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Note

Effects of Food Additives on Susceptibility of Gram Negative Bacteria Derived from Dry-Fermented Sausage

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This study examined the effects of food additives on gram-negative bacteria. The food additives used included synthetic antioxidants (butylated hydroxyanisole, BHA, and butylated hydroxytoluene, BHT), a curing agent and lactic acid with or without a cell-free supernatant (CFS) containing antimicrobial compounds of *Lactobacillus sakei* D-1001. The gram-negative bacteria were selected from dry-fermented sausages and cultured with different food additives for 18 h in nutrient broth, and then another 24 h with or without CFS adjusted (at pH 6.0) to inactivate lactic acid or not adjusted (at pH 4.0). BHA (0.1%) resulted in total viable cell inhibition following 18 h culture. A reduction in cell growth was observed in culture broths with 0.1% lactic acid and synthetic antioxidants at different concentrations. Furthermore, greater susceptibility of gram-negative bacteria could be obtained in 18+24-h cultures with combinations of selected food additives and antimicrobial compounds of *Lactobacillus sakei* D-1001, in a low pH environment depending on the lactic acid concentration.

Keywords: food additive, antimicrobial compounds, gram-negative bacteria

Introduction

Food additives are used extensively in fermented meat products and are involved in various reactions of the meat environment that contribute to the development of taste, texture, consistency, or color. In addition, specific additives used in fermented products have shown important multiple effects for extending shelf life, which is determined by both microbiological (spoilage) and chemical (oxidation and physical) deterioration, such as the production of lactic acid bacteria (LAB) and their antioxidants.

Numerous studies have reported on the spoilage of meat and fermented meat products by pathogenic gram-negative bacteria, including *Escherichia coli* (Glass. Kathleen *et al.*, 1992; Ferreira *et al.*, 2006) and *Salmonella* (Buchanan and Whiting, 1998; Escartin *et al.*, 1999), which produce toxins and cause several diseases in humans (Doyle and Schoeni, 1984; Padhye *et al.*, 1992; Centers for Disease Control, 1995; Pearse *et al.*, 2004). In addition, lipid oxidation in foods may also pose a human health risk (Pearson *et al.*, 1983; Kubow, 1992). To minimize these risks, desirable bacteria that produce antibacterial compounds and food additives have been added to fermented meat products. There are many scientific reports about the positive effects of food additives such as antioxidants (Bozkurt and Erkmen, 2002; Ansorena and Astiasaran, 2004; Lee and Kunz, 2005; Bozkurt, 2006), curing agents (Noil *et al.*, 1990; Olesen *et al.*, 2004) and LAB as starter culture (de Vuyst and Vandamme, 1994; Coffey *et al.*, 1998; Niku-Paavola *et al.*, 1999; Lucke, 2000; Kalalou *et al.*, 2004) in fermented meat products. However, their correlation to such effects as well as the nature of their specific positive actions in the fermented products are not yet clear, especially against food pathogenic gram-negative bacteria. Gram-negative bacteria are highly resistant to foreign molecules such as antimicrobial compounds of LAB (Nikaido, 1989; Gao *et al.*, 1999) due to an effective barrier function of their outer mem-

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branes (Nikaido, 1994), which are absent in gram-positive bacteria.

The aim of this study was to establish a method for reducing the number of gram-negative bacteria either through the use of food additives like lactic acid, butylated hydroxyanisole (BHA), BHA/butylated hydroxytoluene (BHT) and the curing agent alone or in combination with antimicrobial compounds produced by LAB.

Materials and Methods

Food additives Synthetic antioxidants, BHA and BHT, were purchased from MP Biomedicals (Vannes, France) and Nacalai Tesque (Kyoto, Japan), respectively. Lactic acid was obtained from Kanto Chemical (Tokyo, Japan). The curing agent (a mixture containing 5% $NaNO_2$, 10% KNO_3 and 85% NaCl) was obtained from Chiyoda Industry Co. (Tokyo, Japan).

