Cell Transplantation
2019, Vol. 28(12) 1528–1541

DOI: 10.1177/0963689719881366
journals.sagepub.com/home/cll

Measuring the Antimicrobial Activity of Lauric Acid against Various Bacteria in Human Gut Microbiota Using a New Method

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Abstract

Lauric acid (LA) has a broad spectrum of anti-microbiological activities against enveloped viruses and various bacteria, and might be useful to protect against microbial infection and control the balance and distribution of bacteria in human gut microbiota. It is not necessarily more difficult to measure antimicrobial activity the traditional way, but it is, however, more laborious. In the present study, we developed a new method to measure the antimicrobial activity of LA in multiple samples with a microplate reader. A "test complex" (TC) was produced consisting of 100 μL of agar medium with LA in the bottom layer and 300 μL of broth in the top layer in 96-well deep-well microplates. Afterward, analysis of the broth in the top layer showed that the antimicrobial activity was the same as that of the "control complex," (CC) which consisted of 100 μL of agar medium in the bottom layer and 300 μL of broth with LA in the top layer. Furthermore, evaluation of the antimicrobial effect of the TC when using a microplate reader was the same as that with the use of the colony counting method. The colony counting method has confirmed that the antimicrobial activity of LA when bacteria are inoculated into the broth was equivalent between CC and TC, and we validated this by correlating the number of bacteria with absorbance. In addition, the broth itself in TC was transparent enough that the turbidity of broth can be used as an index of the number of bacteria, which enabled the use of a microplate reader for multiple samples. For human gut microbes, LA was shown to have low antimicrobial activity against commensal lactic acid bacteria, but high antimicrobial activity against pathogenic *Bacteroides* and *Clostridium*, suggesting that LA might modulate intestinal health, as confirmed by the proposed method.

Keywords

screening, human gut microbiome, antimicrobial activity, lauric acid (LA), antimicrobial method

Introduction

Fatty acids (FAs) form long hydrocarbon chains capped by carboxyl groups and have unbranched chains of 4 to 28 carbon atoms, which are either saturated or unsaturated. Some medium-chain FAs (MCFAs), such as lauric acid (LA) and caprylic acid (CA), have a broad spectrum of anti-microbiological activities against enveloped viruses and various bacteria in vitro[1–5]. Several studies have suggested that some MCFAs disrupt the bacterial cell wall or membrane to protect host cells against infection[6,7]. FAs, as well as sphingolipids, are involved in the physical, permeability, and immunologic barrier functions of the skin and mucosa[5,8,9], and it is predicted that the antimicrobial activities of MCFAs contribute to these protective functions[10,11].

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Submitted: May 2, 2019. Revised: August 10, 2019. Accepted: September 12, 2019.

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Furthermore, previous studies have shown that MCFAs added to the diet have a protective effect against bacterial invasion of the mucus[3].

In general, in vitro antimicrobial analysis of MCFAs involves a bacterial colony counting method whereby bacteria are inoculated into broth medium containing the indicated concentrations of MCFAs and incubated at 37°C. At the specific time points, the bacterial suspensions are serially diluted by 10-fold and each dilution is plated on an agar plate, which is incubated for a set time, and then the bacterial colonies on the plate are counted. However, this method requires a great number of agar medium plates, and the procedure is rather complicated and requires a relatively long time to complete. Furthermore, with traditional methods, there is a risk that operations for dilutions many times may cause large numerical errors. Therefore, it is difficult to measure and compare the antibacterial activities of many kinds of MCFAs against various types of bacteria in a timely manner and at a higher throughput.

Since the number of bacteria in broth medium is correlated with the turbidity of the media[12], a simple measurement of turbidity with a microplate reader could be used to calculate the number of bacteria. However, the presence of FAs muddies the broth medium, making it impossible to precisely calculate the number of bacteria.

Several studies have reported that the antibacterial activities of antimicrobial hydrophilic materials can be measured with the disc diffusion antimicrobial test[13–16], where the materials penetrate the agar medium to prevent bacterial growth, resulting in the formation of clear zones around each disc that indicate the inability of the test organism to survive in the presence of an antibiotic. Recently, we confirmed that some MCFAs create a clear zone in the disc diffusion antimicrobial test, suggesting that the hydrophilic part of FAs in the medium is able to penetrate the agar and to inhibit bacterial growth. However, it is difficult to quantitatively evaluate the antimicrobial effect of the test samples with the disk diffusion test.

Therefore, we designed an assay where the FAs are encapsulated by the agar medium and the hydrophilic part of the FA diffuses into the broth medium to inhibit bacterial growth (Fig. 1). Then, we assumed that it would be possible to quickly and conveniently measure the antimicrobial effect of FAs using a microplate reader to measure turbidity. The results of the present study showed that it is possible to evaluate the antimicrobial activity of a large quantity of FAs with our proposed method. Furthermore, we applied this method to evaluate the antimicrobial activity of LA against human pathogens and human gut microbes.

Materials and Methods

Bacteria, Media, and Fatty Acids

The bacteria tested in this study are listed in Tables 1 and 2. Cultures of 10 human pathogenic bacteria and *Streptococcus salivarius* were supplemented with a stock solution of 2 × concentrated Todd's Hewitt broth medium (BD Biosciences, Franklin Lakes, NJ, USA), 0.2% yeast extract (THYbroth medium), and glycerol, and then stored at −20°C. The stored bacteria (50 μL) were inoculated into 5 mL of THY broth medium and cultured for 14 h at 37°C for use in the experiments. The human dominant gut bacteria were obtained from the American Type Culture Collection (Manassas, VA, USA), the Japan Collection of Microorganisms (Tsukuba, Japan), and the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Bacterial recovery was performed in accordance with the instructions of the distributors.

Human gut microbes (Table 2), including the most dominant species in the human intestinal indigenous microbiota[17] and bifidobacteria, were pre-cultured as described previously[18] in Gifu anaerobic medium (GAM) at 37°C under anaerobic conditions, because the microbes cannot be grown by THY. For elimination of dissolved oxygen from GAM, GAM bouillon (#05422; Nissui Pharmaceutical, Tokyo, Japan) was completely dissolved in deionized water and sterilized by autoclaving at 115°C for 15 min. When the temperature of the autoclave dropped to 97°C, the GAM broth medium was immediately transferred into an anaerobic chamber (INVIVO2 400; The Baker Company, Sanford, ME, USA) and incubated overnight at 37°C under anaerobic conditions (0% O_2, 5% H_2, 10% CO_2, and 85% N_2) to eliminate any dissolved oxygen.

LA and CA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LA has antimicrobial activity for several *Staphylococcus* including *Staphylococcus aureus* and *Streptococcus* bacteria, whereas CA has antimicrobial activity for *Streptococcus pyogenes* and *Streptococcus sanguinis*, but not *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mutans*, or *Streptococcus salivarius*[1–5]. Since LA, but not CA, inhibits growth of *S. aureus*, we selected the two compounds to show the differentiation of antimicrobial activity for *S. aureus* as shown in the following experiment. The FAs were dissolved in 100% ethanol to final concentrations of 2.5, 0.75, and 0.25 M. The ethanol–FA solutions were concentrated 1000-fold with culture media to 2.5, 0.75, and 0.25 mM, respectively, for use in the antimicrobial experiments. We confirmed that the bacteria were able to grow in media containing 0.1% ethanol to the same extent as with the media alone (data not shown).

