Enhancement of bactericidal effects of sodium hypochlorite in chiller water with food additive grade calcium hydroxide

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ABSTRACT. An alkaline agent, namely food additive grade calcium hydroxide (FdCa(OH)₂) in solution at 0.17%, was evaluated for its bactericidal efficacies in chiller water with sodium hypochlorite (NaOCl) at a concentration of 200 ppm total residual chlorine. Without organic material presence, NaOCl could inactivate Salmonella infantis and Escherichia coli within 5 sec, but in the presence of fetal bovine serum (FBS) at 0.5%, the bactericidal effects of NaOCl were diminished completely. FdCa(OH)₂ solution required 3 min to inactivate bacteria with or without 5% FBS. When NaOCl and FdCa(OH)₂ were mixed at the final concentration of 200 ppm and 0.17%, respectively, the mixed solution could inactivate bacteria at acceptable level (10³ reduction of bacterial titer) within 30 sec in the presence of 0.5% FBS. The mixed solution also inhibited cross-contamination with S. infantis or E. coli on chicken meats. It was confirmed and elucidated that FdCa(OH)₂ has a synergistic effect together with NaOCl for inactivating microorganisms.

KEY WORDS: bacterial disinfection, food additive grade calcium hydroxide, inhibition of cross contamination, sodium hypochlorite, synergistic effect

Recently, food safety has been required at higher level, and hazard analysis and critical control point (HACCP) has been introduced at farm level in Japan [17]. In order to make HACCP work appropriately, it is necessary to enhance biosecurity, such as disinfecting pathogens along the route from farm to table.

In Japan, foodborne illnesses caused by Salmonella spp., Campylobacter and Escherichia coli are still high, with 1,918, 2,089, and 518 patients, respectively, in 2015 [18]. In the United States of America (U.S.A.), Campylobacter and Salmonella are the top 2 bacterial foodborne human enteropathogens associated with poultry-related foodborne illness [3].

Chlorine has been a common antimicrobial utilized for prevention of bacterial carcass cross-contamination in immersion chilling systems and throughout the poultry processing plant [19, 23, 25]. Early studies demonstrated potency of chlorine as an antimicrobial, with only 0.1 mg/l (0.1 ppm) of free chlorine being required to inactivate 99% of Campylobacter strains after 5 min of contact [4]. The relatively low cost and low concentration required for efficacy promoted widespread use of chlorine in processing plants. However, the effectivness of chlorine in immersion tanks (=chiller tanks) has been shown to diminish due to a longer residence time [1, 29], and its effectivness is highly affected by pH changes and the amount of organic matter in the chiller tank [1, 13]. Tsai et al. [27] and Northcutt et al. [22] reported that chiller tanks at the processing plants contained around 0.35 or 0.16% suspended solids, respectively. Although chicken is chemically sanitized during processing and subsequently cleaned before packaging, some microorganisms cannot be removed completely, especially bacteria strongly attached to chicken skin [5, 14, 23, 30]. This is because these microorganisms, including Salmonella and Campylobacter, are able to become entrapped in deep skin layers, crevices, or feather follicles, thus becoming protected from chemical agents [5, 15, 29].

In the European Union (EU), no disinfectant is allowed to be used at the processing plants, especially in chiller tanks [7]. Europe should not authorize chemical washes such as chlorine or peroxyacetic acid on poultry carcasses. In the U.S.A., United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) allows for addition of chlorine to processing waters at levels up to 50 ppm, calculated as free available chlorine in carcass wash applications and chiller make-up water [8].

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FSIS also requires that chlorinated water containing a minimum of 20 ppm available chlorine be applied to all surfaces of carcasses when the inner surfaces have been re-processed (due to carcass contamination), other than solely by trimming [8]. In Japan, NaOCl at 35–200 ppm as total chlorine concentration is used to disinfect poultry carcasses; however, many cross-contaminations still occur, especially during chilling process at the slaughter house, and bacteria from poultry meats are detected after processing, or at the retail markets [19, 25]. Munther et al. [21] also reported that while chlorine control was important for reducing *Escherichia coli* levels during chilling, it played a less significant role in the management of cross-contamination issues. Once the bacteria attach to the surface of chicken meats, it is very difficult to remove or kill these bacteria [1, 14, 30]. It is desirable to kill bacteria before they attach to poultry meats to reduce cross-contamination.

