Influence of Zinc, Sodium Bicarbonate, and Citric Acid on the Antibacterial Activity of Ovotransferrin Against *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Model Systems and Ham

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ABSTRACT The influence of $NaHCO_3$ and citric acid on the antibacterial activity of apo-ovotransferrin in model systems and ham was investigated. The antibacterial activity of 20 mg/mL of ovotransferrin solution with added $NaHCO_3$ (0, 25, 50, or 100 m*M*) or citric acid (0.25 or 0.5%) was evaluated against *Escherichia coli* O157:H7 and *Listeria monocytogenes* in brain heart infusion broth. The antimicrobial activity of ovotransferrin saturated with Fe^{2+} or Zn^{2+} against both pathogens was also measured. In addition, ovotransferrin solutions containing either 100 m*M* $NaHCO_3$ or 0.5% citric acid were applied to commercial hams inoculated with *E. coli* O157:H7 or *L. monocytogenes* and stored at 4°C for 4 wk. The antimicrobial activity of ovotransferrin increased as the concentration of added $NaHCO_3$ increased. Sodium bicarbonate (100 m*M*) significantly improved the antibacterial activity of ovotransferrin against *E. coli* O157:H7 and *L. monocytogenes*. Citric acid (0.5%) combined with ovotransferrin resulted in a synergistic antibacterial effect against *E. coli* O157:H7, and *L. monocytogenes* was susceptible to 0.5% citric acid alone. Sodium bicarbonate diminished the strong antibacterial activity of ovotransferrin + citric acid against *E. coli* O157:H7, and use of sodium citrate instead of citric acid did not produce any antibacterial activity against the pathogens. The antimicrobial activity of ovotransferrin increased significantly under acidic conditions. The Zn-bound ovotransferrin prevented the growth of *L. monocytogenes* as detected in the apo-ovotransferrin combined with 100 m*M* $NaHCO_3$ treatment, but Fe-bound ovotransferrin had little or no inhibitory activity against *E. coli* O157:H7 and *L. monocytogenes*. Ovotransferrin + 100 m*M* $NaHCO_3$ did not exhibit any antibacterial activity against the 2 pathogens in commercial hams, whereas ovotransferrin + 0.5% citric acid suppressed the growth of *L. monocytogenes* in irradiated hams. In conclusion, combinations of ovotransferrin with $NaHCO_3$, citric acid, or Zn^{2+} enhanced the antibacterial activity of ovotransferrin against *E. coli* and *L. monocytogenes,* but there are some limitations as discussed for applying ovotransferrin to meat or meat products.

Key words: antibacterial activity, ovotransferrin, bicarbonate, citric acid, *Escherichia coli* O157:H7

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INTRODUCTION

Ovotransferrin is a 78-kDa monomeric glycoprotein consisting of 686 amino acids. This protein binds reversibly to 2 Fe^{2+} ions per molecule concomitantly with 2 CO^{2-} ions (Abola et al., 1982; Ibrahim, 1997). It is the second major avian egg white protein constituting 12% of total egg white proteins and contributes to the defense of the egg against microbial infection and rotting (Abola et al., 1982). Ovotransferrin has demonstrated antibacterial activity against a wide spectrum of bacteria, including *Micrococcus pyogenes* var. *albus* (Fraenkel-Conrat and Feeney, 1950), *Escherichia coli*, *Pseudomonas* spp., *Streptococcus mutans* (Valenti et al., 1983), *Staphylococcus aureus*, *Bacillus cereus* (Ibrahim, 1997), *Salmonella* Enteritidis (Baron et al., 1997, 2000), and *Candida* (Valenti et al., 1985). The antimicrobial activity of ovotransferrin is influenced by several factors such as $NaHCO_3$ and citrate concentrations, pH conditions, composition of medium or metal irons like Zn^{2+}, as well as bacterial species or strains (Valenti et al., 1981, 1983, 1987; Brock, 1985). Bicarbonate, known as a synergistic anion (Schlabach and Bates, 1975), is a prerequisite for the Fe uptake by transferrin and lactoferrin (Aisen, 1989; Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998), because it inhibits the Fe-chelating ability of citrate (Peaker and Linzell, 1975; Griffiths and Humphreys, 1977). The anion may serve

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as a bridging ligand between proteins and metal ions (Aisen and Listowsky, 1980). Bicarbonate weakly binds to apo-transferrin in the absence of a metal ion (Woodworth et al., 1975), but it is not known if the binding is specific. The affinity of ovotransferrin for $NaHCO_3$ in each lobe is about 3-fold weaker than that of serum transferrin (Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997). Also, carbonate or $NaHCO_3$ (CO_3^{2-} or HCO_3^-) occupies one of the Fe^{3+} coordination sites consisting of 4 lateral chains with 2 tyrosines, 1 histidine,1 aspartate, and a carbonate or $NaHCO_3$ (Bailey et al., 1988; Anderson et al., 1989; Zuccola, 1992; Rawas et al., 1996; Kurokawa et al., 1999).

An excess of citrate can negate the antimicrobial activity of ovotransferrin by chelating Fe, and thus the Fe-binding capacity of ovotransferrin is affected by metal chelaters such as citrate (Phelps and Antonini, 1975). However, the effect of citrate on antibacterial activity of ovotransferrin depends on the strain of microorganism. For example, *E. coli*, which possesses an Fe transport system mediated by citrate (Frost and Rosemberg, 1973), can nullify the antibacterial activity, whereas addition of excess citrate does not influence the antimicrobial activity of ovotransferrin against *Staph. aureus* and *Candida*, which do not have an Fe citrate transport system (Valenti et al., 1980, 1985).

Because ovotransferrin can bind metal ions such as Cu, Al, Zn, and Fe, its antimicrobial effect is logically expected to decrease by binding other metal ions. Generally, Fe, Cu, and Zn have been shown to bind to the same sites in the ovotransferrin molecules (Warner and Weber, 1953). However, ovotransferrin saturated with metals such as Zn and Fe still displayed antimicrobial activities (Valenti et al., 1985, 1987; Ibrahim, 1997). Valenti et al. (1987) reported that Zn^{2+}-loaded ovotransferrin had more bactericidal activity than apo-ovotransferrin and other metal complexes, and the antimicrobial effect of Zn^{2+}-ovotransferrin was attributed to the direct interaction of Zn^{2+}-ovotransferrin with the surface of bacteria rather than Fe depletion from the medium. However, other researchers reported that the antibacterial action of ovotransferrin is due to more complex mechanisms related to direct interactions between ovotransferrin and microorganisms in addition to Fe depletion (Valenti et al., 1985).

Ovotransferrin has a great potential as a natural antimicrobial agent like lactoferrin. However, there is a scarcity of published reports on the use of ovotransferrin to control pathogenic bacteria in meat. To increase the potential of using ovotransferrin as an antibacterial agent in meat, methods to activate or improve antimicrobial capability of natural apo-ovotransferrin against foodborne bacteria is required. In the present study, the influence of $NaHCO_3$ and citric acid as synergistic anions on the antibacterial activity of apo-ovotransferrin was investigated. Also, viable ovotransferrin combined with $NaHCO_3$ and citric acid was applied to ham to determine the possibility of using ovotransferrin as a natural antimicrobial agent in meat processing.