Experimental design Sterile nutrient broths (Merck, Germany) were prepared with each of the following: 3 BHA concentrations (0.01%, 0.05%, and 0.1%), BHA/BHT combination (0.01% BHA + 0.01% BHT), lactic acid at two concentrations (0.05% and 0.1%), the curing agent (0.2%) or a negative control. A gram-negative bacterial suspension was prepared as described below and was inoculated in each broth to a final concentration of 1% and then incubated at 30°C for 18 h. Each culture broth was then divided into three 9-ml portions and 1 ml cell-free supernatant (CFS) prepared as described below was added to two portions and incubated for another 24 h at 30°C; no CFS was added to the third portion. The two portions with CFS were used to determine the combined effects of food additives and CFS at different pH on gram-negative bacteria isolated from dry-fermented sausage. After the final incubation, viable cell counts of gram-negative bacteria were enumerated using the direct plating method on MacConkey agar. Immediately after incubation, pH values were also measured with a pH meter (HM-5S, TOA Electronics, Tokyo, Japan). These experiments were performed in triplicate.

Preparation of bacterial inoculum Dry-fermented sausage (10 g) recovered at the end of processing was analyzed for the presence of gram-negative bacteria. A homogenized sample (1:10 dilution in sterile saline) was used to prepare serial dilutions ($10^{-1} - 10^{-3}$) using sterile saline and 1-ml aliquots of each dilution were incubated with DHL agar (Merck, Darmstadt, Germany). After aerobic incubation of the plates for 24 h at 37°C, 9 colonies were selected from the plates containing 2.9×10^2 colony counts and subcultured onto nutrient agar (Difco, Detroit, MI, USA). Then, cell morphology, gram reaction (Gram stain B&M, Merck), growth on MacConkey agar (Eiken, Tokyo, Japan) and gas production in brilliant green lactose bile broth (Eiken, Tokyo, Japan) were quantified for gram-negative bacteria selection. Bacteria that are rod-shaped, gram-negative, gas producing and pinkish in color on MacConkey agar were selected and stored in nutrient broth supplemented with 30% glycerol at –20°C until further analysis. Just before analysis, frozen cultures were activated in fresh nutrient broth (1%) at 37°C for 24 h and stored at 4°C. To determine numbers of colony-forming units (CFU), diluted culture was plated on MacConkey agar and incubated at 37°C for 24 h. The refrigerated stationary-phase cells were diluted to a cell density of around 8 log CFU/ml and used as the inoculum.

Preparation of crude CFS from LAB L. sakei D-1001 from MMF-161 commercial starter culture (San-ei Sucrochemical Co., Chita, Japan) was cultivated in 100 ml MRS broth at 30°C for 72 h. CFS, which contained antimicrobial compounds from LAB (Daeschel, 1989), was obtained by centrifugation (10,000 × *g*, 20 min, 4°C) and then filtrated through a 0.22-μm pore-size filter (Corning, NY, USA). To determine the effect of pH on the gram-negative bacteria cultured with various additives in nutrient broth, we used CFS at a non-adjusted pH of 4.0 and an adjusted pH of 6.0 with the addition of 10 N NaOH; increasing pH mitigates the antimicrobial effects of organic acids produced in the *Lactobacillus* culture.

Statistical analysis Enumerated populations of gram-negative bacteria were transformed into $\log_{10}$ CFU/ml for data analysis. Analysis of variance (ANOVA) was performed on the data, followed by Turkey's test when significant differences of $p < 0.05$ and $p < 0.01$ were observed. SAS (SAS Institute, Cary, NC, USA) was used for statistical analysis.

Results

pH Changes in pH of broth cultures inoculated with gram-negative bacteria and different food additives are shown in Table 1. After the initial 18-h culture, pH of the culture broths with BHA at different concentrations ranged from 6.19 ± 0.16 to 7.08 ± 0.03. For broths containing 0.05% or 0.1% lactic acid or the curing agent, pH was 5.09 ± 0.01, 4.28 ± 0.02 and 6.43 ± 0.03, respectively. Compared to the control, significantly lower pH ($p < 0.05$) was observed in culture broths with lactic acid, while significantly higher pH ($p < 0.05$) was observed in culture broths with 0.1% BHA. Following the additional 24-h culture, pH changed depending on the presence of CFS. A significant reduction in pH ($p < 0.01$) was observed after the addition of CFS at pH 4.0, compared to both broths with and without CFS at pH 6.0. In addition, all culture broths decreased pH from 4.13 ± 0.03 to 4.78 ± 0.03 after the addition of CFS at pH 4.0. Culture broths with or without CFS at pH 6.0 in combination with

Table 1. pH of the broth cultures inoculated with gram-negative bacteria and different additives (mean ± SD for n=3).

