Antimicrobial Activity of Fatty Acid Salts Against Microbial in *Koji-Muro*

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Abstract. *Aspergillus niger* and *Aspergillus oryzae* are used as *koji* fungi in the spot of the brewing. Since *koji-muro* (room for making *koji*) was a low level of airtightness, microbial contamination has long been a concern to the alcoholic beverage production. Therefore, we focused on the fatty acid salt which is the main component of soap. Fatty acid salts have been reported to show some antibacterial and antifungal activity. This study aimed to find the effectiveness of the fatty acid salt in *koji-muro*. Nine fatty acid salts were tested. The result, C12K was antibacterial effect against *B. subtilis*. C10K and C12K was antifungal effect against *R. oryzae*. These results suggest C12K has potential in the field of *koji-muro*.

1 Introduction

Fungus is ubiquitous in the indoor environment, and breed in the foods such as vegetables and grains, causing corruption and deterioration of these foods in some cases. Bacteria and yeast can be cited as a fellow of other microbial.

Aspergillus species are common contaminants of starchy foods including bread and potatoes. But, *Aspergillus niger* and *Aspergillus oryzae* are used as *koji* fungi in the spot of the brewing. Since *koji-muro* (room for making *koji*) was a low level of airtightness, microbial contamination has long been a concern to the alcoholic beverage production. Typical causes of microbial contamination include *Rhizopus* species in the fungi and *Bacillus* species in the bacteria. *Rhizopus* species are called "*kumonosukabi*" in Japanese, and has been used as *koji* fungi in Asia other than Japan. *Bacillus* species include *Bacillus subtilis* in the typical ones. It is known to shape a durable organ of spores when the growth environment is deteriorated. It has been taken a variety of countermeasure in the current situation to microbial contamination. As an example, the method can be mentioned that using ethanol and invert soap and the fumigation. However, the former is the effect and persistence of low against fungus and spores is a problem. The latter can only be done at the end of the season because it is too powerful.

Therefore, we focused on the fatty acid salt which is the main component of soap. Also, it is an alkali metal salt consisting of alkali and fatty acid (CH_3-(R)-COOH), which is a kind of anionic surfactant. It is used as many industrial material. Fatty acids vary in length and degree of saturation, and naturally occurring fatty acids have a chain length of 4 to 28 carbons, which may be saturated or unsaturated [1]. Moreover, it is known that there is antibacterial activity to bacteria, such as *Staphylococcus aureus* and *Eschrichia coli* [2-4]. So this study examined antimicrobial activities against *Aspergillus oryzae*, *Rhizopus oryzae* and *Bacillus subtilis*.

This study aimed to find the effectiveness of the fatty acid salt in *koji-muro* as antimicrobial agents.

2 Detail Experimental

2.1 Source and preparation of fatty acid salts

Table 1 show the fatty acid salts used in the experiment. Nine fatty acids were tested. Butyric acid (C4:0), caproic acid (C6:0), and linoleic acid (C18:2) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), oleic acid (C18:1), and linolenic acid (C18:3) were obtained from Tokyo Chemical Industry Co., Ltd.

Hydrated mixtures of fatty acid salts in solution were prepared by gravimetric determination, using fatty acids, KOH: potassium hydroxide pellets (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and water.

Samples were prepared at 350 mM concentrations. The samples were then stirred for 2 h at 75 °C. KOH aq was added to yield theoretical neutralization at pH 10.5 of fatty acid salts. Potassium butyrate (C4K), potassium caproate (C6K), potassium caprylate (C8K), potassium caprate (C10K), potassium laurate (C12K), potassium myristate (C14K), potassium oleate (C18:1K), potassium linoleate (C18:2K), potassium linolenate (C18:3K), and the blank were all adjusted using a KOH pH-adjusted solution (pH 10.5). All fatty acid salts and the KOH pH-

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adjusted solution were filter-sterilized at low temperature (4-6 °C) using a 0.20-μm Millipore filter (Toyo Roshi Kaisya, Ltd., Tokyo, Japan) [5].

