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# Growth Inhibition of Foodborne and Pathogenic Bacteria by Conjugated Linoleic Acid

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The influence of conjugated linoleic acid (CLA) on the growth of some foodborne and pathogenic bacteria was examined. A potassium salt of CLA (CLA-K) was tested against three Gram-positive strains (*Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus mutans*) and five Gram-negative strains (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Vibrio parahemolyticus*, *Klebsiella pneumoniae*, and *Proteus mirabilis*). CLA-K-mediated growth inhibition was evident for all tested strains, particularly the Gram-positive strains. The $IC_{50}$ value of CLA-K was 0.3 mM for *B. cereus*, 1.2 mM for *S. aureus*, and 0.3 mM for *S. mutans*, whereas the value was 1.2 mM for *K. pneumoniae*, 1.2 mM for *P. aeruginosa*, 1.8 mM for *S. typhimurium*, 1.8 mM for *V. parahemolyticus*, and 2.4 mM for *P. mirabilis*. The CLA-K delayed the growth of all the tested strains at lower CLA-K concentrations, but completely inhibited the growth at higher concentrations. All cells grown in the medium containing CLA-K contained CLA in their membranes and exhibited irregular cell surface and cell disruption, which were greater in Gram-positive than Gram-negative strains. Higher lactic dehydrogenase activity (LDH), protein content, and malondialdehyde (MDA) content were evident in Gram-positive strains than in Gram-negative strains. These results suggest that the broad spectrum of growth inhibition by CLA mediated through the lipid peroxidation of CLA in the membranes and in the medium.

**KEYWORDS: Conjugated linoleic acid; bactericidal effect; Gram-positive strains; Gram-negative strains; foodborne bacteria; pathogenic bacteria; lipid peroxidation**

## INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term that refers to cis and trans configurations at conjugated double-bond systems (C7,9; C8,10; C9,11; C10,12; C11,13; and C12,14) of octadecadienoic acid. CLA was first isolated from fried ground beef as an anticarcinogen (*1*). Since then, more than 3000 CLA research papers related to biological functions and contents in various food systems have been published. The bulk of the research on CLA has focused on anticarcinogenesis in animals and human cancer cell lines, reduction of body fat in experimental animals and humans, antiatherosclerosis, antidiabetes, and lipid metabolism (*2*−*8*). Meanwhile, attempts have been made to enhance food functionality by the introduction of CLA in foods and to develop analytical methods of CLA detection in meats from ruminants, dairy products, and mother's milk (*9*−*11*).

Despite the plethora of studies on CLA, there have been only a few investigations concerning the antibacterial activity of CLA, which have been confined to the foodborne bacterium *Listeria monocytogenes* (*12, 13*) and *Lactobacillus* spp. (*14*). CLA reduces the growth of *L. monocytogenes* in vitro, but the efficacy is lower than that of lauric acid (C12:0) or linoleic acid (C18:2) (*12*), and can markedly reduce the growth of *L. monocytogenes* in refrigerated milk (*13*). CLA also reduces the growth of *Lactobacillus reuteri*, *Lactobacillus zeae*, *Lactobacillus helveticus*, and *Lactobacillus acidophilus* in MRS broth or milk (*14*). Most long-chain saturated and unsaturated fatty acids

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**Table 1.** Growth Inhibitory Effects of CLA-K on Selected Foodborne and Pathogenic Bacteria Assayed by Agar Diffusion

| microbial strain | | amount[a] per disk | | | |
|---|---|---|---|---|---|
| | | 6.0 μmol | 12.0 μmol | 18.0 μmol | 24.0 μmol |
| Gram-positive | *B.cereus* | 11.2 ± 0.6[b] | 11.9 ± 0.6 | 12.3 ± 0.6 | 13.3 ± 1.0*[c] |
| | *S. aureus* | 9.4 ± 0.6 | 9.7 ± 1.0 | 10.1 ± 1.0 | 12.4 ± 0.6* |
| | *S. mutans* | 12.2 ± 0.6 | 12.0 ± 1.0 | 12.7 ± 1.0 | 15.1 ± 1.0* |
| Gram-negative | *K. pneumoniae* | 9.8 ± 0.6 | 10.1 ± 0.0 | 10.4 ± 1.0 | 12.2 ± 0.6* |
| | *P. aeruginosa* | 9.2 ± 0.0 | 9.0 ± 1.0 | 10.4 ± 0.6 | 11.0 ± 0.6* |
| | *S. typhimurium* | 8.4 ± 1.0 | 8.6 ± 1.0 | 9.0 ± 0.6 | 9.9 ± 0.6* |
| | *V. parahemolyticus* | 8.2 ± 0.6 | 8.2 ± 0.6 | 8.9 ± 0.6 | 9.4 ± 0.6* |
| | *P. mirabilis* | 8.6 ± 0.6 | 9.1 ± 0.6 | 9.8 ± 0.6 | 10.1 ± 0.6* |

[a] Molecular weight of CLA-K is 320. [b] Mean ± SD of the clear zone including disk diameter (8 mm). [c] The asterisk indicates significant difference ($p < 0.05$) of 24.0 vs 6.0 μmol by Student's *t* test.