Broth	18 h	18+24 h with CFS at pH 4.0	18+24 h with CFS at pH 6.0	18+24 h without CFS
control	6.28 ± 0.12^{b}	4.74 ± 0.05^{c}	6.20 ± 0.10^{b}	7.09 ± 0.26^{a}
0.01% BHA	6.19 ± 0.16^{b}	$4.64 \pm 0.04^{c*}$	6.04 ± 0.08^{b}	$6.70 \pm 0.10^{a*}$
0.02% BHA/BHT	6.20 ± 0.10^{b}	$4.65 \pm 0.03^{c*}$	6.08 ± 0.04^{b}	6.78 ± 0.18^{a}
0.05% BHA	6.63 ± 0.56^{a}	$4.60 \pm 0.00^{b*}$	$5.80 \pm 0.08^{a*}$	$6.28 \pm 0.29^{a*}$
0.1% BHA	$7.08 \pm 0.03^{a*}$	$4.59 \pm 0.01^{c*}$	$6.54 \pm 0.02^{b*}$	7.12 ± 0.06^{a}
0.1% lactic acid	$4.28 \pm 0.02^{b*}$	$4.13 \pm 0.03^{c*}$	$4.60 \pm 0.05^{a*}$	$4.27 \pm 0.03^{b*}$
0.05% lactic acid	$5.09 \pm 0.01^{c*}$	$4.43 \pm 0.03^{d*}$	$5.67 \pm 0.06^{b*}$	$6.07 \pm 0.21^{a*}$
0.2% curing agent	6.43 ± 0.03^{b}	4.78 ± 0.03^{c}	$6.37 \pm 0.03^{b*}$	$7.28 \pm 0.09^{a*}$

* Means significantly different in same column with respect to the control ($p < 0.05$).
[a-d] Means in the same row with superscript letters in common are significantly different ($p < 0.01$).

BHA at various concentrations resulted in pH ranges of 5.80 ± 0.08 to 6.54 ± 0.02 and 6.28 ± 0 .29 to 7.12 ± 0.06, respectively. The pH of culture broth containing 0.05% lactic acid with and without CFS at pH 6.0 was 5.67 ± 0.06 and 6.07 ± 0.21, respectively, while that of culture broth containing 0.1% lactic acid with and without CFS was 4.60 ± 0.05 and 4.27 ± 0.03, respectively. The culture broths inoculated with the curing agent with or without CFS at pH 6.0 had the pH of 6.37 ± 0.03 and 7.28 ± 0.09, respectively.

Effects of different food additives in 18-h cultures The populations of gram-negative bacteria in nutrient broths containing the synthetic antioxidant, lactic acid and curing agent are presented in Table 2. For treatments with 0.1% lactic acid, 0.01% or 0.05% BHA and 0.02% BHA/BHT, growth of gram-negative bacteria was significantly ($p < 0.05$) reduced by approximately 4.8, 1, 1.3 and 1.5 log units, respectively, compared to the controls. For 0.1% BHA alone, growth of gram-negative bacteria was completely inhibited in the 18-h culture at pH 7.08. Incubated culture broths with 0.2% curing agent and 0.05% lactic acid showed no significant differences in viable cell counts, compared to the control without additives.

Effect of CFS in 18+24-h culture The effects of food additives on the susceptibility of gram-negative bacteria to antimicrobial compounds from *L. sakei* D-1001 were expressed as the log-reduction of viable cell counts (Table 2). Analysis of this data revealed significant differences among the 18+24-h cultures ($p < 0.01$), depending on the addition of CFS. When CFS at pH 4.0 was added, a substantial reduction in gram-negative bacteria was observed in all culture broths including the control; no viable cells were detected following the use of CFS at pH 4.0 in cultures with either 0.05% BHA or 0.02% BHA/BHT. The viable cell count was markedly reduced to 1.02 ± 0.03 log units in cultures with a combination

Table 2. Viable counts of gram-negative bacteria in broth cultures with different additives (mean $\log_{10}$ CFU/ml ± SD for n=3).

