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Antimicrobial Synergistic Effect of Linolenic Acid and Monoglyceride against *Bacillus cereus* and *Staphylococcus aureus*

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The antimicrobial effect of linolenic acid with or without monoglyceride (glycerol laurate or glycerol myristate) against six food-borne microorganisms was determined in broth medium. Minimum inhibitory concentration of linolenic acid on *Bacillus cereus* and *Staphylococcus aureus* was 20 and 50 ppm, respectively. The growth of *B. cereus* treated with linolenic acid at 10 ppm with 10 ppm monoglyceride was more inhibitory than that of linolenic acid alone, and the viable cell population was reduced 2−4 log cycles compared to that of the control. When linolenic acid was added at that level, the adenosine triphosphate (ATP) concentration of extracellular fluid was drastically increased compared with that of the control, and the combined effect with monoglyceride was higher than that with linolenic acid alone. However, the intracellular ATP concentration decreased compared with that of the control. From these results, we concluded that linolenic acid has a strong antimicrobial activity against *B. cereus* and *S. aureus*, and that linolenic acid combined with monoglyceride showed stronger antimicrobial activity than using linolenic acid alone.

KEYWORDS: Antimicrobial; linolenic acid; monoglyceride; synergistic effect; ATP

INTRODUCTION

Eating habits of the human population have changed significantly with the development of industry and technology, and the consumption of processed food has been increasing. Occurrences of food contamination due to putrefaction and microorganisms during processing, storage, and distribution of food have led to concerns about food safety.

The number of food poisoning cases has been increasing every year in Korea. Among these cases, food poisoning from *Salmonella* accounts for 40−50% of the total. The main contaminated foods were meat and processed meat products, resulting in 30−70% of the cases, and processed fish products, which accounted for 11−37% (*1*).

To reduce health hazards and economic loss due to food-borne microorganisms, research has been undertaken on suppressing or preventing microorganisms causing food poisoning. Studies have been reported on using preservatives such as sodium benzoate and potassium sorbate (*2*) alone or in combination with organic acids such as propionic acid, acetic acid, citric acid, tartaric acid, lactic acid, and malic acid (*3, 4*). Considering the tendency of consumers to avoid foods containing synthetic preservatives, investigations are being pursued on natural antimicrobial substances from animal and plant sources, and promising substances include pine needle extract (*5*), *Sophora flavescens* extract (*6*), *Shizandra chinensis* extract (*7*), protamine, lysozyme, ethanol, hydrolysate of pectin, grapefruit extract, tea extract, and chitosan (*8*).

α-Linolenic acid (9,12,15-octadecatrienoic acid) is abundant in safflower oil, soybean oil, cotton seed oil, perilla oil, and evening primrose, and was identified as the antimicrobial substance in *Mallotus japonicus* Muell (*9*). Linolenic acid is an unsaturated fatty acid not synthesized in the body, and among unsaturated fatty acids, α-linolenic acid and the *r*-linolenic acid are known to lower the levels of serum cholesterol, triglycerides, and LDL-cholesterol, decreasing the risk of arteriolosclerosis, cancer, and allergic diseases (*10, 11*).

A study on the antimicrobial effect of fatty acids showed the antimicrobial effect is far more significant in those connected in straight chain fatty acids rather than isomer fatty acids (*12*). In the ester group of fatty acids composed with 6−18 carbons, a significant antimicrobial effect was present in glycerol caprylate (C_{10}), glycerol laurate (C_{12}), and glycerol myristate (C_{14}) against bacteria, yeasts, and molds (*13*−*15*). The strength of the antimicrobial effect of the fatty acids with 18 carbons and glycerol laurate (C_{12}) against *Bacillus cereus* was in the order of stearic < oleic < lauric < glycerol laurate < linolenic acid (*16*).

In the present study, we measured the antimicrobial activity of linolenic acid against six food-borne microorganisms. The antimicrobial mechanism was also examined through release of ATP, and the stimulation of antimicrobial effect was

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Table 1. Strains and Media Used for Antimicrobial Experiment

microorganisms tested	media used	incubation temp (°C)
Vibrio parahaemolyticus ATCC 33844	TSB & TSA[a] + 3%NaCl	30
Bacillus cereus ATCC 11778	NB & NA[b]	30
Salmonella typhimurium ATCC 14028	NB & NA	30
Staphylococcus aureus ATCC 25923	TSB & TSA	37
Escherichia coli O157:H7 ATCC 43894	TSB & TSA	37
Salmonella enteritidis KCCM 12021	TSB & TSA	37

[a] Tryptic soy broth and Tryptic soy agar (Difco). [b] Nutrient broth and Nutrient agar (Oxoid).

investigated by increasing the solubility of water-insoluble linolenic acid using emulsifying agents (glycerol laurate and glycerol myristate).

