Growth patterns of *Escherichia coli* O157:H7 in ground beef treated with nisin, chelators, organic acids and their combinations immobilized in calcium alginate gels

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Received 30 January 2002; accepted 29 May 2002

Abstract

The effects of antimicrobial substances including nisin, acetic acid, lactic acid, potassium sorbate and chelators (disodium ethylenediamine tetraacetic acid [EDTA] and sodium hexametaphosphate [HMP]), alone or in combination and, with or without immobilization in calcium alginate gels, on the growth of *Escherichia coli* O157:H7 in ground beef were investigated. Results showed that acetic acid and potassium sorbate could inhibit the growth of *E. coli* O157:H7 effectively at 10°C and at 30°C. Both EDTA and HMP did not halt the growth of *E. coli* O157:H7. In an antimicrobial system immobilized with calcium alginate, most of the antimicrobials could not inhibit the growth of *E. coli* O157:H7 in ground beef at 10°C and at 30°C, with the exception of acetic acid and lactic acid. Immobilization did not enhance the effectiveness of acetic acid against *E. coli* O157:H7 in ground beef at 10°C and at 30°C (*P* > 0.05) but it did enhance the effectiveness of lactic acid at 10°C. In a system combining different antimicrobials, treatment with nisin/EDTA or nisin/potassium sorbate at 10°C revealed a significantly lower population change of *E. coli* O157:H7 compared to samples treated with nisin, EDTA or potassium sorbate alone. The use of calcium alginate immobilization further enhanced the effectiveness of the combination system of nisin/EDTA, nisin/acetic acid and nisin/potassium sorbate on the growth of *E. coli* O157:H7 in ground beef at 10°C but it was not effective at 30°C.

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Keywords: *Escherichia coli* O157:H7; Immobilization; Antimicrobial substances; Ground beef

1. Introduction

*Escherichia coli* O157:H7 was identified as a foodborne pathogen in 1982 (Doyle, 1991) and outbreaks caused by this pathogen have been reported worldwide (Akashi et al., 1994; Anonymous, 1991; Williams et al., 2000). Consumption of undercooked ground beef has been linked epidemiologically to some of the outbreaks (Neill, 1989). In addition, milk, hamburgers, cheese, yogurt, apple cider, water, raw pork, raw beef, raw poultry, salad dressings and vegetables are also contaminated potentially with *E. coli* O157:H7 (Anonymous, 1996; Lin et al., 1996; Morgan et al., 1993; Swedlow et al., 1992; Vernozy-Rozand, 1997). An *E. coli* O157:H7 outbreak associated with swimming in an improperly chlorinated swimming pool has also been reported (Friedman et al., 1999).

The growth of *E. coli* O157:H7 in food is affected by several factors. Temperature between 10°C and 45°C is suitable for the growth of some selected *E. coli* strains and these toxigenic strains could produce verotoxin in medium system (Palumbo et al., 1995). Flores et al. (1996) have indicated that the growth of *E. coli* O157:H7 in ground beef patties and raw pork treated with 0.5% phosphate is affected significantly by temperature (*P* < 0.05). In addition, the heat sensitivity of *E. coli* O157:H7 is influenced by food storage conditions (Jackson et al., 1995). In contrast to its sensitivity to heat, as several researchers have shown, *E. coli* O157:H7 has an unusual tolerance to acidic conditions (Brackett et al., 1994; Conner and Kotrola, 1995).

Food preservatives are used for inhibiting the growth of pathogens and spoilage micro-organisms. Among the naturally occurring preservatives, the antimicrobial
substances produced by *Lactococcus lactis* have received great attention by researchers. Nisin, a polypeptide bacteriocin produced by *L. lactis* subsp. *lactis*, is a generally recognized as safe (GRAS) substance (Federal Register, 1988). Fang and Lin (1994a–c, 1995a) have shown that psychrotrophic Gram-positive pathogens such as *Listeria monocytogenes*, which can grow in modified atmosphere packaged food stored at low temperature, could be effectively inhibited by nisin. Gram-negative pathogens are resistant to nisin due to the difference in the structure of the cell wall envelope. The inhibitory activity of nisin on Gram-negative pathogens could be improved by combining it with other antimicrobial factors. Cutter and Siragusa (1995a) reported that nisin together with chelators exhibit a better antimicrobial activity on Gram-negative pathogens in culture media. However, this combination was found to be less effective when applied to real food systems, compared to culture media (Cutter and Siragusa, 1995b).

