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Bacteriology

Microbicidal effects of slightly acidic hypochlorous acid water and weakly acidified chlorous acid water on foulbrood pathogens

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ABSTRACT. *Paenibacillus larvae* and *Melissococcus plutonius* are bacterial pathogens of honey bee brood. As decontamination of beekeeping equipment, including combs, is essential to control these pathogens, we evaluated the disinfecting effects of slightly acidic hypochlorous acid water (SAHAW) and weakly acidified chlorous acid water (WACAW) on the pathogens. Both disinfectants exhibited strong disinfecting effects in suspension tests under no organic matter conditions and reduced both pathogens by >5 $\log_{10}$ CFU/m*l*. Although the microbicidal activity of SAHAW with an available chlorine concentration (ACC) of 10–30 ppm was decreased by organic matter, it reduced viable *P. larvae* spores in combs more efficiently than H_2O when the comb was not as dirty. However, its efficacy on combs decreased at 4°C and when overused or highly contaminated combs were tested. WACAW with an ACC of ≥600 ppm had a higher disinfecting capacity than SAHAW, and efficiently removed *P. larvae* spores from combs even under organic matter-rich and low-temperature conditions. However, even by WACAW, the amount of viable spores in combs was not markedly reduced depending on contamination levels and *P. larvae* genotypes. These results suggest the usefulness of both disinfectants for decontaminating beekeeping equipment depending on the situations expected.

KEY WORDS: disinfection, *Melissococcus plutonius*, *Paenibacillus larvae*, slightly acidic hypochlorous acid water, weakly acidified chlorous acid water

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American foulbrood (AFB) caused by *Paenibacillus larvae* and European foulbrood (EFB) caused by *Melissococcus plutonius* are two major bacterial infectious diseases of honey bee brood. Both diseases are globally distributed including in Japan [6], and may collapse many colonies when outbreaks occur. Thus, these diseases are recognized as economically important for the apiculture industry. In both diseases, strains of the causative pathogens are classified into different genotypes. *P. larvae* strains have been classified into four ERIC types (ERIC I, II, III and IV) by repetitive-element PCR [11] and 24 sequence types (STs) by multilocus sequence typing (MLST) [16, 20, 26] (https://pubmlst.org/plarvae/), whereas *M. plutonius* strains have been classified into more than 30 STs by MLST, and the STs have been further grouped into three clonal complexes (CC3, CC12 and CC13) [2, 5, 15, 24] (https://pubmlst.org/mplutonius/). In both pathogens, strains with different genotypes have different phenotypes, including virulence [2, 11, 21]. In Japan, isolation of *P. larvae* ERIC II strains from AFB cases has been increasing recently [26], and *M. plutonius* CC12 strains have been frequently isolated from EFB cases [24].

P. larvae is a spore-forming bacterium and can produce over one billion spores in each infected larva. The spores are highly resistant to heat and chemical agents and can survive for many years in dried larval scales, hive products and equipment (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.02.02_AMERICAN_FOULBROOD.pdf). Not only in diseased colonies, *P. larvae* spores may also exist in clinically healthy colonies. Indeed, analysis of honey samples that were harvested some years before the outbreak of AFB revealed that colonies were already contaminated with spores several years before the detection of clinical symptoms [27]. It is common in beekeeping to exchange hive material like honey or brood combs between colonies

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in order to reuse hive material when setting up a new colony and to unite weak colonies to build a strong colony [10]. If the exchanged material is contaminated with *P. larvae* spores, the practice causes the spread of AFB from colony to colony. Although *M. plutonius* is not a spore-forming bacterium, it is also a durable organism. In nature, honey bee larvae become infected with *M. plutonius* through the ingestion of brood food contaminated with the pathogen, and the ingested *M. plutonius* multiplies within the larval gut; however, the infection is not always lethal. Infected larvae may survive and deposit *M. plutonius* along with their feces into the brood comb when they pupate [9]. Hence, like AFB, the combs contaminated with *M. plutonius* cause transmission of EFB to other colonies. Therefore, decontamination of beekeeping equipment, including combs, is important for the control of both foulbroods.

In Japan, several disinfection methods, such as gamma irradiation and ethylene oxide sterilization, have been used to decontaminate combs. These methods have been demonstrated to be effective for sterilization of *P. larvae* spore-contaminated materials [4, 23]; however, as dedicated facilities are required for these methods, other disinfection methods that are effective and easily performed on site have been desired. For this purpose, slightly acidic hypochlorous acid water (SAHAW), also referred to as slightly acidic electrolyzed water, has recently been introduced for the decontamination of beekeeping equipment, including combs, in Japan. SAHAW is a chlorine-based disinfectant produced by the electrolysis of dilute hydrochloric acid solution in the electrolytic cell without a separating membrane and is permitted as a food additive by the Ministry of Health, Labour and Welfare (MHLW) of Japan. The principal ingredient of SAHAW with pH values of 5.0–6.5 is hypochlorous acid (HClO), which exhibits a wide spectrum of antimicrobial activity [19, 22, 25, 28]. As SAHAW has strong antimicrobial activity with a relatively low available chlorine concentration (ACC), it has the advantage of being less corrosive for equipment, less irritating for the human body [13, 14, 19] and having less of an impact on the environment. However, in the presence of organic matter, its antimicrobial activity is known to decrease [22].

Chlorous acid ($HClO_2$) water is another chlorine-based disinfectant approved as a food additive by the MHLW of Japan. Weakly acidified chlorous acid water (WACAW) is one of the chlorous acid-based disinfectants. WACAW mainly contains three kinds of oxygenated chlorines ($HClO_2$, ClO_2^- and ClO_2). Under weakly acidified conditions (pH 5.0–6.0), $HClO_2$ plays a major role in microbial killing as a predominant species. In previous studies, WACAW was reported to inactivate a wide variety of microorganisms, including endospores of *Clostridium*, *Bacillus* and *Paenibacillus* species [12, 17]. In addition, WACAW is relatively stable under organic matter-rich conditions and retains its microbicidal activity [12, 17]. Therefore, WACAW may also be used as an effective alternative for reducing pathogen contamination of beekeeping equipment.

However, little or no information is available on the microbicidal efficacy of SAHAW and WACAW on honey bee pathogens. Therefore, we evaluated the microbicidal efficacy of the two disinfectants on representative strains of ERIC I and II *P. larvae* and CC3, CC12 and CC13 *M. plutonius in vitro*. We also used honey bee combs that were used in apiaries for 1–5 years and contained different levels of organic matter, and evaluated the efficacy of SAHAW and WACAW in killing or removing *P. larvae* spores on the combs.

MATERIALS AND METHODS

Bacterial strains and culture conditions

M. plutonius DAT606 (genotype: ST3, CC3), DAT561 (ST12, CC12) and DAT585 (ST26, CC13) and *P. larvae* DTK386 (ERIC I, ST15) and DTK384 (ERIC II, ST10) isolated from diseased European honey bee larvae were used as representative strains from each genetic group in the present study. The genotypes of the strains were obtained from previous studies [16, 24]. *M. plutonius* strains were cultured on KSBHI agar [1] at 35°C under anaerobic conditions using the AnaeroPack System (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *P. larvae* strains were cultured on MYPGP agar [3] or Columbia agar (BBL; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) supplemented with 5% defibrinated sheep blood at 35–37°C under air plus 5% CO_2 conditions.