Table 1. Samples preparation； FAS

C4K : Potassium btyrate
C6K : Potassium caproate
C8K : Potassium caprylate
C10K : Potassium caprate
C12K : Potassium laurate
C14K : Potassium myristate
C18:1K : Potassium oleate
C18:2K : Potassium linoleate
C18:3K : Potassium linolenate
Control : KOH pH-adjusted solution

2.2 Fungal strains and growth conditions

A. oryzae (*Akita konnno* store, Japan), *R. oryzae* NBRC 4716 and *B. subtilis* NBRC 3335 were obtained from the NBRC (Biological Resource Center, NITE, Tokyo, Japan).

The fungi were initially grown on Potato Dextrose Agar (PDA: *Nissui* Pharmaceutical Co., Ltd., Tokyo, Japan) in the dark and the culture stocks were stored at 4 °C. Plant-pathogenic fungi were routinely sub-cultured on PDA in slants in the dark at 30 °C so that the fresh cultures were available for later use [5].

2.3 Preparation of spore suspensions

Fungi were grown on PDA slants at 30 °C until well sporulated, approximately 10-14 d. Spores were harvested in sterile distilled water using a sterile inoculation loop and gentle agitation; a drop of 0.005 % sulfosuccinic acid bis (2-ethylhexyl) ester sodium salt and 0.9 % NaCl were added to aid wetting of the spores. The spore concentration was determined by counting using a hemacytometer (Thoma, Sunlead Glass Corp., Saitama, Japan). The initial spore concentration was adjusted to 3.0×10^4 spores/mL [5].

2.4 Preparation of cell suspensions

The subcultures were incubated at 30 °C for 1 day. Growth was harvested with autoclaved sterilized water. The bacteria were sedimented by centrifugation at 4200 rpm for 20 min. Pellets were washed three times with 30 mL of autoclaved sterilized water each time. Final pellets were suspended in 20 mL of autoclaved sterilized water. The washed suspensions of *Bacillus* were used on the day of preparation [6].

2.5 Effect of fatty acid salts on fungal spores or bacterial cells

Solutions of 400 μL of fatty acid salts (final concentration of 175 mM in the tubes) and 400 μL of the spore suspension (3.0×10^4 spores/mL or 3.0×10^5 CFU/mL) were prepared in 1.5 mL plastic tubes. Spores mixed with the KOH pH-adjusted solution were used as controls. Final pH of all samples were a range of pH 9.2-10.8. The mixtures were incubated at 25 °C. Samples were counted at 0, 10, 60, and 180 min by plating (100 μL) on PDA or NA. Fungal colonies were counted after incubation for 2 d or 10 d at 30 °C. Viable counts ($\log_{10}$ CFU) of spore was subtracted from the viable count of the control ($\log_{10}$ CFU), and the difference was used as a measure of the antifungal activity. All experiments were performed at least thrice.

2.6 Determination of minimum inhibitory concentrations (MICs)

The MIC is defined as the lowest concentration of drug sufficient for inhibiting visible growth of spores after 10 min of incubation. MICs against fungi were determined using the two-fold dilution method [7, 8].

Table 2 show two-fold dilution method of fatty acid salts. Each fatty acid salt was separately inoculated with 400 μL of *R. oryzae* NBRC 4716 or *B. subtilis* NBRC 3335 at 3.0×10^4 spores/mL or 3.0×10^5 CFU/mL. 1.5 mL plastic tubes containing 400μL of each of fatty acid salts were inoculated separately with 400 μL of the fungi or bacteria. The tubes, each containing a total volume of 800 μL, were incubated at 25°C for each organism for 10 min. After incubation, samples were plating on PDA or NA, incubated at 30°C for 7 d, and then examined for the growth of spores. Following incubation, the end point was visually assessed and expressed in mM. The lowest concentration of the antifungal or antibacterial treatment that inhibited visible growth of the fungi or bacteria after incubation was taken as the MIC of the treatment.

Table 2. Two-fold dilution method of fatty acid salts

Two-fold dilution	concentration [mM]				
	175	87.5	43.8	21.9	10.9
fatty acid salts 175 mM	400 μL	200	200	200	200
KOH pH-adjusted solution	-	200	200	200	200

2.7 Other reagents tested

Linear alkylbenzene sulfonate (LAS, *Shabondama* Soap Co., Ltd., Japan) is an anionic surfactant similar to fatty acid salts, and was used in this experiment for comparison (pH 10.5). LAS was obtained from *Shabondama* Soap Co., Ltd., Fukuoka, Japan.

