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Bactericidal efficacy of a quaternary ammonium compound with food additive grade calcium hydroxide toward *Salmonella* Infantis and *Escherichia coli* on abiotic carriers

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ABSTRACT. The bactericidal efficacies of 0.2% food additive grade calcium hydroxide ($FdCa(OH)_2$) solution, a quaternary ammonium compound (QAC) diluted at 1:500 (QACx500) and their mixture–Mix500 ($FdCa(OH)_2$ powder added at final concentration 0.2% to QACx500)–were investigated at two different temperatures (room temperature (RT) (25 ± 2°C) and 2°C), using varying contact time, with or without presence of organic materials (5% fetal bovine serum: FBS), either in suspension or on abiotic carrier (steel, rubber and plastic). In the suspension test, QACx500 could inactivate *Salmonella* Infantis at effective level (≥3 log reductions), within 30 sec and 5 sec, respectively, with or without 5% FBS at RT; however, at 2°C it required 30 min and 1 min, respectively. Mix500 revealed the same efficacy as QACx500 at RT, but, at 2°C it required 1 min and 30 sec, respectively with or without FBS. Whereas, 0.2% $FdCa(OH)_2$ solution alone could inactivate *S.* Infantis within 1 min and 3 min, respectively at RT and 2°C, even with 5% FBS. In the carrier test, single disinfectant required bit more (3 or 5 min) contact time to reduce bacterial load (*S.* Infantis or *Escherichia coli*) down to the effective level on rubber surface than that on steel and plastic surface. However, Mix500 could inactivate both bacteria on carrier surfaces within 1 min, even at 2°C. Thus, synergistic effects were observed in the suspension test and the carrier test at both temperatures toward both bacteria.

KEY WORDS: abiotic carrier, bacterial pathogen, food additive grade calcium hydroxide ($FdCa(OH)_2$), quaternary ammonium compound, synergistic effect

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Salmonellosis and colibacillosis are considered to be major bacterial diseases that are communicable to humans [23]. Salmonellosis is one of the most common food-borne zoonoses and a major public health concern. The main sources of *Salmonella* infection for humans are foods of animal origin, particularly consumption of contaminated poultry meat and eggs [2, 7, 11, 17].

Enhancement of biosecurity at farm level is very important to keep domestic animals free from pathogens. For the proper biosecurity at poultry farm level, disinfection of pathogens on cars, boots or plastic cages is hence essential. Several studies have been carried out on the routes of *Salmonella* Enteritids transmission via infected animals, contaminated materials, insects, and soils [44]; however, plastic poultry transport cages possibly constitute a potential route of *Salmonella* transmission among farms [36, 37, 49]. Therefore, the levels of pathogenic microorganisms on the poultry cages that enter the farm need to be reduced to avoid chick infection. Boots of farm employees and visitors can act as mechanical carriers for pathogens transmission and spreading of diseases among farms or barns [31, 36]. Cross contamination of broilers may occur once flocks are placed into transportation coops containing feces from previously transported broilers. Dirty coops harbor microorganisms on floor surfaces and become a vector for cross contamination [25].

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Disease prevention and control largely depend on biosecurity, and disinfectants are very important components of the biosecurity program designed by the poultry industry [40]. Adequate and correct disinfection procedures can reduce the incidence of infectious diseases and their transmission. Several environmental factors, including organic load, temperature, and contact time are responsible for altering the kinetics of disinfection [20, 35].

Quaternary ammonium compounds (QACs) are cationic detergents, widely used as common disinfectants at animal farms and food processing industries, owing to their relatively low toxicity and broad antimicrobial spectrum, but their inactivation efficacies are usually diminished by organic materials contamination or at low temperature [9, 12, 19, 40]. Sodium hypochlorite (NaOCl) is also popular, but its efficacy is also inadequate in the presence of organic materials [16, 45]. Food additive grade calcium hydroxide ($FdCa(OH)_2$) is a strong alkaline agent with high pH (pH 12.7); it is a relatively novel product among materials that can inactivate pathogens, which demonstrated excellent bactericidal efficacies in the feces, in order to prevent environmental contamination [14, 15, 38, 45]. It could also inactivate bacteria on the egg shell [1]. Recently, we demonstrated the synergistic effect of $FdCa(OH)_2$ together with NaOCl, accounting for their bactericidal efficacies under organic materials contaminated conditions [45], and with QAC accounting for their virucidal efficacies under the low temperature conditions [18].