MATERIALS AND METHODS

Strains and Culture Condition. The six food-borne microorganisms used in this research and their culture conditions are shown in **Table 1**. The bacterial cultures were prepared for 24 h in sterilized broth medium from 1 colony from an agar slant, from which 0.1 mL was transferred to new broth medium and grown for 18 h.

Materials. α-Linolenic acid, glycerol laurate (monolaurin), glycerol myristate (monomyristin), and luciferase-luciferin (L 0633) were purchased from Sigma Chemical Co. (St. Louis, MO) for evaluating use. The purity of these chemicals was 99%.

Measurement of Antimicrobial Activity. α-Linolenic acid (5−1000 ppm), glycerol laurate (5−1000 ppm), and glycerol myristate (5−1000 ppm) were prepared by dissolving them in ethanol. These solutions (0.1 mL) and 0.1 mL of the second culture were transferred to 10 mL of sterile broth medium. The initial population of each inoculum was adjusted to 10^6-10^7 colony forming units. The treated group was injected with 0.3 mL of culture broth into a well of Bioscreen C (Labsystem, Oy, Helsinki, Finland), and then the absorbency was measured (at 600 nm) for 72 h at 12-h intervals. The ethanol (0.1 mL) was added for the control. Incubation temperature for each microorganism is shown in **Table 1**.

Measurement of antimicrobial activity of linolenic acid with monoglyceride was conducted by the same method at a minimum inhibitory concentration (MIC) or under MIC. The MIC was defined in this study as the lowest concentration of an agent which prevented growth on broth medium until 72 h by measurement of absorbency when compared with that of the control.

Viable Cell Population. Linolenic acid, with or without monoglyceride, and 0.1 mL of each activated culture were added into culture broth and incubated for 72 h at the optimum temperatures (**Table 1**), and then the viable cell population was counted at 24 h by the plate count method (*17*).

Measurement of Adenosine Triphosphate (ATP). In food science, assays for measuring ATP are used in detection of bacteria, contaminants in several food systems, and determination of the antimicrobial properties of food (*18*, *19*). There is a high correlation between viable cell populations of bacteria-contaminated food and ATP concentrations (*20*).

In this study, the antimicrobial mechanism was investigated by measuring the intracellular and extracellular ATP concentrations using methods previously described (*21*, *22*).

The culture broths of *B. cereus* and *Salmonella enteritidis* were incubated for 24 h at the optimum temperature (**Table 1**) and then centrifuged for 10 min at 1,000*g*, and the supernatant was removed. The cell pellets were washed with sodium phosphate buffer (0.1 M, pH 7.0) and then recentrifuged under the same conditions. A cell suspension was prepared with 2 mL of sodium phosphate buffer (0.1 M, pH 7.0), divided into four groups, and placed into elpendorf tubes. Linolenic acid (0.05 mL/0.5 mL) with or without emulsifying agent (glycerol laurate and glycerol myristate, 0.05 mL/0.5 mL) was placed into the cell suspension. Ethanol (0.05 mL/0.5 mL) was used for the control. Samples were reacted at room temperature for 30 min, centrifuged for 5 min at 2000*g*, and then incubated in ice to prevent ATP loss. The ATP concentrations of the supernatant, which represents the extracellular concentration, were determined using a luminometer (Lumac bv, Landgraaf, The Netherlands) after the addition of 80 μL of 100 mM glycine buffer to 20 μL of supernatant and 100 μL of the prepared luciferase-luciferin (5 mg/mL). The ATP concentrations representing intracellular content were determined by adding 100 μL of the cell suspension to 25 mM HEPES containing Triton-X 100 and then sonicating (Bransonic 5210R-DTH, Branson Ultrasonics Corp., CT) after resuspending to disrupt the microorganisms. Subsequently, 100 μL of luciferase-luciferin (5 mg/mL) was added to the mixture, and the ATP concentration was measured with a luminometer. To calculate the intracellular and extracellular ATP concentrations, a standard ATP curve ranging from 10^{-1} to 10^{-3} μM portion was used to obtain a linear relationship between ATP concentration (μM) and relative light unit which resulted in a r^2 (coefficient of determination) value of 0.96. The following regression equation was obtained: $\log(y) = 0.766\log(x) + 7.39$.