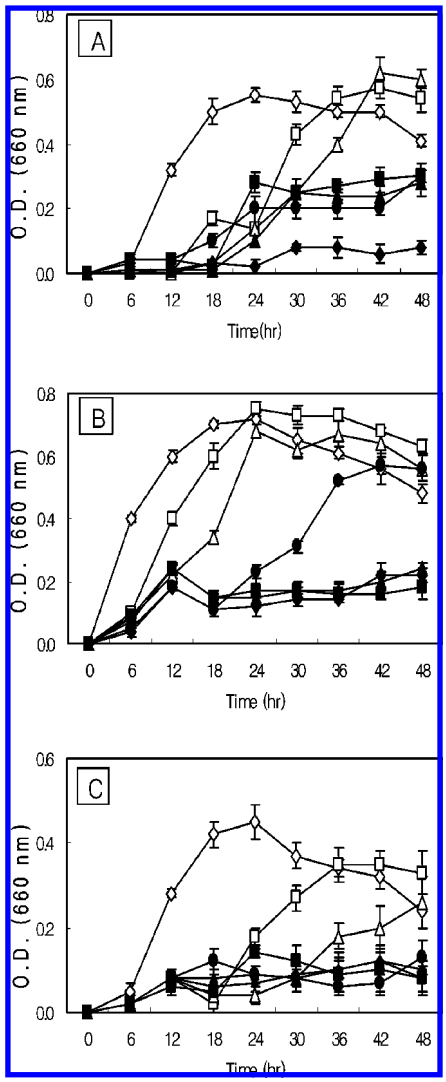


**Figure 1.** Growth inhibition by CLA-K on *B. cereus* (**A**), *S. aureus* (**B**), and *S. mutans* (**C**). Data are expressed as mean ± SD of 0 (control; ◇), 0.3 (□), 0.6 (△), 1.2 (●), 1.8 (▲), 2.4 (■), and 3.0 mM CLA-K (◆) treatments. Growth curves of greater than 1.2 mM CLA-K concentrations on *B. cereus* (**A**) and *S. mutans* (**C**) and greater than 1.8 mM CLA-K concentrations on *S. aureus* (**B**) were significantly different from that of control (*F* test, $p < 0.05$).

exhibit antimicrobial activities (*12*−*18*), yet scant attention has been paid to the antimicrobial potential of CLA, which is a long-chain polyunsaturated fatty acid.

Fatty acids exhibit antibacterial or no effect depending on bacterial strain and types of fatty acid. Long-chain polyunsaturated fatty acids show divergent antibacterial activities against Gram-positive and Gram-negative bacterial strains. For examples, the bactericidal activity of linoleic and linolenic acids against Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* has been well-documented (*17*), whereas long-chain unsaturated fatty acids, including linoleic acid, do not inhibit the growth of Gram-negative bacteria such as *Escherichia coli*, except for *Pseudomonas aeruginosa* (*19, 20*). Hence, it is of significance to evaluate the growth inhibitory effects of CLA against Gram-positive and Gram-negative strains causing food poisoning and disease in humans. The purpose of the present study was to examine the bactericidal effect of CLA for some foodborne bacteria and pathogenic bacteria.

## MATERIALS AND METHODS

**Materials.** Linoleic acid, lactate dehydrogenase (LDH), nicotinamide adenine dinucleotide (reduced) (NADH), potassium pyruvate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 1,1,2,3-tetramethoxypropane (TMP), malondialdehyde (MDA), glutaraldehyde, osmium tetroxide, hexamethyldisilazane, and 1,1,3,3-tetramethylguanidine (TMG) were purchased from Sigma-Aldrich (St. Louis, MO). Nutrient broth (NB), nutrient agar (NA), and brain−heart infusion (BHI) broth/agar were obtained from Difco (Detroit, MI). Paper disks, 8 mm in diameter, were obtained from Advantec Toyo (Tokyo, Japan). All other reagents used were of analytical grade.

**Preparation of CLA and CLA Potassium Salt (CLA-K).** CLA was synthesized from linoleic acid by a previously described alkaline isomerization method (*21*). The composition of the CLA as analyzed by gas chromatography (*21*) was 45% c9,t11 CLA, 47% t10,c12 CLA, and 8% other CLA isomers. CLA-K was prepared by reacting equivalent amounts of CLA and 0.1 M KOH/ethanol solution at 80 °C for 30 min by stirring with a magnetic stirrer bar, followed by adjustment of the pH of the reaction mixture to 7.0 with 0.1 N HCl solution. After removal of unreacted CLA by extraction with hexane, the reaction mixture was freeze-dried, using an Ilshinlab MCFD 5512 freeze-dryer (Yangju, Korea) to obtain CLA-K.

**Bacterial Cultures.** Four foodborne bacteria (*Bacillus cereus*, ATCC 10702; *Staphylococcus aureus*, ATCC 10537; *Salmonella typhimurium*, ATCC 29630; and *Vibrio parahemolyticus*, ATCC 17802) and the causative agents of dermatitis (*Pseudomonas aeruginosa*, ATCC 10145), tooth decay (*Streptococcus mutans*, ATCC 25175), pneumonia (*Klebsiella pneumoniae*, ATCC 13883), and epidemic diarrhea (*Proteus mirabilis*, ATCC 21100) were obtained from the Korea Culture Center of Microorganisms (Seoul, Korea). Each strain was cultured in NB at 37 °C, with the exception of *B. cereus*, which was cultured at 30 °C. *S. mutans* and *V. parahemolyticus* were cultured in BHI broth and 3% NaCl-containing NB, respectively, at 37 °C. All bacteria were cultured in a KSI-200 L temperature-controlled shaking incubator (Koencon, Hanam, Korea) for 18 h at 200 rpm. Each culture was diluted to give a 1.0 absorbance at 660 nm with fresh medium for use as an inoculum for the antibacterial assay.

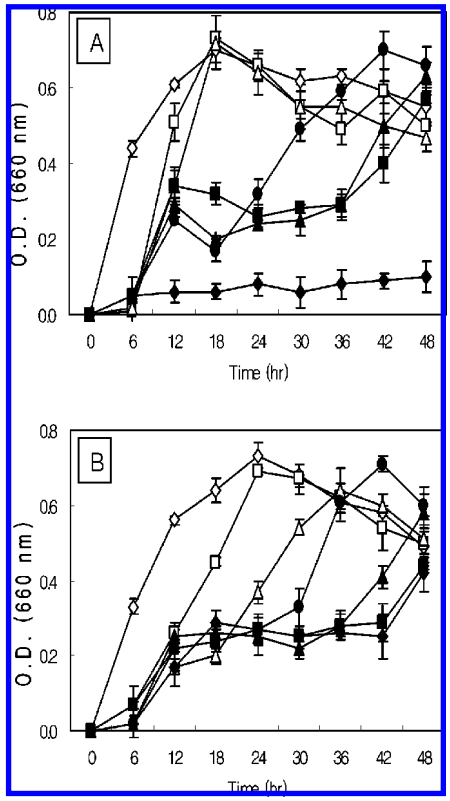


**Figure 2.** Growth inhibition of CLA-K on *K. pneumoniae* (**A**) and *P. aeruginosa* (**B**). Data are expressed as mean ± SD of 0 (control; ◇), 0.3 (□), 0.6 (□), 1.2 (●), 1.8 (▲), 2.4 (■), and 3.0 mM CLA-K (◆) treatments. Growth curve of 3.0 mM CLA-K concentration on *K. pneumoniae* (**A**) was significantly different from that of control (*F* test, $p < 0.05$). No significance was seen in the growth of *P. aeruginosa* (**B**) in the presence and absence of CLA-K.