Organic acids are used historically to control the growth of micro-organisms and prevent food from spoilage. Organic acids used for food preservation are weak acids with the pH range between pH 3 and 5 which possess some levels of buffer activity (Doores, 1993). Acetic acid has demonstrated a better antibacterial ability than other organic acids under the same pH conditions (Abdul-Raouf et al., 1993; Cherrington et al., 1991). Many GRAS organic acids, including lactic acid, and citric acid were not significantly effective in preventing the proliferation of *E. coli* O157:H7 in ground, roasted beef (Abdul-Raouf et al., 1993). However, the antimicrobial activity of organic acids can be enhanced by combining it with other food preservatives or heat (Greer and Dilts, 1992; Marshall and Kim, 1996).

Immobilization techniques have attracted increasing interest in the past decade. Immobilization not only has been applied to microbial system for the production of various enzymes (Basha and Palanivelu, 2000; Lim et al., 2001; Lusta et al., 2000), but also has been applied to pathogen inhibition (Fang and Lin, 1995b; Lante et al., 1994) and development of low-fat ground beef (Brewer et al., 1992; Bullock et al., 1995; Egbert et al., 1992; Lin and Keeton, 1998). We have reported the effects of EDTA and acetic acid on the growth of *E. coli* O157:H7 in ground beef stored under various temperatures by using response surface methodology (Fang and Hsueh, 2000). The study reported here was undertaken to determine the fate of *E. coli* O157:H7 in ground beef as influenced by temperature, chelators and organic acids in the presence of nisin. The effect of the antimicrobials and combination of antimicrobial systems, immobilized by calcium alginate gels, on the growth of this pathogen was investigated as well.

### 2. Materials and methods

#### 2.1. Strain for experiment

*E. coli* O157: H7 ATCC 43894 was obtained from the Biotechnology Laboratory at the Department of Food Science, National Chung Hsing University (Taiwan, Republic of China). Cultures were maintained in 40% glycerol at −20°C. The strain was propagated by adding 0.1 ml of thawed cell suspension to 10 ml tryptic soy broth (TSB) (Difco Laboratories, Detroit, Michigan, USA) and cultured at 37°C for 24 h. After two consecutive transfers, one loop of the culture was inoculated into 50 ml TSB and incubated in a shaker (shaking speed was 150 r min⁻¹) at 37°C for 12–13 h until the cells had reached 9 log₁₀ cfu ml⁻¹. The culture was then serially (1:10) diluted in sterile 0.1% peptone (pH 7.0) to yield 5 log₁₀ cfu ml⁻¹.

#### 2.2. Preparation of antimicrobial substances

Nisin (Nisaplin, 10⁶ IU g⁻¹) was obtained from Aplin & Barrett Ltd (Towbridge, England) and was stored at 4°C. Acetic acid, potassium sorbate, sodium hexametaphosphate (HMP), calcium chloride (anhydrous), sodium alginate and lactic acid (d, l) were purchased from Hayashi Pure Chemical Industries Ltd (Osaka, Japan), while disodium ethylenediamine tetraacetic acid (EDTA) was obtained from Merck Co. (Darmstadt, Germany). A nisin working solution was prepared freshly by dissolving the nisin in 0.02 M potassium phosphate buffered saline. They were stored at 4°C and was used within 4 h. Chelator solutions were prepared by adding disodium EDTA or sodium HMP to 1 M potassium phosphate buffered saline. They were adjusted to pH 5.8 with HCl or NaOH and autoclaved at 121°C for 15 min. Various concentrations of acetic acid, lactic acid and potassium sorbate solutions were prepared in de-ionized water and autoclaved at 121°C for 15 min after adjusting the pH to 5.8. All solutions were stored at 4°C and warmed to room temperature for at least 2 h before using.