Preparation of M. plutonius inoculum

M. plutonius strains cultured for two days were collected by sterile cotton swabs, suspended in sterile H_2O and adjusted to the optical density at 600 nm of approximately 1.98. In order to remove medium components, we collected bacterial cells from 1 m*l* of the suspension by centrifugation (15,000 rpm, 5 min) and removed the supernatants. The *M. plutonius* pellet was then suspended in sterile H_2O and adjusted to approximately 1×10^8 CFU/m*l*.

Preparation of P. larvae spores

P. larvae spores were prepared according to standard methods for American foulbrood research [3] with some modifications. *P. larvae* strains grown on agar plates were suspended in MYPGP broth, and appropriately diluted bacterial suspensions were spread onto MYPGP agar plates. The plates were incubated at 35–37°C for 12–20 days under air plus 5% CO_2 conditions. After sporulation of more than 70–90% cells was confirmed by staining with malachite green, spores were collected from 10 or more agar plates, suspended in cold sterile H_2O and washed four times with cold sterile H_2O by collecting spores via centrifugation (12,000 × g, 15 min, 4°C), discarding the supernatant and suspending the spore pellet in 30 m*l* of cold sterile H_2O. Washed spores were suspended in 5–10 m*l* of cold sterile H_2O and stored at 4°C. One week or more later, the spores were washed several more times with 30 m*l* of cold sterile H_2O to remove organic matter from lysed vegetative cells. The re-washed spores were suspended in 5–10 m*l* of cold sterile H_2O and stored at 4°C until use. In some cases, amphotericin B was added to H_2O for the first washing at a final concentration of 16.8 μg/m*l* to avoid fungal contamination. Heat resistant and germinable spore concentrations were measured

by heating a portion of the spore suspension at 65°C/80°C for 15 min, plating serial dilutions of the spore suspension on MYPGP agar plates and counting colonies on the plates after incubation at 35–37°C for seven days under air plus 5% CO_2 conditions.

Disinfectants

SAHAW (ACC, 10–30 ppm) was purchased from HOCL Inc. (Fujisawa, Japan). WACAW (ACC, 2,400 ppm) was kindly supplied by HONBUSANKEI Co., Ltd. (Osaka, Japan). WACAW was diluted in sterile H_2O as necessary. The disinfectants were protected from light and stored at 4°C until use.

Suspension tests

Approximately 1×10^8 CFU/m*l* of *M. plutonius* inoculum was prepared as described above. Approximately 1×10^7 CFU/m*l* of *P. larvae* spore inoculum was prepared by diluting the spore suspension in sterile H_2O and heating it at 80°C for 15 min. The final bacterial concentration in each inoculum was measured in each test by culturing serial dilutions of the inoculum at 35°C on KSBHI agar for four days under anaerobic conditions (for *M. plutonius*) or MYPGP agar for seven days under air plus 5% CO_2 conditions (for *P. larvae*). The results are shown in Supplementary Tables 1 and 2. As interfering conditions, 5% honey, 1% yeast extract plus 1% bovine serum albumin (BSA) (simulated high-level soiling conditions in the veterinary area [7]), 0.3% BSA (simulated low-level soiling conditions in the veterinary area/dirty conditions in food, industrial, domestic and institutional areas [7, 8]) and 0.03% BSA (simulated clean conditions in food, industrial, domestic and institutional areas [8]) were tested. Yeast extract and BSA were purchased from Becton, Dickinson and Co. and Sigma-Aldrich (St. Louis, MO, USA), respectively. The interfering substances were prepared as 10 × concentrated solutions and sterilized by filtration through a membrane with a pore size of 0.2 or 0.22 μm.

For evaluation of the microbicidal effects of SAHAW and WACAW on the foulbrood pathogens, the bacterial inoculums, 10 × concentrated organic matter solutions/sterile H_2O (for no organic matter conditions) and the disinfectants were mixed in 1.5-m*l* tubes at a ratio of 1:1:8, and the mixtures were incubated at 20°C. As controls, sterile H_2O was used instead of the disinfectants. At the end of each treatment time (5 min, 1 hr, 6 hr and 24 hr), a portion of the mixture was transferred to a sterile tube containing the same volume of neutralizing solution (3% yeast extract plus 3% BSA for SAHAW and 0.1 M Na_2SO_3 for WACAW) to halt disinfection. After neutralization, the number of viable *M. plutonius* cells and *P. larvae* spores in the treated samples was investigated by plating serial dilutions of the neutralized samples onto KSBHI and MYPGP agar plates, respectively, and counting colonies on the plates after incubation under the conditions described above. Tests were independently repeated three times. The efficacy of disinfectants was expressed as $\log_{10}$ CFU/m*l* reduction, which was calculated by the following formula: $\log_{10}$ CFU/m*l* reduction=$\log_{10}$ (the viable cell concentration before the treatment/the viable cell concentration after the treatment).

Carriers used for carrier tests

Four combs (nos. 1–4) used as carriers were kindly provided from two apiaries where no AFB outbreak has been recorded. The absence of *P. larvae* in the combs was confirmed by collecting swab samples from five cells of each comb and culturing the samples on Columbia agar supplemented with 5% defibrinated sheep blood and MYPGP agar at 37°C for five days under air plus 5% CO_2 conditions. As controls to evaluate the efficacy of disinfectants in killing or removing of *P. larvae* spores under no organic matter conditions, 96-well microplates were used as carriers.

ATP-bioluminescence assay of combs

The amount of organic matter that remained on the surface of the combs used in this study was indirectly estimated by measuring the amount of ATP and AMP originating from organisms and their residues. The amount of ATP and AMP was measured by the ATP-bioluminescence assay using Lumitester PD-30 (Kikkoman Biochemifa Co., Tokyo, Japan) according to the manufacturer's instructions, and the results were expressed in relative light units (RLU). With the Lumitester PD-30 system, AMP can also be detected by converting it into ATP by pyruvate orthophosphate dikinase. Organic matter adhered to the internal wall and bottom of cells was collected by inserting the special cotton-tipped swab moistened with sterile H_2O into a cell, pressing the swab lightly on the bottom of the cell and rotating it three times. In total, 12 samples were collected from 12 cells for each comb. The cells used in this assay were not used for the evaluation of disinfection efficiency.

Carrier tests using combs

For the carrier tests, the combs were cut in multiple pieces, and each piece was fixed in a sterile petri dish. After heating a portion of the *P. larvae* spore suspension at 65°C for 15 min, approximately 1×10^5 or 1×10^7 CFU was inoculated in each cell of the combs or in each well of the 96-well microplates and dried in an incubator at 25°C for 17–25 hr. The number of inoculated spores was measured in each test by culturing serial dilutions of the spore suspension on MYPGP agar or Columbia agar supplemented with 5% defibrinated sheep blood at 37°C for seven days under air plus 5% CO_2 conditions, and the results are shown in Supplementary Table 3. The cells and wells were then filled with SAHAW (ACC, 10–30 ppm), WACAW (ACC, 2,400, 1,200, 600 and 300 ppm) or sterile H_2O (ACC, 0 ppm; for control) and incubated at 20°C or 4°C. In some tests, *P. larvae* spore-inoculated cells were prewashed by filling them with 40°C sterile H_2O, incubating at 25°C for 1 hr and removing the water from the cells. Prewashed cells were then dried, filled with disinfectants or H_2O and incubated at 20°C. After a 16-hr incubation, disinfectants and H_2O were removed, and the cells and wells were dried in an incubator at 25°C for approximately 16 hr. The number of viable spores that remained in the cells and wells was measured by adding 100 μ*l* of sterile H_2O into the cells and wells, pipetting up and down 50 times, and culturing serial dilutions of the collected suspensions under the conditions described above.