Chlorine has been shown to kill micro-organisms within very short period (within 5 sec) [10] and this short period allowed chlorine to inactivate Newcastle disease virus (NDV) in the air [12]. However, in the presence of organic matter, the efficacies were diminished [1, 10, 12].

In the present study, food additive grade calcium hydroxide (FdCa(OH)₂) [9] was evaluated for its inactivation efficacy toward *Salmonella Infantis* and *E. coli*, and the efficacy with NaOCl was evaluated, too. To get better efficacy, 17% of Ca(OH)₂ that is saturated concentration was used in this study [2]. And also for the application at poultry processing plants, “food additive grade” was chosen.

**MATERIALS AND METHODS**

**FdCa(OH)₂ powder and NaOCl**

FdCa(OH)₂ powder at pH 13, made of natural calcium carbonates derived from limestone through calcination process, with the average diameter of the powder size at 10 μm, was kindly provided by Fine Co., Ltd. (Tokyo, Japan) [9]. NaOCl (Turukuron®, at 12% chlorine concentration) was purchased from Toagosei Co., Ltd. (Tokyo, Japan). FdCa(OH)₂ powder of 170 mg was prepared in 100 ml of redistilled water (dW₂) and centrifuged at 1,750 × g for 10 min at 4°C. The resulted supernatant was used as saturated 0.17% FdCa(OH)₂ [2]. NaOCl containing 200 ppm chlorine (200 ppm NaOCl) was prepared in dW₂. For the mixed solution (200 ppm NaOCl and 0.17% FdCa(OH)₂), NaOCl was diluted to 200 ppm chlorine in 0.17% FdCa(OH)₂ solution. Solutions containing NaOCl, namely 200 ppm NaOCl and the mixed solution, were used for the experiments immediately within 1 min after preparation.

**Inocula preparation**

Bacterial suspensions of *E. coli* strain NBRC106373 and *S. Infantis* were prepared and enumerated as described previously [11]. In order to make rifampicin resistant bacteria, 100 μl of log phase culture of *E. coli* was employed through its surface plating on deoxycholate hydrogen sulfide-lactose (DHL) agar containing different concentrations of rifampicin (from 0–100 μg/ml), followed by overnight incubation at 37°C. Colonies grown in the area containing higher concentration of rifampicin were picked up and subsequently cultured in the Luria-Bertani (LB) medium containing the same concentration of rifampicin. Log phase of bacteria from the solution culture was used for the experiments, and stocked as EC-Rif in 10% skim milk at −80°C until used. *E. coli*-Rif was enumerated on DHL agar containing 100 μg/ml rifampicin.

**Computation of reduction factor**

Reduction factor (RF) was calculated using the equation below after conversion of sample titer to log₁₀ CFU/ml:

\[
\text{RF} = \frac{\text{tpc}}{\text{ta}}
\]

In the above equation, tpc is bacterial titer from an untreated sample in log₁₀ CFU/ml whereas ta is the titer of recovered bacteria from treated samples. The inactivation rate was acceptable when the RF was greater than or equal to 3 [8, 11, 16, 26].

**Evaluation of bactericidal efficacies in aqueous phase**

Four hundred micro-liter of solutions (0.17% FdCa(OH)₂, 200 ppm NaOCl and the mixture) were mixed with 100 μl of bacteria in a microtube, incubated for indicated time (0, 5, 30 sec, 1 or 3 min) at 4°C and then neutralized by adding 500 μl of blocking solution containing 0.7 M Tris-HCl (pH 7.2) and 30% fetal bovine serum (FBS), which made pH of the tested solutions around 8.2. In the presence of FBS, 2.5μl (0.5%), 5.0μl (1.0%) or 25μl (5.0%) of FBS was added, respectively, into the microtubes before adding bacteria. For the positive control, 100 μl of *E. coli*, *E. coli*-Rif and *S. Infantis* was individually inoculated in 400 μl of PBS then, after 3 min incubation, 500 μl of the blocking solution was added. These experiments were performed in triplicate.