Statistical Analysis. Reported data represent the mean of three different experiments with three measurements in each experiment. Statistical Analysis Systems software (SAS Institute, Cary, NC, 1996) was used for analysis of variance. Values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Antimicrobial Effect of Linolenic Acid. In the liquid media containing linolenic acid at a level between 10 and 1000 ppm, each microorganism was inoculated, and the MICs determined based on absorbance using Bioscreen C for 72 h at 12-h intervals are shown in **Figure 1**.

Among the six food-borne microorganisms, linolenic acid showed an excellent inhibitory effect against *B. cereus* and *S. aureus*, and MICs for these strains were 20 and 50 ppm, respectively. Linolenic acid inhibited the growth of *Vibrio parahaemolyticus* at 25 ppm for 12 h, and a slight inhibitory effect was present for *Salmonella typhimurium*, *S. enteritidis*, and *Escherichia coli* O157:H7 at 1000 ppm. The MIC for *B. cereus* was shown to be 20 ppm in the present study, which is slightly higher than the range of 10−15 ppm that was reported to inhibit spore germination and growth of *B. cereus* (*16*). In addition, Ahn et al. (*9*) reported that the MIC against *Listeria monocytogenes* was 20 ppm.

From these results, we could determine that the antimicrobial activity of linolenic acid is superior against Gram (+) bacteria such as *B. cereus*, *S. aureus*, and *L. monocytogenes*, and is low against Gram (−) bacteria such as *V. parahaemolyticus*, *S. typhimurium*, *E. coli* O157:H7, and *S. enteritidis*. These results were similar to those of Kabara et al. (*13*). This difference is due to the difference in the cell membrane structure of Gram (+) bacteria, in which the cytoplasmic membrane is inside the peptidoglycan layer, whereas in Gram (−) bacteria, the outer membrane is present outside the cell membrane. The resistance by Gram (−) bacteria is due to lipopolysaccharides present in the outer membrane, which form a barrier preventing the penetration of fatty acid into the cell (*23*). Thus, the method of