In carrier tests, neutralization of the disinfectants was not performed in order to remain as close as possible to actual practice. Tests were repeated at least three times using different cells. The efficacy of disinfectants was expressed as $\log_{10}$ CFU/cell or well reduction, which was calculated by the following formula: $\log_{10}$ CFU/cell or well reduction=$\log_{10}$ (the number of viable spores in inoculum/the number of viable spores recovered after the treatment).

Statistical analysis

All the statistical analyses were performed in EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [18], which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). For each experiment, Kruskal-Wallis test followed by Steel-Dwass *post-hoc* test, Welch's *t*-test or one-way ANOVA followed by Bonferroni *post-hoc* test was used depending on the type of variables and the number of levels in factors with a significance level of 0.05. In this study, the minimum detectable value of each test was used for the analyses of data below the detection limit.

RESULTS

Microbicidal activity of SAHAW and WACAW on M. plutonius in suspension tests

In the suspension tests, SAHAW with an ACC of 10–30 ppm and WACAW with that of 2,400 ppm were used, and the microbicidal activity of the disinfectants was evaluated on the representative *M. plutonius* strains of CC3, CC12 and CC13. In the absence of organic matter, both disinfectants inactivated all *M. plutonius* within 5 min (>5 $\log_{10}$ CFU/m*l* reduction) (Fig. 1 and Supplementary Table 1). SAHAW also reduced all three *M. plutonius* strains by >5 $\log_{10}$ CFU/m*l* within 5 min under 5% honey and 0.03% BSA conditions (Fig. 1A and Supplementary Table 1). Under these conditions, regardless of the *M. plutonius* genotypes, microbicidal activity of the disinfectants was significantly higher than that of H_2O (Welch's *t*-test, $P<0.01$ [in all CCs]) (Fig. 1). However, microbicidal activity of SAHAW decreased with increasing concentrations of organic matter. As the viability of some *M. plutonius* strains decreases under hypotonic conditions, viable bacterial counts decreased with time under 0.3% BSA conditions even in the absence of disinfectants (0.02–3.15 $\log_{10}$ CFU/m*l* reduction at 24 hr); however, the reduction was not promoted by the addition of SAHAW under these conditions (0.57–3.69 $\log_{10}$ CFU/m*l* reduction at 24 hr) (Welch's *t*-test at 24 hr, $P>0.15$ [in all CCs]) (Fig. 1A and Supplementary Table 1). Moreover, in the presence of 1% yeast extract plus 1% BSA, SAHAW had little microbicidal activity (0.63 $\log_{10}$ CFU/m*l* or lower reduction at 24 hr), and the reduction was not significantly different from that of H_2O (0.55 or lower at 24 hr) (Welch's *t*-test at 24 hr, $P>0.44$ [in all CCs]) (Fig. 1A and Supplementary Table 1).

WACAW was more stable under organic matter-rich conditions than SAHAW. WACAW inactivated *M. plutonius* CC3 and CC13 strains within 5 min and the CC12 strain within 1 hr (>5 $\log_{10}$ CFU/m*l* reduction) even in the presence of 0.3% BSA, and the reduction was significantly higher than that of H_2O (Welch's *t*-test, $P<0.01$ [in all CCs]) (Fig. 1B and Supplementary Table 1). Under 1% yeast extract plus 1% BSA conditions, the microbicidal effects of WACAW varied among *M. plutonius* strains. WACAW reduced viable CC13 cells with time and achieved a reduction of >5 $\log_{10}$ CFU/m*l* (i.e., below the detection limit) after 24-hr treatment. The $\log_{10}$ CFU/m*l* reduction of CC13 cells was always significantly higher than that of H_2O (Welch's *t*-test, $P<0.05$) (Fig. 1B and Supplementary Table 1). WACAW also reduced the number of viable CC3 cells to a certain extent (1.93–2.21 $\log_{10}$ CFU/m*l* reduction at 24 hr), and significantly efficient microbicidal activity was observed at 6 and 24 hr (Welch's *t*-test, $P<0.05$). However, viable CC3 cells were still detected even after 24-hr treatment (Fig. 1B and Supplementary Table 1). The CC12 strain was the most resistant to WACAW among the three strains. Although significant difference in the $\log_{10}$ CFU/m*l* reduction between WACAW and H_2O was detected at 24 hr (Welch's *t*-test, $P=0.03$), the reduction by WACAW was only 0.55 or lower (Fig. 1B and Supplementary Table 1). That is, the CC12 strain was hardly killed under the 1% yeast extract plus 1% BSA conditions.

Sporicidal activity of SAHAW and WACAW on P. larvae in suspension tests

SAHAW with an ACC of 10–30 ppm and WACAW with that of 2,400 ppm were also used for *P. larvae* spores, and their sporicidal activity was evaluated on the representative ERIC I and ERIC II strains. *P. larvae* spores were more resistant to the disinfectants than *M. plutonius*. Although SAHAW inactivated *P. larvae* spores (>5 $\log_{10}$ CFU/m*l* reduction) in the absence of organic matter, it took 1 hr for the ERIC I strain and 6 hr for the ERIC II strain (Fig. 2A and Supplementary Table 2). In the presence of organic matter, even in small amounts, SAHAW exhibited almost no sporicidal effects and achieved a reduction of only a 0.64 $\log_{10}$ CFU/m*l* at 24 hr (Fig. 2A and Supplementary Table 2).

Similar to the effects on *M. plutonius*, WACAW exhibited stronger sporicidal activity than SAHAW. In the presence of 5% honey and 0.03% BSA as well as under no organic matter conditions, WACAW exhibited significant sporicidal activity in 5 min (Welch's *t*-test, $P<0.001$) and completely sterilized spores of both genotypes (>5 $\log_{10}$ CFU/m*l* reduction) within 1 hr (Fig. 2B and Supplementary Table 2). However, even using WACAW with a high ACC (2,400 ppm), *P. larvae* spores were hardly inactivated under the 0.3% BSA and 1% yeast extract plus 1% BSA conditions (0.53 $\log_{10}$ CFU/m*l* or lower reduction at 24 hr) (Fig. 2B and Supplementary Table 2).