MATERIALS AND METHODS

Ovotransferrin

Apo-ovotransferrin used in this study was produced by the method of Ko and Ahn (unpublished data). Holo-ovotransferrin was prepared by adding $NaHCO_3$ and $FeCl_3$ to the egg white solution diluted with 2 volumes of distilled water. The pH of the egg white solution was adjusted to pH 9.0, 43% ethanol added, centrifuged at 3,220 × *g* for 20 min, and the supernatant was collected (first supernatant). The precipitate was reextracted with 43% ethanol and centrifuged. The second supernatant collected was combined with the first supernatant, the ethanol concentration was adjusted to 56% using 100% ethanol, and was then centrifuged at 3,220 × *g* for 20 min. The precipitate (holo-ovotransferrin) was collected and dissolved in distilled water. After pH adjustment of holo-ovotransferrin to 4.7 with 200 m*M* citric acid, apo-ovotransferrin was obtained by removing the Fe molecules bound to holo-ovotransferrin using AG 1-X_2 resin (chloride form, Bio-Rad, Hercules, CA). The apo-ovotransferrin was freeze-dried and used in the microbial experiments. The dried apo-ovotransferrin was dissolved in distilled water, the pH was adjusted to 7.4, and NaCl was added to 0.15 *M*. The purity of prepared ovotransferrin was around 80%. The content of residual Fe in the prepared apo-ovotransferrin solution was less than 0.5 mg/kg. Before using it in microbial studies, it was sterilized by filtering through a 0.45-μm (pore size) filter.

Bacterial Strains and Culture Conditions

A 5-strain mixture of *Listeria monocytogenes*, including strains H7962 serotype 4b, H7762 serotype 4b, H7969 serotype 4b, H7764 serotype 1/2 a, and Scott A NADC 2045 serotype 4b, or *E. coli* O157:H7 (ATCC 43890, C467, FRIK 125, ATCC 43895, and 93-062) was used in this study. *Listeria monocytogenes* Scott A was obtained from Irene Wesley at the National Animal Disease Center, Agricultural Research Service, USDA (Ames, IA). All other *L. monocytogenes* strains were obtained as clinical isolates from the multistate outbreak of 1998 and 1999 (Centers for Disease Control and Prevention, Atlanta, GA). *Escherichia coli* O157:H7 strains were obtained from the culture collection of the Microbial Food Safety Laboratory in Food Science and Human Nutrition at Iowa State University. Each culture was maintained as frozen (−70°C) stock in brain heart infusion broth (**BHI**, Difco Laboratories, Detroit, MI) supplemented with 10% glycerol until used. Before each experiment, individual stock cultures were transferred twice in 10 mL of BHI (Remel Inc., Lenexa, KS) and incubated at 35°C for 20 h.

Preparation of Inocula

For each pathogen, equal amounts of each strain were combined to prepare a 5-strain mixture of *L. monocyto-*

genes or *E. coli* O157:H7. Cells from the mixture were harvested by centrifugation (10,000 × *g*, 10 min, 4°C) in a Sorvall Super T21 centrifuge (DuPont Instruments, Wilmington, DE) and washed once in sterile 0.85% (wt/vol) NaCl (saline). The cell pellet was suspended in fresh saline, and this suspension was used as the inoculum. The following method was used to determine the number of bacteria used in each experiment: after serial dilution with a 1-mL aliquot of the suspension into 0.1% peptone water (Difco Laboratories), 0.1 mL of the appropriately diluted solution was surface-plated on the BHI agar media. After incubating at 35°C for 24 to 48 h, the numbers of *E. coil* O157:H7 and *L. monocytogenes* colonies were counted.

$NaHCO_3$ Effect

Disc diffusion assays were performed using ovotransferrin (5, 10, 15, or 20 mg/mL) and $NaHCO_3$ (30, 40, or 50 m*M*) in combination to determine optimal inhibitory concentrations of ovotransferrin and $NaHCO_3$ against *E. coli* O157:H7 and *L. monocytogene*s. Ovotransferrin solution (20 mg/mL) was used to determine the effect of $NaHCO_3$ on antibacterial activity of ovotransferrin. After preparing sterilized ovotransferrin (40 mg/mL), the same volume of 2× strength BHI broth and then 2 *M* sterilized $NaHCO_3$ solution were added to make a final $NaHCO_3$ concentration of 0, 25, 50, or 100 m*M*. Also, 10^3 to 10^4 concentrations of viable *E. coli* O157:H7 and *L. monocytogenes* were inoculated into the sterilized BHI broth media containing ovotransferrin and $NaHCO_3$. After inoculation, the broth culture was incubated at 35°C for 1, 2, 4, 6, and 8 d and then the turbidities of BHI broth culture were measured at 620 nm to estimate the change in population of *E. coli* O157:H7 and *L. monocytogenes* in each treatment.

Citric Acid Effect

The effect of citrate or citric acid on the antibacterial activity of ovotransferrin was investigated. Apo-ovotransferrin solution (40 mg/mL) was added to the same volume of 2×-concentrated BHI broth. Stock citric acid solution was added to the BHI broth to yield an ovotransferrin solution containing 0.25 or 0.5% citric acid. Brain heart infusion broth culture with citric acid was inoculated with the test strains and used as a control. Also, 25 m*M* sodium citrate, equivalent to 0.5% citric acid, was added to the BHI broth containing ovotransferrin and $NaHCO_3$ (50 m*M*) to determine if the effect of citric acid is due to the acidic pH or the role of the synergistic anions. Viable *E. coli* O157:H7 and *L. monocytogenes* at 10^4 to 10^5 cfu/mL were inoculated aseptically into each BHI broth culture. After mixing the broth culture, they were incubated at 35°C for 1, 2, 4, and 8 d. An aliquot (1 mL) of the culture solution was serially diluted with 0.1% peptone water, and 0.1 mL of each serially diluted solution was spread homogenously onto the BHI agar plate. Colonies were enumerated after incubating the BHI agar plates at 35°C for 24 to 48 h. Four sets of samples were prepared and analyzed at each sampling time.

Antimicrobial Activity of Apo-, Fe-, and Zn-Ovotransferrin

To saturate apo-ovotransferrin with Fe^{3+} or Zn^{2+}, 0.15 mL of 0.2 *M* $FeCl_3 \cdot 6H_2O$ or 0.16 mL of 0.2 *M* $ZnCl_2$ was added to 30 mL of ovotransferrin-$NaHCO_3$ solution (40 mg/mL-50 m*M*, pH 7.4), because one 80 kDa ovotransferrin molecule binds 2 metal ions. Four replications were prepared. After adding $FeCl_3$ or $ZnCl_2$ to ovotransferrin solution, the prepared solutions were left standing for a few hours for complete saturation and then dialyzed to remove $NaHCO_3$. The Zn-ovotransferrin, Fe-ovotransferrin, or apo-ovotransferrin solution was added to the same volume of 2×-concentrated BHI broth culture, 50 m*M* and 100 m*M* $NaHCO_3$, and then 10^4 to 10^5 cfu/mL of viable *E. coli* O15:H7 or *L. monocytogenes* strains were inoculated and the solution held at 35°C for 2 d. Viable cells from each treatment were then determined after surface-plating on BHI agar and incubated at 35°C for 24 h for *E. coli* O157:H7 and 48 h for *L. monocytogenes*.