**Evaluation of inhibition of cross-contamination**

Commercial chicken thigh meats, purchased at a retail market were cut into ten gram pieces and each piece was spiked with *E. coli*-Rif or *S. Infantis* at 1 ml of 10⁸ CFU/ml for 15 min. The spiked chicken meat piece was placed in a 250 ml glass beaker containing 40 ml of each tested solution at 4°C, namely 0.17% FdCa(OH)₂, 200 ppm NaOCl and the added mixture with 0.2% FBS, plus dW₂ containing 0.2% FBS as control; then the non-spiked chicken meat piece was placed into the same beaker immediately (within 1 sec) and incubated for 5 min with shaking at 100 rpm in Bio-Shaker BR-22UM (TAITEC Co., Saitama, Japan). The non-spiked piece was taken out from the beaker and rinsed with 40 ml of dW₂ twice, transferred into a stomacher.

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INACTIVATION OF BACTERIA IN CHILLER WATER

Table 1. Bactericidal efficacies of 0.17% FdCa(OH)₂ toward *Salmonella* Infantis and *Escherichia coli*

| Bacteria         | FBS (%) | Control<sup>b)</sup> | RF<sup>a)</sup> | 0<sup>c</sup> sec | 5 sec | 30 sec | 60 sec | 180 sec |
|------------------|---------|----------------------|----------------|-------------------|-------|--------|--------|---------|
| *Salmonella* Infantis | 0       | 8.60 ± 0.08<sup>d)</sup> | 0.00 ± 0.00 | 0.91 ± 0.22       | 1.86 ± 0.10 | 2.11 ± 0.17 | ≥6.00 ± 0.08 |
|                  | 1       | 1.19 ± 0.16          | 0.00 ± 0.00 | 0.90 ± 0.20       | 1.87 ± 0.33 | 2.33 ± 0.27 | ≥6.00 ± 0.08 |
|                  | 5       | 0.55 ± 0.03          | 0.00 ± 0.00 | 1.28 ± 0.04       | 2.25 ± 0.07 | ≥5.69 ± 0.07 |
| *Escherichia coli* | 0       | 8.29 ± 0.03          | 0.00 ± 0.00 | 0.51 ± 0.02       | 1.22 ± 0.06 | 1.94 ± 0.15 | ≥5.69 ± 0.07 |
|                  | 1       | 0.44 ± 0.01          | 0.00 ± 0.00 | 0.95 ± 0.03       | 1.65 ± 0.08 | 5.30 ± 0.16 |

a) Reduction factor (RF)=log<sub>10</sub> (titer of control/ml) − log<sub>10</sub> (titer of treated samples/ml). b) Titer of bacteria in the control (log<sub>10</sub> CFU/ml). c) Contact times. d) Data represent means ± standard deviation of three different experiments.

Table 2. Bactericidal efficacies of 0.17% FdCa(OH)₂, 200 ppm NaOCl or the mixture solution toward *Salmonella* Infantis and *Escherichia coli*

| Tested solution | Bacteria         | FBS (%) | Control<sup>b)</sup> | RF<sup>a)</sup> | 5<sup>c</sup> sec | 30 sec |
|-----------------|------------------|---------|----------------------|----------------|-------------------|--------|
| *Salmonella* Infantis | 0.17% FdCa(OH)₂ | 0.5     | 0.72 ± 0.14          | 0.98 ± 0.10 | 0.72 ± 0.14       | 0.98 ± 0.10 |
|                  | 200 ppm NaOCl    | 0.5     | 5.41 ± 0.27          | 5.41 ± 0.27 | 0.80 ± 0.37       | 1.14 ± 0.52 |
|                  | Mixture          | 0.5     | 5.41 ± 0.27          | 5.41 ± 0.27 | 1.31 ± 0.25       | 5.41 ± 0.27 |
|                  | 0.17% FdCa(OH)₂ | 0.5     | 0.49 ± 0.07          | 1.19 ± 0.05 | 0.49 ± 0.07       | 1.19 ± 0.05 |
|                  | 200 ppm NaOCl    | 0.5     | 5.53 ± 0.15          | 5.53 ± 0.15 | 0.36 ± 0.07       | 0.47 ± 0.19 |
|                  | Mixture          | 0.5     | 3.17 ± 0.82          | 5.53 ± 0.15 | 0.32 ± 0.23       | 1.29 ± 0.08 |

a) Reduction factor (RF)=log<sub>10</sub> (titer of control/ml) − log<sub>10</sub> (titer of treated samples/ml). b) Titer of bacteria in the control (log<sub>10</sub> CFU/ml). c) Contact times. d) Data represent means ± standard deviation of three different experiments.