The efficacy of SAHAW and WACAW for the decontamination of honey bee combs

As stated above, both SAHAW and WACAW had the ability to kill foulbrood pathogens in suspension tests; however, *P. larvae* spores were more resistant to the disinfectants than *M. plutonius*, and their microbicidal activity was greatly affected by organic matter. In order to investigate the potential of these disinfectants to reduce the contamination level of combs with *P. larvae* spores, we next evaluated the efficacy of SAHAW and WACAW in killing or removing *P. larvae* spores in cells of the combs. For this test,

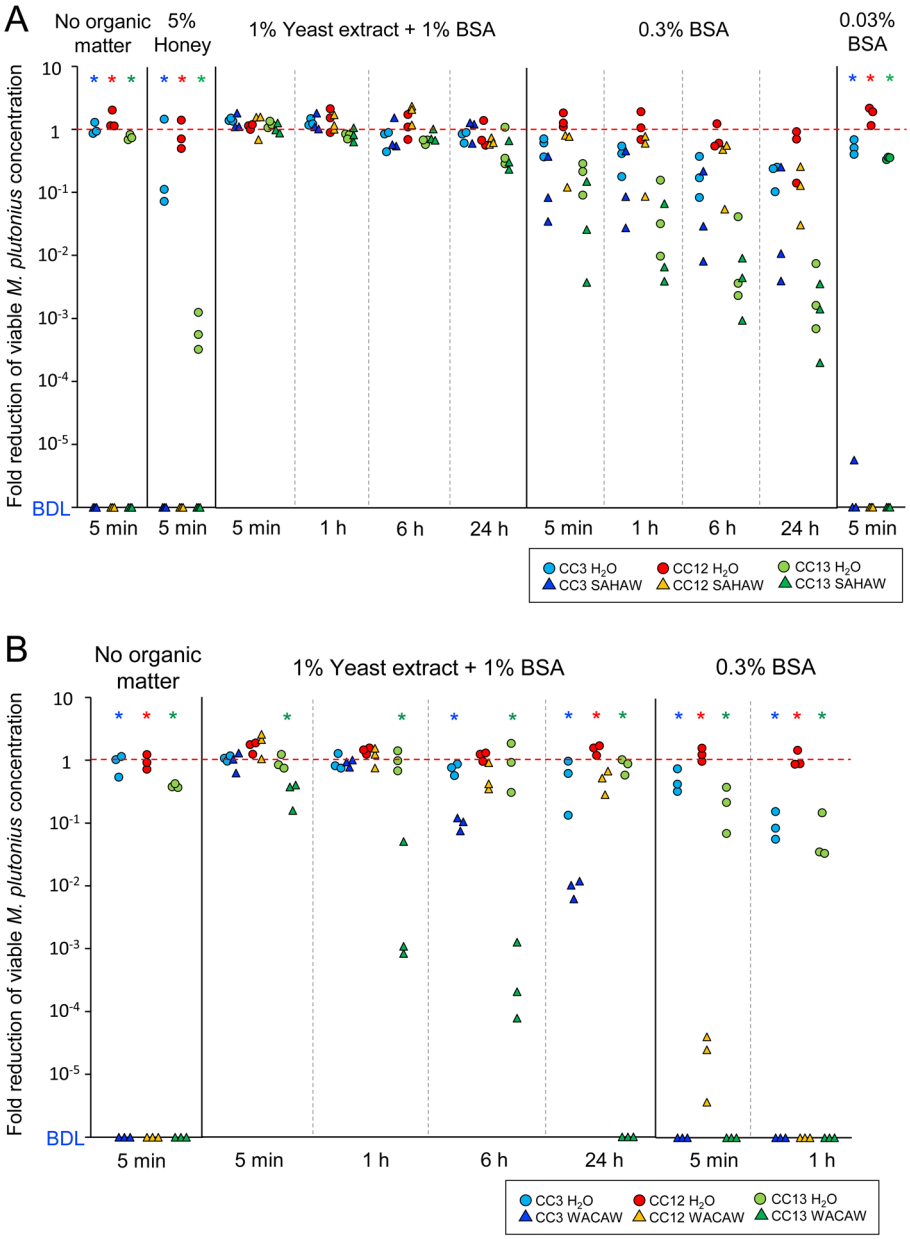

Fig. 1. Microbicidal effects of slightly acidic hypochlorous acid water (SAHAW) (available chlorine concentration [ACC], 10–30 ppm) (A) and weakly acidified chlorous acid water (WACAW) (ACC, 2,400 ppm) (B) on *Melissococcus plutonius* in suspension tests. Approximately 1×10^8 CFU/m*l* of *M. plutonius* cells, 10 × concentrated organic matter solutions/sterile H_2O and the disinfectants were mixed at a ratio of 1:1:8, and incubated at 20°C. As controls, sterile H_2O was used instead of the disinfectants. At the end of each treatment time, a portion of the mixture was mixed with the same amount of neutralizing solution. The number of remaining viable bacterial cells was investigated as described in MATERIALS AND METHODS. Data were collected from three independent tests, and the survival of *M. plutonius* strains was expressed as fold changes in viable cell concentrations. BDL represents "below the detection limit" of the test. Asterisks indicate statistically significant efficacy (P<0.05; disinfectants vs. H_2O) against clonal complex (CC) 3 (blue asterisks), CC12 (red asterisks) and CC13 (green asterisks) strains.

we used combs that have been used for one (comb nos. 1 and 2), three (comb no. 3) or five (comb no. 4) years in AFB-free apiaries in Japan. Although the amount of organic matter remaining in the cells of the combs varied according to the cells tested, the cells of the comb used for five years contained more organic matter (32,305.08 ± 6,709.82 RLU) than those of the combs used for one (5,186.08 ± 2,919.13 [no. 1] or 11,009.58 ± 1,247.41 [no. 2] RLU) or three (3,306.17 ± 714.64 RLU) years (Kruskal-Wallis test and Steel-Dwass *post-hoc* test, P<0.01) (Supplementary Fig. 1). Therefore, we regarded the combs used for one and three years and that used for five years as clean and dirty combs, respectively, for convenience. For no organic matter controls, wells of 96-well microplates were used as dummy cells. As it took a long time to inactivate all ERIC II *P. larvae* spores in suspension tests even under no organic matter conditions, we treated spore-inoculated carriers with disinfectants for 16 hr in this study.

In our carrier tests, after incubation of spore-inoculated cells/wells with disinfectants or sterile H_2O, the liquid was removed