Antibacterial Activity of Ovotransferrin with Citrate or $NaHCO_3$ on Ham

Commercial hams were purchased from local retail stores. Hams were sliced to 0.2-cm-thick pieces and vacuum-packaged (−1 bar of vacuum with 10 s of dwell time) in low oxygen-permeable bags (nylon-polyethylene, 9.3 mL of O_2/m_2 per 24 h at 0°C; Koch, Kansas City, MO).

After randomly separating the sliced hams into 2 groups, the samples were irradiated at 5 kGy using a linear accelerator (Circe IIIR; Thomason CSF Linac, Saint-Aubin, France) to destroy background microflora. Irradiation of hams was introduced to investigate if the natural strains in commercial hams affect the antibacterial activity of ovotransferrin. Hams inoculated with the test strains but no antimicrobial agents were used as controls. The energy and power concentration were 10 MeV and 10 kW, respectively, and the average dose rate was 88.3 kGy/min. To confirm the target dose, 2 alanine dosimeters were attached to the top and bottom surface of a sample per cart. The alanine dosimeter was read using a 10^4 Electron Paramagnetic Resonance Instrument (Bruker Instruments Inc., Billerica, MA). After irradiation, the irradiated and nonirradiated hams were transferred to the microbiology laboratory and stored at 4°C. Viable *E. coli* O157:H7 and *L. monocytogenes* cocktail stock suspension (0.1 mL) were inoculated aseptically on the surface of a sliced ham (6.5 × 4.0 × 0.2 cm) to yield a population of 10^6 cfu/cm^2. After inoculation, ham samples were manually mixed for 30 s to evenly distribute the inocula, and then pack-

aged samples were further separated into 3 groups. One milliliter of the ovotransferrin solutions (20 mg/mL) containing 100 m*M* $NaHCO_3$ or 0.5% of citric acid was distributed evenly on the same surface of sliced ham, manually mixed for 30 s, vacuum-packaged (Multivac A300/16, Sepp Haggenmuller KG, Wolfertschwenden, Germany) in nylon-polyethylene bags, and stored in a refrigerator.

The number of surviving target pathogens was enumerated after refrigeration (4°C) for 0, 5, 10, 15, 22, 29, and 34 d. Each package was aseptically opened using alcohol-sterilized scissors. Sterile 0.1% peptone water (30 to 50 mL) was added to each bag and pummeled at normal speed for 1 min in a stomacher. Samples were serially diluted with 0.1% peptone water, and 0.1 mL of the diluted *E. coli* O157:H7 culture solution was spread onto a MacConkey sorbitol agar plates (Difco Laboratories), whereas samples containing *L. monocytogenes* were spread onto a MOX media. The number of *E. coli* O157:H7 and *L. monocytogenes* colonies appearing on MacConkey sorbitol agar and MOX media were enumerated after incubation for 24 to 48 h at 35°C. Four sets of samples were prepared for each treatment and analyzed each sampling time.

Statistical Analysis

All statistics were replicated 4 times, and data were analyzed using the JMP software (version 5.1.1; SAS Institute., Cary, NC). Differences in the mean values were compared by Tukey's honestly significant differences test, and mean and SEM values were reported (Kuehl, 2000). The Tukey-Kramer test was performed to determine whether the treatment means differ from the controls. Statistical significance for all comparisons was set at $P < 0.05$.

RESULTS AND DISCUSSION

Antibacterial Activity of Natural Apo-Ovotransferrin

Natural apo-ovotransferrin showed a weak antibacterial property against *E. coli* O157:H7 after 6 to 8 d of incubation (Figure 1A), but its antimicrobial activities against *L. monocytogenes* were apparent after 4 d of refrigerated storage (Figure 1B). The antibacterial activity of natural apo-ovotransferrin varied with microorganisms. Valenti et al. (1983) reported that *Pseudomonas* spp., *E. coli,* and *Strep. mutans* were susceptible, whereas *Staph. aureus, Proteus* spp., and *Klebsiella* were relatively resistant to ovotransferrin. Viable lactoferrin is reported to have greater antibacterial effectiveness toward *L. monocytogenes* than that of *E. coli* O157:H7 (http://www.newswise.com/articles/view/525381). Similar to the antibacterial activity of lactoferrin, apo-ovotransferrin was more effective in inhibiting the growth of *L. monocytogenes* than *E. coli* O157:H7.

$NaHCO_3$

The influence of $NaHCO_3$ against the growth of *E. coli* O157:H7 and *L. monocytogenes* was investigated before identifying its synergistic effect with ovotransferrin. As the concentration of $NaHCO_3$ increased, the turbidity of BHI broth culture inoculated with *E. coli* O157:H7 decreased. However, turbidity value of 0.6 obtained from 100 m*M* $NaHCO_3$ alone at d 2 (Figure 1A) indicated that $NaHCO_3$ did not restrain the growth of *E. coli* O157:H7. Corral et al. (1988) reported that 120 m*M* (1% wt/vol) $NaHCO_3$ produced a 4-log reduction of the initial number of *E. coli* in stationary phase by the changes in physicochemical environment of the mi-