**Antibacterial Assay.** *Agar Diffusion Method.* The antimicrobial activity of CLA-K on NA was determined as previously described method (*22*) with slight modification. A 0.5 mL volume of the bacterial inoculum and top agar (4.5 mL of NB containing 0.8% agar at 45 °C) was carefully poured onto NA. After solidification, paper disks were placed on the agar surface, followed by pouring of 10 μL of a CLA-K sample solution (0, 6.0, 12.0, 18.0, or 24.0 μmol of CLA-K/10 μL dimethyl sulfoxide) on each paper disk. After 18 h of incubation, a clear zone induced by each sample solution was measured for calculation of antibacterial activity of samples by subtracting the clear zone induced by dimethyl sulfoxide as the control solvent. All experiments were performed in triplicate.

*Liquid Culture Method.* Reaction mixtures were maintained at a concentration of 0, 0.3, 0.6, 1.2, 1.8, 2.4, or 3.0 mM CLA-K in 10 mL of NB containing 10 μL of the bacterial inoculum. Each reaction mixture in a culture tube was incubated at the optimal cultural condition for the particular bacterial strain for 48 h. During the incubation period, aliquots were withdrawn from each test tube at 6 h intervals, and the absorbance at 660 nm was determined using a Beckman DU-8 spectrophotometer (Fullerton, CA). All experiments were performed in triplicate.

**Growth of Bacteria for Scanning Electron Microscopy (SEM) and Biochemical Analyses.** Representative Gram-positive (*B. cereus* and *S. aureus*) and Gram-negative bacterial strains (*P. aeruginosa* and *K. pneumoniae*) were cultured in their optimum culture conditions with CLA-K at a concentration of 0.3 mM for *B. cereus*, 1.2 mM for *S. aureus*, 1.2 mM for *P. aeruginosa*, and 1.2 mM for *K. pneumoniae* or without CLA-K. The cultures were terminated at the stationary phase after initiation of culture by addition of inocula and then centrifuged at 8000*g* for 15 min, using a model PK121R (ALC International Srl, MI, Italy), to obtain bacteria cells and supernatants.

*SEM Analysis.* The morphological changes of bacterial cells treated with or without CLA-K were investigated by SEM as previously

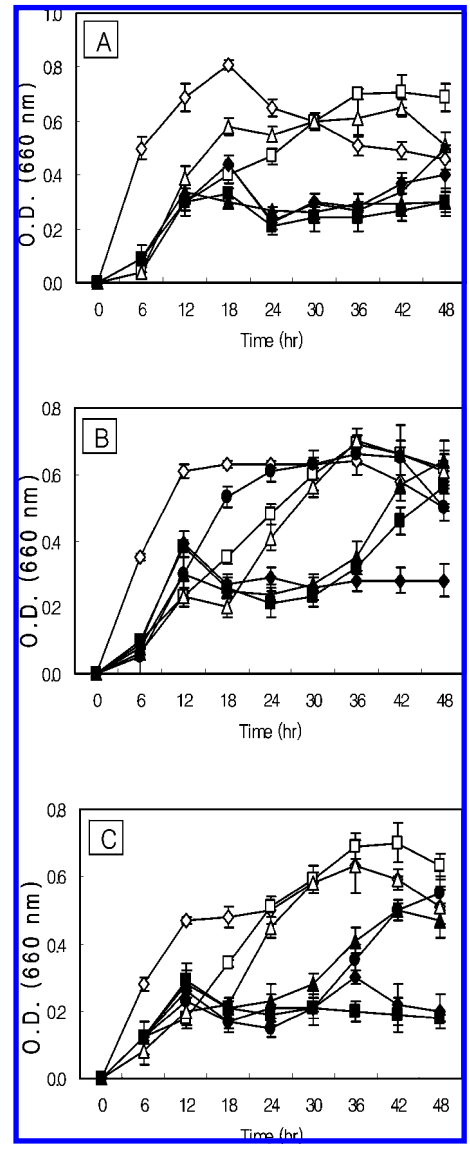


**Figure 3.** Growth inhibition of CLA-K on *S. typhimurium* (**A**), *V. parahemolyticus* (**B**), and *P. mirabilis* (**C**). Data are expressed as mean ± SD of 0 (control; ◇), 0.3 (□), 0.6 (△), 1.2 (●), 1.8 (▲), 2.4 (■), and 3.0 mM CLA-K (◆) treatments. Growth curves of greater than 1.8 mM CLA-K concentrations on *S. typhimurium* (**A**), 3.0 mM CLA-K concentration on *V. parahemolyticus* (**B**), and greater than 2.4 mM CLA-K concentrations on *P. mirabilis* (**C**) were significantly different from that of control (*F* test, $p < 0.05$).

described method (*18*). Briefly, the bacterial cells were washed gently with phosphate-buffered saline (PBS; 50 mM, pH 7.2), fixed with 0.5 mL of 2.5% glutaraldehyde at 4 °C for 2 h, and postfixed with 0.1 mL of 2% osmium tetroxide in PBS at room temperature for 1 h. The specimen was dehydrated using sequential ethanol concentrations ranging from 50 to 100% with 10 min of exposure per concentration, with ethanol finally replaced by acetone. After dehydration, each specimen was dried at 4 °C for 1 h with 0.1 mL of hexamethyldisilazane. Finally, each specimen was sputter-coated with gold in an ion coater for 5 min, followed by microscopic examination using a Philips XL30 S FEG field emission scanning electron microscope (Amsterdam, The Netherlands).