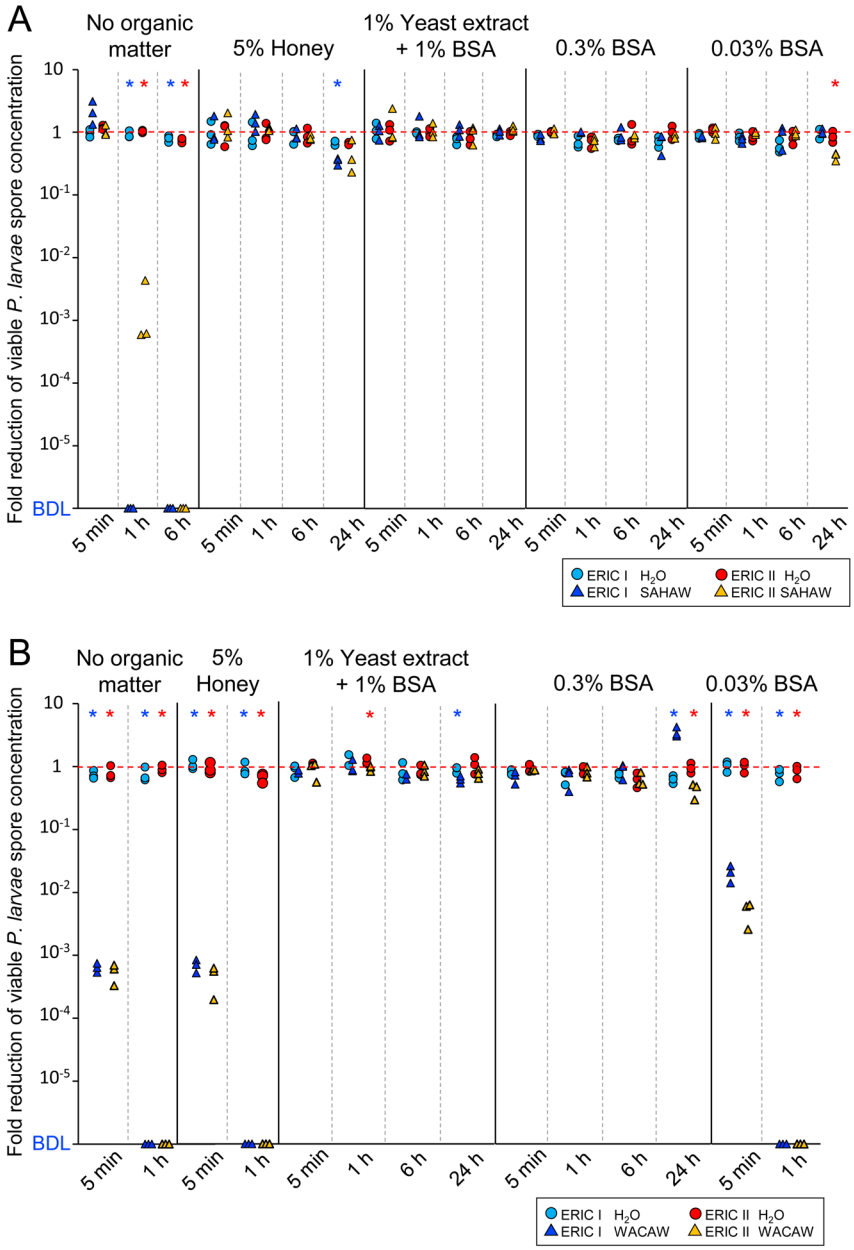

Fig. 2. Sporicidal effects of slightly acidic hypochlorous acid water (SAHAW) (available chlorine concentration [ACC], 10–30 ppm) (A) and weakly acidified chlorous acid water (WACAW) (ACC, 2,400 ppm) (B) on *Paenibacillus larvae* spores in suspension tests. Approximately 1×10^7 CFU/m*l* of *P. larvae* spores, 10 × concentrated organic matter solutions/sterile H_2O and the disinfectants were mixed at a ratio of 1:1:8, and incubated at 20°C. As controls, sterile H_2O was used instead of the disinfectants. At the end of each treatment time, a portion of the mixture was mixed with the same amount of neutralizing solution. The number of remaining viable spores was investigated as described in MATERIALS AND METHODS. Data were collected from three independent tests, and the survival of spores was expressed as fold changes in viable spore concentrations. BDL represents "below the detection limit" of the test. Asterisks indicate statistically significant efficacy (P<0.05; disinfectants vs. H_2O) against ERIC I (blue asterisks) and ERIC II (red asterisks) spores.

from the cells/wells and absorbed by paper towels by covering the cells/wells with paper towels and plastic lids, turning them over and shaking the liquid off from the cells/wells thoroughly. Therefore, even by H_2O, some of the inoculated spores were washed off, and the contamination levels with spores decreased to some extent. Indeed, when microplate wells inoculated with *P. larvae* spores at doses of 0.18–5.0 × 10^5 CFU/well were treated at 20°C for 16 hr, a reduction of 1.62 $\log_{10}$ CFU/well was observed even with H_2O (Fig. 3 and Supplementary Table 3). However, as both SAHAW (ACC, 10–30 ppm) and WACAW (ACC, 2,400 ppm) significantly more efficiently removed *P. larvae* spores from the wells of the microplates (>3.26 $\log_{10}$ CFU/well reduction) than H_2O (one-way ANOVA and Bonferroni test, P<0.001 [in both ERIC I and II]) (Fig. 3 and Supplementary Table 3), these disinfectants were confirmed to have sporicidal activity even on solid substance surfaces under the no organic matter conditions.

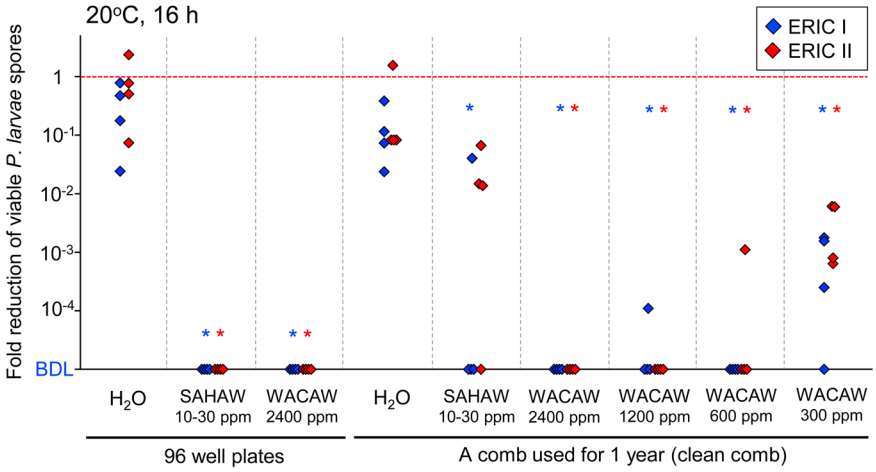

Fig. 3. Efficacy of slightly acidic hypochlorous acid water (SAHAW) (available chlorine concentration [ACC], 10–30 ppm) and weakly acidified chlorous acid water (WACAW) (ACC, 300–2,400 ppm) in killing or removing *Paenibacillus larvae* spores in cells of a comb used for one year (a clean comb). As controls, 96-well microplates were also used as carriers. Approximately 1×10^5 CFU of *P. larvae* spores was inoculated in each cell of the comb or in each well of 96-well microplates. After drying the cells and wells, they were filled with disinfectants and incubated at 20°C for 16 hr. As controls, sterile H_2O was used instead of the disinfectants. The number of remaining viable spores was investigated as described in MATERIALS AND METHODS. Data were collected from four independent tests, and the survival of spores was expressed as fold changes in viable spore numbers. BDL represents "below the detection limit" of the test. Asterisks indicate statistically significant efficacy of disinfectants (P<0.05; disinfectants vs. H_2O) against ERIC I (blue asterisks) and ERIC II (red asterisks) spores.

When a clean comb inoculated with *P. larvae* spores at doses of 0.18–5.0 × 10^5 CFU/cell was used, some of the spores were also removed from the cells physically by H_2O after incubation at 20°C for 16 hr; however, the $\log_{10}$ CFU/cell reduction was only 1.63 or lower (Fig. 3 and Supplementary Table 3). Under the same conditions, SAHAW achieved a reduction of >3.89–4.08 $\log_{10}$ CFU/cell (below the detection limit) in four of the eight cells used for the test, but the efficacy differed according to the cells, and SAHAW did not efficiently remove the spores from the other four cells (1.18–1.86 $\log_{10}$ CFU/cell reduction) (Fig. 3 and Supplementary Table 3). The efficacy of SAHAW may also differ according to the *P. larvae* genotypes. When ERIC I spores were inoculated, viable spores were eliminated from three of the four cells by SAHAW, and the efficacy was significantly higher than that of H_2O (Welch's *t*-test, P=0.03). On the other hand, although SAHAW always reduced ERIC II spores (1.18–>4.04 $\log_{10}$ CFU/cell reduction) more efficiently than H_2O (−0.19–1.08 $\log_{10}$ CFU/cell reduction) (Supplementary Table 3), statistically significant difference was not detected between SAHAW and H_2O (Welch's *t*-test, P=0.098). In contrast, WACAW removed *P. larvae* spores significantly more efficiently than H_2O regardless of its ACC and ERIC types (one-way ANOVA and Bonferroni test, P<0.05), and viable spores were eliminated (i.e., >3.26–4.70 $\log_{10}$ CFU/cell reduction) from most of the cells tested after treatment of the cells with WACAW at an ACC of 600–2,400 ppm (Fig. 3 and Supplementary Table 3).