In the present study, the bactericidal efficacies of QAC and of $FdCa(OH)_2$ alone and their combination were evaluated at two different temperatures (room temperature (RT) (25 ± 2°C) and 2°C), with varying contact times, either with or without organic loads in suspension test, toward *S.* Infantis. In addition, the synergistic bactericidal efficacies between QAC and $FdCa(OH)_2$ were evaluated toward *S.* Infantis and *Escherichia coli* on abiotic carriers (rubber, steel and plastic).

MATERIALS AND METHODS

Application of chemical disinfectants and neutralizers

A QAC (Rontect®), kindly supplied by Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan), was diluted 1:500 (QACx500) with redistilled water (dW_2) to obtain a final concentration of 200 ppm didecyl-dimethylammonium chloride (DDAC), as recommended by the manufacturer. $FdCa(OH)_2$ powder at pH 13, made of natural calcium carbonates derived from limestone through calcination process, with average diameter of powder particles being 10 μm [15, 45], was kindly provided by Fine Co., Ltd. (Tokyo, Japan). For making 0.2% $FdCa(OH)_2$ solution, 200 mg of $FdCa(OH)_2$ powder was added to 100 m*l* of dW_2 or 100 m*l* of QACx500 and then centrifuged at 1,750 × g for 10 min at 4°C. The resulting supernatants were used as 0.2% $FdCa(OH)_2$ solution or QAC and $FdCa(OH)_2$ mixture (Mix500), respectively. For experiments at RT, all solutions were kept at RT for at least 30 min. The RT was maintained at 25 ± 2°C using an air-conditioner. For low temperature experiments, all solutions and bacteria were kept on ice for at least 30 min, until the temperature of the solutions became 2 ± 0.5°C. These solution temperatures were confirmed with bar thermometers. Chemical neutralizers, namely, a blocking solution, was prepared by adding 30% fetal bovine serum (FBS) in 0.7 M Tris–HCl (pH 7.2), and used after fixed contact time of disinfectant application, to stop the inactivation reaction.

Preparation of bacteria-contaminated carriers

Three kinds of carriers with different surface structure types, namely, rubber, steel and plastic, were purchased from local markets and used in this study. These materials are commonly used in the vehicle tires, boots, farm equipments or tracks and livestock farm (feeder, water pots, egg trays and chicken transport cages), respectively. All the carrier coupons were cut into small pieces (around 5.0 × 5.0 cm). The pieces were washed with tap water first, and then with sterile dW_2, to remove visible dirt from the carrier surfaces, if any. All the washed carriers were dried in a laminar flow hood for at least 15 min, and stored at RT until used. On the day of the experiment, carriers were washed with dW_2 again, dried and autoclaved at 121°C for 15 min, but in case of plastic coupons, they were sterilized by microwave oven for 3 min.

Bacterial suspensions of *E. coli* strain NBRC106373 purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC) (Chiba, Japan) and *S.* Infantis (kindly provided by Prof. Hiroshi Fujikawa, Laboratory of Public Health, Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Tokyo, Japan) were prepared and enumerated as described previously [13].

Each sterile carrier was placed separately in a 90 mm petri-dish under a biological safety cabinet; then, 100 μ*l* of aforementioned freshly prepared bacterial suspensions (approximately 8 $\log_{10}$ CFU/m*l*) mixed with 5% FBS were inoculated, respectively on the surfaces of each carrier. Then, the bacterial suspension was spread by sterile glass spreader and air-dried inside the biological safety cabinet for 60 min at RT, using air-flow with fluorescence light off, for the proper attachment of bacteria onto carrier surfaces. After drying, these artificially contaminated carriers were used for the treatment purpose so as to explore the inactivation efficiency against bacteria of the above mentioned treatment solutions at two different temperatures (RT and 2°C) with various contact times (as detailed below).