Production of the Test Complex and Control Complex, and Measurements of Turbidity and FA Content in the Broth Portion of Both Complexes

Two types of complexes were added to the wells of 96-deep-well plates (Fig. 1). The bottom layer of the Test Complex (TC) consisted of 100 μL of medium (either THY or GAM) supplemented with 2% agar and various concentrations of LA or CA, and the top layer consisted of 300 μL of broth

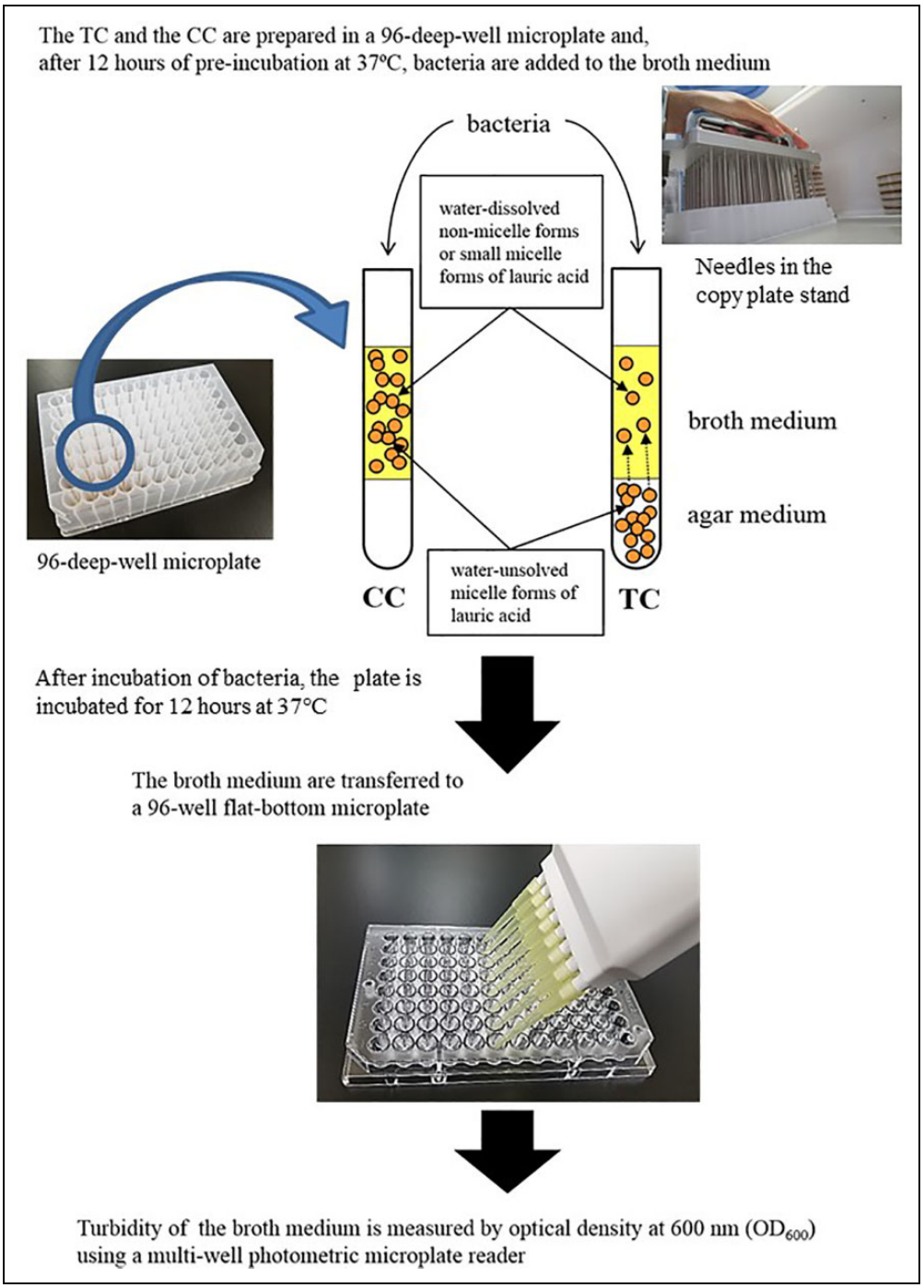

Figure 1. Production of the test complex (TC) and control complex (CC), and measurements of turbidity and amount of FA in the broth portion of the complexes. Two types of test media were developed and added to the wells of 96-well deep-well plates. For turbidity in the top layer of both complexes, the complexes were incubated at 37°C. Then, at the indicated time, the top layer portions of both complexes were harvested to measure the turbidity at OD_{600} using a multi-well photometric microplate reader.

medium. The bottom layer of the Control Complex (CC) consisted of 100 µL of agar medium (either THY or GAM) in the bottom layer, and the top layer consisted of 300 µL of broth medium with various concentration of LA and CA. To prepare the agar medium of both complexes, the agar medium was autoclaved (THY at 121°C for 15 min; GAM at 115°C for 15 min). The agar medium was autoclaved and allowed to cool to 50°C. Then, a 1/1000 volume of 100% ethanol or 100% ethanol and 1000-fold concentrated MCFAs were added to the cooled medium, which was gently agitated. Then, 100 µL of the solution was pipetted into the wells of a 96-well deep-well microplate. Human gut

Table 1. List of 10 Human Pathogenic Bacteria and *Streptococcus Salivarius* that were used in this Study.

Species	Strain
Staphylococcus aureus	ATCC6538P
Streptococcus agalactiae	A909
Streptococcus mutans	MT8148
Streptococcus pneumoniae	TIGR4
Streptococcus pyogenes	NIH35
Streptococcus salivarius	HHT
Streptococcus sanguinis	ATCC10558
Escherichia coli	ATCC35218
Klebsiella oxytoca	K7
Klebsiella pneumoniae	ATCC4352
Serratia marcescens	ATCC8100

microbial species as described in Table 2 can be grown by GAM medium under anaerobic condition. For the GAM medium, the above procedure was performed under anaerobic conditions as described previously.

For measurement of the turbidity of the broth medium in the top layer of both complexes, the complexes were incubated at 37°C. Then, at the indicated times, the top layer portions of the complexes were harvested to measure the turbidity at the optical density of 600 nm (OD_{600}) using a multi-well photometric microplate reader (Multiskan GO; Thermo Fisher Scientific, Waltham, MA, USA). To measure the amount of FAs in the THY broth medium and GAM broth medium portion of the TC, the complex was incubated at 37°C for the indicated time (10 min, 2, 6, 8, 12, and 24 h) and the THY broth medium and GAM broth medium portion was harvested. Then, the amount of FAs in the medium was measured using the Free Fatty Acid Assay Kit (STA-618; Cell Biolabs, Inc., San Diego, CA, USA), in accordance with the manufacturer's instructions.

Inoculation of S. aureus *in the Complexes and Bacterial Colony Counting*

S. aureus is very common human pathogenic bacteria, and widely used as a test organism in antimicrobial screening methods. The stored *S. aureus* was grown for 14 h in THY broth medium. Then, the culture medium containing the bacteria was inoculated into the 300 μL top layer portion of the TC and CC, as the starting concentration of bacteria in the top layer portion is fixed to 0.05–0.06 of OD_{600}, which is 1–3 $\times$ 10^5 CFU/mL. The complexes were produced with various concentrations of LA and CA and pre-incubated at 37°C for 12 h before bacterial inoculation. After inoculation of 0.3–1 $\times$ 10^5 CFU bacteria into the 300 μL top layer portion (1–3 $\times$ 10^5 CFU/mL), the complexes were incubated at 37°C for 12 h. Afterward, the bacterial suspensions in the broth portion of the complexes were serially diluted by 10-fold and plated on the THY agar plates. After incubation

Table 2. List of Human Gut Bacterial Species and Strains.

	Species	Strain
	Bacteroides caccae	JCM9498
Most dominant species in human gut microbes	*Bacteroides dorei*	JCM13471
	Bacteroides finegoldii	JCM13345
	Bacteroides fragilis	JCM11019
	Bacteroides intestinalis	JCM13265
	Bacteroides ovatus	JCM5824
	Bacteroides stercoris	JCM9496
	Bacteroides thetaiotaomicron	JCM5827
	Bacteroides uniformis	JCM5828
	Bacteroides vulgatus	JCM5826
	Bacteroides xylanisolvens	JCM15633
	Blautia hansenii	JCM14655
	Clostridium asparagiforme	DSM15981
	Clostridium nexile	ATCC27757
	Collinsella aerofaciens	JCM7790
	Coprococcus comes	ATCC27758
	Dorea formicigenerans	ATCC27755
	Dorea longicatena	DSM13814
	Eubacterium cylindroides	JCM10261
	Eubacterium siraeum	ATCC29066
	Parabacteroides distasonis	JCM5825
	Parabacteroides johnsonii	JCM13406
	Parabacteroides merdae	JCM9497
	Roseburia intestinalis	DSM14610
	Ruminococcus gnavus	ATCC29149
	Ruminococcus lactaris	ATCC29176
	Ruminococcus productus	JCM1471
	Ruminococcus torques	ATCC27756
Lactic acid bacteria	*Enterococcus faecalis*	ATCC700802
	Lactobacillus casei subsp. casei	JCM1134
	Lactobacillus casei subsp. rhamnosus	ATCC7469
	Lactobacillus gasseri	JCM1130
	Lactobacillus johnsonii	JCM8794
	Lactobacillus plantarum	JCM1158
	Lactobacillus reuteri	JCM1149
	Lactococcus lactis	JCM1112
	Leuconostoc mesenteroides subsp. mesenteroides	JCM6124
Bifidobacteria	*Bifidobacterium adolescentis*	JCM1275
	Bifidobacterium animalis subsp. lactis	JCM10602
	Bifidobacterium bifidum	JCM1254
	Bifidobacterium breve	JCM1192
	Bifidobacterium catenulatum	JCM1194
	Bifidobacterium infantis	ATCC15697
	Bifidobacterium longum	JCM1217
	Bifidobacterium pseudocatenulatum	JCM1200
	Bifidobacterium pseudolongum	JCM1205
Butyrate-producing bacteria	*Clostridium bolteae*	JCM12243
	Clostridium indolis	JCM1380
	Clostridium ramosum	JCM1298
Pathogen	*Clostridium difficile*	JCM1296
	Clostridium perfringens	JCM1290
	Fusobacterium nucleatum subsp. nucleatum	JCM8532

at 37°C for 24 h, the number of bacterial colonies was counted.