On the other hand, when a dirty comb inoculated with *P. larvae* spores at doses of 0.18–5.0 × 10^5 CFU/cell was used, SAHAW did not exhibit strong spore-removal effects in all tests. The $\log_{10}$ CFU/cell reduction by SAHAW (0.18–2.08) was similar to that by H_2O (−0.38–2.02) (Fig. 4 and Supplementary Table 3), and no significant difference was observed in the reduction between SAHAW and H_2O (Welch's *t*-test, P=0.95 [in ERIC I] and 0.36 [in ERIC II]). In contrast, the $\log_{10}$ CFU/cell reduction by WACAW at an ACC of 2,400, 1,200 or 600 ppm (2.97–>4.70, 3.36–>4.30 and 2.95–>4.30, respectively) was significantly higher than that of H_2O and SAHAW (one-way ANOVA and Bonferroni test, P<0.01 [in both ERIC I and II]). However, viable spores were not completely removed from approximately half of the cells tested. At an ACC of 300 ppm, viable spores were detected from all the treated cells (the $\log_{10}$ CFU/cell reduction, 0.74–4.30), and no significant difference was observed in the reduction when compared with that of H_2O (one-way ANOVA and Bonferroni test, P=0.60 [in ERIC I] and 0.13 [in ERIC II]) (Fig. 4 and Supplementary Table 3).

As combs may be maintained, including disinfection, during winter, we also evaluated the efficacy of SAHAW and WACAW at 4°C. For this test, a clean comb was used as a carrier. *P. larvae* spores were inoculated to wells/cells at doses of 1.2–3.6 × 10^5 CFU/well or cell, and the carriers were treated with SAHAW or WACAW for 16 hr. In 96-well microplates, both disinfectants reduced viable spores (≥4.0 $\log_{10}$ CFU/well reduction) more efficiently than H_2O (0.25–1.44 $\log_{10}$ CFU/well reduction) (one-way ANOVA and Bonferroni test, P<0.001 [in both ERIC I and II]), and no viable spore was detected from most of wells after treatment with the disinfections (Fig. 5 and Supplementary Table 3). However, under the low temperature conditions, SAHAW did not decontaminate the comb efficiently, and the $\log_{10}$ CFU/cell reduction (0.70–1.90) was similar to that by H_2O (0.37–1.31) (Fig. 5 and Supplementary Table 3) (Welch's *t*-test, P=0.24 [in ERIC I] and 0.30 [in ERIC II]). In contrast, WACAW more efficiently removed *P. larvae* spores from the comb than SAHAW even at 4°C, and viable spores in inoculated cells became undetectable (>4.08 $\log_{10}$ CFU/cell reduction) after treatment at an ACC of 1,200–2,400 ppm. At 600 ppm, WACAW reduced ERIC I and ERIC II *P. larvae* by 3.43–>4.30 and 1.94–>4.20 $\log_{10}$ CFU/cell, respectively, and the efficacy was significantly higher

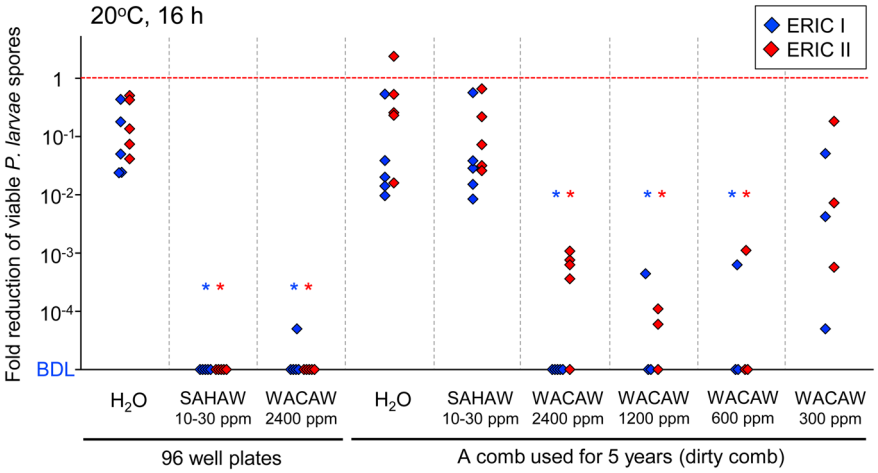

Fig. 4. Efficacy of slightly acidic hypochlorous acid water (SAHAW) (available chlorine concentration [ACC], 10–30 ppm) and weakly acidified chlorous acid water (WACAW) (ACC, 300–2,400 ppm) in killing or removing *Paenibacillus larvae* spores in cells of a comb used for five years (a dirty comb). As controls, 96-well microplates were also used as carriers. Approximately 1×10^5 CFU of *P. larvae* spores was inoculated in each cell of the comb or in each well of 96-well microplates. After drying the cells and wells, they were filled with disinfectants and incubated at 20°C for 16 hr. As controls, sterile H_2O was used instead of the disinfectants. The number of remaining viable spores was investigated as described in MATERIALS AND METHODS. Data were collected from at least three independent tests, and the survival of spores was expressed as fold changes in viable spore numbers. BDL represents "below the detection limit" of the test. Asterisks indicate statistically significant efficacy of disinfectants (P<0.05; disinfectants vs. H_2O) against ERIC I (blue asterisks) and ERIC II (red asterisks) spores.

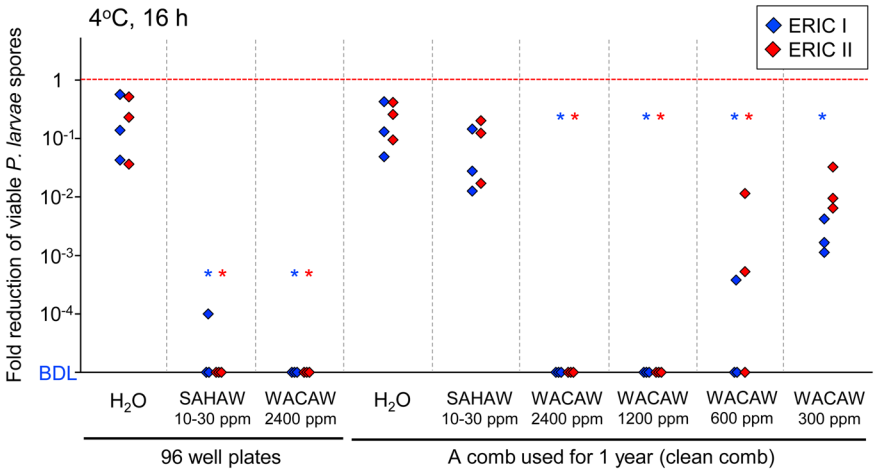

Fig. 5. Efficacy of slightly acidic hypochlorous acid water (SAHAW) (available chlorine concentration [ACC], 10–30 ppm) and weakly acidified chlorous acid water (WACAW) (ACC, 300–2,400 ppm) in killing or removing *Paenibacillus larvae* spores in cells under low temperature conditions (i.e., simulated winter conditions). A comb used for one year (a clean comb) and 96-well microplates were used as carriers. Approximately 1×10^5 CFU of *P. larvae* spores was inoculated in each cell of the comb or in each well of 96-well microplates. After drying the cells and wells, they were filled with disinfectants and incubated at 4°C for 16 hr. As controls, sterile H_2O was used instead of the disinfectants. The number of remaining viable spores was investigated as described in MATERIALS AND METHODS. Data were collected from three independent tests, and the survival of spores was expressed as fold changes in viable spore numbers. BDL represents "below the detection limit" of the test. Asterisks indicate statistically significant efficacy of disinfectants (P<0.05; disinfectants vs. H_2O) against ERIC I (blue asterisks) and ERIC II (red asterisks) spores.