Suspension test for evaluating the bactericidal activities of the treatment solutions against S. Infantis in aqueous phase

For the inactivation evaluation toward *S.* Infantis in aqueous phase, two different reaction temperatures (RT and 2°C) and a number of contact times were investigated. In the absence of organic materials, four hundred micro-litters of each tested solution (QACx500, 0.2% $FdCa(OH)_2$ solution or Mix500) were mixed with 100 μ*l* of *S.* Infantis in a microtube, incubated at indicated time (5, 30 sec, 1 and 3 min) at RT or on ice. Following incubation, inactivation of bacteria was stopped by adding 500 μ*l* of the blocking solution [33]. On the other hand, to evaluate the inactivating capacity of these solutions in the presence of organic materials, 25 μ*l* of FBS (5% at the final concentration in the reaction) was added into the microtube containing 375 μ*l* of each

Table 1. Bactericidal efficacies of $FdCa(OH)_2$, a quaternary ammonium compound and their mixture toward *Salmonella* Infantis in the aqueous phase

Solution	Temp (°C)	FBS (%)	Positive control	Bacterial titer (log_{10} CFU/m*l*) at different contact times						
				0 sec	5 sec	30 sec	1 min	3 min	20 min	30 min
$FdCa(OH)_2$[a)]	25	0	8.88 ± 0.05[d)]	8.65 ± 0.10	NT[e)]	5.97 ± 0.25	4.39 ± 0.46*	≤2.60 ± 0.00	NT	NT
QACx500[b)]				8.62 ± 0.12	3.04 ± 0.24*	≤2.60 ± 0.00	NT	NT	NT	NT
Mix500[c)]				8.76 ± 0.06	3.26 ± 0.19*	≤2.60 ± 0.00	NT	NT	NT	NT
$FdCa(OH)_2$		5	8.80 ± 0.08	8.63 ± 0.04	NT	6.53 ± 0.17	5.13 ± 0.07*	3.83 ± 0.11*	NT	NT
QACx500				8.58 ± 0.04	NT	3.44 ± 0.48*	≤2.60 ± 0.00	NT	NT	NT
Mix500				8.71 ± 0.07	NT	3.10 ± 0.30*	≤2.60 ± 0.00	NT	NT	NT
$FdCa(OH)_2$	2	0	8.87 ± 0.06	8.65 ± 0.10	NT	6.30 ± 0.25	5.28 ± 0.20*	4.30 ± 0.25*	NT	NT
QACx500				8.69 ± 0.03	7.30 ± 0.25	6.01 ± 0.28	4.31 ± 0.18*	NT	NT	NT
Mix500				8.82 ± 0.02	5.94 ± 0.04	4.16 ± 0.25*	≤2.60 ± 0.00	NT	NT	NT
$FdCa(OH)_2$		5	8.91 ± 0.03	8.63 ± 0.04	NT	6.93 ± 0.17	5.71 ± 0.04	4.58 ± 0.12*	NT	NT
QACx500				8.80 ± 0.02	NT	NT	NT	NT	6.12 ± 0.27	4.73 ± 0.14*
Mix500				8.79 ± 0.06	NT	6.20 ± 0.10	4.71 ± 0.58*	≤2.60 ± 0.00	NT	NT

a) Food additive grade calcium hydroxide powder (200 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). b) A quaternary ammonium compound (QAC) diluted by 1:500 in redistilled water (QACx500). c) $FdCa(OH)_2$ powder (200 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Bacterial titer (log_{10} CFU/m*l*). e) Not tested. *Single asterisk indicates effective bacterial reduction (≥3 log_{10} CFU/m*l*). Bacterial titer ≤2.6 log_{10} CFU/m*l* indicates bacterial reduction to the undetectable level. Both effective and undetectable level bacterial reductions are significantly different (P<0.05) from positive control titer.

tested solution, then100 *µl* of bacteria was added and incubated for 30 sec and 1, 3, 20 and 30 min at RT or on ice and inactivation reaction was stopped by aforementioned way. The viable bacteria in each sample including positive control were counted (log_{10} CFU/m*l*) by plating 25 *µl* portions on DHL agar plate after making serial ten-fold dilution with sterile phosphate buffered saline, followed by 24 hr incubation at 37°C.

To confirm the effect of the blocking solution, the blocking solution was added to each solution before adding bacteria (treatment for 0 sec). For the positive control, 100 *µl S.* Infantis was inoculated in 400 *µl* of PBS and 500 *µl* of the blocking solution, making serial 10-fold dilutions.

Each solution was repeatedly tested 3 times (in triplicates), and one representative data from the experiments is shown in the result section, after statistical analyses. Three repeated experiments showed a very similar trend in the results, with statistical significance.