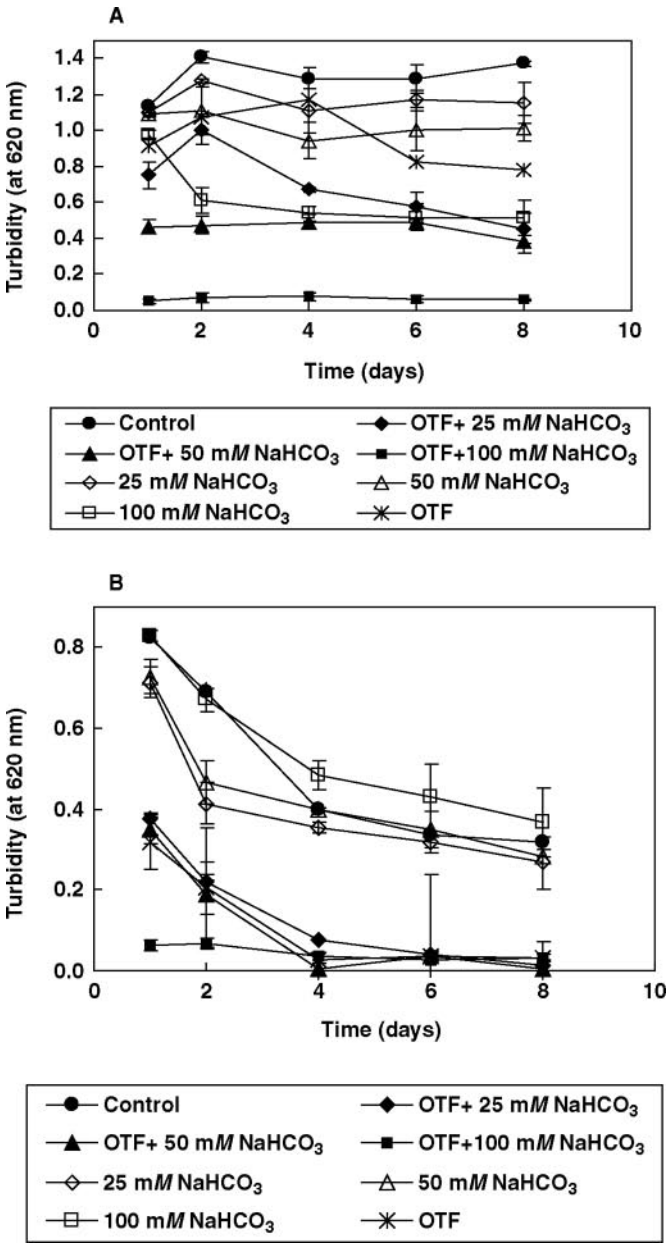

Figure 1. The effect of $NaHCO_3$ on antibacterial activity of ovotransferrin against *Escherichia coli* O157:H7 (A, 10^3 cell inoculation) and *Listeria monocytogenes* (B, 10^4 cell inoculation) during 35°C incubation in model systems. Control was inoculated with only *E. coli* O157:H7. OTF = 20 mg/mL of ovotransferrin, n = 4.

croorganisms. Our result, however, indicated that *E. coli* O157:H7 was relatively resistant to $NaHCO_3$. These results might be due to the fact that we used 5 different strains of *E. coli* O157:H7, which have different susceptibility against $NaHCO_3$.

In contrast to *E. coli* O157:H7, as the concentration of $NaHCO_3$ increased, the turbidity of BHI broth culture with *L. monocytogenes* increased, and the turbidity of sample with 100 m*M* $NaHCO_3$ had the greatest value (Figure 1B). Czuprynski and Faith (2002) reported that 50 μL of 10% $NaHCO_3$ (wt/vol) enhanced the virulence of *L. monocytogenes* EGD or *L. monocytogenes* strain Scott A in mice. *Escherichia coli* O157:H7 showed some susceptibility to 100 m*M* $NaHCO_3$, whereas *L. monocytogenes* exhibited little resistance to the $NaHCO_3$.

$NaHCO_3$ on Antibacterial Activity of Ovotransferrin

Sodium bicarbonate (25, 50, or 100 m*M*) was added to BHI broth containing apo-ovotransferrin to improve its antibacterial activity against *E. coli* O157:H7 and *L. monocytogenes*. The antibacterial activity of ovotransferrin + $NaHCO_3$ depended on the concentration of $NaHCO_3$ added (Figure 1). As the concentration of $NaHCO_3$ increased, antimicrobial activity of ovotransferrin + $NaHCO_3$ increased. According to many reports, Fe^{3+}-specific binding capacity of transferrin is promoted at constant pH as the concentration of $NaHCO_3$ increased (Aisen, 1989; Pakdaman and El Hage Chahine, 1997; Abdallah and Chahine, 1998). Like transferrin, $NaHCO_3$ increased the antibacterial effect of apo-ovotransferrin against *E. coli* and *L. monocytogenes*. Also, with *E. coli* O157:H7, the turbidity of ovotransferrin solutions containing $NaHCO_3$ was lower than that of apo-ovotransferrin or $NaHCO_3$ alone (Figure 1A). This indicated that $NaHCO_3$ alone had no effect on the antibacterial activity of apo-ovotransferrin against *E. coli* O157:H7. The combination of ovotransferrin with 100 m*M* $NaHCO_3$ was the best condition for inhibiting the growth of *E. coli* O157:H7. Ovotransferrin + 25 m*M* $NaHCO_3$ or ovotransferrin + 50 m*M* $NaHCO_3$ did not inhibit the growth of *L. monocytogenes* during the first 4 d of incubation, whereas ovotransferrin containing 100 m*M* $NaHCO_3$ prevented the growth of *L. monocytogenes* from the beginning. There was an apparent synergistic effect of 100 m*M* $NaHCO_3$ on the antimicrobial activity of apo-ovotransferrin against *L. monocytogenes* (Figure 1B). Considering ovotransferrin + 100 m*M* $NaHCO_3$ has the lowest turbidity value in 2 strains (Figure 1), 100 m*M* $NaHCO_3$ was the most appropriate concentration to improve the antibacterial activity of apo-ovotransferrin against *E. coli* O157:H7 and *L. monocytogenes*.

Carbonate anions interact directly with the complex of transferrin- or lactoferrin-bound metals such as Fe and Cu (Eaton et al., 1990). Apo-transferrin is also known to interact with 2 HCO_3^- or CO_3^{2-}. These carbonic ions react directly with arginine residues of transferrin in binding sites of 2 lobes (Bellounis et al., 1996; Pakdaman et al., 1998). Sodium bicarbonate at 50 m*M* increased the antibacterial activity of ovotransferrin against *E. coli* W1485, whereas ovotransferrin without $NaHCO_3$ did not show antibacterial activity (Valenti et al., 1983). The present study suggests that a certain concentration of $NaHCO_3$ can increase the antibacterial effectiveness of ovotransferrin toward *E. coli* O157:H7 and *L. monocytogenes*.

Citric Acid and Citrate

The effects of citric acid on the growth of *E. coli* O157:H7 and *L. monocytogenes* were different: *L. monocytogenes* was more sensitive to citric acid than *E. coli* O157:H7 (Figure 2). Also, the antibacterial activity of citric acid depended upon the concentration of citric acid. Citric acid at a 0.5% concentration showed much greater antibacterial activity than 0.25% citric acid. Also, 0.5% citric acid alone did not completely inhibit the growth of *E. coli* O157:H7 until 8 d of incubation (Figure 2A and Table 1), but induced s 2.5- ~ 3-log reduction of viable *L. monocytogenes* cells after 2 d of incubation (Figure 2B). Lee et al. (2001, 2002) reported that sodium citrate has strong antimicrobial effects against gram-positive strains but has weak antibacterial activity against gram-negative microorganisms. At the present study, citric acid showed stronger antibacterial activities against *L. monocytogenes* than *E. coli* O157:H7. Considering the report that sodium citrate has antibacterial activity against gram-positive microorganisms, the cause of antimicrobial action of citric acid against *L. monocytogenes* was not ascribed to pH.