than that of H_2O and SAHAW (one-way ANOVA and Bonferroni test, P<0.05 [in both ERIC I and II]) (Fig. 5 and Supplementary Table 3). Even at 300 ppm, ERIC I and ERIC II spores were reduced by 2.38–2.95 and 1.49–2.19 $\log_{10}$ CFU/cell, respectively; however, when compared with H_2O, significant reduction was detected only in ERIC I spores (one-way ANOVA and Bonferroni test, P<0.001 [in ERIC I] and P=0.31 [in ERIC II]) (Fig. 5 and Supplementary Table 3).

P. larvae can produce over one billion spores in each infected larva (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.02.02_AMERICAN_FOULBROOD.pdf); therefore, we inoculated high doses ($0.78–4.0 \times 10^7$ CFU) of *P. larvae* spores to each cell of clean combs assuming highly contaminated combs and investigated the efficacy of the disinfectants on decontamination

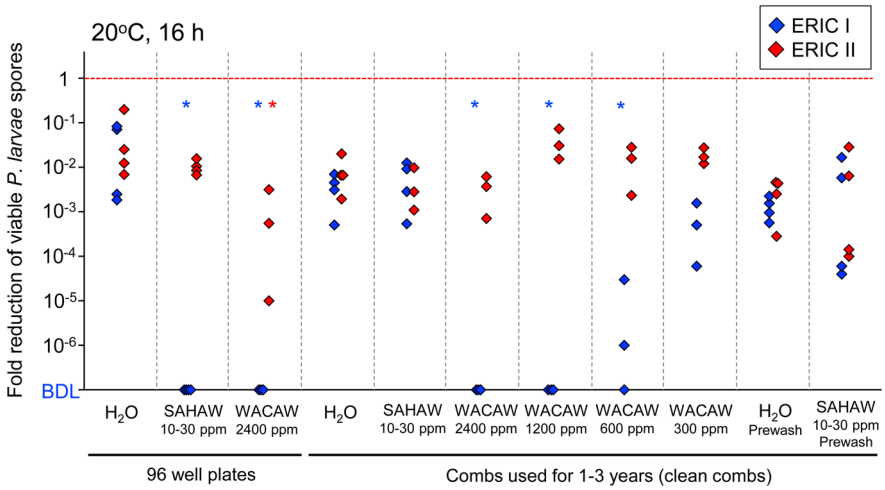

Fig. 6. Efficacy of slightly acidic hypochlorous acid water (SAHAW) (available chlorine concentration [ACC], 10–30 ppm) and weakly acidified chlorous acid water (WACAW) (ACC, 300–2,400 ppm) in killing or removing a large quantity of *P. larvae* spores in cells. Combs used for 1–3 years (clean combs) and 96-well microplates were used as carriers. Approximately 1×10^7 CFU of *P. larvae* spores was inoculated in each cell of the combs or in each well of 96-well microplates and dried. In some tests, cells inoculated with *P. larvae* spores were prewashed with 40°C sterile H_2O. The cells and wells were then filled with disinfectants and incubated at 20°C for 16 hr. As controls, sterile H_2O was used instead of the disinfectants. The number of remaining viable spores was investigated as described in MATERIALS AND METHODS. Data were collected from at least three independent tests, and the survival of spores was expressed as fold changes in viable spore numbers. BDL represents "below the detection limit" of the test. Asterisks indicate statistically significant efficacy of disinfectants ($P<0.05$; disinfectants vs. H_2O) against ERIC I (blue asterisks) and ERIC II (red asterisks) spores.

of the cells. Under no organic matter conditions (i.e., in microplate wells), viable ERIC I spores became undetectable (>5.89 $\log_{10}$ CFU/well reduction) following treatment with SAHAW (ACC, 10–30 ppm) or WACAW (ACC, 2,400 ppm), and the efficacy was significantly higher than that of H_2O (one-way ANOVA and Bonferroni test, $P<0.001$) (Fig. 6 and Supplementary Table 3). However, both disinfectants did not work well on ERIC II spores. The $\log_{10}$ CFU/well reduction by SAHAW (1.81–2.17) was not significantly different from that by H_2O (0.70–2.16) (one-way ANOVA and Bonferroni test, $P=1$) (Fig. 6 and Supplementary Table 3). In addition, although WACAW reduced ERIC II spores more efficiently (2.51–4.90 $\log_{10}$ CFU/well reduction) than H_2O (one-way ANOVA and Bonferroni test, $P<0.05$), it could not eliminate viable spores completely.

On the highly contaminated combs, SAHAW was ineffective against both ERIC I and ERIC II spores (1.91–3.28 and 2.01–2.97 $\log_{10}$ CFU/cell reduction, respectively), and the degree of reduction was similar to that by H_2O (2.16–3.30 and 1.70–2.72 $\log_{10}$ CFU/cell reduction for ERIC I and ERIC II, respectively) (one-way ANOVA, $P>0.5$ [in both ERIC I and II]) (Fig. 6 and Supplementary Table 3). Before SAHAW treatment, we added a prewashing step with 40°C sterile H_2O as described in MATERIALS AND METHODS; however, the prewashing of the combs did not improve the disinfecting effects by SAHAW (one-way ANOVA, $P>0.5$ [in both ERIC I and II]) (Fig. 6 and Supplementary Table 3). In contrast, WACAW at an ACC of 600–2,400 ppm sterilized or sanitized the highly contaminated combs more efficiently than H_2O, but only when they were contaminated with ERIC I spores ($\log_{10}$ CFU/cell reduction, >5.89 at 2,400 and 1,200 ppm [below the detection limit] and ≥4.48 at 600 ppm) (one-way ANOVA and Bonferroni test, $P<0.001$). Consistent with the results in microplate wells, ERIC II spores were more resistant to WACAW than ERIC I spores under the highly contaminated conditions, and even at an ACC of 2,400 ppm, the degree of reduction by WACAW (2.21–3.15 $\log_{10}$ CFU/cell reduction) was similar to that by H_2O (1.70–2.72 $\log_{10}$ CFU/cell reduction) (one-way ANOVA and Bonferroni test, $P=1$) (Fig. 6 and Supplementary Table 3).