Carrier tests for evaluating the bactericidal activities of the treatment solutions toward S. Infantis and E. coli on carriers

In the carrier experiments, the aforesaid two different reaction temperatures and four contact times were evaluated. At RT, bacteria contaminated carrier coupons were placed separately in a petri-dish within a safety cabinet, then five hundreds micro-litters of each tested solution (QACx500, 0.2% $FdCa(OH)_2$ solution or Mix500) were added separately onto contaminated surfaces (either *S.* Infantis or *E. coli* contaminated) of each carrier, and incubated for 30 sec, 1, 3 and 5 min. After incubation, the bactericidal efficacies of the tested solutions were blocked by placing the carrier into stomacher bags containing 2 m*l* of the blocking solution. For the experiments at low temperature, the Petri-dishes containing each contaminated carrier, tested solutions and the blocking solution were kept for at least 30 min on ice before the evaluation. The temperatures of the tested solutions were recorded prior to use. Afterward, five hundreds micro-litters of each treatment solution were added separately onto contaminated carrier on ice, and incubated for designated time (30 sec, 1, 3 and 5 min). Following incubation, inactivation reaction was stopped by placing the carrier into stomacher bag, as described above.

Subsequently, each carrier surfaces were rubbed vigorously by hand over the bag and scrapping with sterile pipettes to dislodge the bacterial cells from the carrier surfaces into fluids. Then, the fluids were transferred from the stomacher bag into a microtube separately and diluted in serial 10 fold dilution in PBS. Alongside, as a control, five hundreds micro-litters of dW_2 (dW_2 control) were added onto each contaminated carrier and kept in contact for 5 min at RT or on ice. The viable bacteria in each sample was counted (log_{10} CFU/m*l*), as described above. Each test was carried out three times in triplicate as shown in the suspension test, and one representative data from the experiments is shown in the result section, after statistical analyses.

Inactivation analysis

Inactivation efficacy against the bacteria was determined by calculating the reduction factor (RF), using the following equation: RF=tpc−ta, where tpc is the number of bacteria of the untreated or dW_2 treated sample in log_{10} units, and ta is the number of the recovered bacteria of the treated samples. Inactivation was considered to be effective when RF was ≥3, indicating a reduction of the number of bacteria greater than 1,000 times [22, 41, 43].

Statistical analysis

The RF values of each experiment were analyzed independently and shown as mean ± standard error (SE). One-way analysis of

Table 2. Bactericidal efficacies of the treatment solutions toward *S.* Infantis on carriers at room temperature

Solution	Type of carrier	dW_2 Control log_{10} CFU/m*l*	Bacterial titer (log_{10} CFU/m*l*) at different contact times			
		3 min	30 sec	1 min	3 min	5 min
$FdCa(OH)_2$[a)]	Rubber	7.54 ± 0.07[d)]	NT[e)]	4.78 ± 0.09	3.43 ± 0.52*	3.10 ± 0.35*
QACx500[b)]			5.30 ± 0.35	4.64 ± 0.03	3.05 ± 0.31*	≤2.60 ± 0.00
Mix500[c)]			4.60 ± 0.08	3.10 ± 0.30*	≤2.60 ± 0.00	NT
$FdCa(OH)_2$	Steel	7.62 ± 0.03	5.14 ± 0.36	4.16 ± 0.17*	3.25 ± 0.33*	≥2.60 ± 0.00
QACx500			4.96 ± 0.10	3.39 ± 0.40*	≤2.60 ± 0.00	NT
Mix500			3.45 ± 0.19*	≤2.60 ± 0.00	NT	NT
$FdCa(OH)_2$	Plastic	7.17 ± 0.18	5.38 ± 0.26	4.29 ± 0.15	3.38 ± 0.17*	2.76 ± 0.16*
QACx500			4.68 ± 0.28	3.21 ± 0.31*	2.82 ± 0.23*	≤2.60 ± 0.00
Mix500			3.49 ± 0.21*	≤2.60 ± 0.00	NT	NT

a) Food additive grade calcium hydroxide powder (200 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). b) A quaternary ammonium compound (QAC) diluted by 1:500 in redistilled water (QACx500). c) $FdCa(OH)_2$ powder (200 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Bacterial titer (log_{10} CFU/m*l*). e) Not tested. *Single asterisk indicates effective bacterial reduction (≥3 log_{10} CFU/m*l*). Bacterial titer ≤2.6 log_{10} CFU/m*l* indicates bacterial reduction to the undetectable level. Both effective and undetectable level bacterial reductions are significantly different (P<0.05) from dW_2 control titer.

variance (ANOVA) *post hoc* test (SPSS, Armonk, NY, U.S.A.) was performed to determine statistical significance of differences in disinfection efficacy between the positive control or dW_2 control and treatment group, and also among the treatment group. Significant difference was noticed while the associated P value was less than 0.05.