LAS composed a sodium salt consisting of linear alkyl benzene sulfonic acid, a number of compounds as with soap. 98% of compound elaborated LAS are linear alkyl benzene sulfonic acid sodium of 12 carbon chain, the rest is included other carbon chain length.

3 Results and Discussion

3.1 Antibacterial activity of saturated fatty acid salts against *B. subtilis*

Figure 1 show the antibacterial activity of saturated fatty acid salts against *B. subtilis*. The average initial population of bacteria at 0 min in all samples was approximately 3.0×10^5 CFU/mL. Bacteria was incubated for 1 d. Final concentration of saturated fatty acid salts were 175 mM. C12K produced a 5 log-units reduction in the growth of *B. subtilis* NBRC 3335 after incubation for 10 min. Thus, C12K, C18:1K, C18:2K and C18:3K suppressed 99.999% of bacterial growth. Against *B. subtilis* NBRC 3335, C10K produced an antibacterial effect of 5 log-units (suppressing 99.999 % of growth) following incubation for 180 min. C8K and C14K produced an antibacterial effect of 4 log-units (suppressing 99.99 % of growth) following incubation for 180 min. However, C4K, C6K, and pH-adjusted solution (control) were ineffective after 180 min. Saturated fatty acid salts exerted an antibacterial activity, and no effect was produced by the pH-adjusted solution alone.

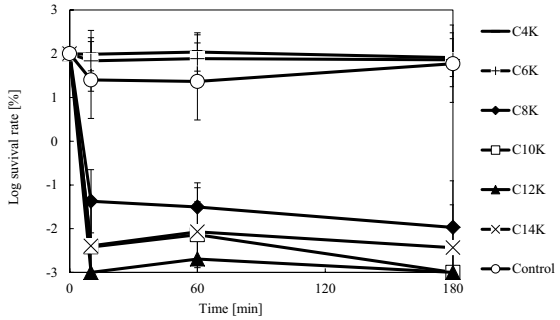

Figure 1. Antibacterial activity of saturated fatty acid salts against *B. subtilis*
Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30°C for 1 d were enumerated at the specified time points on NA. Symbols: －, C4K; +, C6K; ◆, C8K; □, C10K; ▲, C12K; ×, C14K; ○, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

Antibacterial activity of saturated fatty acid salts were found to have defferent intensities of the activity by the defference in the carbon chain. In addition, it was revealed that the antibacterial activity of the relatively long saturated fatty acid salts having a carbon chain is high. Thus, we consider the reason why the difference in antibacterial activity by the difference in the number of carbon chain. Hydrophobic groups of the saturated fatty acid salt is fatty acid. Fatty acid has been considered increasing destabilization of cell membranes by being inserted into the phospholipid bilayer of the cell membrane of the bacteria [9, 10]. Therefore, the saturated fatty acid salts of the short chain saturated fatty acid salts for the hydrophobic group is short, be suspected that did not result in increased destabilization of cell membranes in *B. subtilis*. The saturated fatty acid salt have a medium-chain of hydrophobic group is considered increasing destabilization of cell membranes by a hydrophobic group is inserted into the phospholipid bilayer of the cell membrane of *B. subtilis*, it is believed to have shown an antibacterial effect.

3.2 Antibacterial activity of unsaturated fatty acid salts against *B. subtilis*

Figure 2 show the antibacterial activity of unsaturated fatty acid salts against *B. subtilis*. The average initial population of bacteria at 0 min in all samples was approximately 3.0×10^5 CFU/mL. *B. subtilis* was incubated for 1 d. Final concentration of unsaturated fatty acid salts were 175 mM. C18:1K, C18:2K and C18:3K produced a 5 log-units reduction in the growth of *B. subtilis* NBRC 3335 after incubation for 10 min. Thus, C18:1K, C18:2K and C18:3K suppressed 99.999% of bacterial growth. However, pH-adjusted solution (control) were ineffective after 180 min. Unsaturated fatty acid salts exerted an antibacterial activity, and no effect was produced by the pH-adjusted solution alone.