DISCUSSION

SAHAW is recognized as a safe disinfectant [13, 14, 19] and can be produced on-site using purchasable compact generators at a relatively low cost, thus it began to be used to decontaminate beekeeping equipment in Japan. When a questionnaire survey on disinfectants was carried out in a region in Japan, 30% of respondent beekeepers used SAHAW and 23% of respondents planned on using it (Ohashi, I., unpublished data). In our study, under no organic matter conditions, SAHAW sterilized *M. plutonius* quickly (Fig. 1A). This disinfectant was also effective for *P. larvae* spores if the spores were exposed to it for several hours (Fig. 2A). Even under 5% honey and 0.03% BSA conditions, SAHAW exhibited strong antimicrobial activity against *M. plutonius* (Fig. 1A). In addition, SAHAW reduced the contamination levels of *P. larvae* spore-inoculated cells more efficiently than H_2O when a relatively clean comb was used as a carrier (Fig. 3). These results suggest that SAHAW is worth considering as an option to reduce the number of foulbrood pathogens in combs when the combs are relatively clean and not old. However, the efficacy of SAHAW is not 100% even under such desirable conditions. Indeed, some cells in the relatively clean comb were not effectively decontaminated by

SAHAW (Fig. 3). As the amount of remaining organic matter varied among cells in a comb, as shown in Supplementary Fig. 1, the cells that were not well decontaminated by SAHAW may contain more organic matter than well decontaminated cells. In addition, the efficacy of SAHAW markedly decreased when disinfection was performed at 4°C and when overused or highly contaminated combs were used as carriers (Figs. 4–6). Of note, comb no. 2 was used under low temperature conditions. Although it was only used for one year and categorized as a clean comb, its cells contained slightly more organic matter than those of the other clean combs (nos. 1 and 3) (Supplementary Fig. 1). In addition, even under the low temperature conditions, SAHAW removed *P. larvae* spores from 96-well microplates significantly more efficiently than H_2O (one-way ANOVA and Bonferroni test, $P<0.001$) (Fig. 5); therefore, we cannot rule out the possibility that the reduced effects of SAHAW at 4°C were partially due to the organic matter remaining in comb no. 2. Even if this is the case, when low temperature conditions are expected, the reduction of SAHAW efficacy needs to be taken into consideration to prevent AFB efficiently.

WACAW had a higher disinfecting capacity than SAHAW, especially under the organic matter-rich conditions and at low temperatures (Figs. 4 and 5). The ACC of WACAW used in this study (300–2,400 ppm) was higher than that of SAHAW (10–30 ppm). As the bactericidal effects of chlorine-based disinfectants usually increase with a higher ACC, the high disinfecting capacity of WACAW may have simply been due to the high ACC. In addition, the stable microbicidal activity of WACAW under organic matter-rich conditions may have resulted from the large dissociation of total chlorine level and free available chlorine (FAC) level in the disinfectant [12]. WACAW mainly contains $HClO_2$, ClO_2^- and ClO_2 as oxygenated chlorines. ClO_2^- has very weak oxidative potential and makes only a minor contribution to the FAC level and antimicrobial killing. In WACAW, the ClO_2^- content is considered to be much higher than that of $HClO_2$ and ClO_2 [12]. This ClO_2^- may continuously supply $HClO_2$ even after FAC is consumed from contact with microorganisms or organic matter [12]. Although WACAW has not been used in apiculture, it was approved as a food additive in 2013 in Japan and has since been applied to food and environmental sanitation [12, 17]. The maximum $HClO_2$ concentration approved as a food additive is 400 ppm, which corresponds to an ACC of approximately 800 ppm according to the manufacturer (HONBUSANKEI Co., Ltd.). In the present study, WACAW with an ACC of 600 ppm worked well for removing viable *P. larvae* spores from combs, suggesting WACAW as an alternative safe and promising disinfectant for beekeeping equipment. However, for efficient decontamination of combs, all cells in the combs have to be filled with disinfectants, thus a relatively large amount of disinfectants is needed in apiaries. WACAW products are purchasable, but it cannot be produced on-site because its generator is not on the market; therefore, the use of WACAW is currently more expensive than SAHAW.

Of note, the efficacy of the disinfectants varied between different strains. In suspension tests using *M. plutonius*, the representative strain of CC12 was the most resistant to the disinfectants among the three strains tested and was hardly killed under the 1% yeast extract plus 1% BSA conditions even by WACAW with an ACC of 2,400 ppm (Fig. 1B). Microbicidal effects of the disinfectants tested are attributable to their oxidative potential. Interestingly, although *M. plutonius* CC3 and CC13 strains tested (DAT606 and DAT585, respectively) cannot grow under aerobic conditions, DAT561 of CC12 can grow aerobically [1], implying that DAT561 is more resistant to oxidative stress than DAT606 and DAT585. This different oxidative stress susceptibility might have resulted in different disinfectant susceptibility between the strains. In suspension tests with *P. larvae*, inactivation of the ERIC II spores by SAHAW took longer than inactivation of the ERIC I spores under no organic matter conditions (Fig. 2A). Moreover, it was more difficult to remove viable ERIC II spores from highly contaminated combs than ERIC I spores (Fig. 6). Of note, it was more difficult to produce spores of the representative ERIC II strain used in this study on MYPGP agar plates than the ERIC I strain. Although we devised methods for spore preparation to reduce remaining vegetative cells and their debris by extending the culture time of the ERIC II strain and washing spore solutions repeatedly at intervals of one or more weeks, we cannot rule out the possibility that ERIC II spore solutions contained more remaining impurities (vegetative cells and their debris) than ERIC I spore solutions, and the impurities may have reacted with available chlorine, resulting in the loss of sporicidal activity of the disinfectants. To show clear correlation between bacterial genotypes and disinfectant susceptibility, it is necessary to test at least several strains for each genotype. As we tested only a single strain from each genetic group, this result may have nothing to do with the genotypes. However, *M. plutonius* CC12 and *P. larvae* ERIC II strains have been involved in many foulbrood cases in Japan [24, 26], thus the lower disinfectant susceptibility of strains with these genotypes than the others may become a problem for efficient prevention of AFB in the field.

In conclusion, if combs to be disinfected are not old and relatively clean (e.g., when the average RLU of the cells by the Lumitester PD-30 system is approximately 10,000 or less), SAHAW, which is available at a relatively low cost, will be a useful option to reduce contamination levels of foulbrood pathogens. However, even under such favorable conditions, disinfection with SAHAW should be carried out on a warm day. In addition, although prewashing of combs did not improve the disinfecting effects by SAHAW on highly contaminated combs in our study (Fig. 6), we consider prewashing of combs to be prerequisite for removing visible content from the cells and maximizing the effects of SAHAW. If the combs are old or not clean, WACAW with an ACC of 600 ppm or higher will more effectively reduce contamination levels than SAHAW. Strong spore-removal effects of WACAW can also be expected at low temperatures. However, even if WACAW is used, it may not be sufficient for the prevention of foulbroods depending on contamination levels and genotypes of bacterial strains. Therefore, for effective control of foulbroods, it is important to use different disinfectants and other control methods (e.g., replacement of combs, gamma irradiation and ethylene oxide sterilization) properly according to the conditions and available budget. Honey bees are affected not only by foulbroods but also other infectious diseases, including chalkbrood by the fungus *Ascosphaera apis*, nosemosis by the microsporidian *Nosema apis* and *N. ceranae* and various virus infections. The two disinfectants used in this study may also be useful to control these diseases, although there is little or no information regarding the effects of SAHAW and WACAW on other honey bee pathogens. Further studies using the disinfectants will provide additional useful information for infectious disease control in apiculture.

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