RESULTS

Evaluation of bactericidal efficacies of tested solutions against S. Infantis in aqueous phase

Table 1 shows the inactivation activity of 0.2% $FdCa(OH)_2$, QACx500 and Mix500 toward *S.* Infantis in liquid either at RT or on ice (2°C). When the blocking solution was added to the tested solutions prior to adding the bacteria (0 sec), almost no bacterial titer reduction was revealed (P>0.05), comparing to positive control (Table 1), which ensured that the blocking solution stopped inactivation reaction of the tested solutions completely.

As shown in Table 1, QACx500 could inactivate *S.* Infantis from 8.80 and 8.88 log_{10} CFU/m*l* to 3.44 (RF=5.36) and 3.04 (RF=5.84) log_{10} CFU/m*l* within 30 and 5 sec, respectively with or without 5% FBS; however, for the reduction to undetectable level (≤2.6 log_{10} CFU/m*l*) it required 1 min (RF≥6.20) and 30 sec (RF≥6.28), respectively, at RT. Mix500 showed similar bactericidal efficacy to QAC at the same contact time. At 5 and 30 sec, the RF value was found to be statistically significant (P<0.05) when compared with 0 sec contact time or the positive control. $FdCa(OH)_2$ solution alone could inactivate bacteria at effective level (RF≥3), within 1 min, regardless of the presence of 5% FBS.

At low temperature (2°C), the bactericidal efficacy of QACx500 was reduced and it required 30 min to inactivate *S.* Infantis (RF=4.18) at effective level in the presence of 5% FBS. Conversely, Mix500 inactivated this bacterium to the undetectable level within 3 min (RF≥6.31) and 1 min (RF≥6.27), respectively with or without 5% FBS (Table 1). Nonetheless, $FdCa(OH)_2$ solution could inactivate *S.* Infantis at effective level within 3 min, in the presence of 5% FBS. The data was found statistically significant (P<0.05) when ≥3log_{10} CFU/m*l* bacterial reduction (RF≥3) was noticed the between control and the treatment groups. From the above findings, it was confirmed and elucidated that $FdCa(OH)_2$ has a synergistic effect together with QAC for inactivating *S.* Infantis.

Evaluation of bactericidal efficacies of the tested solutions on carriers

As shown in Tables 2 and 3, the recovered number of bacteria from *S.* Infantis contaminated carrier after dW_2 control wash were 7.54, 7.62 and 7.17 log_{10} CFU/m*l* respectively from rubber, steel and plastic at RT but at 2°C the number of bacteria were 7.21, 7.05 and 7.36, respectively. At RT, QACx500 inactivated *S.* Infantis to the undetectable level (RF≥4.94) on rubber surface within 5 min; however, at 2°C it cannot inactivate this bacterium to the undetectable level within the same time. Conversely, Mix500 could inactivate *S.* Infantis to the undetectable level within 3 min even at 2°C (Tables 2 and 3). $FdCa(OH)_2$ solution alone could inactivate bacteria effectively within 3 min (RF=4.11) and 5 min (RF=3.43), respectively at RT and 2°C on rubber surface. In steel surface, $FdCa(OH)_2$, QAC and Mix500 inactivated *S.* Infantis to the undetectable level (RF≥5.02) within 5, 3 and 1 min, respectively at RT, but in plastic, QAC and Mix500 could inactivate this bacterium to the undetectable level (RF≥4.57) within 5 and 1 min, respectively (Table 2). At 2°C, only Mix500 could inactivate bacteria to the undetectable level (RF 4.61, RF 4.45, and RF>4.76), respectively, on rubber, steel and plastic surfaces within 3 min (Table 3).

At RT, Table 4 shows the recovered number of bacteria from *E. coli* contaminated carrier after dW_2 treatment were 6.82, 7.24 and 7.37 log_{10} CFU/m*l* from rubber, steel and plastic respectively, but at 2°C, the number of bacteria were 6.63, 7.29 and 6.92, respectively (Table 5). QACx500 could inactivate *E. coli* to the undetectable level on rubber (RF≥4.22), steel (RF≥4.64) and plastic (RF≥4.77) surfaces within 3 min at RT; but, at 2°C, it could not inactivate bacteria to the undetectable level within 3 min.