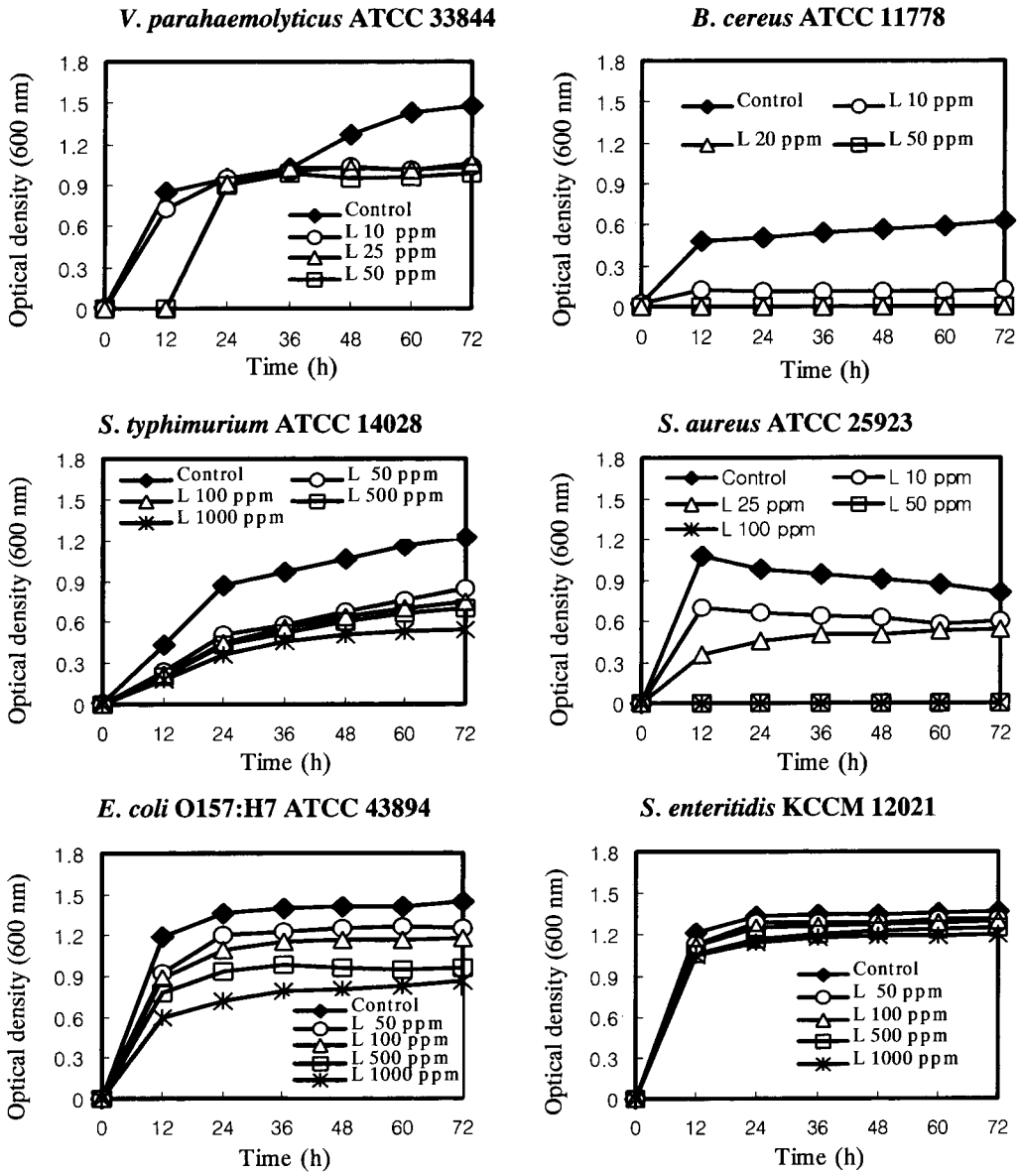

Figure 1. Growth inhibition of linolenic acid (L) on several food-borne microorganisms.

Table 2. Inhibitory Effects of Glycerol Laurate against Food-Borne Microorganisms

	glycerol laurate (ppm)				
microorganisms tested	25	50	100	500	1000
V. parahaemolyticus ATCC 33844	+[a]	+	+	+	+
B. cereus ATCC 11778	++[b]	++	++	++	++
S. typhimurium ATCC 14028	N[c]	N	N	N	N
S. aureus ATCC 25923	++	++	++	++	++
E. coli O157:H7 ATCC 43894	N	N	N	N	N
S. enteritidis KCCM 12021	N	N	N	N	N

[a] +, Optical density value is zero after 12 h (inhibitory effects). [b] ++, Optical density value is zero after 72 h (inhibitory effects). [c] N, No inhibitory effects.

Table 3. Inhibitory Effects of Glycerol Myristate against Food-Borne Microorganisms

	glycerol myristate (ppm)				
microorganisms tested	25	50	100	500	1000
V. parahaemolyticus ATCC 33844	N[a]	N	+[b]	+	+
B. cereus ATCC 11778	N	++[c]	++	++	++
S. typhimurium ATCC 14028	N	N	N	N	N
S. aureus ATCC 25923	N	N	++	++	++
E. coli O157:H7 ATCC 43894	N	N	N	N	N
S. enteritidis KCCM 12021	N	N	N	N	N

[a] N, No inhibitory effects. [b] +, Optical density value is zero after 12 h (inhibitory effects). [c] ++, Optical density value is zero after 72 h (inhibitory effects).

removing the lipopolysaccharide wall using chelating agents for the penetration of antimicrobial substances into cells after the adsorption into cell surface was suggested (*13*, *24*). On the basis of these observations, we assumed linolenic acid effectively inhibited Gram (+) bacteria.

Antimicrobial Effect of Emulsifying Agents. MIC was obtained by measuring the absorbance with Bioscreen C for 72 h at 12-h intervals. The six food-borne microorganisms were inoculated into liquid media with glycerol laurate (GA) and glycerol myristate (GM) at concentrations between 10 and 1000 ppm as shown in **Tables 2** and **3**.

MIC of GA for *B. cereus* was 25 ppm, and of GM was 50 ppm. In the case of *S. aureus*, GA was 25 ppm, and GM was 100 ppm. As for *V. parahaemolyticus*, GA was 25 ppm, and GM at 100 ppm delayed the cellular growth for 12 h. The growth of other strains was slightly inhibited at 1000 ppm.