*LDH Activity and Protein Content.* LDH activity and protein content were measured in the supernatant obtained following the harvesting of each cell culture as described above (*23*). Briefly, 0.2 mL of sample was added to 1.0 mL of reaction solution containing 0.159 μmol of NADH and 0.75 μmol of potassium pyruvate in PBS at 25 °C. LDH activity was determined by measuring the rate of decrease of NADH

**Table 2.** Effects of CLA-K on Stationary Phase and $IC_{50}$ Value for the Tested Bacterial Strains

| bacteria strain | | stationary phase[a] (h) | $IC_{50}$[b] (mM) |
|---|---|---|---|
| Gram-positive | *B. cereus* | 24 | 0.3 |
| | *S. aureus* | 24 | 1.2 |
| | *S. mutans* | 24 | 0.3 |
| Gram-negative | *K. pneumoniae* | 18 | 1.2 |
| | *P. aeruginosa* | 24 | 1.2 |
| | *S. typhimurium* | 18 | 1.8 |
| | *V. parahemolyticus* | 24 | 1.8 |
| | *P. mirabilis* | 36 | 2.4 |

[a] Incubation time to attain the stationary phase of given bacterial strain culture free-CLA-K during incubation of 48 h. [b] $IC_{50}$ represents a minimum concentration that inhibits the growth of given bacterium by >50% against that of control treatment at stationary phase.

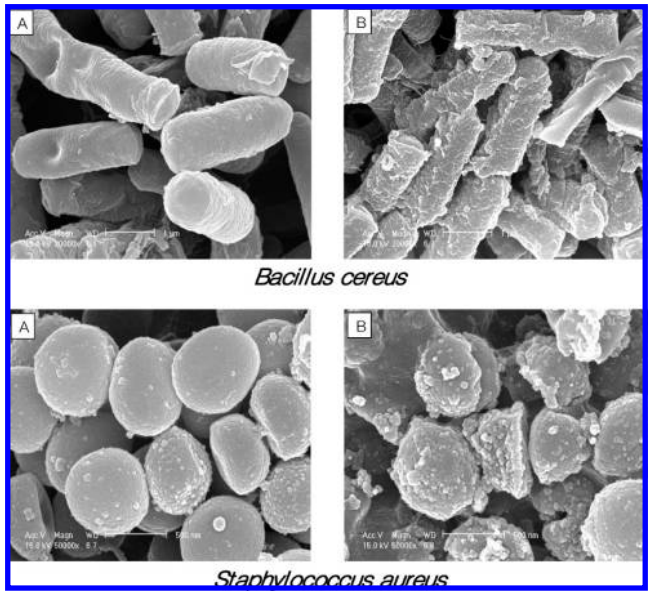


**Figure 4.** Scanning electron micrographs of *B. cereus* (top panels) and *S. aureus* (bottom panels). Bacteria were incubated in the absence (**A**) or presence (**B**) of CLA-K with concentrations of CLA-K corresponding to the $IC_{50}$ values for the time to attain stationary phase (**Table 2**).

concentration at 340 nm for 1 min. Protein content was measured by monitoring the absorption at 280 nm of the supernatant.

*MDA Assay.* Bacterial cell culture was used to evaluate lipid peroxidation by a previously described TBA assay (*24*). Briefly, 0.5 mL of bacterial cell culture was mixed with 0.5 mL of 20% TCA to destroy the bacterial cell wall. The entire 1.0 mL volume was centrifuged for 10 min at 12000*g* at 4 °C, using a model PK121R (ALC International Srl). After centrifugation, the supernatant was suspended in 0.5 mL of PBS, followed by the addition of 1.0 mL of 0.67% TBA. The reaction mixture was heated for 20 min at 90 °C, and absorption at 535 nm was measured. MDA concentration ($\mu$M) was calculated with a standard curve created with TMP. Culture medium was used as a blank.

*Analysis of Fatty Acid Profile.* Bacteria obtained as described earlier in the text were washed gently with PBS. Total lipid was extracted from bacterial cells in chloroform/methanol (2:1. v/v) by homogenization using a model Ultraturax T25 (IKA-WERKE, Staufen, Germany) as described previously (*25*) and was methyl esterified with 20% TMG solution (*11*). The fatty acid profile of bacterial cells was analyzed by gas chromatography using a Sherlock 4.0 program (MIDI, Newark, DE) as previously described (*26*).

**Statistical Analysis.** Data are expressed as mean ± SD. Data were analyzed by ANOVA, and significance at $p < 0.05$ or $p < 0.01$ was examined by Student's *t* test and *F* test (*27*).

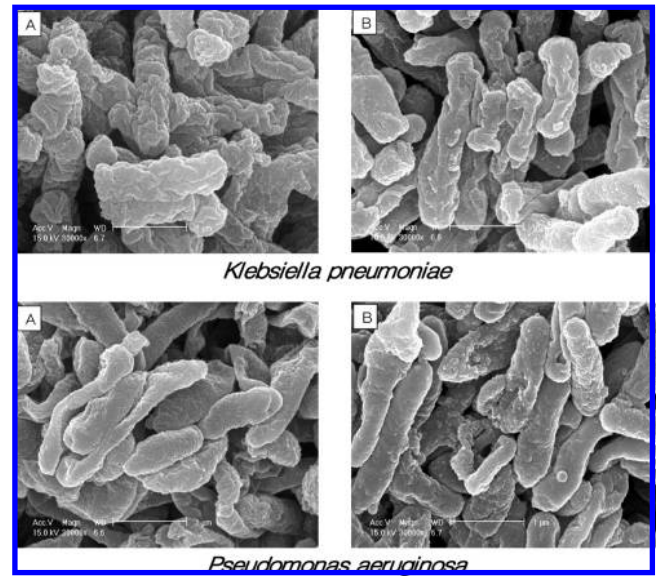


**Figure 5.** SEM of *K. pneumoniae* (top panels) and *P. aeruginosa* (bottom panels). Bacteria cells were incubated in the absence (**A**) or presence (**B**) of CLA-K with concentrations of the CLA-K corresponding to the $IC_{50}$ value for the time to attain stationary phase (**Table 2**).

## RESULTS

**Growth Inhibition of Bacteria.** As determined by an established agar diffusion method, CLA-K inhibited the growth of all tested Gram-positive and Gram-negative strains (**Table 1**). The efficacy of CLA-K increased in a concentration-dependent manner, with a significant difference ($p < 0.05$) evident between the highest (24 $\mu$mol) and lowest (6 $\mu$mol) CLA-K concentrations for all strains. Of the Gram-positive bacteria, *S. mutans* was most sensitive to 24 $\mu$mol of CLA-K, with a 15 mm diameter clear zone, followed by *B. cereus*, with a 13 mm diameter clear zone. Gram-negative bacteria were less sensitive to CLA-K; 24 $\mu$mol of CLA-K maximally inhibited *K. pneumoniae* with a 12 mm diameter clear zone, followed by *P. aeruginosa* with 11 mm diameter clear zone.