#### 2.3. Decontamination of beef

Fresh raw beef was purchased from the Jiann-Gwo market, Taichung, Taiwan, and was transported to the laboratory under low temperature. The raw beef was immersed into boiling water for 30 s and was then transferred quickly to a sterile platform. The denatured protein on the meat surface was removed with a sterilized dissection knife. The meat was then disinfected under UV light (60-W germicidal bulbs, 51-cm distance...
from beef) exposure for 20 min (Cutter and Siragusa, 1995b) in a laminar flow hood. The treated beef was ground using a sterile stainless-steel grinder, was packed into sterile bags and stored at -20°C. Prior to the experiment, the ground beef was defrosted in a 25°C water bath for 20 min.

2.4. Treatment protocols

A flow chart of the treatment procedure is given in Fig. 1. Samples of ground beef (25 g each) in triplicate were randomly assigned to untreated controls and various treatments. Treated groups were then inoculated with 0.2 ml of strain suspension (viable count was about 5 log_{10} cfu ml^{-1}), aseptically mixed, and treated with 0.5 ml of different concentrations of agents as shown in Fig. 1. Nisin (0, 10^2, 10^3, 10^4 IU g^{-1}), EDTA (0, 50, 100, 150 µg g^{-1}), HMP (0, 1000, 2000, 3000 µg g^{-1}), acetic acid (0%, 1.0%, 1.5%, 2.0%, w/v), lactic acid (0%, 1.0%, 1.5%, 2.0%, w/v) and potassium sorbate (0, 500, 1000, 2000 µg g^{-1}) were added to the ground beef from stock solutions to obtain the desired concentration.

Immobilization experiments were performed by preparing nisin (10^3 IU g^{-1}), EDTA (100 µg g^{-1}), HMP (3000 µg g^{-1}), acetic acid (2.0%), lactic acid (2.0%) and potassium sorbate (2000 µg g^{-1}) in different concentrations of sodium alginate (0%, 0.5%, 1.0%, 1.5%). The inoculation of the pathogen and the addition of antimicrobial agents were the same as above except that 90 mM CaCl_2 was added to each solution so that a calcium alginate gel would form in the ground beef. Three immobilized combination systems were used in this investigation: (1) nisin (10^3 IU g^{-1}) + EDTA (50 mM); (2) nisin (10^3 IU g^{-1}) + acetic acid (2.0%); (3) nisin (10^3 IU g^{-1}) + potassium sorbate (2000 µg g^{-1}) (Fig. 1). All treatments were aseptically conducted in a laminar flow hood at room temperature. After mixing the ground beef thoroughly, treated samples were placed in sterile Petri dishes, sealed with Parafilm® (American National Can™, Chicago, Illinois, USA) and stored at 10°C and 30°C. Samples were stored at 10°C for up to 10 days; while those at 30°C were stored for up to 24 h. Two replications were performed for each sampling time for bacterial count and pH measurement.

![Flow chart and treatment diagram of antimicrobial system application protocols.](Fig 1)
2.5. Analysis of samples

Test samples were diluted 10 fold in 0.1% peptone-water, placed in sterile stomacher bags and then blended at high speed with a stomacher (Model 400, Seward Medical, London, UK) for 2 min. After making a serial dilution, the sample was inoculated to tryptic soy agar (TSA) (Difco Laboratories) using a Spiral Plater (model DU2, Spiral Biotechnology, Cincinnati, Ohio, USA) (Fang and Hsueh, 2000). Plates were incubated for 18–24 h at 37°C before counting. The logarithm of the difference in population (DP) of the test strain was calculated using the formula (Fang and Lin, 1995a)

$$\log \text{DP} = \log \left( \frac{N}{N_0} \right) = (\log N) - (\log N_0),$$

where $N$ and $N_0$ represent the bacterial populations (cfug$^{-1}$) at times $t$ and zero, respectively. The pH values of 10 fold dilution samples were also measured, in duplicate, using a pH meter (Hanna 8417, Italy).

2.6. Statistical analysis of data

For each treatment, the data from the independent replicate trials were analysed statistically by using the General Linear Models procedures of SAS (SAS Institute, 1982, Statistical Analysis System, Cary, North Carolina, USA). Significant differences between mean DP of $E. \text{coli}$ O157:H7 in each treatment were determined using Duncan’s multiple range test. Repeated measurement test was used to determine the significant difference among treatments. Significance was expressed at 5% or 1% level.