Bacterial Test and Turbidity Measurements

The turbidity of the bacterial cultures was measured as shown in Fig. 1. The bacteria presented in Table 1 were grown for 14 h in THY broth medium. The bacterial culture was inoculated into 300 μL of the top layer of the TC containing various concentrations of MCFAs in the bottom layer. The concentrations of the indicated bacteria are fixed at 0.05–0.06 of OD_{600}, and the number of colonies depends on the bacterial species: *S. aureus*, *S. agalactiae*, *Streptococcus pneumoniae*, *S. pyogenes*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*, 0.3–1 × 10^5 CFU/300 μL of the top layer; *S. salivarius*, *S. sanguinis*, *Escherichia coli*, and *Serratia marcescen*s, 0.3–1 × 10^7 CFU/300 μL of the top layer. Three wells were inoculated with bacteria but did not contain MCFAs in the bottom layer, serving as a bacterial growth controls, and the three wells were not inoculated with any bacteria but did not contain MCFAs in the bottom layer portion, serving as a blank. The plate was then incubated at 37°C and after 12 h the bacterial culture was harvested from the top layer and transferred to a microtiter plate for turbidity measurement. The turbidity was measured at OD_{600} using a microplate reader (Multiskan GO microplate reader, Thermo Fisher Scientific). The amount of bacteria in the broth medium of the top layer in the TC was assessed using the colony counting method and a correlation diagram was used to relate the number of bacterial colonies to the turbidity. The bacterial culture in the top layer was harvested and the turbidity was measured at OD_{600}. The number of bacteria in the top layer portion of the TC was measured by the colony counting method and the turbidity in the top layer portion without MCFAs in the lower layer was measured with a microplate reader. With the use of a correlation diagram, the number of bacterial colonies in the culture broth was calculated from the turbidity of the bacterial culture in the top layer of the TC with the MCFAs in the bottom layer.

For measuring the turbidity of cultures of human gut microbes, the TC contained 100 μL of oxygen-free GAM agar medium(bottom layer portion) and 300 μL of oxygen-free GAM broth medium (top layer portion) in the wells of 96-deep-well plates. The pre-cultures of bacteria at the starting point (0.05–0.06 of OD_{600}) indicated in Table 2 were inoculated in the GAM broth medium using a copy plate stand (Tokken, Chiba, Japan) and cultured under anaerobic conditions for 24 h (Fig. 1). Bacterial growth was estimated by measurement of turbidity at OD_{600} using a Multiskan GO microplate reader (Thermo Fisher Scientific). The plateau of bacterial growth in the absence of LA greatly differed among bacterial species (data not shown). Therefore, the relative growth was calculated, which indicates bacterial growth in the presence of LA, as compared with no LA.

Statistical Evaluations

All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 24.0 (IBM Corporation, Armonk, NY, USA). Statistical comparisons among the groups were performed with the Student's *t*-test and multiple intergroup comparisons were made using one-way analysis of variance followed by Tukey's multiple comparison test. A probability (p) value of <0.05 was considered statistically significant.

Results

Comparison of Turbidity by Adding MCFAs between the TC and CC

Turbidity of broth medium in TC and CC after adding MCFAs was measured. Furthermore, the turbidity of the TC was compared with that of the CC in which MCFAs were suspended in the broth medium portion for 12 h.

The turbidity of the comparatively water-soluble CA when suspended in the top layer portion was not changed by increasing the concentration of CA (Fig. 2A). Meanwhile, the turbidity of LA, which is poorly water soluble, had increased to more than 0.17 at 0.75 mM or more in the top layer portion of the CC. However, the turbidity of the top layer in the TC did not increase as much as the concentration of LA (0.75 mM and equivalent to 2.5 mM) (Fig. 2B).

The change in turbidity of the top layer portion was measured when a higher concentration of LA was added to the bottom layer portion of the TC. As shown in Fig. 2C, the turbidity of the top layer portion in the TC increased with the concentration of LA. However, the turbidity of 20 mM LA in the TC was still lower than that at 2.5 mM in the CC.

Amount of MCFAs in the Top Layer Portion of the TC

Next, after adding LA to the TC, the amount of LA in the top layer portion was examined. LA concentrations of 0.25, 2.5, and 5 mM were added to the bottom layer portion of the TC, which was then incubated at 37°C. Then, at 2, 4, 6, 8, and 12 h, the top layer portion was harvested and the amount of LA was measured. As shown in Fig. 3A, the amount of LA migrating to the top layer portion of the TC gradually increased with time. When 5 mM of LA was added, the amount of LA in the top layer portion was 1.1 mM at 6 h after the addition. Furthermore, the amount of LA in the top layer portion increased to nearly 44 μM when added at 0.25 mM and to 440 μM when added at 2.5 mM, respectively, at 12 h after the addition. However, after 12 h, there was no increase in the amount of LA in the top layer portion, which remained almost constant until after 24 h (Fig. 3B). The results showed that the amount of LA diffusing from the bottom layer portion to the top layer portion was almost 17–18% of the total. The amount of LA in the top layer portion was also determined after adding LA to the CC, and

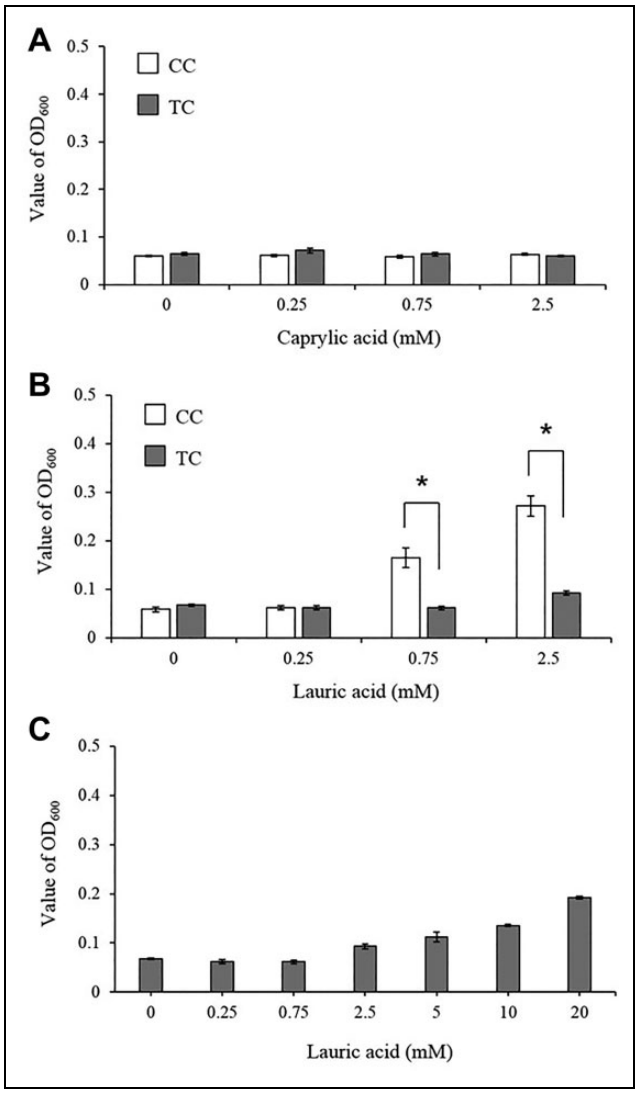

Figure 2. Turbidity of the broth medium portion of the TC and CC complexes after incubation with MCFAs. In the TC, MCFAs were encapsulated in the THY agar medium, while in the CC, MCFAs were suspended in the THY broth medium. Both the TC and CC complexes were then incubated at 37°C for 12 h. Then, the turbidity of the broth medium portion in the TC and in the CC complexes were measured and compared. The turbidity of CA and LA in the broth medium of the two complexes is shown in (A) and (B), respectively. (C) shows the turbidity of TC with increasing concentrations of LA. Data are presented as mean ± SEM of six samples (two independent experiments performed in triplicate). The Student's *t*-test was used to evaluate differences between groups. *$p < 0.05$.