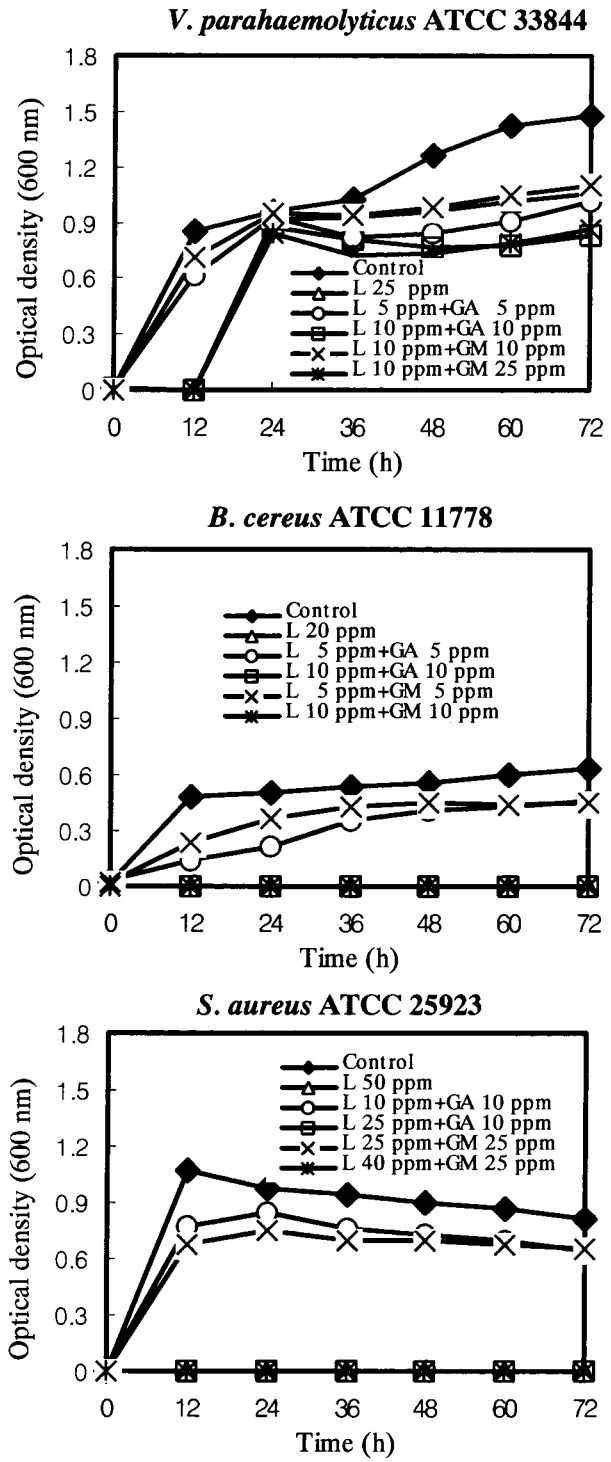

Figure 2. Growth inhibition of linolenic acid (L) with glycerol laurate (GA) or glycerol myristate (GM) on several food-borne microorganisms.

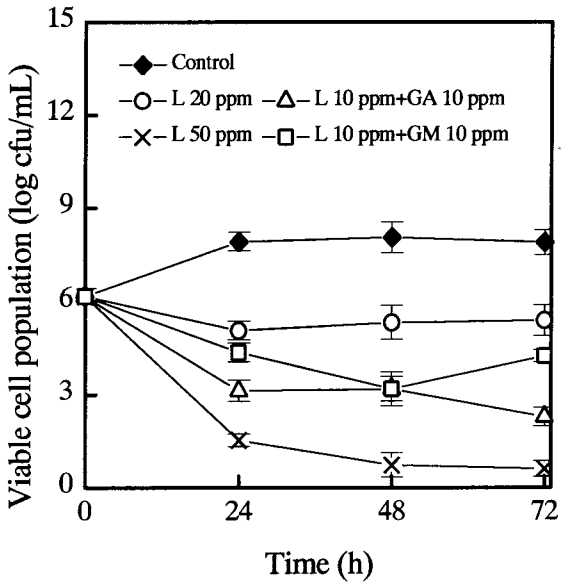

Figure 3. Growth inhibition of linolenic acid (L) with or without glycerol laurate (GA) and glycerol myristate (GM) on *Bacillus cereus* ATCC 11778. Vertical bars indicate ±SD. Experiments were carried out in triplicate.