3. Results

3.1. Growth and pH values in ground beef treated with various agents at 10°C and 30°C

The influences of nisin, EDTA, HMP, acetic acid, lactic acid, potassium sorbate (0, 500, 1000, 2000 μg g$^{-1}$) and sodium alginate (0%, 0.5%, 1.5%, 2.0%) on the growth of $E. \text{coli}$ O157:H7 in ground beef stored at 10°C and 30°C are shown in Figs. 2 and 3, respectively. Samples were stored at 10°C for 96 h; while at 30°C, treated samples were stored for 24 h. After 96 h incubation at 10°C, untreated samples had a DP of 2.13. Those treated with 102, 103 and 104 IU nisin g$^{-1}$ had DP of 2.11, 1.95 and 1.91 log10 cfug$^{-1}$, respectively (Fig. 2). Treatment with nisin had no significant effect on the growth of $E. \text{coli}$ O157:H7. Similarly, when samples were incubated at 30°C, there was no effect on growth.

Differences in population of 2.48, 2.56, 1.73 and 1.69 log10 cfug$^{-1}$ were found in groups, which were treated with 0, 50, 100 and 150 μg g$^{-1}$ of EDTA and stored at 10°C for 96 h (Fig. 2). On the other hand, 5.60, 5.35, 5.23 and 5.46 log10 cfug$^{-1}$ of DP were recorded in samples treated with the same concentrations of EDTA and stored at 30°C for 24 h (Fig. 3). Only samples treated with 100 and 150 μg g$^{-1}$ of EDTA and incubated at 10°C were significantly different ($P \leq 0.05$) when compared with the untreated sample, while no significant difference ($P > 0.05$) was found among groups treated with EDTA and incubated at 30°C. For samples treated with HMP, no significant difference ($P > 0.05$) was found between groups treated with 1000 or 2000 μg g$^{-1}$ HMP and control at 30°C (Fig. 3) but at 10°C concentration of HMP of 1000, 2000 and 3000 μg g$^{-1}$ resulted in significant reductions in...
population. No significant difference ($P > 0.05$) was found when the beef was treated with various concentrations of lactic acid and stored either at 10°C for 96 h or 30°C for 24 h. Potassium sorbate inhibited significantly ($P \leq 0.05$) the growth of E. coli in ground beef both at 10°C and at 30°C. The differences in population of this pathogen in beef stored at 30°C were 1.69, 0.34 and 0.11 log$_{10}$cfu g$^{-1}$ when treated with 500, 1000 and 2000 µg potassium sorbate g$^{-1}$, respectively (Fig. 2). At 30°C the differences were 3.61, 2.62 and 0.84 log$_{10}$cfu g$^{-1}$, respectively (Fig. 3). Calcium alginate (1.5%) lowered significantly ($P \leq 0.05$) the growth of E. coli in ground beef incubated at 10°C compared to the untreated control (1.75 vs 1.94 log$_{10}$cfu g$^{-1}$) (Fig. 2) but calcium alginate seemed to promote the growth of E. coli O157:H7 in ground beef at 30°C (Fig. 3). Acetic acid was the most effective agent in inhibiting the growth of this pathogen in ground beef at 10°C and 30°C. The DP of E. coli O157:H7 in ground beef treated with 1% acetic acid incubated at 10°C for 96 h was $-0.14 \log_{10}$cfu g$^{-1}$, compared to the untreated sample in which the DP was 1.86 log$_{10}$cfu g$^{-1}$ (Fig. 2). The DP was 1.68 log$_{10}$cfu g$^{-1}$ compared to the untreated sample (5.52 log$_{10}$cfu g$^{-1}$) when samples were incubated at 30°C for 24 h (Fig. 3). Significant differences ($P \leq 0.01$) were found among the samples treated with acetic acid both at 10°C and 30°C when compared to untreated samples (Figs. 2 and 3).

Although the pH values of beef inoculated with E. coli O157:H7 following different treatments and stored at 10 and 30°C were measured, no correlation between change in pH and changes in DP of E. coli O157:H7 were observed (data not shown).