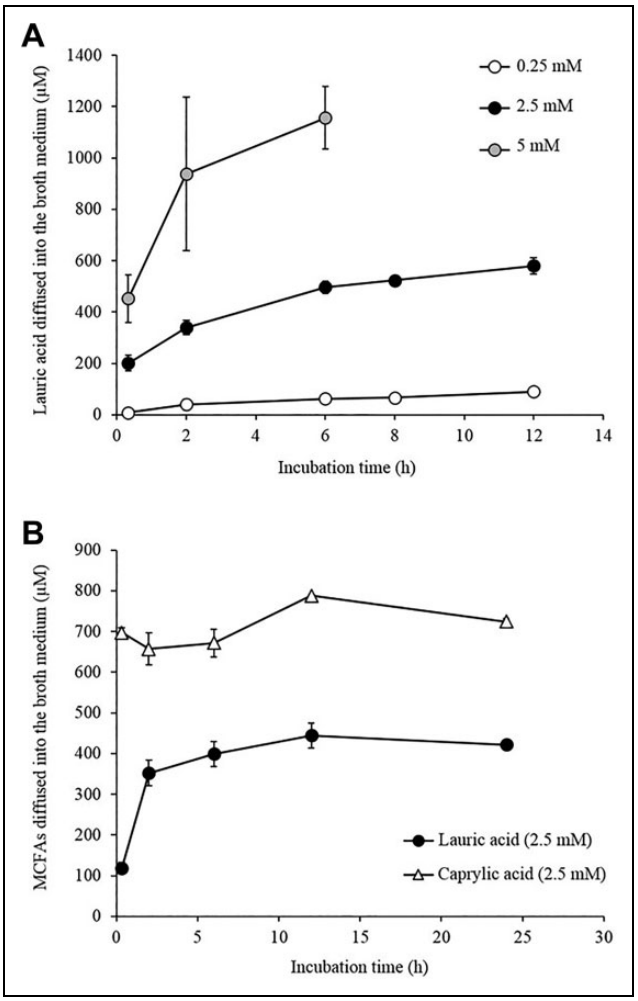

Figure 3. MCFA content in the top layer portion. (A) LA equivalent to concentrations of 0.25, 2.5, and 5 mM was added to the bottom layer portion of the TC and incubated at 37°C. Then, at 10 min, 2, 6, 8, and 12 h, the top layer portion was harvested and the amount of LA in the THY broth medium was measured. (B) LA and CA equivalents to concentrations of 2.5 mM were added to the bottom layer portion of the TC and incubated at 37°C. Then at 10 min, 2, 6, 12, and 24 h, the top layer portion was harvested and the amount of the FAs in the medium was measured. Data are presented as mean ± SEM of six samples (two independent experiments performed in triplicate).

the concentration in the top layer portion at 2, 6, 8, 12, and 24 h after the addition was the same as that when initially added (data not shown).

With the comparatively water-soluble CA, 0.65 mM was present in the top layer portion at 2 h after the addition of 2.5 mM, and there was hardly any change in the concentration until after 24 h (Fig. 3B).

Comparison of the Antibacterial Effects of MCFAs between the TC and CC

Since it is well known that LA has antimicrobial effects against *S. aureus*[4,16,19,20], the activity of LA against *S. aureus* between the TC and CC was compared. LA was added at concentrations of 0.25, 0.75, and 2.5 mM to the TC and CC, which were then incubated at 37°C for 12 h. Thereafter, the complex was mixed gently and *S. aureus* ($0.3\text{-}1 \times 10^5$ CFU) was added to the top layer portion. After incubation at 37°C for 12 h, the number of bacterial colonies in the top layer portion was counted by the colony counting method. Surprisingly, the broth medium with

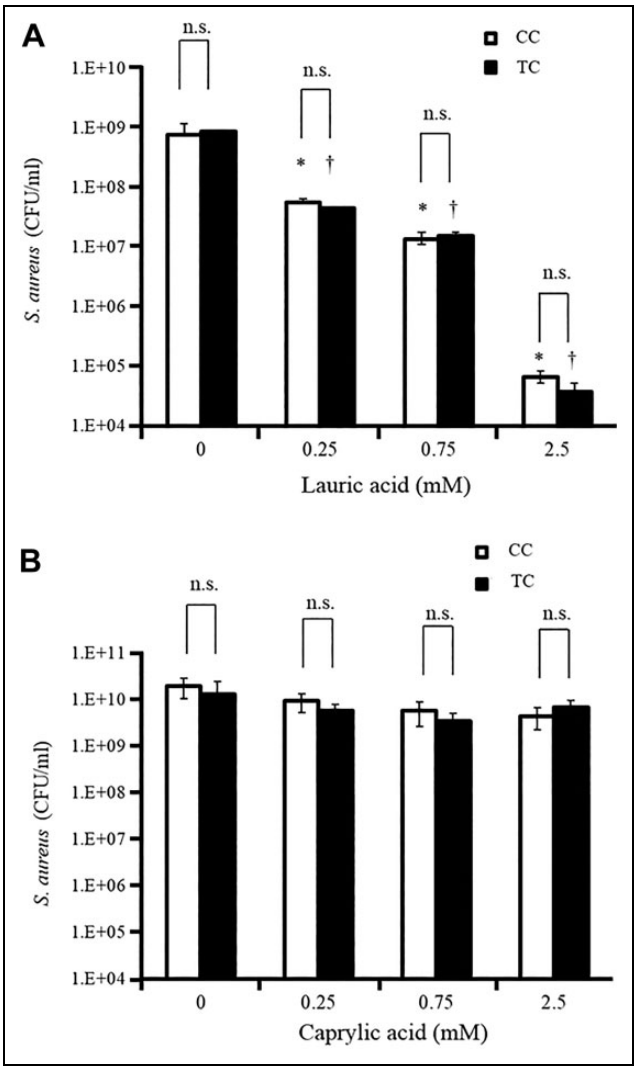

Figure 4. Comparison of antibacterial effects of MCFAs between the TC and CC. LA (A) and CA (B) were added at 0.25, 0.75, and 2.5 mM in both complexes, and incubated at 37°C for 12 h. Thereafter, the complex was mixed gently and *S. aureus* ($0.3–1 \times 10^5$ CFU) was added to the top layer portion. After incubation at 37°C for 12 h, the number of bacterial colonies in the top layer portion was counted with the colony counting method. Data are presented as mean ± SEM of six samples (two independent experiments performed in triplicate). $^*p < 0.05$ compared with the white-colored bar data at LA 0; $^\dagger p < 0.05$ compared with the black-colored bar data at LA 0; n.s. = not significant.

LA diffused from the bottom layer portion in TC and broth medium with LA in CC inhibited growth of *S. aureus* equally. (Fig. 4A). In addition, CA, which has no antimicrobial effect against *S. aureus*, did not suppress the growth of *S. aureus* in either complex (Fig. 4B).

Antimicrobial Activity of LA Determined by Absorbance Measurement

The method we developed allows us to assess the antimicrobial activity of the water-soluble part of MCFAs based on an absorbance measurement. The TC containing LA was added to the wells of 96-deep-well microplates. After incubation at 37°C for 12 h, *S. aureus* was inoculated into the top layer portion of the TC. At 0, 2, 6, and 12 h of incubation, the top layer portion containing the bacteria was harvested and used to count the number of *S. aureus* by a colony count method and to measure absorbance at OD_{600}. As shown in Figs. 5A and B, the number of bacteria and the OD_{600} decrease with increasing amounts of LA added to the agar. Furthermore, the OD_{600} values at the indicated concentrations of LA were in proportion to the number of bacteria (Fig. 5C). The same results have been shown in the case of *S. sanguinis* and *E. coli* (Supplemental Fig. 1), suggesting that the absorbance measurements can provide quite an accurate estimate of the number of bacterial cells.