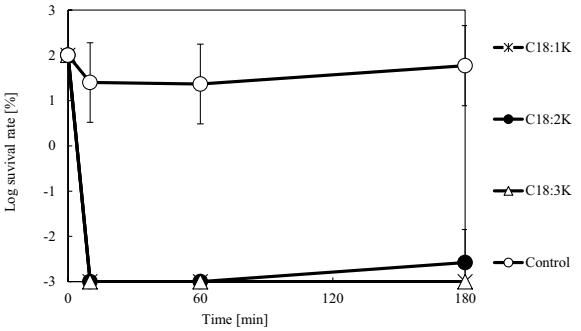

Figure 2. Antibacterial activity of unsaturated fatty acid salts against *B. subtilis*
Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30°C for 1 d were enumerated at the specified time points on NA. Symbols: ＊, C18:1K; ●, C18:2K; △, C18:3K; ○, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

Antibacterial activity of unsaturated fatty acid salts were found to have defferent intensities of the activity by the short saturated fatty acid salts. Thus, we consider the reason why the difference in antibacterial activity by the short saturated fatty acid salts. Hydrophobic groups of the unsaturated fatty acid salt is fatty acid same as saturated fatty acid salts. Fatty acid has been considered increasing destabilization of cell membranes by being inserted into the phospholipid bilayer of the cell membrane of the bacteria [9]. The unsaturated fatty acid salt have long-chain of hydrophobic group. So we considered increasing destabilization of cell membranes by a hydrophobic group is inserted into the phospholipid bilayer of the cell membrane of *B. subtilis*, it is believed to have shown an antibacterial effect [3].

3.3 Antifungal activity of saturated fatty acid salts against *R. oryzae*

Figure 3 show the antifungal activity of saturated fatty acid salts against *R. oryzae* NBRC 4716. The average initial population of fungus at 0 min in all samples was approximately 3.0×10^4 spores/mL. Fungus was incubated for 1 d. Final concentration of fatty acid salts were 175 mM. C12K produced a 4 log-units reduction in the growth of *R. oryzae* NBRC 4716 after incubation for 10 min. Thus, C12K suppressed 99.99% of fungal growth. Against *R. oryzae* NBRC 4716, C10K produced an antifungal effect of 4 log-units (suppressing 99.99 % of growth) following incubation for 180 min. C14K produced an antifungal effect of 3 log-units (suppressing 99.9 % of growth) following incubation for 180 min. However, C4K, C6K, C8K and pH-adjusted solution (control) were ineffective after 180 min. Saturated fatty acid salts exerted an antifungal effect, and no effect was produced by the pH-adjusted solution alone.

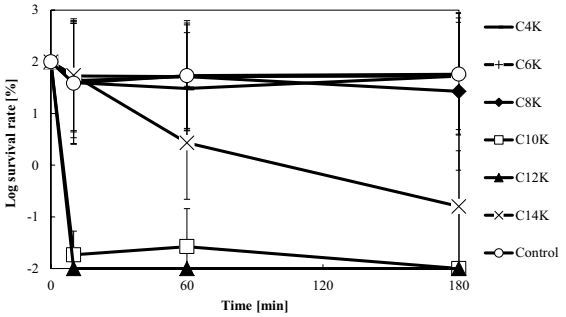

Figure 3. Antifungal activity of saturated fatty acid salts against *R. oryzae*
Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30°C for 1 d were enumerated at the specified time points on PDA. Symbols: －, C4K; +, C6K; ◆, C8K; □, C10K; ▲, C12K; ×, C14K; ○, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

Antifungal activity of saturated fatty acid salts were found to have defferent intensities of the activity by the defference in the carbon chain. The antimicrobial effect of fatty acids, which are the raw material for the production of fatty acid salts, decrease with increasing chain length, and medium-chain fatty acids exhibit stronger activity than longer chain fatty acids [11]. Thus, the reason for the difference in antifungal activity caused by the difference in the number of carbon chain, it is suspected that showed antifungal activity in fungas spores for the same reason as that discussed antibacterial activity of the fatty acid salt against *B. subtilis*. However, some of the saturated fatty acid salt against *R. oryzae* did not show high antifungal activity. *B. subtilis* are classified as Gram-negative bacteria among the bacteria, so this cell walls is thin compared to the fungas [3, 12]. Thus, we considered result did not increase the destabilization of the cell membrane of *R. oryzae* because C8K is not a sufficient length of the hydrophobic group.

3.4 Antifungal activity of unsaturated fatty acid salts against *R. oryzae*

Figure 4 show the antifungal activity of unsaturated fatty acid salts against *R. oryzae* NBRC 4716. The average initial population of fungus at 0 min in all samples was approximately 3.0×10^4 spores/mL. Fungus was incubated for 1 d. Final concentration of unsaturated fatty acid salts were 175 mM. C18:1K, C18:2K, C18:3K and pH-adjusted solution (control) were ineffective after 180 min. Unsaturated fatty acid salts exerted an antifungal effect, and no effect was produced by the pH-adjusted solution alone.