The growth inhibitory effects of CLA-K were also measured against the same Gram-positive (**Figure 1**) and Gram-negative strains (**Figures 2** and **3**) in liquid cultures using 0, 0.3, 0.6, 1.2, 1.8, 2.4, and 3.0 mM CLA-K. A concentration-dependent action of CLA-K was again evident, but efficacy was also dependent on the bacterial strains and was greater in the tested Gram-positive bacteria than in the Gram-negative bacteria. **Figure 1** depicts the growth curves of *B. cereus*, *S. aureus*, and *S. mutans* in the presence of the various concentrations of CLA-K. As seen in **Figure 1A**, the stationary phase of the untreated control cultures of *B. cereus* was attained by 24 h, whereas the incubation time necessary to reach the stationary phase was prolonged to 42 h in the presence of 0.3 mM and 0.6 mM CLA-K. Higher CLA-K concentrations almost completely inhibited growth. Similar results were seen in the growth curves of *S. aureus* (**Figure 1B**). Stationary phase was attained by 24 h in both control and 0.3 and 0.6 mM CLA-K-treated cultures, whereas attainment of stationary phase in the presence of 1.2 mM CLA-K required 42 h, with growth being inhibited in the presence of >1.8 mM CLA-K. The growth inhibitory efficacy of CLA-K on *S. mutans* was similar to that of *B. cereus* and *S. aureus* at higher concentration of CLA-K, but *S. mutans* was the most sensitive to lower concentrations of CLA-K (**Figure 1C**).

The growth curves of *K. pneumoniae* and *P. aeruginosa* are shown in panels **A** and **B** of **Figure 2**, respectively. An

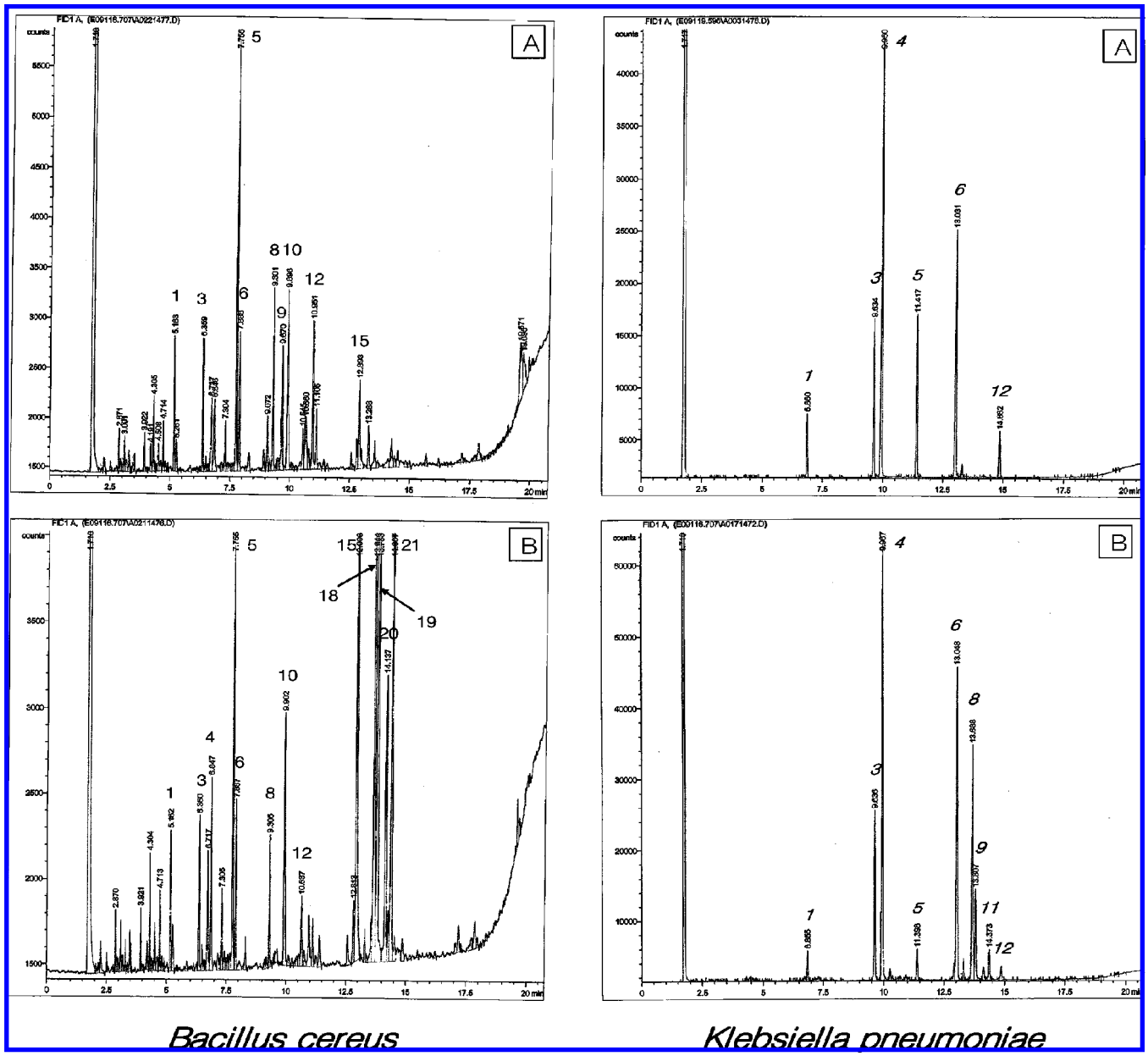


**Figure 6.** Gas chromatograph spectra of *B. cereus* (left panels) and *K. pneumoniae* (right panels). Bacteria cells were incubated in the absence (**A**) or presence (**B**) of CLA-K with concentrations of CLA-K corresponding to the $IC_{50}$ value for the time to attain stationary phase (**Table 2**). Peak numbers were identified as in **Table 3** for *B. cereus* and in **Table 4** for *K. pneumoniae*.