Citric Acid and Citrate Effect on Antibacterial Activity of Ovotransferrin

Even though ovotransferrin + 0.25% citric acid did not show antibacterial activity, ovotransferrin added with 0.5% citric acid showed bactericidal effect against *E. coli* O157:H7. Also, 0.5% citric acid and ovotransferrin combination produced a larger reduction in the number of viable *E. coli* O157:H7 cells than that with 0.5% citric acid alone (Figure 2A). This indicated that 0.5% citric acid and ovotransferrin combination has some synergistic effect against *E. coli* O157:H7. Ovotransferrin + 0.25% citric acid did not show significant difference in bactericidal effect against *L. monocytogenes*. In fact, the antibacterial activity of 0.25% citric acid against *L. monocytogenes* decreased when ovotransferrin was combined. Ovotransferrin containing 0.5% citrate showed bactericidal effect against *L. monocytogenes*, but its antimicrobial activity was lower than that of 0.5% citric acid alone after 4 d of incubation at 35°C (Figure 2B). Therefore, the bactericidal effect of ovotransferrin solution added with 0.5% citric acid against *L. monocytogenes* was attributed to anti-

Table 1. Influence of citric acid and citrate on antibacterial activity of ovotransferrin against a 10^5 population of *Escherichia coli* O157:H7 in brain heart infusion broth on d 1, 2, 4, and 8 of storage[1]

	Number of viable cells ($\log_{10}$ cfu/mL)			
Treatments	d 1	d 2	d 4	d 8
Citric acid	$7.90^{c,w} \pm 0.65$	$6.88^{c,wx} \pm 0.43$	$5.90^{b,x} \pm 1.00$	$2.23^{c,y} \pm 0.43$
OTF + citric acid	$4.71^{d,w} \pm 0.09$	$3.92^{d,x} \pm 0.10$	$2.04^{c,y} \pm 0.00$	$1.00^{d,z} \pm 0.00$
OTF + citric acid +$NaHCO_3$	$8.64^{ab,x} \pm 0.10$	$8.41^{b,x} \pm 0.36$	$9.34^{a,w} \pm 0.19$	$8.74^{a,x} \pm 0.15$
OTF + Na citrate	$9.24^{a,w} \pm 0.06$	$9.35^{a,w} \pm 0.08$	$8.97^{a,x} \pm 0.05$	$8.22^{b,y} \pm 0.07$
OTF + $NaHCO_3$	$8.40^{bc,w} \pm 0.10$	$8.64^{ab,w} \pm 0.39$	$8.69^{ab,w} \pm 0.18$	$7.75^{b,x} \pm 0.03$

[a–d]Different letters within a column with the same storage day are different ($P < 0.05$). n = 4.

[w–z]Different letters within a row with the same treatment are different ($P < 0.05$).

[1]This study used 0.5% citric acid, 25 m*M* sodium citrate, and 50 m*M* $NaHCO_3$. OTF = ovotransferrin (20 mg/mL).

bacterial activity of 0.5% citric acid rather than their synergistic effect. In addition, the citric acid effects on antibacterial activity of apo-ovotransferrin against *E. coli* O157:H7 and *L. monocytogenes* were lost in the presence of 50 m*M* $NaHCO_3$. Also, the number of viable cells in ovotransferrin + 0.5% citric acid in the presence of 50 m*M* $NaHCO_3$ was similar to that of ovotransferrin with 50 m*M* $NaHCO_3$ in both pathogens. Ovotransferrin + 25 m*M* sodium citrate solution did not inhibit the growth of the 2 pathogens in contrast to ovotransferrin + 0.5% citric acid (Tables 1 and 2).

Bishop et al. (1976) report that as the molar ratio of citrate to apo-lactoferrin increased, the antibacterial capacity of apo-lactoferrin toward coliform bacteria decreased. Reiter et al. (1975) reported that the presence of citrate in bovine colostrum counteracted the antibacterial effectiveness of lactoferrin against *E. coli*. Frost and Rosemberg (1973) reported that an excess of citrate annulled the antibacterial activity of ovotransferrin against *E. coli* O157:H7, because it possesses an Fe transport system mediated by citrate. Also, an anionic chelating by citrate occurred at high pH values. As 1 Fe molecule binds 2 citrate molecules, the Fe-binding capacity by ovotransferrin is affected by the metal chelater (Phelps and Antonini, 1975). The effect of sodium citrate on antibacterial effectiveness of ovotransferrin is dependent upon its concentration. The growth of *E. coli* is recovered better in the presence of 50 m*M* sodium citrate than 10 m*M* sodium citrate (Valenti et al., 1983). The Fe release of transferrin was faster in the presence of citrate than formate or acetate, because citrate interacts with decarbonated monoferric proteins with high affinity. In the absence of citrate, however, Fe release occurs at lower pH, and the intermediate tertiary complex was not formed (Abdallah and el Hage Chahine, 1999).

Generally, Fe-binding capacity of ovotransferrin is known to decrease under acidic pH (Warner and Weber, 1953; Butterworth et al., 1975; Williams et al., 1978). As 0.5% citric acid decreases the pH of ovotransferrin solution to around 4 to 5, the antibacterial activity of ovotransferrin should be decreased due to its low Fe-binding capacity. However, this study indicated that 0.5% citric acid enhanced the antibacterial activity of ovotransferrin against *E. coli* O157:H7. Ovotransferrin

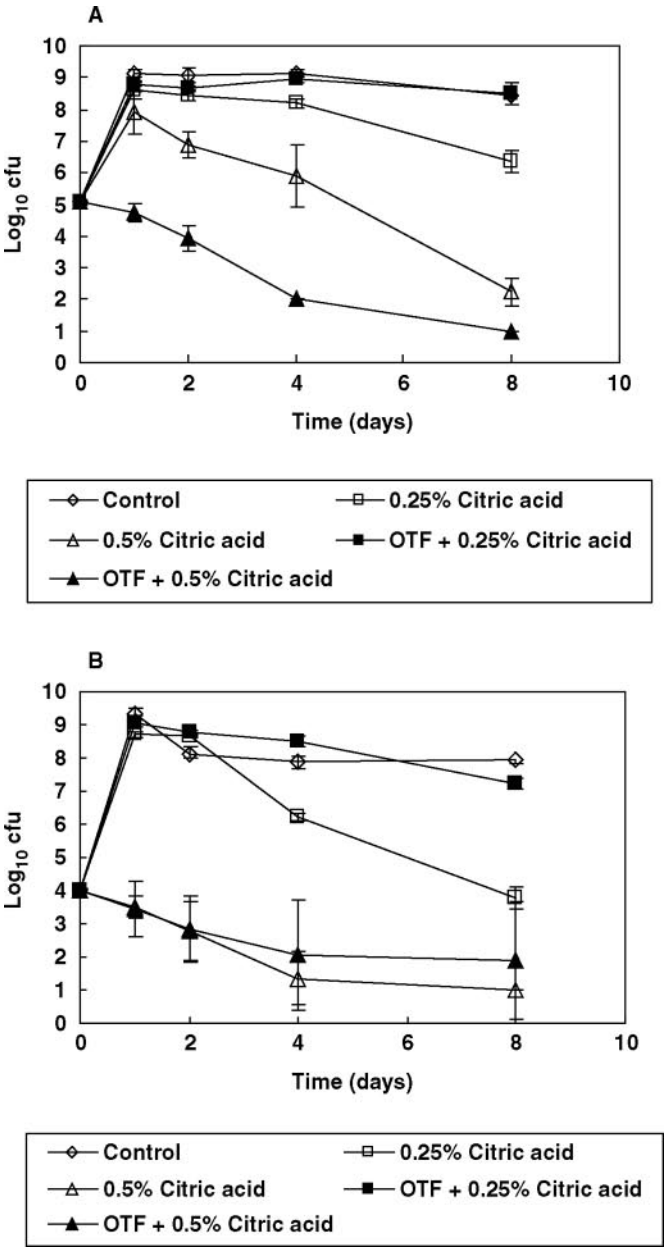

Figure 2. The influence of citric acid on antibacterial activity of ovotransferrin against 10^5 cfu/mL of *Escherichia coli* O157:H7 (A, 20 mg/mL of ovotransferrin) and 10^4 population of *Listeria monocytogenes* in model systems. Control was inoculated with only *E. coli* O157:H7. OTF in A = 20 mg/mL of ovotransferrin; OTF in B = 15 mg/mL of ovotransferrin, n = 4.