3.2. Growth pattern of E. coli in ground beef treated with various immobilized antimicrobials at 10°C and 30°C

The differences in population of E. coli O157:H7 in ground beef treated with nisin (10$^3$ IU g$^{-1}$), EDTA (100 µg g$^{-1}$), HMP (3000 µg g$^{-1}$), acetic acid (2%), lactic acid (2%) and potassium sorbate (2000 µg g$^{-1}$), which were immobilized by various concentrations of alginate at 10°C are shown in Fig. 4. The incubation times were 216 h for nisin, acetic acid, lactic acid and potassium sorbate. For EDTA and HMP the incubation times were 120 and 192 h, respectively. Fig. 5 shows the growth of E. coli in ground beef treated with the same conditions as above, except that they were stored at 30°C for 24 h. Nisin immobilized with 1.5% alginate showed significantly lower ($P \leq 0.05$) reduction of E. coli population in beef stored at 10°C compared to the non-immobilized control (3.95 vs 4.71 log$_{10}$cfu g$^{-1}$) (Fig. 4). On the other hand, samples treated with nisin immobilized with 0.5% and 1.0% calcium alginate and incubated at 30°C for 24 h showed DP of 4.89 and 5.25 log$_{10}$cfu g$^{-1}$, which were significantly lower ($P \leq 0.05$) than the untreated group (5.41 log$_{10}$cfu g$^{-1}$) (Fig. 5).

Immobilized EDTA and HMP promoted the growth of E. coli O157:H7 in ground beef both at 10°C and 30°C (Figs. 4 and 5). Differences between samples treated with EDTA immobilized in 1.5% calcium alginate and untreated samples were significant at both 10°C and 30°C. At 30°C there were also significant differences ($P \leq 0.05$) between groups treated with EDTA immobilized with 0.5% and 1.0% calcium alginate and the control group. For samples treated with immobilized HMP, significant differences ($P \leq 0.05$) were found between the treated groups and the untreated control at both 10°C and 30°C (Figs. 4 and 5). Alginate did not enhance the effectiveness of acetic acid against E. coli O157:H7 in ground beef at 10°C ($P > 0.05$) when compared to the efficacy of non-immobilized acetic acid. Acetic acid was the agent most effective at inhibiting E. coli O157:H7 in this study. On the other hand, alginate enhanced the effectiveness of lactic acid against E. coli O157:H7 in ground beef stored.
at 10°C. The DP of the pathogen were 2.81, 2.82 and 2.48 log10 cfu g⁻¹, which were significantly lower \((P \leq 0.05)\) than the population in the control group \((3.32 \log_{10} \text{cfu g}^{-1})\), when treated with lactic acid immobilized with 0.5%, 1.0% and 1.5% calcium alginate gel, respectively (Fig. 4). For samples treated with immobilized lactic acid and stored at 30°C, the differences were significant \((P \leq 0.05)\) only between groups treated with lactic acid immobilized with 1.5% calcium alginate and the control sample \((4.63 \text{ vs } 5.11 \log_{10} \text{cfu g}^{-1})\) (Fig. 5). As in the samples treated with immobilized EDTA and HMP, potassium sorbate immobilized with various concentrations of calcium alginate gel did not show enhanced antimicrobial activity, but was found to promote the growth of \(E. \text{coli O157:H7}\) in ground beef stored at 10°C and 30°C (Figs. 4 and 5). After the beef samples were treated and stored at 10°C for 216 h, the DP of \(E. \text{coli}\) were 4.08, 4.05 and 3.59 log10 cfu g⁻¹ in the samples treated with potassium sorbate immobilized by 0.5%, 1.0% and
1.5% of calcium alginate, respectively, which were significantly higher \((P < 0.05)\) than the DP of the control group \((1.98 \log_{10} \text{cfu g}^{-1})\). The same pattern was also observed in samples treated with immobilized potassium sorbate and stored at 30°C for 24 h (Fig. 5).