RESULTS

Evaluation of bactericidal efficacies of 0.17% FdCa(OH)₂

Table 1 shows the capacity of 0.17% FdCa(OH)₂ to inactivate bacteria in liquid. When the blocking solution was added to the solution before adding bacteria (0 sec), no reduction was observed. Regardless of the presence or absence of FBS, 0.17% FdCa(OH)₂ inactivated *Salmonella* Infantis from 10<sup>8.60</sup> PFU/ml to below the detection limit (≤10<sup>2.6</sup> CFU/ml) (RF≥6.00), as well as *E. coli* from 10<sup>8.29</sup> PFU/ml to below the detection limit (≤10<sup>2.6</sup> CFU/ml) (RF≥5.69), except presence of 5% FBS (RF=5.3). However, it took more than 1 min for 0.17% FdCa(OH)₂ to inactivate bacteria.

Enhancement of bactericidal efficacies of NaOCl with 0.17% FdCa(OH)₂

Table 2 summarizes the results of the mixture of NaOCl and FdCa(OH)₂. In the absence of organic materials, namely without FBS, 200 ppm NaOCl inactivated *S. Infantis* within 5 sec from 10<sup>8.01</sup> PFU/ml to below the detection limit (≤10<sup>2.6</sup> CFU/ml) (RF≥5.41), as well as *E. coli* from 10<sup>8.13</sup> PFU/ml to below the detection limit (≤10<sup>2.6</sup> CFU/ml) (RF≥5.3). With 0.5% FBS, 200 ppm NaOCl could not inactivate bacteria within 30 sec. As shown in Table 1, 0.17% FdCa(OH)₂ could not inactivate bacteria within 30 sec. However, when these 2 materials were mixed, the mixture could inactivate bacteria within 30 sec in the presence of 0.5% FBS. The mixture could not inactivate bacteria in 1.0% FBS.

Evaluation of inhibition of cross-contamination

When bacteria spiked chicken meats were pooled with non-spiked chicken meats for 5 min in dW₂ containing 0.2% FBS at
4°C, the spiked bacteria were detected in the non-spiked meats at $10^{4.74}$ PFU/ml for *S. Infantis* and $10^{5.21}$ PFU/ml for *E. coli*-Rif as shown in Table 3. This demonstrated that the cross-contamination of bacteria from contaminated meats to non-contaminated meats occurred in dW₂. When the meats were dipped in 200 ppm NaOCl containing 0.2% FBS, bacteria were detected in the non-spiked meats in the same order as dW₂, namely $10^{4.74}$ PFU/ml for *S. Infantis* and $10^{5.20}$ PFU/ml for *E. coli*-Rif (Table 3). With 0.17% FdCa(OH)₂ containing 0.2% FBS, bacteria detected in the non-spiked meats were $10^{5.33}$ PFU/ml for *S. Infantis* and $10^{5.90}$ PFU/ml for *E. coli*, respectively. In the mixed solution containing 0.2% FBS, bacteria detected in the non-spiked meats were $10^{5.76}$ PFU/ml for *S. Infantis* and $10^{5.51}$ PFU/ml for *E. coli*, and the RF was 2.98 and 2.71, respectively.

**DISCUSSION**

Cross-contamination occurs at the processing plants of poultry especially in the chiller tank [19, 25], and it was also demonstrated in the present study as shown in Table 3 even with 200 ppm NaOCl. Poultry meats will be contaminated with bacteria through attachment of feces during the processing course, and the contaminated meats will release these bacteria into the environment, and non-contaminated meats will thereby be contaminated [19, 25]. Once bacteria attached to the meats, it was difficult to kill the bacteria on meats [14, 30]. It is desirable to kill bacteria immediately before attachment to the other meats.