Table 2. Effect of citric acid and citrate on antibacterial activity of ovotransferrin against a 10^4 population of *Listeria monocytogenes* in brain heart infusion broth on d 1, 2, 4, and 8 of storage[1]

	Number of viable cell ($\log_{10}$ cfu/mL)			
Treatments	d 1	d 2	d 4	d 8
Citric acid	$3.52^{b,w} \pm 0.32$	$2.78^{b,w} \pm 1.14$	$1.52^{b,x} \pm 0.96$	$1.00^{b,x} \pm 0.00$
OTF + citric acid	$3.52^{b,w} \pm 0.83$	$2.86^{b,w} \pm 0.99$	$2.05^{b,w} \pm 1.67$	$1.91^{b,w} \pm 1.78$
OTF + citric acid + $NaHCO_3$	$8.86^{a,w} \pm 0.14$	$8.60^{a,wx} \pm 0.41$	$8.14^{a,xy} \pm 0.29$	$7.67^{a,y} \pm 0.60$
OTF + Na citrate	$9.23^{a,w} \pm 0.11$	$8.62^{a,x} \pm 0.17$	$8.57^{a,x} \pm 0.23$	$8.43^{a,x} \pm 0.31$
OTF + $NaHCO_3$	$8.60^{a,w} \pm 0.19$	$7.74^{a,y} \pm 0.01$	$8.14^{a,x} \pm 0.07$	$7.13^{a,z} \pm 0.04$

[a,b]Different letters within a column with the same storage day are different ($P < 0.05$). n = 4.

[w–z]Different letters within a row with the same treatment are different ($P < 0.05$).

[1]This study used 0.5% citric acid, 25 m*M* sodium citrate, and 50 m*M* $NaHCO_3$. OTF = ovotransferrin (15 mg/mL).

+ 25 m*M* sodium citrate did not show any antibacterial activity against the 2 tow pathogens, and $NaHCO_3$ annulled the antimicrobial activity of ovotransferrin + 0.5% citric acid. Considering these results, the antimicrobial action of ovotransferrin was enhanced by acidic pH conditions. The synergistic effect of citric acid on antibacterial activity of ovotransferrin against *E. coli* O157:H7 was due to proton formation, because addition of $NaHCO_3$ or sodium citrate did not result in acidic environments. As ovotransferrin reacted directly to bacterial membrane, the permeability of outer membrane was assumed to be increased. Under such a circumstance, many protons provided by citric acid can enter inside the cell membrane. Thus, the proton motive force of *E. coli* O157:H7 could have been destroyed and resulted in cell death. This suggested that the synergistic effect of citric acid on antibacterial activity of ovotransferrin is not related to the Fe-binding capability of ovotransferrin. Viable lactoferrin is reported to interact directly with bacterial membrane materials including lipopolysaccharides (Appelmelk et al., 1994; Elass-Rochard et al., 1995), glycosaminoglycans (Mann et al., 1994), and cell-type-specific receptors on epithelial and immune cells (Baveye et al., 1999; Brock, 2002; Suzuki et al., 2002; Ward et al., 2002). Also, specific binding of lactoferrin against outer membrane proteins of gram-negative bacteria can lead to inhibition of various cellular functions and deregulation of adhesion-fimbrial synthesis and increase of permeability in bacterial outer membrane, which increase the susceptibility of cell membrane to hydrophobic antibiotics and lysozyme and facilitate the release of lipopolysaccharides molecules from cell membrane (Ellison et al., 1988; Ellison and Giehl, 1991; Naidu, 2002).

Antibacterial Activity of Apo-, Fe-, and Zn-Ovotransferrin

To see if the antibacterial activity of ovotransferrin is related to its metal-binding property, the antibacterial activity of ovotransferrin saturated with Fe^{2+} or Zn^{2+} against *E. coli* O157:H7 and *L. monocytogenes* was determined. The content of residual Fe in apo-ovotransferrin used in this study was around 0.38 mg/kg. As expected, natural apo-ovotransferrin showed little antibacterial activity, but ovotransferrin added with 50 m*M* $NaHCO_3$ has antibacterial activity against *E. coli* O157:H7 and *L. monocytogenes* (Figure 3). Ovotransferrin + 100 m*M* $NaHCO_3$ showed the strongest bacteriostatic activity against both pathogens. Ovotransferrin saturated with Fe had little or no bactericidal activity against *E. coli* O157:H7 and *L. monocytogenes* (Figures 3A and 3B). Ovotransferrin bound with Zn also did not show antibacterial activity against *E. coli* O157:H7 (Figure 3A), but it had a similar degree of antibacterial activity as apo-ovotransferrin added with 50 m*M* $NaHCO_3$ against *L. monocytogenes* (Figure 3B). These results did not agree with those of Valenti et al. (1987), who reported that ovotransferrin saturated with Zn had greater antibacterial activity against *E. coli* than apo-ovotransferrin. Also, Ibrahim (1997) reported that ovotransferrin saturated with Fe showed more powerful bactericidal effect against *E. coli* than Fe-free ovotransferrin, even though ovotransferrin inhibited bacterial growth by chelating Fe with high affinity. He also indicated that the treatment has a strong antimicrobial activity against *Staph. aureus* regardless of the amount of Fe available. Several other reports also suggested that ovotransferrin bound with metals such as Zn and Fe had antibacterial activity (Valenti et al., 1985, 1987; Ibrahim, 1997). However, the present study demonstrated that Fe-bound ovotransferrin had little antimicrobial effect and the antibacterial activity of Zn-bound ovotransferrin varied with microorganisms. It was found that Zn^{2+} affected antibacterial action of ovotransferrin against *L. monocytogenes* but not *E. coli* O157:H7. Considering Zn-bound ovotransferrin possesses bacteristatic activity, it is not likely to convince such a conclusion that the cause of antibacterial activity of ovotransferrin is attributed to Fe deprivation or metal-binding capacity. Valenti et al. (1982) suggested that the antibacterial effectiveness of ovotransferrin is not due simply to the Fe deprivation from media. Many recent reports indicated that antibacterial activity of ovotransferrin may be associated with direct interactions between ovotransferrin and microorganism, regardless of its Fe-binding properties (Arnold et al., 1980, 1981; Valenti et al., 1985). According to these reports, ovotransferrin saturated with Fe still displayed antifun-

gal activity toward *Candida* (Valenti et al., 1985). Ibrahim et al. (2000) suggested that the N-terminal lobe of ovotransferrin showed more potent bactericidal activity against *Staph. aureus* than the C-terminal lobe regardless of the degree of Fe saturation.