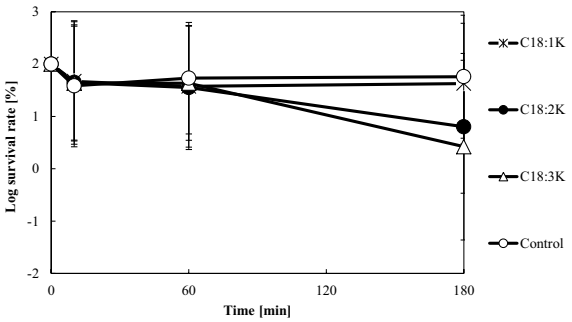

Figure 4. Antifungal activity of saturated fatty acid salts against *R. oryzae*
Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30°C for 1 d were enumerated at the specified time points on PDA. Symbols: ＊, C18:1K; ●, C18:2K; △, C18:3K; ○, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

Antifungal activity of unsaturated fatty acid salts were found to have different intensities of the activity by the saturated fatty acid salts. However, the reason have not been clarified.

3.5 Antifungal activity of fatty acid salts against *A. oryzae*

Figure 5 show the antifungal activity of fatty acid salts against *A. oryzae*. The average initial population of fungus at 0 min in all samples was approximately 3.0×10^4 spores/mL. Fungus was incubated for 2 d. Final concentration of fatty acid salts were 175 mM. No obvious change was observed in tested fatty acid salts against *A. oryzae*. Antifungal effect was not produced by the pH-adjusted solution alone.

So far, fatty acid salts have been observed antimicrobial activity against *R. oryzae* and *B. subtilis* in present study and *Penicillium* [5] and oral bacteria [13] in previous study, but did not observed antifungal activity against *A. oryzae*.

For future work, we would like to examine the reason for fatty acid salt against *A. oryzae* spores did not showed antifungal activity.

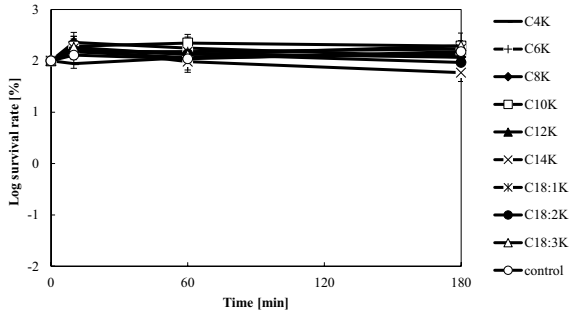

Figure 5. Antifungal activity against *A. oryzae*
Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30°C for 2 d were enumerated at the specified time points on PDA. Symbols: －, C4K; +, C6K; ◆, C8K; □, C10K; ▲, C12K; ×, C14K; ∗, C18:1K; ●, C18:2K; △, C18:3K; ○, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

3.6 Antimicrobial activity of other reagents against *B. subtilis*, *R. oryzae* and *A. oryzae*

Figure 6 show antimicrobial avtivity of LAS against *B. subtilis* NBRC 3335, *R. oryzae* NBRC 4716 and A. oryzae (*Akita konnno* store). The average initial population of bacteria or fungas at 0 min in all samples was approximately 3.0×10^5 CFU/mL or 3.0×10^4 spore/mL. Bacteria was incubated for 1 d. *R. oryzae* was incubated for 1 d. *A. oryzae* was incubated for 2 d. Final concentration of fatty acid salts and LAS was 175 mM. LAS produced a 5 log-units reduction in the growth of *B. subtilis* NBRC 3335 after incubation for 10 min. Thus, LAS suppressed 99.999 % of bacterial growth. Similar results were obtained when the result of C12K, C18:1K, C18:2K and C18:3K against *B. subtilis* NBRC 3335. We would like to examine the reason for the LAS showed high antibacterial activity. LAS is synthetic detergent so it includes a sulfonate salt having an alkyl group with a carbon chain 10-14. Therefore, it suspected that LAS showed antibacterial activity against *B. subtilis* because it include same carbon chain as fatty acid salts.