### 3.3. Effect of immobilized combination systems on the growth profile of \(E. coli\) in ground beef stored at 10°C and 30°C

Three combination systems including (1) nisin \((10^3 \text{IU g}^{-1})\) + EDTA \((50 \text{mM})\); (2) nisin \((10^3 \text{IU g}^{-1})\) + acetic acid \((2\%)\); (3) nisin \((10^3 \text{IU g}^{-1})\) + potassium sorbate \((2000 \text{mg g}^{-1})\) were used in this investigation. These systems were either immobilized with 1.5% calcium alginate gel or used without immobilization. Fig. 6 shows the effect of these combination systems, alone and immobilized with calcium alginate, on the growth of \(E. coli\) O157:H7 in ground beef stored at 10°C. In the ground beef treated with nisin + EDTA, the DP of \(E. coli\) population was \(3.34 \log_{10} \text{cfu g}^{-1}\) after 10 days of storage at 10°C. Populations of samples treated with nisin and EDTA alone at 10°C for 10 days were found to be 3.48 and \(3.47 \log_{10} \text{cfu g}^{-1}\), respectively (data not shown). The immobilization treatment of nisin/EDTA with 1.5% calcium alginate gel showed lower DP than the non-immobilized sample \((3.01 \text{ vs } 3.34 \log_{10} \text{cfu g}^{-1})\) (Fig. 6). The initial pH values of samples treated with the nisin/EDTA combination system were 2.41 and 2.42 for the non-immobilized and immobilized samples, respectively. After 10 days of storage at 10°C, pH values were found to be 5.60 and 5.56, respectively (Table 1). In the combination systems of nisin/acetic acid and nisin/potassium sorbate, immobilization with 1.5% calcium alginate gel also enhanced the inhibitory effect at 10°C. Statistical analysis showed significant difference between these combination systems and the immobilized combination systems at 10°C for nisin/EDTA \((P < 0.05)\), nisin/acetic acid \((P < 0.01)\) and nisin/potassium sorbate \((P < 0.01)\) (Table 2). The sample treated with immobilized nisin/acetic acid at 30°C seemed to have higher DP compared to the non-immobilized sample \((1.15 \text{ vs } 0.53 \log_{10} \text{cfu g}^{-1})\) (Fig. 7), although this enhancement was not significant \((P > 0.05)\) (Table 2). However, significant differences \((P < 0.05)\) were found between the group treated with immobilized nisin/potassium sorbate and the non-immobilized group at 30°C for 24 h (Table 2). The initial pH values of samples treated with the combination system of nisin/potassium sorbate were 5.66 and 5.68 for non-immobilized and immobilized samples, respectively. After 24 h of storage at 30°C, pH values decreased to 5.38 and 5.31, respectively (Table 1). Again, the pH was found to be inconsistent with the changes of cumulative population of \(E. coli\) O157:H7 in combination systems with or without immobilization.

### 4. Discussion

Our previous reports have indicated that psychrophilic Gram-positive pathogens such as \(L. monocytogenes\),
which can grow in modified-atmosphere packaged food stored at low temperature, could be effectively inhibited by nisin (Fang and Lin, 1994a). However, nisin is not generally active against Gram-negative bacteria, yeasts and fungi. The outer membrane of Gram-negative bacteria does not allow molecules like nisin to reach their site of action, which is the cell membrane. Research including this investigation shows that nisin is ineffective at suppressing the growth of *E. coli* O157:H7 (Cutter and Siragusa, 1995b; Zhang and Mustapha, 1999). The outer membrane permeability can be altered by treatment with chelators such as EDTA or high hydrostatic pressure resulting in increased sensitivity towards nisin (Hauben et al., 1996; Vaara, 1992). In this study, samples treated with EDTA at 100 and 150 μg ω−1 in the absence of nisin showed significant inhibitory effect to *E. coli* O157:H7 in ground beef at 10°C which is probably due to its high chelating activity (Boziaris and Adams, 1999). Hathcox and Beuchat (1996) have reported that EDTA at 300 and 600 μg ml−1 inhibited the growth of *E. coli* O157:H7 in TSB. EDTA concentrations ranging from 20 to 500 μgω−1 have been chosen to investigate its effect on *E. coli* O157:H7 by some researchers (Cutter and Siragusa, 1995a, b; Zhang and Mustapha, 1999). However, since the legal limit for EDTA as a food additive in Taiwan is 100 μg ω−1, EDTA up to 150 μgω−1 was used in this investigation, since the effect of up to 150 μg EDTA on the growth of *E. coli* O157:H7 has been established (data not shown). Cutter and Siragusa (1995a, b) have reported that nisin and nisin with EDTA are less effective against *E. coli* O157:H7 and *Salmonella typhimurium* attached