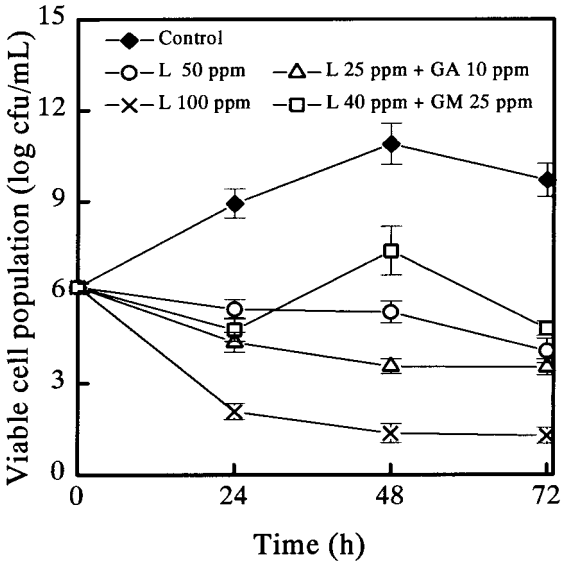

Figure 4. Growth inhibition of linolenic acid (L) with or without glycerol laurate (GA) and glycerol myristate (GM) on *Staphylococcus aureus* ATCC 25923. Vertical bars indicate ±SD. Experiments were carried out in triplicate.

Wang et al. (*15*, *25*) and Oh et al. (*26*) reported that GA and GM have antimicrobial effect against *L. monocytogenes*. Further, GA inhibited growth for 72 h at 25 ppm, and GM was inhibitory at 25 ppm for 24 h and at 50 ppm for 72 h.

Emulsifying agents are composed of molecules having a hydrophilic group and a lipophilic group. Glycerin esters of fatty acids containing 12−18 carbons are commonly used as emulsifying agents; glycerin esters of short-chain fatty acid with 3−10 carbons have a weak emulsifying capacity, but have an antimicrobial effect. The mechanism of the antimicrobial effect by emulsifying agents has been reported to be due to inhibition by GA and GM, which attach to the bacterial cell surface, preventing normal action of cell membrane or inactivating enzyme after penetration into cells, and denaturing the nucleus or protein, preventing growth (*26*−*28*).

Antimicrobial Effect When Combining Linolenic Acid and Emulsifying Agent. On the basis of the results obtained from adding linolenic acid or monoglyceride separately into each strain culture, we selected a MIC or a concentration under MIC and observed the synergistic effect of linolenic acid combined with GA or GM. Results are shown in **Figure 2**.

In the case of *B. cereus*, when linolenic acid was added alone the MIC was 20 ppm, but when linolenic acid (10 ppm), which showed no growth inhibition alone, was added with 10 ppm GA or 10 ppm GM, an excellent synergistic effect was observed with significant growth inhibition. Although the growth inhibition concentration of linolenic acid for *S. aureus* was 50 ppm,

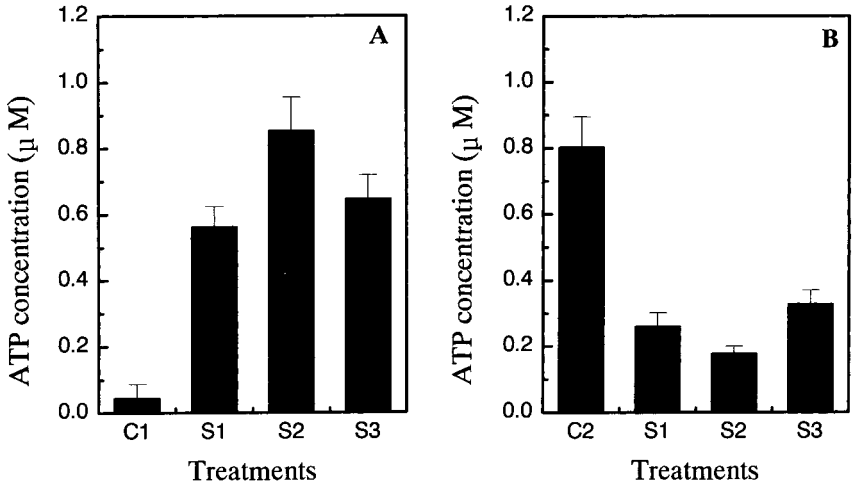

Figure 5. Adenosine triphosphate (ATP) concentration in culture supernatants and cell pellets of *Bacillus cereus* ATCC 11778: (A) Concentration of extracellular ATP, (B) Concentration of intracellular ATP. C1, Culture supernatant control; C2, cell pellet control; S1, addition of 20 ppm linolenic acid; S2, addition of linolenic acid 10 ppm with glycerol laurate 10 ppm; S3, addition of linolenic acid 10 ppm with glycerol myristate 10 ppm. Vertical bars indicate ±SD. Experiments were carried out in triplicate.