LAS produced a 4 log-units reduction in the growth of *R. oryzae* NBRC 4716 after incubation for 10 min. Thus, LAS suppressed 99.99 % of fungal growth. Similar results were obtained when the result of C12K against *R. oryzae* NBRC 4716. We would like to examine the reason for the LAS showed high antifungal activity. The reason for the antifungal activity is suspected same reason as that antibacterial activity of LAS against *B. subtilis*.

No obvious change was observed in tested in LAS against *A. oryzae* (*Akita konnno* store). The reason for the result is suspected same reason as that antifungal activity of fatty acid salts against *A. oryzae* (*Akita konnno* store).

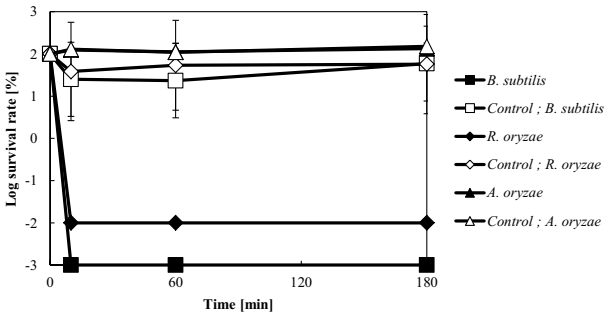

Figure 6. Antimicrovial activity of LAS against *B. subtilis*, *R. oryzae* and *A. oryzae*
Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30°C for 1 d or 2 d were enumerated at the specified time points on NA or PDA. Symbols: ■, *B. subtilis*; □, Control of *B. subtilis*; ◆, *R. oryzae*; ◇, Control of R. oryzae; ▲, *A. oryzae*; △, Control of *A. oryzae*. Experiments were performed in triplicate, and the error bars represent the standard deviation.

3.7 MIC of fatty acid salts and LAS

Two-fold dilution samples of the 175 mM solution inoculated with fungus or bacteria were incubated for 10 min, and then applied to the agar medium, and MICs were determined after 7 d of culture. The results are shown in Table 3.

First, we describe the result of MIC of fatty acid salts against *B. subtilis*. All samples of fatty acid salts concentrations > 175 mM were required to inhibit fungal growth. Sample of LAS concentrations > 175 mM were required to inhibit fungal growth.

Next, we describe the result of MIC of fatty acid salts against *R. oryzae*. C10K and C12K at 175 mM inhibited the growth of *R. oryzae* NBRC 4716 for 7 d. However, concentrations > 175 mM of the other samples were required to inhibit fungal growth. LAS at 87.5 mM inhibited the growth of *R. oryzae* NBRC 4716 for 7 d.

Table 3. MIC against *B. subtilis*

FAS	MIC [mM]	
	B. subtilis	*R. oryzae*
C4K : Potassium btyrate	>175	>175
C6K : Potassium caproate	>175	>175
C8K : Potassium caprylate	>175	>175
C10K : Potassium caprate	>175	175
C12K : Potassium laurate	>175	175
C14K : Potassium myristate	>175	>175
C18:1K : Potassium oleate	>175	>175
C18:2K : Potassium linoleate	>175	>175
C18:3K : Potassium linolenate	>175	>175
LAS	>175	87.5

4 Conclusions

In this study, C12K, C18:1K, C18:2K and C18:3K showed the highest antibacterial activity (5 log-units) against *B. subtilis* NBRC 3335, suppressing 99.999% of bacterial growth (Figure 1, 2). A control solution at the same pH as the fatty acid salt solutions did not affect bacterial growth. So we concluded that the antibacterial activity was due to the fatty acid salts themselves, not pH. Also, from the determination of MIC, it was found to be bacteriostatic action. In addition, the antibacterial activity of LAS was same as fatty acid salts.

Next, C12K showed the highest antifungal activity (4 log-units) against *R. oryzae* NBRC 4716, suppressing 99.99 % of fungal growth (Figure 3, 4). A control solution at the same pH as the fatty acid salt solutions did not affect fungal growth. So we concluded that the antifungal activity was due to the fatty acid salts themselves, not pH. Also, the antibacterial activity of LAS was same as fatty acid salts.

No obvious change was observed in tested fatty acid salts against *A. oryzae* (*Akita konnno* store) (Figure 5, 6).

Additional research is required to have high antimicrobial activity with other reagent.

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