Antimicrobial Activity of LA against Human Pathogenic Bacteria and Oral Streptococci

Numerous studies have reported the antibacterial effects of LA[13,15,16,21,22] and some have demonstrated that LA has antimicrobial effects against Gram-positive streptococci, but not many Gram-negative bacilli (i.e., *E. coli*, *K. oxytoca*, *K. pneumoniae*, and *S. marcescens*). Therefore, we investigated whether the antimicrobial effects against these bacteria can be accurately assessed with our novel measurement method.

Nine human pathogenic bacteria and *S. salivarius* were seeded in the TC containing various concentrations of LA, and the turbidity change of the broth medium portion after incubation at 37°C for 12 h was measured. The concentrations of the indicated bacteria at the starting point (0.05–0.06 of OD_{600}) is the following: *S. aureus*, *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *K. pneumoniae*, and *K. oxytoca*, $1–3 \times 10^5$ CFU/mL; *S. salivarius*, *S. sanguinis*, *E. coli*, and *S. marcescens*, $1–3 \times 10^7$ CFU/mL. As shown in Fig. 6, the proliferation of *S. agalactiae*, *S. mutans*, *S. pneumoniae*, *S. pyogenes*, *S. salivarius*, and *S. sanguinis* was significantly inhibited by the addition of 0.25 mM and 2.5 mM LA (Fig. 6). On the other hand, the growth of *E. coli*, *K. oxytoca*, *K. pneumoniae*, and *S. marcescens* was not significantly suppressed even with the addition of 2.5 mM LA (Fig. 6). We had confirmed that LA affects the growth of *S. aureus*, *S. mutans*, *S. pneumoniae*, *S. pyogenes*, *S. salivarius*, and *S. sanguinis*, but not that of *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *S. marcescens* using a disk diffusion antibacterial test[16]. These results suggest that our method can correctly evaluate the antimicrobial effects of LA.

Antimicrobial Effects of LA on Human Gut Microbes

Measurement of the antimicrobial activity of LA by the colony count method or diffusion disk test is time-consuming and laborious; thus, it is extremely difficult to assess the concentrations of many types of bacteria simultaneously. Since the proposed measuring method can easily assess the antimicrobial activities of LA against a large

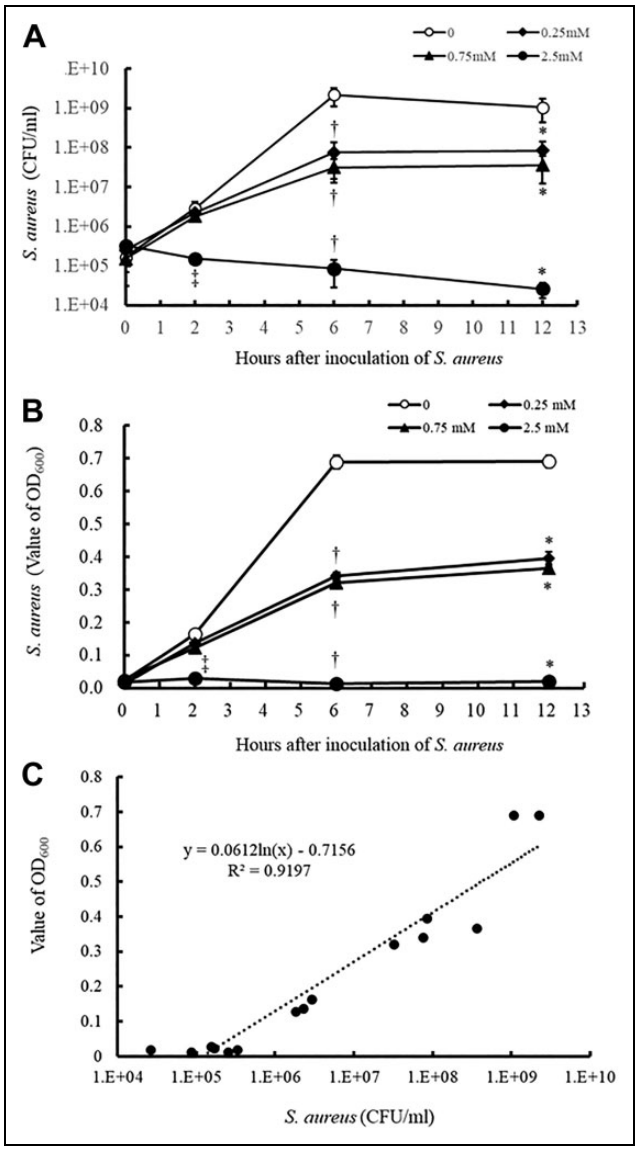

Figure 5. Antimicrobial activity of LA assessed by colony counting methods and absorbance measurement. The TC containing LA was placed in the wells of 96-well deep-well microplates. After incubation for 12 h at 37°C, *S. aureus* was inoculated into the top layer portion of the TC. After incubation for 0, 2, 6, and 12 h, the top layer portion containing the bacteria was harvested and the number of *S. aureus* colonies was counted by the colony count method (A) and absorbance at OD_{600} was measured (B). Panel C shows the correlation between the numbers of bacteria by the colony count method and the OD_{600} values from the data of panels A and B. Data in (A) and (B) are presented as mean ± SEM of six samples (two independent experiments performed in triplicate). *$p < 0.05$ compared with the data of LA 0 at 12 h; †$p < 0.05$ compared with the data of LA 0 at 6 h; ‡$p < 0.05$ compared with the data of LA 0 at 2 h.

amount of bacteria in a short time, we examined the antimicrobial activities of LA against human gut microbes.

First, we investigated changes in penetration into the top layer of LA when the medium in TC was changed from THY to GAM. In Fig. 7A, LA in the bottom layer in GAM penetrated into the top layer and in THY; however, the diffused amount of LA in GAM was nearly 50% of that in THY. In order to examine how the decrease in the amount of LA in the top layer affects the antibacterial effect in TC, we created TC and CC in GAM and compared the antibacterial effect in both colony counts. Fig. 7B shows that the antimicrobial effects of *Enterococcus faecalis* in the broth medium of the LA-containing top layer diffused from the bottom layer in the TC and broth medium in the top layer; the antibacterial effects of the liquid medium due to the addition of LA in the CC that was equally inhibited were also evaluated. Moreover, LA did not suppress the growth of *E. coli*, which is resistant to LA, in either complex (Fig. 7B). We also showed that the antimicrobial effect obtained with the proposed method is comparable to that observed when LA is suspended in the top layer with the bacteria (Fig. 7C). The results suggest that it is possible to detect the antimicrobial activity in our system by GAM.

The plateau of bacterial growth in the absence of LA greatly differed among bacterial species (data not shown). Therefore, we calculated the relative growth of the bacteria in the presence of LA, as compared with that without LA (Fig. 8).

The growth of all of the gut microbes tested was suppressed by LA, although there were variations in the extent of suppression. The growth of gut microbes belonging to the genera *Bacteroides* and *Parabacteroides* was mostly suppressed by the addition of 2.5 mM LA to the TC (Fig. 8). In contrast, the growth of lactic acid bacteria (genus *Lactobacillus*) in the presence of 2.5 mM LA in the TC was in the range of 15% (*Lactobacillus casei* subsp. *casei*) to 60% (*Lactobacillus reuteri*), as compared with that without the addition of LA (Fig. 8). The growth of *E. faecalis* in the presence of 2.5 mM LA in the TC was reduced by 75%, while the growth of *Lactococcus lactis* was completely inhibited. In both of the *Eubacterium* species tested, the growth in the presence of 2.5 mM LA in the TC was reduced by about 70%, as compared with that without the addition of LA (Fig. 8). The growth of *Bifidobacterium adolescentis*, *Blautia hansenii*, *Clostridium ramosum*, *Collinsella aerofaciens*, *Coprococcus comes*, and *Dorea longicatena* was reduced by about 75% by the addition of 2.5 mM LA to the TC. Meanwhile, the growth of *Clostridium perfringens* and *Fusobacterium nucleatum* subsp. *nucleatum* was completely inhibited by the addition of 2.5 mM LA to the TC, and the growth of *Clostridium difficile* was reduced by about 80% of that without LA (Fig. 8).