incubation period of 18 h was required to attain stationary phase in control culture of *K. pneumoniae* and cultures treated with 0.3 and 0.6 mM CLA-K (**Figure 2A**). In the presence of 1.2 mM CLA-K, cultures required 42 h of incubation to reach stationary phase, whereas stationary phase was not achieved within 48 h for cultures generated in the presence of 1.8 and 2.4 mM CLA-K, and complete inhibition of growth was evident in the presence of 3.0 mM CLA-K. The incubation times for *P. aeruginosa* cultures to reach stationary phase were 24, 24, 36, and 42 h in the presence of 0, 0.3, 0.6, and 1.2 mM CLA-K, respectively (**Figure 2B**). Stationary phase was not attained within 48 h in the presence of >1.8 mM CLA-K, but growth steadily increased during this period. CLA-K inhibited the growth of *S. typhimurium*, *V. parahemolyticus*, and *P. mirabilis* to various degrees of concentration-related efficacy, with 3.0 mM CLA-K almost always proving to be growth inhibitory (**Figure 3**).

To measure the growth inhibition efficacy of CLA-K for the tested bacteria, the $IC_{50}$ values (defined as the lowest CLA-K concentration inhibiting >50% of control growth at an incubation time sufficient to attain stationary phase in untreated cultures) were determined and are summarized in **Table 2**. The $IC_{50}$ values were higher for the tested Gram-negative bacteria than for the Gram-positive bacteria.

**Bacterial Morphology.** The morphology of two representative Gram-positive strains (*B. cereus* and *S. aureus*) and two Gram-negative strains (*P. aeruginosa* and *K. pneumoniae*) treated with CLA-K at their determined $IC_{50}$ concentrations and grown to stationary phase for the incubation times shown in **Table 2** was ascertained by SEM. In general, CLA-K altered the morphology of bacterial cells in comparison to their controls (**Figures 4** and **5**). All control bacteria displayed a regular surface, whereas CLA-K-treated bacteria presented severe membrane alterations consistent with disruption of membrane integrity and resulting lysis (*18, 24, 28*). Treated *B. cereus* and *S. aureus* displayed a collapse of large areas of their surface and also wrinkled abnormalities distributed uniformly over the surface (**Figure 4**). CLA-K exerted a milder effect on morpho-

**Table 3.** Composition of Major Fatty Acids in Bacterial Cells Cultured with or without CLA-K

| | | | Gram-positive[c] | | | |
|---|---|---|---|---|---|---|
| | | | *B. cereus* | | *S. aureus* | |
| peak | retention time[a] (min) | fatty acid[b] | control (%) | treatment (%) | control (%) | treatment (%) |
| 1 | 5.16 | C13:0 (iso) | 3.7[d] | 1.6 | | |
| 2 | 5.26 | C13:0 (anteiso) | 1.1 | 0.7 | | |
| 3 | 6.36 | C14:0 (iso) | 3.7 | 1.8 | 0.7 | 0.5 |
| 4 | 6.72 | C14:1 (5c) | 2.8 | 1.9 | 1.0 | 1.7 |
| 5 | 7.76 | C15:0 (iso) | 13.1 | 5.5 | 5.4 | 3.4 |
| 6 | 7.91 | C15:0 (anteiso) | 4.4 | 2.3 | 41.9 | 20.2 |
| 7 | 9.07 | C16:1 (iso I)/C14:0 (3OH) | 1.8 | | | |
| 8 | 9.31 | C16:0 (iso) | 6.1 | 1.8 | 0.9 | |
| 9 | 9.67 | C15:0 (iso 2OH)/C16:1 (7c) | 4.3 | | 4.5 | 4.0 |
| 10 | 9.90 | C16:0 | 7.7 | 4.6 | 4.8 | 8.1 |
| 11 | 10.64 | C16:0 (10 methyl) | | 1.2 | 0.7 | 1.1 |
| 12 | 10.96 | C17:0 (iso) | 5.6 | 1.1 | 3.8 | 1.1 |
| 13 | 11.11 | C17:0 (anteiso) | 2.3 | 0.8 | 16.2 | 3.6 |
| 14 | 12.81 | C18:2 (6,9c)/C18:0 (ante) | 1.2 | 1.0 | | |
| 15 | 12.90 | C18:1 (9c) | 3.5 | 11.0 | 1.3 | 1.6 |
| 16 | 13.30 | C18:0 | 1.6 | | 8.8 | 12.7 |
| 17 | 13.56 | C17:0 (iso 3OH) | 1.6 | 0.8 | 0.6 | 0.7 |
| 18 | 13.64 | c9,t11 CLA | | 18.1 | | 12.2 |
| 19 | 13.77 | t10,c12 CLA | | 19.2 | | 8.8 |
| 20 | 14.13 | C19:1 (iso) | 0.9 | 6.1 | | 0.7 |
| 21 | 14.38 | C19:0 (Iso) | 0.7 | 8.3 | 1.4 | 1.6 |
| 22 | 16.72 | C20:0 | | | 4.4 | 2.4 |
| 23 | 17.05 | unidentified | | | | 4.4 |
| 24 | 17.19 | unidentified | | | | 4.4 |
| 25 | 17.76 | unidentified | | | | 1.6 |

[a] Retention time seen in **Figure 6**. [b] Fatty acid peaks were identified by comparing their retention times with those of standard compounds. Peak area percent was calculated as peak area of the interest peak divided by the total area of all peaks multiplied by 100. [c] Each strain was cultured in the absence (control) or presence of CLA-K (treatment) at 37 °C for 24 h with the exception of *B. cereus*, which was cultured at 30 °C. The concentrations of CLA-K corresponded to the $IC_{50}$ value for their corresponding stationary phase as shown in **Table 2**. Fatty acid profile was measured in the bacteria mass of each strain. [d] Values >1.0% are reported in this table.