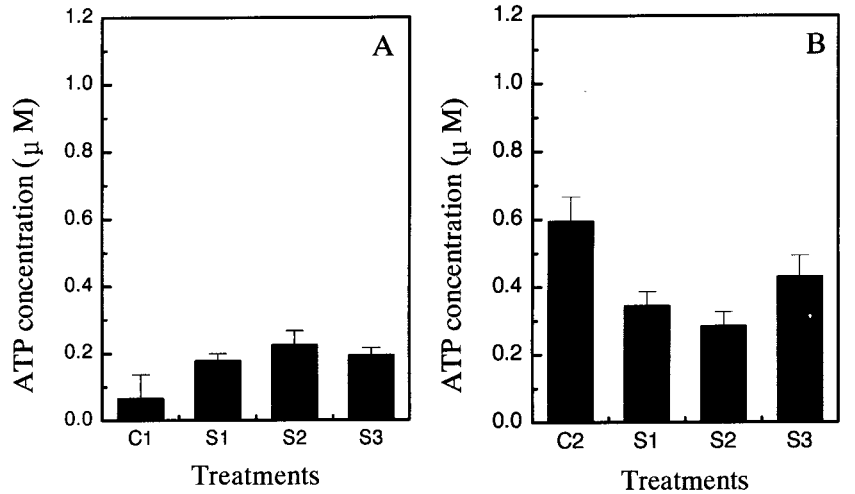

Figure 6. Adenosine triphosphate (ATP) concentration in culture supernatants and cell pellets of *Salmonella enteritidis* KCCM 12021: (A) Concentration of extracellular ATP, (B) Concentration of intracellular ATP. C1, Culture supernatant control; C2, cell pellet control; S1, addition of 100 ppm linolenic acid; S2, addition of linolenic acid 100 ppm with glycerol laurate 100 ppm; S3, addition of linolenic acid 100 ppm with glycerol myristate 100 ppm. Vertical bars indicate ±SD. Experiments were carried out in triplicate.

an excellent antimicrobial effect was observed with 25 ppm linolenic acid and 10 ppm GA, 40 ppm linolenic acid and 25 ppm GM, and 30 ppm linolenic acid and 50 ppm GM. Linolenic acid at a concentration of 25 ppm retarded cell growth of *V. parahaemolyticus* for 12 h, and 10 ppm linolenic acid with 10 ppm GA or 25 ppm GM also inhibited growth for 12 h. In other strains, cellular growth was slightly reduced when one of these emulsifying agents was added with linolenic acid (data not shown). From these results, we could determine that the antimicrobial effect of linolenic acid is better when used together with an emulsifying agent rather than when used alone, suggesting that the synergistic effect is due to the emulsifying agent making the adsorption of linolenic acid to cell surface or penetration of linolenic acid into cells possible.

Measurement of Viable Cell Population. Because the bacterial concentration under 10^5 cfu/mL does not show a proliferation pattern by measurement of optical density, we added linolenic acid and GA or GM into the culture medium and counted viable cells for 72 h at 24-h intervals with the plate count method as the accurate method.

In the case of *B. cereus* (**Figure 3**), we observed a 2.5 log cycles reduction compared with the control at the MIC of linolenic acid of 20 ppm at 72 h incubation, and a 7.29 log cycles reduction with linolenic acid at a concentration of 50 ppm at 72 h, a bactericidal effect was observed. Further, we observed a 3.65 log cycles reduction when 10 ppm of linolenic acid was added with 10 ppm of GM at 72 h, confirming the synergistic effect, and we observed a 5.61 log cycles reduction when 10 ppm of linolenic acid was added with 10 ppm of GA at 72 h, showing a superior synergistic effect.

The cell growth of *S. aureus* (**Figure 4**) decreased by 3.59−5.00 log cycles with the addition of 50 ppm of linolenic acid compared with the control, and decreased by 7.94−8.58 log cycles with the addition of 100 ppm of linolenic acid. Cell growth decreased by 6.17 log cycles when 25 ppm of linolenic acid and 10 ppm of GA at 72 h were added, and by 4.90 log cycles when 40 ppm of linolenic acid and 10 ppm of GM at 72 h were added.