Discussion

In the present study, a novel method is described for the measurement of the antimicrobial activity of LA with the use of a microplate reader. With this assay system, the antimicrobial activity of LA diffusing from the bottom layer portion in TC was comparable to that of LA dissolved in the top layer portion in CC. Importantly, we showed that the

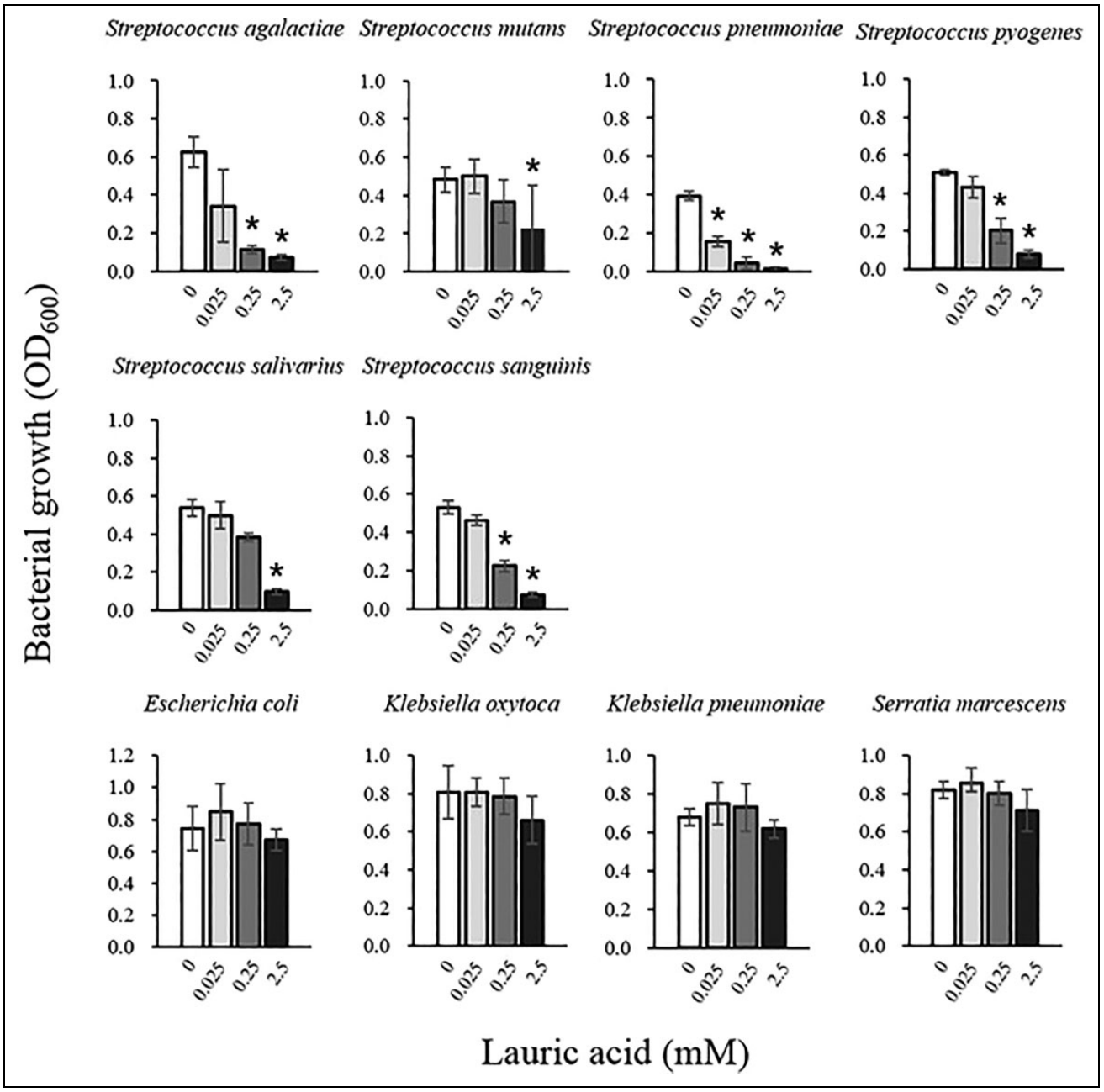

Figure 6. Antimicrobial activity of LA against human pathogenic bacteria and oral streptococci. LA equivalent to concentrations of 0.25, 2.5, 5 mM was added to the bottom layer portion of the TC in the wells of 96-well deep-well microplates. After incubation for 12 h at 37°C, nine human pathogenic bacteria and *S. salivarius* were inoculated into the top layer portion of the TC. After incubation for 12 h, the top layer portion containing the bacteria was harvested and the turbidity was measured at OD_{600}. Data are presented as mean ± SEM of six samples (two independent experiments performed in triplicate). *$p < 0.05$ compared with the data of LA 0.

amount of bacteria in the top layer portion of the TC was corrected with turbidity.

The disc diffusion antimicrobial test has traditionally been used to evaluate the antibacterial activity of MCFAs[16]. This measurement method utilizes the phenomenon that penetration of LA in a disk to agar medium plate which contains 1.5–2% agar inhibits bacterial growth on the plate[13–16]. We confirmed the formation of a clear zone around the disk infiltrated with LA on *S. aureus* seeded in agar medium. It was further confirmed that the agar medium in the inhibition zone was not cloudy. Based on these results, a novel measuring method using TC was devised (Fig. 1). In the present study, LA in the bottom layer portion of the TC penetrated the top layer portion and suppressed bacterial growth in the top layer portion (Fig. 4). However, the LA contained in the agar did not cloud the top layer portion (Fig.

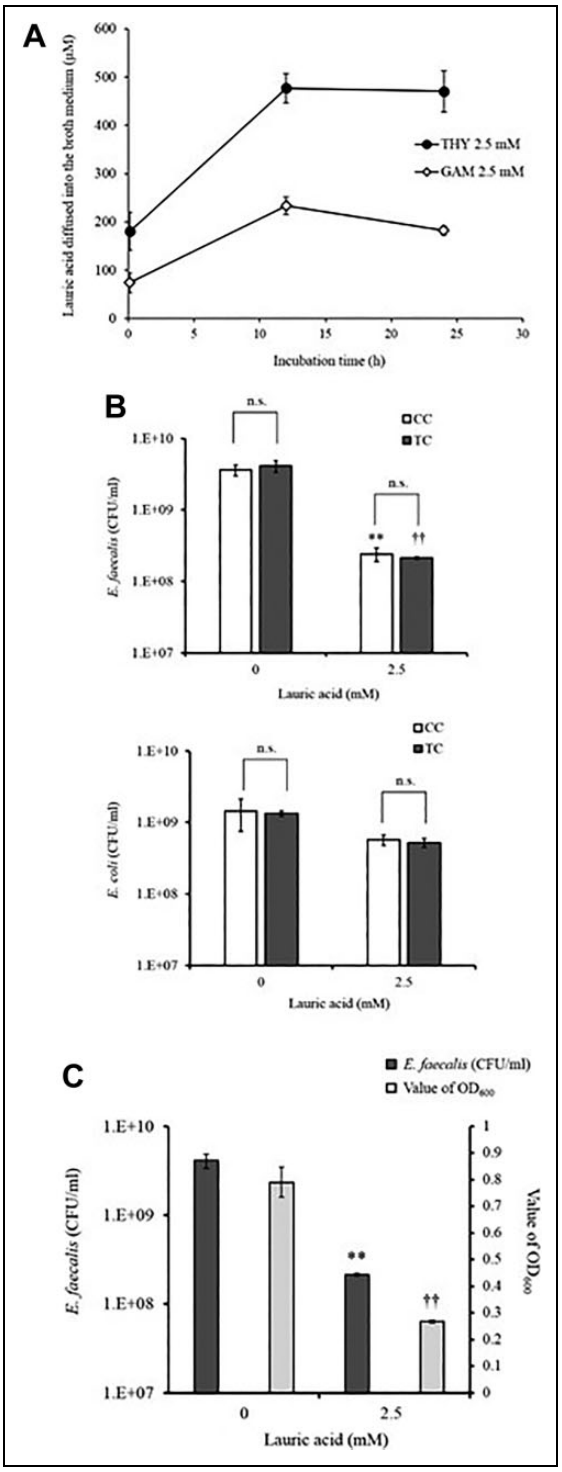

Figure 7. LA content in the top layer and antimicrobial activity in GAM. (A) LA with 2.5 mM concentrations was added to the bottom layer of the TC and incubated at 37°C. Then, at 10 min, 12 h, and 24 h, the upper portion was harvested, and the amount of LA in the medium was measured. Closed circles are for THY 2.5 mM, and open circles are for GAM 2.5 mM. (B) LA was added at 0 and 2.5 mM in both complexes and incubated at 37°C for 12 h. Thereafter, the complex was gently mixed, and *E. faecalis* and *E. coli* ($0.3–1 \times 10^7$ CFU) were added to the top layer. After incubation at 37°C for 12 h, (*to be Continued.*)