**Table 4.** Composition of Major Fatty Acids in Bacteria Cultured with or without CLA-K

| | | | Gram-negative[c] | | | |
|---|---|---|---|---|---|---|
| | | | *K. pneumoniae* | | *P. aeruginosa* | |
| peak | retention time[a] (min) | fatty acid[b] | control (%) | treatment (%) | control (%) | treatment (%) |
| 1 | 6.85 | C14:0 | 4.5 | 1.5 | 1.1 | 1.3 |
| 2 | 9.63 | C16:1 (7c)/C15:0 (iso 2OH) | | | 7.6 | 10.5 |
| 3 | 9.67 | C15:0 (iso 2OH)/C16:1 (7c) | 13.1 | 10.6 | 6.3 | 3.4 |
| 4 | 9.90 | C16:0 | 40.3 | 32.3 | 35.2 | 30.5 |
| 5 | 11.39 | C17:0 (cyclo) | 14.0 | 1.8 | 0.9 | |
| 6 | 12.99 | C18:1 (7c) | 22.8 | 23.3 | 45.8 | 40.7 |
| 7 | 13.30 | C18:0 | 1.1 | 1.3 | 1.6 | 1.7 |
| 8 | 13.64 | c9,t11 CLA | | 13.5 | | 3.0 |
| 9 | 13.77 | t10,c12 CLA | | 5.8 | | 1.3 |
| 10 | 14.13 | C19:1 (iso I) | | 1.1 | | 0.8 |
| 11 | 14.38 | C19:0 (iso) | | 2.2 | | 2.9 |
| 12 | 15.02 | C19:0 | 4.2 | 0.8 | | |

[a] Retention time seen in **Figure 6**. [b] Fatty acid peaks were identified by comparing their retention times with those of standard compounds. Peak area percent was calculated as peak area of the interest peak divided by the total area of all peaks multiplied by 100. [c] Each strain was cultured in the absence (control) or in the presence of CLA-K (treatment) at 37 °C for 24 h with the exception of *K. pneumoniae*, which was cultured for 18 h. The concentrations of the CLA-K correspond to the $IC_{50}$ value corresponding to the stationary phase as shown in **Table 2**. Fatty acid profile was measured in the bacterial mass of each strain. [d] Values >1.0% are reported.

logical changes of *P. aeruginosa* and *K. pneumoniae* (**Figure 5**). These observations on the morphological changes of *S. aureus* and *P. aeruginosa* treated with CLA-K are in agreement with results reported by Shin et al. (*18*).

**Changes in Fatty Acid Composition and Biochemical Markers.** Membrane fatty acid profile, MDA content, LDH activity, and protein content in each bacterial culture were determined for *B. cereus*, *S. aureus*, *P. aeruginosa*, and *K. pneumoniae* treated with CLA-K and grown to stationary phase as described in the preceding section.

Membrane fatty acid compositions of the tested bacteria were determined after incubation in the absence or presence of CLA-K by gas chromatography (**Figure 6** and **Tables 3** and **4**). Under control conditions, no CLA isomers were detected in the membranes of all tested strains, whereas upon addition of CLA-K to the medium, all strains increased the level of CLA (c9,t11 and t10,c12 CLA isomers) in their membranes to some extent. The membrane CLA level was 37.3% in *B. cereus*, 21.0% in *S. aureus*, 19.3% *K. pneumoniae*, and 4.3% in *P. aeruginosa*. Interestingly, *S. aureus* cultured in the medium

**Table 5.** Effects of CLA-K on MDA Production

| bacterial strain[a] | | MDA[b] ($\mu$M) control | MDA[b] ($\mu$M) treatment |
|---|---|---|---|
| Gram-positive | *Bacillus cereus* | 0.10 ± 0.07[c] | 1.10 ± 0.05***[d] |
| | *Staphylococcus aureus* | 0.17 ± 0.05 | 1.52 ± 0.37* |
| Gram-negative | *Klebsiella pneumoniae* | 0.25 ± 0.06 | 0.92 ± 0.21* |
| | *Pseudomonas aeruginosa* | 0.15 ± 0.06 | 0.55 ± 0.14* |

[a] Each strain was cultured in NB at 37 °C for 24 h with the exceptions of *B. cereus*, which was cultured at 30 °C, and *K. pneumoniae*, which was cultured for 18 h. [b] MDA was determined in the bacteria cell culture of each strain using TMP as a standard. [c] Mean ± SD of four independent experiments. [d] * and *** represent significant difference at $p < 0.05$ and $p < 0.001$, respectively, of control treatment against CLA-K treatment by Student's *t* test.

containing CLA-K contained various unidentified fatty acids as compared to control cells (**Table 3** and **Figure 6**).

Production of MDA in cultures after exposure to CLA-K is summarized in **Table 5**. The quantity of MDA rather did not change in any of the cultures when CLA-K was absent. In contrast, the amounts of MDA increased significantly ($p < 0.05$, $p < 0.001$) in CLA-K-containing cultures. MDA production by *B. cereus* and *S. aureus* was superior to that by *K. pneumoniae* and *P. aeruginosa*. The leakage of cellular contents induced by CLA-K was compared (**Table 6**). Changes of LDH activity and protein in supernatants after exposure of the tested bacteria to CLA-K were determined. Both LDH activity and protein quantity significantly ($p < 0.05$, $p < 0.001$)increased in the presence of CLA-K as compared to bacteria grown in control. The increases in both LDH activity and protein content were greater for *B. cereus* and *S. aureus* than for *K. pneumoniae* and *P. aeruginosa*.

## DISCUSSION

The present study demonstrates that CLA inhibits the growth of both Gram-positive bacteria (*B. cereus*, *S. aureus*, and *S. mutans*) and Gram-negative bacteria (*S. typhimurium*, *V. parahemolyticus*, *P. aeruginosa*, *K. pneumoniae*, and *P. mirabilis*). The bactericidal effect of CLA was evident for all tested strains, particularly Gram-positive bacteria, although variations in the effective concentration were apparent, perhaps reflecting the fact that CLA is a polyunsaturated fatty acid. The delayed growth apparent at some lower CLA-K concentrations and the varying $IC_{50}$ values are not consistent with the previously reported tendency of polyunsaturated fatty acids to solely reduce growth of Gram-positive bacteria (*17*) and of the ineffectiveness of long-chain polyunsaturated fatty acids on Gram-negative bacteria as a consequence of the presence of lipopolysaccharide (LPS) in the outer membrane (*16*, *18*, *19*, *29*).