One log cycle reduction was observed in *V. parahaemolyticus* with the addition of 25 ppm of linolenic acid. In *S. typhimurium*,

E. coli O157:H7, and *S. enteritidis* addition of 1,000 ppm of linolenic acid (data not shown) showed growth reduction.

In conclusion, compared with using linolenic acid alone, the antimicrobial effects increased with the addition of the emulsifying agents GA and GM, and a greater antimicrobial effect was observed with the addition of GA than with the addition of GM.

Intracellular and Extracellular ATP Concentration. To observe the antimicrobial mechanism associated with combination of linolenic acid and emulsifying agent, we measured the intracellular and extracellular ATP concentrations of the Gram (+) bacteria *B. cereus* and the Gram (−) bacteria *S. enteritidis*.

Linolenic acid (**Figure 5**) showed excellent antimicrobial effect in *B. cereus*; and for the extracellular ATP, concentration was 0.565 μM with the treatment of MIC of linolenic acid alone at 20 ppm, and 0.855 μM with the treatment of 10 ppm linolenic acid with 10 ppm GA (showing a relatively high concentration compared with the other groups), and 0.649 μM with the addition of 10 ppm linolenic acid and 10 ppm GM. As for the intracellular ATP, concentration decreased to 0.262 μM with the addition of linolenic acid, 0.180 μM with the addition of linolenic acid together with GA, and 0.330 μM with the addition of linolenic acid together with GM.

In *S. enteritidis* (**Figure 6**), the tendency of increased extracellular ATP concentration and decreased intracellular ATP concentration was observed. The extracellular ATP concentration increased to 0.180 μM with the addition of linolenic acid at a concentration of 100 ppm, and to 0.227 μ M and 0.196 μM with the addition of 100 ppm linolenic acid and 100 ppm GA, and with the addition of 100 ppm linolenic acid and 100 ppm GM, respectively. The intracellular ATP concentration at the same conditions decreased to 0.289 μM with the addition of linolenic acid, 0.347 μM with the addition of linolenic acid and GA, and to 0.433 μM with the addition of linolenic acid and GM.

In the case of *B. cereus*, an excellent antimicrobial effect was shown with the addition of 20 ppm linolenic acid. Viable cell populations decreased by 3.65−5.61 log cycles with the addition of emulsifying agents, and the extracellular ATP concentration increased significantly. On the other hand, the extracellular ATP concentration in *S. enteritidis* showed a slight decrease with the addition of 100 ppm linolenic acid, and a decrease in cell growth.

The above results indicate that the extracellular ATP concentration increased in *B. cereus* and *S. enteritidis* with the addition of the emulsifying agents in combination with linolenic acid compared to addition of linolenic acid alone, and the extracellular ATP concentration in *B. cereus* showed an excellent antimicrobial effect. This was significantly superior to that in *S. enteritidis*, suggesting that the extracellular ATP concentration increased significantly in those strains with a large antimicrobial effect.

These results were similar to the experiment by Ahn et al. (*21*) who observed allyl isothiocyanate resulted in the intracellular ATP elution in *L. monocytogenes* and observed the destruction of cell membrane using a transmission electron microscope. The experiment by Han (*22*) reported that the antimicrobial substance of *Dryopteris crassirhizoma* Nakai increased the extracellular ATP concentration of *L. monocytogenes*, and that the phenomenon of decreasing the intracellular ATP concentration causes the destruction of, or abnormality in, cell membrane, which resulted in the antimicrobial effect through extracellular elution of intracellular ATP.

The antibacterial activity of fatty acids is probably due to the ability of these compounds to disrupt the membranes of bacterial cells and cause lysis of the cells (*23*). From these results, linolenic acid is assumed to exert an antimicrobial effect by causing cell surface or intracellular abnormality. When an emulsifying agent is used, the solubility of the insoluble linolenic acid increases to change cell membrane structure, eluting intracellular ATP to outside of cells as a result of the antimicrobial effect.

From these experiments, we conclude that linolenic acid has an antimicrobial effect for some strains of bacteria that cause food poisoning. In addition, its antimicrobial effect was synergistic with addition of the emulsifying agents glycerol laurate and glycerol myristate. We believe that further studies using SEM and TEM are needed for an in-depth look at the antimicrobial mechanism of linolenic acid.

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