2). These results suggest that the proposed novel method was sufficient to monitor changes in bacterial growth in response to LA using a spectrophotometer. In our experiments, the bottom layer portion is set at 2%. Because of its fragility, 1.5% agar was not used during the experiment. In addition, we use 100 μL of agar medium and 300 μL of broth medium because of technical requirements. We make the TC and CC in each well (500 μL capacity) of a 96-well deep-well plate. Next, we inoculate bacteria in each well using the "copy plate stand" shown in Fig. 1. In this inoculation process, the height of the bottom portion should be sufficiently low so that the needles in the copy plate stand (Fig. 1) do not stab the agar. Furthermore, the volume of the broth medium was limited to 300 μL to prevent overflow during bacterial inoculation. We performed the experiments under these conditions.

The evaluation of the antimicrobial effect with the proposed method yields similar results as the colony counting method (Fig. 4). Furthermore, regarding the antibacterial effects of LA against each tested bacterial species, the results of the proposed measuring method were comparable as previously reported[13,15,16,21,22]. The antimicrobial activities of MCFAs are conventionally assessed by a bacterial colony counting method, as described in the introduction section. However, the bacterial colony counting method requires a great deal of agar medium and is energy- and time-consuming. Therefore, it is rather difficult to measure and compare the antibacterial activities of various FAs against many bacterial species simultaneously. The method proposed here can be used to evaluate the antimicrobial effects of FA against many bacterial species simultaneously, thanks to the 96-well plate setting. In addition, since the OD_{600} value correlates with the number of colonies, the antimicrobial activity can be assessed easily and accurately by measuring absorbance in a microplate reader.

Since the number of bacteria in the broth medium is correlated with the turbidity of the media, a simple measurement of turbidity with a microplate reader is generally used to calculate the number of bacteria. However, the presence of FAs muddies the broth medium, making it impossible to precisely calculate the number of bacteria. In contrast, one might believe that we have good control when we only add FA and then validate by plating out the bacteria from the other wells; thus, we can somehow correct the "muddiness" created by the FA. However, we do not agree with this opinion. We showed that adding 0.75 mM and 2.5 mM of LA to the broth medium increased the turbidity at 0.2 and 0.3

Figure 7. (Continued). the number of bacterial colonies in the top layer was counted using the colony counting method. Panel C shows the correlation between the numbers of *E. faecalis* by the colony count method and the OD_{600} values in TC. Data are presented as mean ± SEM of six samples (two independent experiments performed in triplicate). $^{**}p < 0.05$ compared with the white-colored bar data at LA 0; $^{\dagger\dagger}p < 0.05$ compared with the black-colored bar data at LA 0; n.s., not significant.

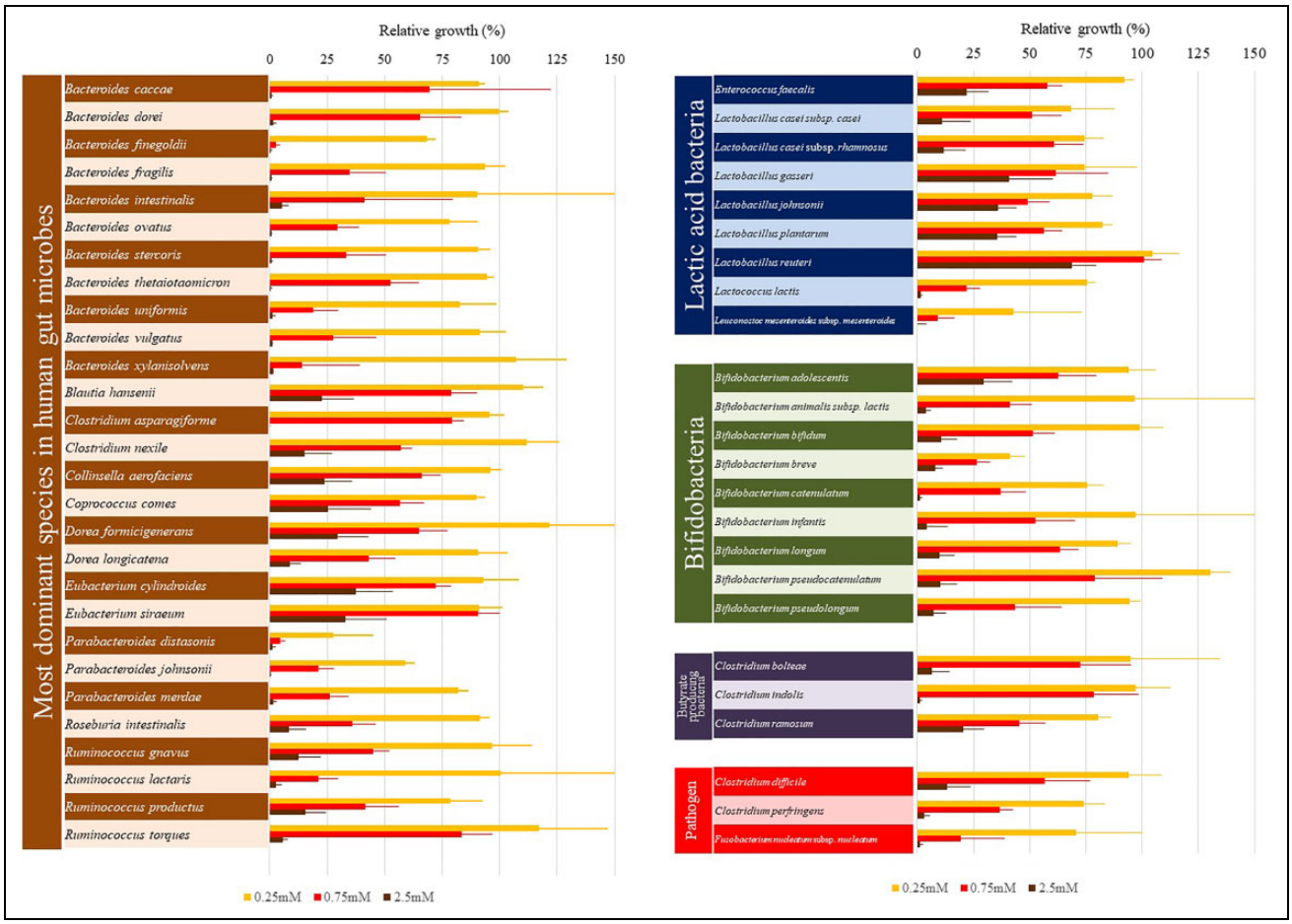

Figure 8. Antimicrobial effect of LA on human gut microbes. LA equivalent to concentrations of 0.25, 2.5, 5 mM was added to the bottom layer portion of the TC in the wells of 96-well deep-well microplates. After incubation for 12 h at 37°C, human gut microbes were inoculated into the top layer portion of the TC to fix 0.05–0.06 of OD_{600}. After incubation for 12 h, the top layer portion containing the bacteria was harvested and the turbidity was measured at OD_{600}. LA was not added to some wells; these were used as controls for each bacterial growth. The relative bacterial growth in the presence vs. absence of LA was calculated. Data are presented as the mean ± SEM of six samples (two independent experiments performed in triplicate).