Broth	18 h	18+24 h with CFS at pH 4.0	18+24 h with CFS at pH 6.0	18+24 h without CFS
control	8.57 ± 0.15^{a}	6.77 ± 0.32^{b}	8.90 ± 0.10^{a}	9.07 ± 0.21^{a}
0.01% BHA	7.60 ± 0.30^{a} *	$1.02 \pm 0.03^{b*}$	$8.17 \pm 0.21^{a*}$	$8.20 \pm 0.36^{a*}$
0.02% BHA/BHT	7.07 ± 0.32^{b} *	ND	$8.20 \pm 0.10^{a*}$	$8.10 \pm 0.26^{a*}$
0.05% BHA	$7.23 \pm 0.71^{ab*}$	ND	$7.80 \pm 0.36^{a*}$	$6.37 \pm 0.21^{b*}$
0.1% BHA	ND	ND	ND	ND
0.1% lactic acid	3.77 ± 0.95^{a} *	ND	ND	$4.37 \pm 0.51^{a*}$
0.05% lactic acid	8.23 ± 0.06^{a}	$4.00 \pm 0.69^{b*}$	8.53 ± 0.12^{a}	8.73 ± 0.32^{a}
0.2% curing agent	8.67 ± 0.32^{a}	$3.20 \pm 1.57^{b*}$	8.87 ± 0.25^{a}	9.10 ± 0.10^{a}

ND, no detection (<30 CFU/mL).
* Means significantly different in the same column, compared to the control ($p < 0.05$).
[a,b] Means in the same row with different letters are significantly different ($p < 0.01$).

of 0.01% BHA and CFS at pH 4.0. In contrast, the viable cell counts of gram-negative bacteria from 18-h cultures were increased in 18+24-h culture broths regardless of the presence or absence of CFS at pH 6.0, except for culture broth with 0.05% BHA alone, which had a slightly decreased viable cell count.

The viable cell count was reduced to 3.77 ± 0.95 log units by 0.1% lactic acid in the 18-h culture and then was slightly increased to 4.37 ± 0.51 log units after the additional 24-h culture without CFS. For this additive, the gram-negative bacterial population was absent when CFS was added at either pH. On the other hand, when a lower concentration of lactic acid was used, CFS at pH 4.0 reduced the viable cell count from 8.23 ± 0.06 log units to 4.0 ± 0.69 log units (a decrease of approximately 4.23 log units). In contrast, the same additive with or without CFS at pH 6.0 showed an increase in the viable cell count; a similar trend was seen for cultures with the curing agent.

Discussion

This study examined the effects of various food additives on gram-negative bacteria in broth. Gram-negative bacteria were isolated from dry-fermented sausages made without starter culture. The fermented meat products might be spoiled by pathogens without a heating step to control pathogen populations (Rodel, 1992; Gill and Landers, 2003). However, in many cases of spoilage, bacteria can be reduced in number, either by inhibition or elimination by various manufacturing methods including the use of LAB in fermentation (Takeo *et al.*, 1994; Coffey *et al.*, 1998; Gonzalez and Diez, 2002; Kalalou *et al.*, 2004). In this study, *L. sakei* was selected because it 1) is more competitive than other lactobacilli, 2) is most commonly found in dry-fermented sausages (Rantsiou and Cocolin, 2006), 3) has a shorter lag phase, 4) has a higher maximum growth rate, and 5) has higher final cell density (Dossmann *et al.*, 1996), which all contribute to the hygienic quality of meat products by producing various antimicrobial compounds including organic acid, hydrogen peroxide, carbon dioxide, diacetyl and high molecular mass compounds like bacteriocins towards undesirable bacteria such as gram-positive bacteria (Mataragas *et al.*, 2003). However, Tantillo *et al.* (2002) reported that gram-negative bacteria were resistant to antimicrobial activity of *L. sakei*. Lactic acid produced by LAB in sausage fermentation reduces pH at which point it has a preservative effect; pH values below 5.2 in dry-fermented sausage are favorable for preservation and its hygienic stability (Leistner, 1995). Among all culture additives tested in this study, culture broths with 0.1% lactic acid showed the lowest pH. A pronounced decrease in pH was observed in all culture broths incubated with CFS at pH 4.0.