Table 3. Bactericidal efficacies of the treatment solutions toward *S.* Infantis on carriers at 2°C

Solution	Type of carrier	dW_2 Control $\log_{10}$ CFU/m*l*	Bacterial titer ($\log_{10}$ CFU/m*l*) at different contact times			
		3 min	30 sec	1 min	3 min	5 min
$FdCa(OH)_2$[a)]	Rubber	7.21 ± 0.18[d)]	NT[e)]	5.96 ± 0.04	4.73 ± 0.06	3.78 ± 0.13*
QACx500[b)]			5.53 ± 0.18	4.92 ± 0.05	4.27 ± 0.22*	3.37 ± 0.16*
Mix500[c)]			4.89 ± 0.06	3.71 ± 0.05*	≤2.60 ± 0.00	NT
$FdCa(OH)_2$	Steel	7.05 ± 0.20	NT	5.11 ± 0.36	3.92 ± 0.26*	3.05 ± 0.31*
QACx500			NT	4.67 ± 0.14	3.43 ± 0.19*	2.99 ± 0.21*
Mix500			4.46 ± 0.17	3.38 ± 0.17*	≤2.60 ± 0.00	NT
$FdCa(OH)_2$	Plastic	7.36 ± 0.20	NT	5.59 ± 0.30	4.44 ± 0.10	2.93 ± 0.33*
QACx500			NT	5.07 ± 0.13	4.16 ± 0.25*	2.88 ± 0.28*
Mix500			4.82 ± 0.10	3.80 ± 0.11*	≤2.60 ± 0.00	NT

a) Food additive grade calcium hydroxide powder (200 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). b) A quaternary ammonium compound (QAC) diluted by 1:500 in redistilled water (QACx500). c) $FdCa(OH)_2$ powder (200 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Bacterial titer ($\log_{10}$ CFU/m*l*). e) Not tested. *Single asterisk indicates effective bacterial reduction (≥3 $\log_{10}$ CFU/m*l*). Bacterial titer ≤2.6 $\log_{10}$ CFU/m*l* indicates bacterial reduction to the undetectable level. Both effective and undetectable level bacterial reductions are significantly different (P<0.05) from dW_2 control titer.

Table 4. Bactericidal efficacies of the treatment solutions toward *E. coli* on carriers at room temperature

Solution	Type of carrier	dW_2 Control $\log_{10}$ CFU/m*l*	Bacterial titer ($\log_{10}$ CFU/m*l*) at different contact times		
		3 min	30 sec	1 min	3 min
$FdCa(OH)_2$[a)]	Rubber	6.82 ± 0.20[d)]	NT[e)]	4.24 ± 0.32	2.76 ± 0.16
QACx500[b)]			4.84 ± 0.12	3.15 ± 0.08*	≤2.60 ± 0.00
Mix500[c)]			3.17 ± 0.30*	≤2.60 ± 0.00	NT
$FdCa(OH)_2$	Steel	7.24 ± 0.22	NT	4.80 ± 0.36	≤2.60 ± 0.00
QACx500			4.55 ± 0.20	3.31 ± 0.38*	≤2.60 ± 0.00
Mix500			3.48 ± 0.25*	≤2.60 ± 0.00	NT
$FdCa(OH)_2$	Plastic	7.37 ± 0.20	NT	4.76 ± 0.40	≤2.60 ± 0.00
QACx500			5.06 ± 0.11	3.58 ± 0.53*	≤2.60 ± 0.00
Mix500			3.85 ± 0.52*	≤2.60 ± 0.00	NT

a) Food additive grade calcium hydroxide powder (200 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). b) A quaternary ammonium compound (QAC) diluted by 1:500 in redistilled water (QACx500). c) $FdCa(OH)_2$ powder (200 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Bacterial titer ($\log_{10}$ CFU/m*l*). e) Not tested. *Single asterisk indicates effective bacterial reduction (≥3 $\log_{10}$ CFU/m*l*). Bacterial titer ≤2.6 $\log_{10}$ CFU/m*l* indicates bacterial reduction to the undetectable level. Both effective and undetectable level bacterial reductions are significantly different (P<0.05) from dW_2 control titer.