To recover bacteria in meats, the homogenized method was used in the present study because the wipe or rinse methods could not recover bacteria in the deep layer of skin or meats [5, 29]. As shown in Table 1, the saturated concentration, 0.17%, FdCa(OH)₂ can kill bacteria even in the presence of 5% FBS, although it takes more than 1 min to inactivate them even without FBS. So the concentration 0.17% was used for the further experiments. In Japan, 200 ppm NaOCl is used as disinfectant in the chiller tank at processing plants, but many reports demonstrated that it is difficult to inactivate bacteria in the chiller tanks because of organic material contamination in the tanks [1, 19, 25]. In the present study, as shown in Table 2, 200 ppm NaOCl could inactivate bacteria within 5 sec. In the preliminary experiment, 200 ppm NaOCl could inactivate bacteria in the presence of 0.15% FBS within 5 sec (data not shown). However in the presence of 0.5% FBS that mimics the chiller tank containing about 0.35% suspended solids [22, 27], the inactivation activity of 200 ppm NaOCl was diminished (Table 2). The mixed solution containing 0.17% FdCa(OH)₂ and 200 ppm NaOCl could inactivate bacteria within 30 sec to the undetectable level even in the presence of 0.5% FBS. However, with 1.0% FBS, the mixture could not inactivate bacteria within 30 sec (Table 2). These data demonstrated a synergistic effect of NaOCl and Ca(OH)₂ for inactivation of bacteria. The mechanism of the synergistic effect seems to be that high pH and calcium made the bacterial cell membrane damaged [20] and OCI⁻ ion could pass the damaged membrane and denature proteins in cytosol of bacteria. Normally, in 200 ppm NaOCl solution around pH 8.0–9.0, HOCl molecule composes about 10% while hypochlorite ion (OCl⁻) composes about 90%, and the molecule only can pass through the cell membrane [28]. However, under high pH condition with Ca(OH)₂ all of the latter becomes OCl⁻ and can enter the bacterial cells through the damaged membrane. In the present study, it was shown that it took 5 to 10 sec to make the damage of the bacterial cell membrane. As shown in Table 2, in the 0% FBS condition, 200 ppm NaOCl could inactivate *E. coli* under detectable level within 5 sec, while the mixture inactivated partially within 5 sec. The reason seemed to be that in high pH all became OCl⁻ but could not enter bacterial cytosol, because little damage of the cell membrane was created. At 10 sec post-incubation, *E. coli* was inactivated to the undetectable level in the mixture in the 0% FBS condition (data not shown).

In the chiller tank, cross-contamination occurs easily as shown in Table 3, and implementation of logistic slaughter (where *Campylobacter*-negative flocks are slaughtered first) was suggested [25]; however, there is conflict regarding logistic slaughter with respect to *Salmonella* and with respect to *Campylobacter* status [6, 24]. Inhibition of cross-contamination through immediate bacterial inactivation seems to be applicable by adding FdCa(OH)₂ in chiller tanks containing NaOCl, as shown in Table 3. In chiller tanks, organic materials may be released from chicken meats so the organic material contamination will be more than 0.2% FBS [22, 27]. In the present experiments for "**Evaluation of inhibition of cross-contamination**", 0.2% FBS, but not 0.5%, was used to mimic field conditions, because the experiments contained chicken meats. It is also important to clean the processing plant environment with materials such as FdCa(OH)₂, that can have effect even in the presence of organic materials.

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**Table 3. Bacterial cross-contamination of the non-spiked chicken meats**

| Tested solution with 0.2% FBS | 200 ppm NaOCl | 0.17% FdCa(OH)₂ | Mixture | dW₂ |
|-------------------------------|----------------|-----------------|---------|-----|
| *Salmonella Infantis*⁴ | Homogenate | 4.74 ± 0.15⁵ | 3.51 ± 0.58 | 1.76 ± 0.07 | 4.74 ± 0.07 |
| | RF⁶ | 0.00± 0.07 | 1.24 ± 0.52 | 2.98 ± 0.07 | - |
| *E. coli*-Rif⁴ | Homogenate | 5.20 ± 0.18 | 2.90 ± 0.21 | 2.51 ± 0.40 | 5.21 ± 0.01 |
| | RF⁶ | 0.01 ± 0.08 | 2.32 ± 0.20 | 2.71 ± 0.30 | - |

a) Titer of SI in the inoculum was 8.62 ± 0.07 and that of Ec-Rif was 8.80 ± 0.04. b) Reduction factor (RF)=log₁₀(titer in dW₂/ml)−log₁₀(bacterial titer in treated/ml). c) Data represent means ± standard deviation of three different experiments.
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