Increasing BHA concentration demonstrated a predictable trend in decreasing viable cell counts. BHA has antibacterial effects; however, 0.1% BHA, which is lethal and effective at total inhibition of gram-negative bacteria, currently exceeds the legal limitations for use in food products (FDA: http://www.cfsan.fda.gov/~dms/opa-appa.html). However, a combination of this synthetic antioxidant at lower concentrations (0.01%-0.05%) with CFS at pH 4.0 has the ability to suppress gram-negative bacteria. In this study, reduction in bacterial number was observed for combinations of CFS at pH 4.0 with this synthetic antioxidant even at the lowest concentration of 0.01%. It has previously been reported that the inhibitory effect of this synthetic antioxidant on pathogenic bacteria is due to its ability to disrupt cytoplasmic membranes of cells (Branen *et al.*, 1980; Degre and Sylvestre, 1983; Degre *et al.*, 1983). To our knowledge, this is the first report on the effects of a combination of BHA and CFS against gram-negative bacteria.

In general, lactic acid is produced by LAB in fermented meat products and produces primarily antimicrobial effects against spoilage bacteria (Daeschel, 1989). In our study, the effects of changes in the lactic acid concentration alone or in combination with the addition of CFS on gram-negative bacteria in broth were examined. For 0.1% lactic acid alone, viable cell counts were markedly reduced following 18 h culture, but increased after an additional 24 h culture. In contrast, for a combination of 0.1% lactic acid and CFS, no viable cells were observed, and for a combination of 0.05% lactic acid with CFS at pH 4.0 only, a reduction in viable cells was observed. Furthermore, we observed that control cultures with CFS at pH 4.0 were also more efficient than those with or without CFS at pH 6.0. These findings confirmed results of several reports on the inhibitory effects of lactic acid (Van Netten *et al.*, 1995; Pipek *et al.*, 2005) and the fact that lactic acid increases the outer membrane permeability of gram-negative bacteria (Alakomi *et al.*, 2000).

Curing agents are commonly used in dry-fermented sausage to aid in flavor development and to prevent lipid rancidity (Noil *et al.*, 1990; Olesen *et al.*, 2004). Curing agent concentration in this study was fixed at 0.2% because of results in our previous experiment (Mikami *et al.*, 2004). In this study, no antimicrobial effect was seen with 0.2% curing agent alone, which is similar to the control without any additives. However, the combination of 0.2% curing agent with CFS at pH 4.0 appears to prevent gram-negative bacteria growth. Leroy *et al.* (1999) suggested that nitrite, which is a component of the curing agent, has a synergistic action with lactic acid that is commonly found in CFS (Lindgren and Dobrogosz, 1990). This explains our observation that gram-

negative bacteria are susceptible to the curing agent when combined with CFS at a high lactic acid concentration.

In this study, combinations of antimicrobial treatments at pH at 4.78 ± 0.03 or less were effective at inhibiting gram-negative bacteria derived from dry-fermented sausage compared with using single treatments, except in the case of 0.1% BHA, which alone inhibited all viable cells. These results partially agree with those of Leistner *et al.* (1995), which showed that two or more antimicrobial agents acting synergistically at suboptimal levels are more effective than each of them alone at the optimal level.

Therefore, using food additives can increase gram-negative bacteria susceptibility to antimicrobial compounds of *L. sakei* D-1001, but only in low pH environments that depend on the lactic acid concentration. In addition, food additives at concentrations permitted by the FDA, in combination with antimicrobial compounds of LAB can be useful in devising control strategies for pathogenic gram-negative bacteria in acidic foods such as fermented meat products. Recently, naturally occurring antioxidants are being replaced by synthetic antioxidants because they exhibit similar antioxidant potency (Ramarathnam *et al.*, 1995; Zein, 2000; Bozkurt, 2006). Additional studies are needed to investigate the efficiency of combining natural antioxidants and LAB compounds on gram-negative bacteria.

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