of OD_{600}, respectively (Fig. 2), and turbidity indicates that the numbers of *S. aureus* are usually 3×10^6 CFU/mL and 10^7 CFU/mL, respectively (Fig. 5). This means that bacterial growth cannot be recognized when the number of bacteria is not increased to $> 3 \times 10^6$ CFU/mL and 10^7 CFU/mL. Since the inoculation amount of *S. aureus* in the bacterial growth test is $1–3 \times 10^5$ CFU/mL (0.05–0.06 of OD_{600}), in terms of turbidity values, the addition of LA indicates significant bacterial growth. Furthermore, the maximum value of OD_{600} in some bacteria, i.e., many strains of genus *Streptococcus*, is 0.4–0.6, which is similar to the turbidity level of the broth medium with LA. Therefore, we cannot determine the bacterial growth or exact level of inhibitory bacterial growth by LA. We believe that the presence of FAs, including LA, muddies the broth medium, making it impossible to precisely calculate the number of bacteria. Since LA is only partially soluble in water, the majority of the FA forms micelles in water. However, in the TC, the top layer did not become cloudy, most likely because only the soluble portion of the LA diffused from the bottom layer to the top layer. This resulted in 17%–18% (in the case of THY) and 8%–9% (in the case of GAM) of the total LA inoculated in the bottom layer being present in the top layer, where it consequently induced antibacterial activity. We also showed that the antimicrobial effect obtained with the proposed method is comparable to that observed when LA is suspended in the top layer with the bacteria. The carbon chain and methyl carboxylate of FAs bind and show no antimicrobial activity, whereas binding of the carbon chains of FAs to the carboxyl group conveys antibacterial activities[23]. In addition, Prasad et al. demonstrated that physical features in the agar–fatty acid complex are different from that in agar alone[24]. The agar gel in the presence of some MCFAs induces decreased surface tension activity, enhanced hydrophobicity, decreased water retention, and decreased gel strength. However, the amount of FAs chemically bound in the agar–fatty complex

is very few (almost 0.1–0.2%) when adding FAs to liquefied agar to make the agar–fatty acid complex[24]. The results suggest that formation of the agar–fatty acid complex releases water and hydrophilic substances, including hydrophilic groups of the FAs, out of the agar due to reduced water retention; thus, the presence of the hydrophilic group of the FAs greatly affects antibacterial activity. However, it is not clear how MCFAs encapsulated in agar actually come out in liquid medium; thus, in order to further improve the accuracy of this assay, it is necessary to clarify how MCFAs encapsulated in agar come out in the liquid medium, which is a subject to be examined in the future.

In the present study, we determined the antibacterial effect in THY and GAM. Although the amount of LA penetration to the top layer of the TC in the GAM is less than that in the THY medium, the antibacterial effect in TC was similar to that in the case where LA was directly added to the GAM in CC (Fig. 7). It is still unclear why the antimicrobial activity between the two experimental systems was not different even though the penetrated amount is decreased; thus, it is necessary to clarify the reason in the future. However, the results may suggest that various media may be screened for the antibacterial effects. A previous study reported the protective effects of the oral administration of LA against *C. difficile* in a mouse model of inflammation[22], suggesting that LA and some MCFAs found in foods may be able to escape digestion and absorption, at least partially, and reach the intestinal lumen. Furthermore, in the present study, LA was shown to inhibit the growth of the dominant bacterial species in the human gut microbe, including *Bacteroides caccae* and *Bifidobacterium dorei* (Fig. 8). It was previously reported that *B. caccae* degrades the colonic mucus barrier and enhances pathogen susceptibility when the host takes in food lacking dietary fiber[25], and that early colonization by *B. dorei* may contribute to autoimmune diseases in humans through the inhibition of innate immune signaling and endotoxin tolerance[26]. Therefore, it is possible that the intake of LA may promote the health of the host via inhibition of bacterial growth, especially that of species belonging to the genus *Bacteroides*. Moreover, the oral administration of *B. fragilis* was shown to correct gut permeability, alter microbial composition, and ameliorate autism spectrum disorder-related defects in maternal immune-activated mice[27]. Therefore, it is also possible that intake of LA may adversely affect the gut–microbiome–brain connection because the growth of *Bacteroides fragilis* was completely inhibited in the presence of 2.5 mM LA in the present study. These are, of course, hypotheses that should be further investigated; the effects of LA in the gut may anyway be complicated by the presence of many different species, and by the fact that they act in a complex community of microbes, where the effects of many substances introduced with the diet are overlapping.

Lactic acid bacteria are probiotic bacteria contained in fermented foods such as yogurt and pickles. The results of the present study revealed that LA has low antimicrobial activity against *Lactobacilli* and *E. faecalis* (Fig. 8). Lactic acid bacteria are the predominant species in the human small intestine and inhibit enteritis through the induction of interferon β[28]. Therefore, LA may enhance this anti-inflammatory activity through antimicrobial activities against other bacteria that compete with lactic acid bacteria. In humans, although colonic microbiota lactic acid bacteria are non-dominant, it may be possible to increase the concentrations of prebiotic lactic acid bacteria with the selective antimicrobial activity of LA. *B. adolescentis*, which is relatively resistant to LA (Fig. 8), is the sixth most dominant bacterial species in the feces of healthy Japanese adults[29], and this bacterium strongly induces the proliferation of intestinal Th17 cells when colonized alone in the murine intestine. The induction of Th17 cells bolsters the host mucosal defenses or may lead to the development of autoimmune diseases[30]. Since LA has antimicrobial activities against many other bacterial species in the colonic microbiota that compete with *B. adolescentis*, it is possible that LA may support the growth/colonization of *B. adolescentis* in the colon and therefore induce proliferation of Th17 cells. *C. ramosum* and *C. comes* produce butyric acid[18,31], and are believed to contribute to the suppression of enteritis through induction of regulatory T cells in the host intestinal tract. Therefore, it is not necessarily that they would proliferate in the presence of LA, but maybe they simply would not be negatively affected by it. *C. perfringens* is a causative agent of food poisoning and it was previously reported that *F. nucleatum* subsp. *nucleatum* is associated with colorectal cancer in addition to periodontal disease[32]. Interestingly, in this study we observed that both these bacteria (*C. perfringens* and *F. nucleatum*) were completely inhibited by LA (Fig. 8). Future studies are necessary to indicate whether the suppression of these bacteria would be associated with a change in disease risk, and whether LA could play a role in this modulation.

Some MCFAs have antimicrobial activities against various pathogenic bacterial constituents of the skin and may contribute to the control of the skin bacterial flora [5,10,11,33]. These molecules are also contained in some foods such as coconut oil and milk[34–38]. Thus, the diversity of the antibacterial effects of FAs on various bacteria might contribute to modulate the composition of the intestinal flora and promote health. There are not only LA but also many antimicrobial free fatty acids and monoglycerides[39]; thus, it still remains unknown whether the suppression of these bacteria would be associated with a change in disease risk, and whether LA could play a role in this modulation. Future studies are warranted to investigate the antibacterial activities of other FAs against different bacteria, both individually and in complex cultures. The future studies that we indicated above will bring important suggestions. In other words, if our method could measure the accumulated bacterial inhibition by FAs and other compounds using bacterial mixtures, it may be possible to test new hypotheses regarding LA and other compounds in gut health and change of in vivo disease risk.

Conclusion

In conclusion, the proposed method for measuring the antimicrobial effect of LA can be used to quickly and simultaneously evaluate a large number of types of FAs with very simple preparation steps as compared with more conventional methods. In addition, the antimicrobial activity of LA against human gut microbes was determined with the proposed method, which showed that LA has low antimicrobial activity against lactic acid bacteria, but not *Bacteroides* and *Clostridium*. These results suggest that LA might contribute to intestinal health, as confirmed by the proposed method.

Acknowledgments

We would like to thank Ms. Yoshie Yuasa, Mr. Yuusuke Kotani, and Mr. Kazuki Hayashi for the technical assistance. We also thank Enago for the English editing.

Ethical Approval

All methods and experimental protocols for using bacteria were approved by Kanazawa University Microorganisms Safety Management Regulations and Ishikawa Prefectural University Microorganisms Safety Management Regulations, and all experiments were performed in the Biosafety level 2 (BSL2) rooms that were approved by the Regulations.

Statement of Human and Animal Rights

Statements of Human and Animal Rights is not applicable for this article.

Statement of Informed Consent

Statement of Informed Consent is not applicable for this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of article: This work was supported by grants from the Japan Society for the Promotion of Scieence (KAKENHI): 17H04428, 26462806, and Organization of Frontier Science and Innovation (O-FSI), Kanazawa University (Sakigake Project).

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Supplemental Material

Supplemental material for this article is available online.

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