<table>
<thead>
<tr>
<th>Combination systema</th>
<th>Immobilized with calcium alginate (%)</th>
<th>pH of beef containing test chemicals</th>
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<tr>
<td></td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Nisin + EDTA</td>
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<td></td>
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<td>Nisin + acetic acid</td>
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<td></td>
<td>1.5</td>
<td>5.25</td>
</tr>
<tr>
<td>Nisin + potassium sorbate</td>
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</tr>
<tr>
<td></td>
<td>1.5</td>
<td>5.68</td>
</tr>
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</table>

*aSamples were treated with nisin, EDTA, acetic acid and potassium sorbate at concentrations of 10^3 IU g−1, 50 mM, 2% and 2000 μg g−1, respectively.

*bThe ground beef was stored at 10°C for 240 h.

cThe sample was stored at 30°C for 24 h.

The samples were treated with a combination of antimicrobials and were stored at 10°C or 30°C.

Table 2

Effect of treatments with combined antimicrobial substances immobilized by alginate on *E. coli* O157:H7 growth in ground beef stored at 10°C or 30°C

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Temperature (°C)</th>
<th>P &gt; Fb</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Nisin (1)+EDTA (2)+calcium alginate</td>
<td>10</td>
<td>0.0001**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0001**</td>
</tr>
<tr>
<td>Nisin (1)+AA (2)+calcium alginate</td>
<td>10</td>
<td>0.0001**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0001**</td>
</tr>
<tr>
<td>Nisin (1)+PS (2)+calcium alginate</td>
<td>10</td>
<td>0.0001**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0001**</td>
</tr>
</tbody>
</table>

*aNisin 1000 IU g−1; EDTA, 50 mM; AA, acetic acid 2%; PS, potassium sorbate 2000 μg g−1. The combination system was immobilized in 1.5% calcium alginate gel.

bP value of I, antimicrobial (1) vs antimicrobial (1)+(2); II, antimicrobial (2) vs antimicrobial (1)+(2); III, antimicrobial combination system vs immobilized antimicrobial combination system.

*P ≤ 0.05.

**P ≤ 0.01.
to beef than when grown in broth culture. They have indicated that these reductions were not greater than $0.42 \log_{10} \text{cfu cm}^{-2}$. In this study, however, when combinations of nisin and EDTA were used, significant differences were found compared to the samples treated with nisin or EDTA alone at 10°C. Applying the immobilization technique with calcium alginate gel at 10°C could further enhance this effect. In our previous study, pork tissue treated with calcium alginate immobilized nisin ($10^4 \text{IU g}^{-1}$) and stored at 4°C for 14 days had greater reduction of counts of *L. monocytogenes* when compared to non-immobilized controls ($1.74 \text{ vs } 0.53 \log_{10} \text{cfu g}^{-1}$) (Fang and Lin, 1995b). Similar results were found in this investigation regarding the population reduction of *E. coli* in ground beef treated with immobilized nisin at 10°C. Since nisin is not effective against Gram-negative bacteria, this is probably due to the population reduction effect of calcium alginate gel at low temperature rather than nisin itself.

On the other hand, immobilized EDTA promoted the growth of *E. coli* O157:H7 in ground beef both at 10°C and 30°C. It has been shown that the presence of divalent cations in the environment could lower the ability of chelators such as EDTA to chelate effectively the divalent cations from the LPS layer of the Gram-negative cells (Vaara, 1992). In the calcium alginate gel formation, divalent calcium ions might also be present which could have lowered the chelating ability of EDTA, and thereby lowered its effect on the membrane permeability.

In this study, sodium HMP was more effective at reducing *E. coli* O157:H7 growth in ground beef at lower temperature. Ground beef treated with 1000, 2000, or 3000 µg HMP g$^{-1}$ incubated at 10°C had DP values that were significantly lower ($P \leq 0.05$) than the untreated control. The legal limit for sodium HMP as a food additive in Taiwan is 3000 µg g$^{-1}$ and within this limit, HMP could reduce the growth of *E. coli* O157:H7 in ground beef stored at low temperature. It has been demonstrated that sodium HMP affects the permeability of the outer membrane of Gram-negative bacteria, thereby allowing hydrophobic antibiotics or detergents to inhibit these organisms effectively (Vaara and Jaakkola, 1989). Cutter and Siragusa (1995a) have also demonstrated that treatments with HMP caused the release of cellular components and population reductions compared to buffer control. Our results showed that immobilized HMP enhanced the growth of *E. coli* in ground beef both at 10°C and 30°C, probably for the same reason as mentioned for immobilized EDTA.