CLA, which is a long-chain unsaturated fatty acid, was presently bactericidal to both Gram-positive and Gram-negative bacteria (**Table 2**). Similar to the previous report, polyunsaturated fatty acids are growth inhibitory in the early stage of bacterial growth, with growth recovery occurring subsequently (*24*). We presently demonstrated that low concentrations of CLA-K also can transiently inhibit the growth of bacteria at an early growth stage (**Figures 1**−**3**). Recovery from the diminished growth was delayed at higher CLA-K concentrations, perhaps reflecting a greater perturbance of membrane integrity (**Figures 4** and **5**). Unlike other long-chain polyunsaturated fatty acids, the inhibitory effect of CLA was restricted to Gram-positive and Gram-negative bacteria, which is indicative of a broad spectrum bactericidal effect.

The bactericidal efficacy of CLA-K on the Gram-positive bacteria was superior to that on the Gram-negative bacteria (**Table 2**). Gram-negative bacteria such as *P. aeruginosa* and *Salmonella* spp. have a hydrophilic surface due to the LPS, so that hydrophobic molecules such as lipids have difficulty entering the bilayer, presumably also because of the low fluidity of the hydrocarbon chains in the LPS leaflet and the strong lateral interactions between the LPS molecules (*29*, *30*). In contrast, Gram-positive bacteria have a relatively hydrophobic surface and are easily killed by fatty acids (*18*). This could explain the presently observed enhanced bactericidal efficacy of CLA-K on Gram-positive bacteria.

A positive correlation between fatty acid inhibitory activity and the level of the particular fatty acid in the bacterial membrane has been reported (*31*), and changes of membrane permeability due to the incorporation of polyunsaturated fatty acids into microbial cell membranes leading to cell death have been proposed by many investigators (*18*, *24*, *28*, *31*). Similarly, we observed that the amount of CLA incorporated into the membrane of the tested bacteria positively correlated to their CLA-K susceptibility (**Table 2** and **Figure 6**). Hence, it is presumed that the bactericidal efficacy of CLA on the bacterial strains tested might be associated with the amounts of CLA incorporated into their membranes. As shown in **Tables 3** and **4**, CLA incorporation into membranes induced changes in fatty acid profiles and production of unidentified fatty acid as compared to control membranes, which prompted changes in membrane permeability and fluidity that cause cell death. In contrast to this hypothesis, it has been reported that exogenous CLA causes an increase in the percentage of membrane CLA of *L. reuteri* spp., but no association between level of CLA incorporation into the membrane and inhibition by that fatty acid was observed (*14*). Further studies are needed to resolve this dichotomy.

The mode of the bactericidal action of CLA against the presently tested Gram-positive and Gram-negative bacteria is

**Table 6.** Effects of CLA-K on the LDH Activity and Protein Content

| bacterial strain[a] | | LDH activity[b] (U/mL) control | LDH activity[b] (U/mL) treatment | protein[c] control | protein[c] treatment |
|---|---|---|---|---|---|
| Gram-positive | *B. cereus* | 0.82 ± 0.01[d] | 0.95 ± 0.01***[e] | 0.43 ± 0.04 | 0.93 ± 0.03*** |
| | *S. aureus* | 0.77 ± 0.02 | 0.90 ± 0.01** | 0.39 ± 0.06 | 0.89 ± 0.07*** |
| Gram-negative | *K. pneumoniae* | 0.90 ± 0.02 | 0.95 ± 0.01* | 0.90 ± 0.03 | 1.06 ± 0.03* |
| | *P. aeruginosa* | 0.88 ± 0.01 | 0.91 ± 0.01* | 0.83 ± 0.06 | 3.39 ± 0.37** |

[a] Each strain was cultured in NB at 37 °C for 24 h with the exceptions of *B. cereus*, which was cultured at 30 °C, and *K. pneumoniae*, which was cultured for 18 h. [b] LDH activity was determined in the supernatant of bacteria cell culture of each strain. U represents the unit of LDH to oxidize NADH to NAD as compared to standard enzyme (1000 U/mL). [c] Protein represents a relative concentration, which was determined in the supernatant of each bacterial culture by measuring absorption at 280 nm. [d] Mean ± SD of four independent experiments. [e] *, **, and *** represent significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, of control treatment against CLA-K treatment by Student's *t* test.

completely unknown; however, the mechanistic action of CLA on the bacteria might involve either the incorporation of the compound into the cell wall or bacterial peroxidation. It is evident that CLA-K exposure produced a large-scale surface collapse of the affected bacteria as well as development of a wrinkled surface (**Figures 4** and **5**), which might be associated with the incorporation of CLA into the cell membranes (**Tables 3** and **4**) and resulting lipid peroxidation (**Table 5**). This indicates that CLA-K induces the lytic death of bacteria. This hypothesis is further supported by the leakage of LDH and protein from the cytosol of CLA-treated bacteria (**Table 5**). Our hypothesis for the mechanistic action of CLA on Gram-positive and Gram-negative bacterial strains is consistent with reports that long-chain polyunsaturated fatty acids including linoleic, $\gamma$-linoleic, arachidonic, and eicosapentaenoic acids exhibit antibacterial activities on Gram-positive and Gram-negative bacteria through either their incorporation into the cell wall or their peroxidation (*24*, *25*, *32*). This hypothesis is insufficient to explain the results observed in the present study; hence, there are some other mechanisms to be clarified.

Bacteria incubated with exogenous fatty acids incorporate the fatty acids into their cell membranes, thereby repressing de novo fatty acid biosynthesis (*17*, *33*, *34*). Furthermore, the antibacterial action of polyunsaturated fatty acids such as palmitoleic, linoleic, linolenic, and arachidonic acids is mediated by the inhibition of the enoyl-acyl carrier protein reductase (FabI) of *S. aureus* and *E. coli*, an essential component of bacterial fatty acid synthesis (*17*). The present data indicate that substantial amounts of CLA are readily incorporated into the membranes of bacteria to various extents depending on the bacterial strains and that CLA-K exhibits various strain-dependent bactericidal effects on both Gram-positive and Gram-negative bacteria. CLA, which closely resembles linoleic acid in chemical structure, might have inhibited FabI of the Gram-positive and Gram-negative bacteria tested in the present study.

## NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP March 24, 2009, a correction was made to the caption of Figure 1; the corrected version was reposted March 26, 2009.

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