Table 5. Bactericidal efficacies of the treatment solutions toward *E. coli* on carriers at 2°C

Solution	Type of carrier	dW_2 Control $\log_{10}$ CFU/m*l*	Bacterial titer ($\log_{10}$ CFU/m*l*) at different contact times		
		3 min	30 sec	1 min	3 min
$FdCa(OH)_2$[a)]	Rubber	6.63 ± 0.18[d)]	NT[e)]	5.29 ± 0.33	3.32 ± 0.15*
QACx500[b)]			5.13 ± 0.08	3.92 ± 0.05	3.09 ± 0.29*
Mix500[c)]			4.49 ± 0.25	3.23 ± 0.32*	≤2.60 ± 0.00
$FdCa(OH)_2$	Steel	7.29 ± 0.13	NT	5.55 ± 0.22	3.32 ± 0.36*
QACx500			5.28 ± 0.29	4.37 ± 0.24	2.88 ± 0.28*
Mix500			4.78 ± 0.11	3.88 ± 0.10*	≤2.60 ± 0.00
$FdCa(OH)_2$	Plastic	6.92 ± 0.28	NT	4.69 ± 0.31	3.53 ± 0.25*
QACx500			4.97 ± 0.20	4.10 ± 0.14	3.04 ± 0.24*
Mix500			4.16 ± 0.25	3.20 ± 0.39*	≤2.60 ± 0.00

a) Food additive grade calcium hydroxide powder (200 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). b) A quaternary ammonium compound (QAC) diluted by 1:500 in redistilled water (QACx500). c) $FdCa(OH)_2$ powder (200 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Bacterial titer ($\log_{10}$ CFU/m*l*). e) Not tested. *Single asterisk indicates effective bacterial reduction (≥3 $\log_{10}$ CFU/m*l*). Bacterial titer ≤2.6 $\log_{10}$ CFU/m*l* indicates bacterial reduction to the undetectable level. Both effective and undetectable level bacterial reductions are significantly different (P<0.05) from dW_2 control titer.

Conversely, Mix500 required only 1 min for the inactivation of *E. coli* to the undetectable level on each carrier surface at RT; however, at 2°C it could inactivate to the undetectable level within 3 min (Tables 4 and 5). $FdCa(OH)_2$ could inactivate *E. coli* to the undetectable level on steel and plastic surfaces within 3 min at RT but not on rubber surface; however, at 2°C it could not

inactivate bacteria to the undetectable level within 3 min.

We also observed that Mix500 showed synergistic effects and required very short contact time for the reduction to the undetectable level decontamination on carrier surfaces at both temperatures. Statistically significant (P<0.05) difference was observed between dW_2 control and treatment group (RF≥3).

DISCUSSION

Disinfection is important for breaking down the infection chain of pathogens by reducing the risk of cross-contamination. Disinfectants are used in different fields for the purpose of disease prevention and control [8, 29]. The efficacy of disinfectant is often tested against laboratory bacterial suspensions [6, 34], but for the application at fields it is desirable to test disinfectants toward bacteria on surfaces. In our study we tested the efficacy of disinfectants against laboratory bacterial suspensions and on artificially contaminated carriers.

QACs are the most commonly used disinfectants all over the world at poultry farms as car gate sprays, washing of equipments, foot baths, or washing of farm premises; however, their biocidic efficacies at lower temperature are reduced [19, 40, 46]. In the present study, we demonstrated a strategy for enhancement of QAC activities under cold conditions, in the presence of organic materials. From our results, it was established that the activities of QACs could be synergistically enhanced with $FdCa(OH)_2$ solution (Tables 1–5). At 2°C, in the presence of organic materials, QACx500 needs 30 min to inactivate bacteria at effective level but cannot kill the bacteria to the undetectable level. However, at low temperature, even in the presence of high amount of organic loads (5% FBS), Mix500 could inactivate bacteria to the undetectable level within 3 min, as shown in Table 1. $FdCa(OH)_2$ alone could require 3 min to inactivate bacteria at effectively but not to the undetectable level. Alkaline agents (lime) are eminent for their strong bactericidal properties, even in the presence of organic materials [4, 5, 24, 32, 43]. Ca $(OH)_2$ at 0.17% is the saturated solution [3, 45], a higher percentage (>0.17%) of solution worked better than a saturated solution because of containing many nano-particles in the solution [42]. Toyofuku *et al.* [45] reported that saturated 0.17% $FdCa(OH)_2$ could not capable to inactivate bacteria within 30 sec.