Considering that ovotransferrin has a similar structural similarity to that of human lactoferrin, which can react directly to bacterial surface besides the role of Fe deprivation, it is not difficult to assume that the antimicrobial action of ovotransferrin could be intimately related to direct reactions to bacterial membrane rather than Fe-deprivation property of ovotransferrin alone (Valenti et al., 1987). Ovotransferrin may permeate bacterial outer membranes, reach the inner membrane, and lead to permeation of other ions, which dissipate electrical potential so that it can exert antibacterial action against gram-negative bacteria (Aguilera et al., 2003).

Application of Ovotransferrin to Ham

Based on the results obtained from in vitro tests, the ovotransferrin solutions added with 100 m*M* $NaHCO_3$ or 0.5% citric acid were applied on commercial hams to determine their antibacterial activity. During storage at 4°C for 4 wk, the number of viable *E. coli* O157:H7 cells decreased slowly (Table 3), whereas that of *L. monocytogenes* resulted in 1 to 2 log increase (Table 4) even though Amézquita and Brashears (2002) reported that *L. monocytogenes* showed bacteriostatic activity in cooked ham. This indicated that *E. coli* O157:H7 is more sensitive to low temperature than *L. monocytogenes.* Moreover, commercial hams contain a variety of antibacterial substances such as nitrite, salt, and low water activity, but *L. monocytogenes* is resistant to low temperature and grew slowly. There was little significant difference in the numbers of viable *E. coli* O157:H7 cells between nonirradiated and irradiated hams during 4°C storage (Table 3). The number of viable *L. monocytogenes* cells between nonirradiated and irradiated hams treated with ovotransferrin + 100 m*M* $NaHCO_3$ was not different. Ovotransferrin + 0.5% citric acid did not exhibit any antibacterial activity in nonirradiated hams but inhibited the growth of *L. monocytogenes* in irradiated hams (Table 4). Zhu et al. (2004) found 10^2 to 10^3 cfu/cm^2 of natural *L. monocytogenes* in nonirradiated vacuum-packaged turkey hams and the *L. monocytogenes* grew during the 28 d of refrigerated storage. This indicated that the *L. monocytogenes* existed originally in commercial hams affected the antibacterial activity of ovotransferrin + 0.5% citric acid in this study.

The ovotransferrin + 100 m*M* $NaHCO_3$ treatment had a greater number of viable *L. monocytogenes* cells than control at 10, 15, 22, and 29 d of storage. However, combination of ovotransferrin with 0.5% citric acid exhibited 1.1 log lower viable *L. monocytogenes* cells than control at d 29 (Table 4). This indicated that ovotransferrin combination with 100 m*M* $NaHCO_3$ did not have any antibacterial activity against *E. coli* O157:H7 and *L. monocytogenes,* but the combination of ovotransferrin with 0.5% citric acid suppressed the survival of *L. monocytogenes* in hams (Tables 3 and 4). Figures 1 and 2 showed that the antibacterial activities of ovotransferrin + 100 m*M* $NaHCO_3$ and ovotransferrin + 0.5% citric acid against 2 pathogens in nonirradiated hams and model system are significantly different. Such differences might be attributed to media or matrix compositions, difficulties in treatment distribution, or binding of ovotransferrin to matrix. Because hams have a variety of factors that affect antibacterial activity, the antibacterial activity of ovotransferrin on hams was modulated by these factors.

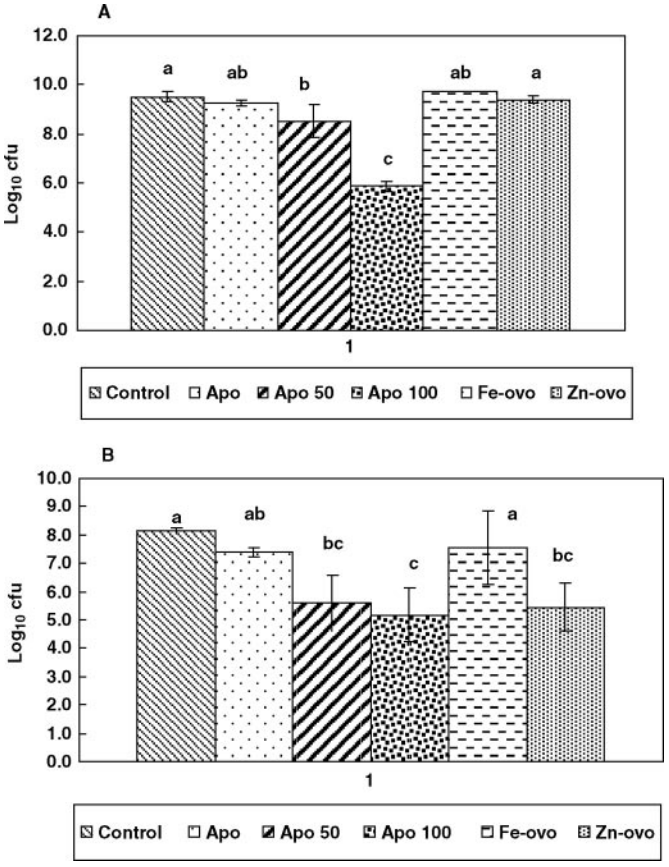

Figure 3. Antibacterial activity of apo-ovotransferrin (OTF), Fe-OTF, and Zn-OTF against (A) 10^4 cfu/mL of *Escherichia coli* O157:H7 and (B) 10^5 cfu/mL of *Listeria monocytogenes* in model systems. Control = only *E. coli* O157:H7; Apo = 20 mg/mL of apo-OTF; Apo 50 = Apo + 50 m*M* $NaHCO_3$; Apo 100 = Apo + 100 m*M* $NaHCO_3$; Fe-ovo = 20 mg/mL of Fe-bound OTF; Zn-ovo = 20 mg/mL of Zn-bound OTF. $^{a-c}$Letters on bars indicate significant differences ($P < 0.05$), n = 4.

Ellison et al. (1988) reported that the antibacterial effectiveness of lactoferrin was not achieved in food or complex media because of divalent cations such as Ca^{2+} and Mg^{2+}. Shimazaki (2000) reported that divalent cations changed the tertiary structure of lactoferrin, which prevented antibacterial action of lactoferrin. Besides these ions, the stability of ovotransferrin in matrix is a critical factor in antibacterial activity of ovotransferrin. Valenti et al. (1982) reported that Sepharose-bound

Table 3. Antibacterial activity of ovotransferrin solutions (20 mg/mL) + either 100 m*M* $NaHCO_3$ or 0.5% citric acid on nonirradiated and irradiated vacuum-packaged hams inoculated with *Escherichia coli* O157:H7 during d 0, 5, 10, 15, 22, and 29 of storage at 4°C[1]