Acetic acid was found to be the most effective agent in the inhibition of the growth of *E. coli* in ground beef stored at 10°C and 30°C in this investigation. Hathcox and Beuchat (1996) indicated that acetic acid (0.1%) had a significant ($P \leq 0.05$) inhibitory effect on the growth of this pathogen in TSB at 37°C. However when beef tissue was inoculated with *E. coli* O157:H7 and washed with distilled water, 1% acetic acid and 1% lactic acid, no significant difference ($P > 0.05$) in the populations of this micro-organism was found among these treatments (Dickson and Siragusa, 1994). Our previous study has shown that acetic acid affects significantly the maximum population density (MPD).
of \textit{E. coli} O157:H7 in ground beef (Fang and Hsueh, 2000). In the present investigation, alginate application did not enhance the action of acetic acid against \textit{E. coli} in ground beef. However, significant differences ($P \leq 0.01$) were found between the immobilized combination systems of nisin/acetic acid incubated at 10°C and the non-immobilized one. The immobilization technique enhanced significantly the inhibition of this pathogen by lactic acid in this investigation. Siragusa and Dickson (1993) have claimed that alginate application enhanced significantly the inhibition of lactic acid of \textit{L. monocytogenes} on lean beef. In their study, when alginate/acetic acid and acetic acid treatments alone were applied, \textit{E. coli} populations of 1.51 and $2.33 \log_{10} \text{cfu g}^{-1}$ were found, respectively.

Sorbic acid and its salts are among the most widely used food preservatives in the world; the most commonly used forms include sorbic acid and potassium salt. Sorbate is applied either as a direct additive onto the product by dipping or spraying, or is incorporated into the protective wrapping (Davidson and Juneja, 1990). Our previous study has shown that the growth of \textit{Staphylococcus aureus} and \textit{E. coli} \textit{O157:H7} in ground beef (Fang and Hsueh, 1990). Our previous study has shown that the growth of \textit{Staphylococcus aureus} and \textit{Bacillus cereus} on vegetarian food could be reduced significantly ($P \leq 0.05$) by the combination system of nisin and potassium sorbate (Fang et al., 1997). In this study, samples treated with potassium sorbate alone showed significant reduction of the population of \textit{E. coli} in ground beef at 10°C and 30°C. However, applying calcium alginate gel promoted the growth of this pathogen in ground beef compared to the non-immobilized sample at both temperatures. In addition, the immobilized combination system of nisin/potassium sorbate reduced significantly the DP of \textit{E. coli} O157:H7 compared to the non-immobilized nisin/potassium sorbate at 10°C. It has been shown that the permeability of the calcium alginate film to potassium sorbate increased proportionally with increasing water activity and potassium sorbate concentration in the system (Wong et al., 1996). Growth promotion of \textit{E. coli} in ground beef caused by immobilized potassium sorbate needs to be further investigated.

5. Conclusions

Our study provides information on the efficacy of nisin combined with other preservative factors against \textit{E. coli} O157:H7 using ground beef as a food model system. The results suggest that, depending on the antimicrobial agents, immobilization with calcium alginate gel does not enhance necessarily their inhibitory effect to \textit{E. coli} O157:H7 in ground beef. In addition, we have demonstrated that immobilized combinations of nisin and antimicrobials (or chelators) at low temperature are effective in reducing \textit{E. coli} O157:H7 on ground beef. Such combination systems may be useful as a multi-hurdle approach to improve the microbial quality and ultimately, the safety and shelf-life of meat products.

Acknowledgements

This work was supported by Department of Health, Republic of China (Project DOH87-TD-1093). Any findings, conclusions and recommendations expressed in this paper are those of the authors and do not necessarily reflect the official views of their sponsors.

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