when stored in air (A), 100% CO_2 (B), and 80% CO_2+20% air (C). Nisin concentration: 0 IU/ml (-■-), 10^4 IU/ml (◆), and 5 × 10^4 IU/ml (✱) at 20°C.

indicated that the increase in aerobic plate counts was reduced by more than 4 $\log_{10}$ CFU/g when raw chicken was stored in a modified atmosphere compared to air at 4°C. The total number of aerobic bacteria on the pork treated with nisin (10^4, 5 × 10^4 IU/ml) combined with MAP was similar to the total aerobic counts of the samples stored under MAP alone. Microbiogical changes of pork loin cuts stored under nisin/MAP combination system are currently under investigation in this laboratory.

This study shows that nisin at a level of 5 x 10^4 IU/ml was useful in controlling *L. monocytogenes* on the raw tenderloin stored under MAP. Chung et al.[19] found that 1 × 10^4 IU of nisin per ml of HCl-NaCl solution was sufficient to decrease the population of *L. monocytogenes* by more than 3 $\log_{10}$ CFU/ml in 24 h. They also indicated that nisin had an inhibitory effect on other gram positive bacteria such as *Staphylococcus aureus* and *Streptococcus lactis*, but did not have an inhibitory effect on gram negative bacteria such as *Salmonella typhimurium, Serratia marcescens,* and *Pseudomonas aeruginosa*, when they were attached to meat. Nisin at a level of 4 x 10^4 IU/ml was also found to be useful in controlling *L. monocytogenes* on the surface of meat[20]. The count of *L. monocytogenes* on meat surface was decreased by 1.06 $\log_{10}$ CFU/ml in 48 h in the investigation conducted by El-Khateib et al.[20].

Table 3 shows changes in headspace composition for meats stored under MA and air conditions at 4°C. Percent O_2 decreased for all air-stored samples while percent CO_2 increased consistently under the same treatments. There were little changes in percent N_2 in the headspace of air-stored samples (date not shown). In the MA-treated samples stored at 4°C, a decrease in percent CO_2 was found. Similar results were reported by MuMullen and Stiles[30] when fresh pork loin cuts were stored under MA conditions. Ingham and Potter[34], in their investigation, also found a significant decrease in percent CO_2 for samples stored at 5°C under MA conditions. Decreasing headspace levels of CO_2 indi-

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Table 3. Headspace composition of bags of pork tenderloin inoculated with *L. monocytogenesand* stored under nisin/MAP combination system at 4°C.

Treatments	Gas compositions	Gas composition(%) at n days of storage					
		0	6	12	18	24	30
Air/No nisin	CO_2	0.00	17.07	25.50	21.64	25.67	31.32
	O_2	19.35	3.25	0.00	0.00	0.00	0.00
Air/10^4IU/ml	CO_2	0.00	18.54	23.40	24.40	24.90	25.04
nisin sol'n	O_2	19.35	3.76	0.00	0.00	0.00	0.00
Air/5 × 10^4IU/ml	CO_2	0.00	16.92	24.00	26.60	24.18	22.14
nisin sol'n	O_2	19.35	4.98	0.00	0.00	0.00	0.00
100%CO_2/No	CO_2	94.40	82.80	83.40	74.35	70.69	60.93
nisin	O_2	0.00	0.00	0.44	0.00	0.00	0.00
100%CO_2/10^4IU/	CO_2	94.40	92.45	83.40	74.98	68.44	58.99
ml nisin sol'n	O_2	0.00	0.00	0.00	0.00	0.00	0.00
100%CO_2/5 × 10^4	CO_2	94.40	88.15	93.15	81.14	71.22	63.86
IU/ml nisin sol'n	O_2	0.00	0.00	0.13	0.00	0.00	0.00
80%CO_2/20%air/	CO_2	78.52	76.58	80.23	61.42	62.24	58.19
No nisin	O_2	4.11	1.94	0.24	0.00	0.00	0.00
80%CO_2/20%air/	CO_2	78.52	78.44	72.65	60.48	59.60	54.49
10^4IU/ml nisin sol'n	O_2	4.11	1.83	0.32	0.00	0.00	0.00
80%CO_2/20%air/	CO_2	78.52	73.48	76.55	66.36	63.48	59.35
5 × 10^4IU/ml nisin sol'n	O_2	4.11	3.20	0.00	0.00	0.00	0.00

Table 4. Residual nisin acivity on pork treated by different concentrations of nisin solution at 4 and 20°C.

Initaial nisin activity (IU/ml)	Temperature(°C)	Remaining nisin activity (IU/g) at n days of stoage						
		0	1	2	5	7	10	20
10^4	4	843.9	n.d.[a]	n.d.	213.8	n.d.	45.6	20.0
	20	843.9	190.3	40.0	n.d.	20.0	n.d.	n.d.
5 × 10^4	4	2015.6	n.d.	n.d.	350.0	n.d.	171.8	59.6
	20	2015.6	410.2	179.6	n.d.	70.1	n.d.	n.d.

[a] not determined. Data are means of duplicate samples.

cated that CO_2 was dissolving in the sample moisture since O_2 impermeable films were used in this study. A decrease in CO_2 in headspace gases of MAP meats is common and has been attributed to diffusion through the package[35] and absorption by meat[36].

The effects on the activity of nisin of incubation at 4 and 20°C on pork samples are indicated in Table 4. The activity of the nisin remaining on the meat decreased rapidly after incubation at 20 and 4°C. The decrease of nisin activity on meat was consistent with the antimicrobial activity to *L. monocytogenes* (Figure 3). Similar results were also indicated by Chung et

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al.[19]. Results of this investigation show that nisin is unstable on pork. The activity of nisin decreased rapidly with time, possibly as the result of binding of nisin with meat particles[37] or by low solubility and uneven distribution of nisin in meat[19]. Recovery of nisin from meat and meat emulsions had been poor and variable[38]. We experience similar difficulties. The method of nisin determination on meat that we used in this study is merely an indication of the extractable nisin activity that is active against the *L. monocytogenes*.

Application of MAP/nisin combination system can improve the safety of meat. Although nisin is effective for inhibition of *L. monocytogenes* growth, it is unstable on pork. Since the bactericidal activity of bacteriocins may be enhanced by lactic acid[39], a supplement of this acid to the system used in this study may be beneficial and further investigations are needed.

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以調氣包裝及乳酸鏈球菌素抑制生豬肉中李斯特單胞菌之生長

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摘　要

本研究探討調氣包裝及乳酸鏈球菌素之使用對生豬肉中李斯特菌生長之影響。所使用之調氣組成包含100%二氧化碳、80%二氧化碳/20%空氣、及100%空氣。生豬肉先經不同濃度之乳酸鏈球菌素溶液(0, 1 × 10^4, 及5 × 10^4IU/ml)處理，經表面接入李斯特菌後再用上述調氣組成以PET/A1/PE袋包裝，並分別於4及20°C下貯存30及10天。結果顯示若無乳酸鏈球菌素之存在，則總菌數及李斯特菌於上述調氣包裝及培養溫度中均能生長。若混合使用乳酸鏈球菌素及調氣包裝，李斯特菌之生長則受到限制，且此作用於4°C下較為明顯。此一混合系統對李斯特菌之抑制作用隨二氧化碳及乳酸鏈球菌素濃度之提高而增強。