	Number of viable cell (log_{10} cfu/mL)					
Sample treatments	d 0	d 5	d 10	d 15	d 22	d 29
Non-IR						
Control[2]	$6.1^{a,x} \pm 0.21$	$5.5^{a,y} \pm 0.22$	$5.3^{ab,yz} \pm 0.12$	$5.2^{a,yz} \pm 0.10$	$5.0^{b,z} \pm 0.08$	$5.5^{a,yz} \pm 0.01$
OTF + $NaHCO_3$	$6.0^{a,x} \pm 0.11$	$5.7^{a,y} \pm 0.50$	$5.2^{b,z} \pm 0.01$	$5.2^{a,z} \pm 0.17$	$5.0^{b,z} \pm 0.10$	$5.0^{c,z} \pm 0.01$
OTF + citric acid	$5.9^{a,x} \pm 0.10$	$5.7^{a,xy} \pm 0.04$	$5.2^{b,yz} \pm 0.05$	$5.1^{a,z} \pm 0.42$	$5.0^{b,z} \pm 0.07$	$5.1^{c,yz} \pm 0.11$
IR						
Control	$6.0^{a,x} \pm 0.0$	$5.5^{a,y} \pm 0.06$	$5.5^{a,y} \pm 0.05$	$4.9^{a,y} \pm 0.32$	$5.3^{a,y} \pm 0.17$	$5.4^{ab,y} \pm 0.01$
OTF + $NaHCO_3$	$6.1^{a,x} \pm 0.19$	$5.6^{a,y} \pm 0.08$	$5.5^{a,yz} \pm 0.06$	$4.9^{a,z} \pm 0.19$	$5.0^{b,z} \pm 0.07$	$5.3^{ab,yz} \pm 0.04$
OTF + citric acid	$6.0^{a,x} \pm 0.0$	$5.7^{a,x} \pm 0.05$	$5.5^{a,xy} \pm 0.08$	$5.0^{a,y} \pm 0.40$	$5.0^{b,y} \pm 0.02$	$5.3^{ab,xy} \pm 0.10$

[a–c]Different letters within a column with the same storage day are different ($P < 0.05$). n = 4.

[x–z]Different letters within a row with the same treatment are different ($P < 0.05$).

[1]This study used 0.5% citric acid and 100 m*M* $NaHCO_3$. OTF = ovotransferrin (20 mg/mL); non-IR = nonirradiated ham; IR = irradiated ham.

[2]Only *E. coli* O157:H7 inoculation.

ovotransferrin showed greater antimicrobial activity than free ovotransferrin, because matrix-bound proteins have greater stability than soluble ovotransferrin. Direct application of lactoferrin to food systems decreases antibacterial activity of lactoferrin, because active substances in complex media such meat or meat products neutralize the antibacterial effectiveness of lactoferrin. Also, the viable lactoferrin can be easily diffused into hams, and the antibacterial activity of lactoferrin on meat surface is difficult to maintain. Incorporation of lactoferrin into film or finding a film matrix appropriate to maintain the antibacterial activity by preventing diffusion of lactoferrin into meat has been discussed (http://www.newswise.com/articles/view/525381).

In conclusion, the antibacterial activity of natural apo-ovotransferrin against *E. coli* O157:H7 and *L. monocytogenes* in model systems increased as the concentration of $NaHCO_3$ increased. Sodium bicarbonate at 100 m*M* markedly increased antibacterial activity of ovotransferrin against *E. coli* O157:H7 and *L. monocytogenes*. Citric acid at 0.5% enhanced antibacterial activity of apo-ovotransferrin against *E. coli* O157:H7, but 0.5% citric acid alone also showed a strong bactericidal activity against *L. monocytogenes.* Addition of $NaHCO_3$ negated the strong antibacterial activity of ovotransferrin + citric acid against the 2 pathogens. The antimicrobial activity of ovotransferrin was greatly enhanced by acidic pH conditions. Zinc-bound ovotransferrin produced a bacteriostatic effect against *L. monocytogenes*, but Fe-bound ovotransferrin had little or no antibacterial activity against *E. coli* O157:H7 and *L. monocytogenes.* Considering these results, Fe bind capacity of ovotransferrin is not the major cause of antibacterial action of ovotransferrin. Previous studies indicate that ovotransferrin directly interacts with bacterial membranes causing a variety of physiochemical changes that affect the survival of microorganisms. Ovotransferrin + 100 m*M* $NaHCO_3$ did not exhibit any antibacterial activity against 2 pathogens in commercial hams, whereas ovotransferrin + 0.5% citric acid suppressed *L. monocytogenes* in irradiated hams but not in nonirradiated hams. There are some limitations of using ovotransferrin to control pathogens in meat or meat products. To overcome these problems, further studies are needed to determine the mechanisms of antibacterial activity of ovotransferrin and to identify various factors that can improve the antibacterial activity of ovotransferrin.

Table 4. Antibacterial activity of ovotransferrin solution (20 mg/mL) + either 100 m*M* sodium carbonate or 0.5% citric acid on nonirradiated and irradiated vacuum-packaged hams inoculated with *Listeria monocytogenes* during d 0, 5, 10, 15, 22, and 29 of storage at 4°C[1]

	Number of viable cell (log_{10} cfu/mL)					
Sample treatments	0	5	10	15	22	29
Non-IR						
Control[2]	$6.5^{a,x} \pm 0.15$	$6.3^{ab,x} \pm 0.01$	$6.5^{b,x} \pm 0.03$	$6.9^{b,yx} \pm 0.10$	$7.5^{ab,y} \pm 0.10$	$8.2^{a,z} \pm 0.09$
OTF + $NaHCO_3$	$6.4^{ab,x} \pm 0.11$	$6.4^{a,x} \pm 0.04$	$7.7^{a,y} \pm 0.09$	$7.6^{a,y} \pm 0.09$	$8.3^{a,z} \pm 0.21$	$8.4^{a,z} \pm 0.05$
OTF + citric acid	$6.4^{ab,x} \pm 0.14$	$6.5^{a,x} \pm 0.04$	$6.6^{b,x} \pm 0.14$	$7.1^{ab,y} \pm 0.03$	$7.3^{ab,y} \pm 0.12$	$8.2^{a,z} \pm 0.10$
IR						
Control	$6.4^{ab,x} \pm 0.04$	$6.2^{b,x} \pm 0.08$	$6.4^{b,x} \pm 0.13$	$6.6^{b,yz} \pm 0.25$	$7.0^{b,y} \pm 0.08$	$8.0^{a,z} \pm 0.30$
OTF + $NaHC0_3$	$6.2^{b,x} \pm 0.14$	$6.5^{a,yz} \pm 0.01$	$7.5^{a,xy} \pm 0.23$	$7.7^{a,xy} \pm 0.45$	$7.7^{ab,xy} \pm 0.63$	$8.3^{a,z} \pm 0.47$
OTF + citric acid	$6.2^{ab,x} \pm 0.18$	$6.2^{b,x} \pm 0.03$	$6.4^{b,x} \pm 0.07$	$6.5^{b,x} \pm 0.22$	$6.9^{b,x} \pm 0.53$	$6.9^{b,x} \pm 0.57$

[a,b]Different letters within a column with the same storage day are different ($P < 0.05$). n = 4.

[x–z]Different letters within a row with the same treatment are different ($P < 0.05$).

[1]This study used 0.5% citric acid and 100 m*M* $NaHCO_3$. OT = ovotransferrin (20 mg/mL); non-IR = nonirradiated ham; IR = irradiated ham.

[2]Only *L. monocytogenes* inoculation.

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