In the field conditions, carrier tests are more relevant for assessing the activity of chemical disinfectants [21]. Pathogens adhere firmly to equipment surfaces through organic or cellular debris [27, 39]. Dried *Salmonella* cells are capable to stay alive on paper discs for 2 years at 4°C [47]. Disinfectants that are competent against bacterial suspensions but may have a reduced effect on bacteria that adhere to any surfaces [30]. The purpose of any disinfection process is to prevent, reduce, or wipe out microbial populations on inanimate objects, surfaces, or the premises [10]. From our study results, it was demonstrated that QACx500 could inactivate *S.* Infantis and *E. coli* to the undetectable level on steel surfaces within 3 min, but Mix500 could require 1 min at RT; however, at lower temperature only Mix500 could inactivate both bacteria to the undetectable level within 3 min but QAC and $FdCa(OH)_2$ are not able to inactivate bacteria to the undetectable level even within 5 min (Tables 2–5). On rubber and plastic surfaces, QAC showed bacterial inactivation to the undetectable level within 5 and 3 min, respectively, and toward *S.* Infantis and *E. coli* at RT but at 2°C, it could not inactivate to the undetectable level within the same time. Conversely, Mix500 could inactivate both bacteria to the undetectable level within 3 min on rubber and plastic surfaces at 2°C (Tables 2–5). But, $FdCa(OH)_2$ alone could not inactivate bacteria to the undetectable level on steel, rubber and plastic surfaces at lower temperature. Møretrø *et al.* [28] described that the tolerance of attached *Salmonella* to disinfectants on a dried surfaces is higher than *Salmonella* in suspension. Møretrø *et al.* [27] found that out of nine disinfectants, only one (70% ethanol) eliminated (>4 $\log_{10}$ reduction) *Salmonella* dried on stainless steel, while all nine disinfectants resulted in elimination of *Salmonella* (>5 $\log_{10}$ reduction) in suspension tests. In our study, we found that all disinfectants could eliminate *Salmonella* (>4 $\log_{10}$ reduction) on steel even at lower temperature, but in suspension test, QAC and Mix500 could inactivate *Salmonella* to the undetectable level (>6 $\log_{10}$ reduction) at RT even in the presence of organic materials, but at lower temperature only Mix500 could eliminate bacteria to the undetectable level (>6 $\log_{10}$ reduction). Statistically significant (P<0.05) difference was noticed between dW_2 control and treatment groups (≥3 $\log_{10}$ reduction).

In our study, Mix500 disinfectant showed the best bactericidal efficacies within very short contact time either in suspension test or on bacteria-contaminated carrier surfaces (*S.* Infantis or *E. coli*) either at RT or low temperature. Bacteria on the steel were more sensitive for decontamination compared to bacteria on rubber and on plastic. A study by Jang *et al.* [20] found that most disinfectants were more effective on stainless steel surface than on wooden surface. Another study narrated by Yilmaz *et al.* [48] found that disinfection of porous surfaces is more difficult than that of non-porous surfaces. In general, disinfectants cannot reach pathogens that hide into the pores of the surfaces. But, on the smooth surfaces, there is no niche where pathogens reside. Therefore, disinfection of pores surfaces is more difficult than that of non-porous surfaces and the bactericidal efficacy on wood was lower than that on steel.

QACs are membrane-active agents interacting with the cytoplasmic membrane (lipid or protein) of bacteria, followed by disorganization of membrane; leakage of intracellular material; degradation of proteins and nucleic acids; and lysis of cell wall caused by autolytic enzymes [12]. At the lower temperature, cytoplasmic membrane becomes rigid and resistant toward QACs [18]. The mechanism of the synergistic effect appears to be that high pH and calcium made the disruption of bacterial cell membrane [26], thereby allowing QAC to pass through the damage membrane and interact with intracellular materials (proteins and nucleic acids), and denature proteins in the cytoplasm of bacteria. A similar synergistic mechanism between NaOCl and $FdCa(OH)_2$ was also speculated towards bacterial cell membrane by Toyofuku *et al.* [45]. Ito *et al.* [18] demonstrated similar synergistic efficacies of QAC and $FdCa(OH)_2$ toward avian influenza and Newcastle disease viruses.

In conclusion, our study findings demonstrate that the mixture of QAC and $FdCa(OH)_2$ has synergistic effects that make

possible acquiring an upgraded bactericidal agent, with broad spectrum of pathogen inactivation. The efficacy of most disinfectants was decreased by low temperatures during carrier disinfections and suspension test, but Mix500 showed excellent efficacy on contaminated carrier, as well as in suspension test at low temperature, even in the presence of organic materials. Thus, environmental temperature, disinfection duration, and target surface should be considered for successful disinfection in field situations.

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