Ethylendiaminetetraacetate and lysozyme improves antimicrobial activities of ovotransferrin against *Escherichia coli* O157:H7

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**ABSTRACT** The aim of this study was to evaluate the effect of EDTA, lysozyme, or the combination of EDTA and lysozyme on the antibacterial activity of ovotransferrin against *Escherichia coli* O157:H7. Ovotransferrin solutions (20 mg/mL) containing 100 mM NaHCO3 (OS) with added EDTA (2.0 or 2.5 mg/mL), lysozyme (1.0, 1.5, or 2.0 mg/mL), or both were prepared. The antibacterial activities of OS, OSE (OS + EDTA), or OSL (OS + lysozyme) against *E. coli* O157:H7 in model systems were investigated by turbidity and viability tests. In addition, OSE, OSL, or OSEL (OS + EDTA + lysozyme) was applied to irradiated pork chops and commercial hams to determine whether the solutions had antibacterial activity on meat products. The effect of the initial cell population on the antibacterial activity of OSE, OSL, and OSEL was determined. Ethylenediaminetetraacetate at 2 mg/mL plus OS induced a reduction of approximately 3 to 4 log in viable *E. coli* O157:H7 cells in brain heart infusion broth media, and 1 mg/mL of lysozyme plus OS resulted in a reduction of approximately 0.5 to 1.0 log during a 36-h incubation at 35°C. However, neither OSE nor OSEL showed a significant antibacterial effect on pork chops and hams during storage at 10°C. The initial cell number in media did not affect the antibacterial activity of OSE or OSEL against *E. coli* O157:H7. This study demonstrates that combinations of ovotransferrin, NaHCO3, and EDTA have the potential to control *E. coli* O157:H7.

**Key words:** ovotransferrin, antibacterial activity, ethylenediaminetetraacetate, lysozyme, *Escherichia coli* O157:H7

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**INTRODUCTION**

Natural antimicrobials that are environmentally friendly, medically acceptable, highly effective, and economical to manufacture have gained attention because of the consumer desire for natural food products (Payne et al., 1994). Ovotransferrin, one of the main iron-binding glycoproteins present in egg white, transports and scavenges Fe(III) in poultry eggs (Kurokawa et al., 1995). It is a major contributor to the defense of the egg against microbial infection and rotting. Many studies have described the antibacterial properties of ovotransferrin against various microorganisms, including *Escherichia coli* (Schade and Caroline, 1944), *Pseudomonas* spp., *Streptococcus mutans* (Valenti et al., 1983), *Staphylococcus aureus, Bacillus cereus* (Ibrahim, 1997), *Salmonella Enteritidis* (Baron et al., 2000), and *Candida* (Valenti et al., 1985). Initially, the iron-binding capability of ovotransferrin, which limits the availability of iron required for microbial growth, was considered the major antibacterial mechanism of ovotransferrin (Alderton et al., 1946; Fraenk-Conrat and Feeney, 1950; Arnold et al., 1980). However, subsequent studies have suggested that the antimicrobial action of ovotransferrin could be intimately related to direct interactions of ovotransferrin with the bacterial surface, resulting in damage to the outer membranes of microorganisms or destruction of their microbial function, such as the proton motive force (Arnold et al., 1981; Ibrahim, 1997; Ibrahim et al., 2000; Aguilera et al., 2003; Ahlborn and Sheldon, 2006). In addition, Ko and Ahn (2008) recently developed a simple purification method for the large-scale production of ovotransferrin, and the application of ovotransferrin as a natural antimicrobial agent in the food or meat industry seems to be of interest.

*Escherichia coli* O157:H7 possesses specific cell-surface appendages such as fimbria or colonization factor antigens that facilitate their adhesion to host tissue-matrix components such as fibronectin, collagens, elastin, and mucin so that it can attach tightly to beef tissues (Naidu, 2002). In addition, illnesses caused by *E. coli* O157:H7 are frequently linked to cattle and cattle
products (e.g., undercooked ground beef and raw milk; Weeratna and Doyle, 1991; Bell et al., 1994).

Combinations of antimicrobials with different mechanisms can increase their antibacterial effectiveness. Ethylenediaminetetraacetate has been used in several food products as a chelating agent to prevent oxidative and deteriorative reactions that are catalyzed by metal ions (Hansen et al., 2001). In addition, EDTA is known to enhance the effectiveness of antimicrobials and antibiotics, especially against gram-negative bacteria. For instance, EDTA promotes the antimicrobial activity of nisin, lysozyme, and monolaurin against gram-negative microorganisms (Hughey and Johnson, 1987; Stevens et al., 1991; Razavi-Rohani and Griffiths, 1994). Ethylenediaminetetraacetate destabilizes the outer membrane of gram-negative bacteria by chelating Ca2+ and Mg2+ salts, which function as bridges between lipopolysaccharides (LPS) in the microbial outer membrane, resulting in the release of LPS from gram-negative bacteria (Vaara, 1992).

Lysozyme catalyzes the hydrolysis of 1,4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in cell wall peptidoglycan. Because the cell walls of gram-negative bacteria are protected by an outer membrane, gram-negative microorganisms are relatively resistant to the antimicrobial activities of lysozyme; thus, the application of lysozyme in foods has been limited (Gill and Holley, 2003). However, lysozyme has gained considerable interest for use in food systems because it is a natural enzyme produced by many animals and its activity targets a specific cellular structure of microorganisms (Proctor and Cunningham, 1988).

Ovotransferrin alone has shown little antibacterial activity against E. coli O157:H7 in previous studies. According to Ko et al. (2008), an ovotransferrin solution with 100 mM NaHCO3 added (OS) was found to be bacteriostatic against the pathogen, but not bactericidal. The aim of the present study was to promote the antimicrobial activity of OS against E. coli O157:H7 by combining OS with EDTA, lysozyme, or both and to identify the possibility of applying this combination (i.e., EDTA or lysozyme, ovotransferrin, and 100 mM NaHCO3) on commercial hams and irradiated pork chops to control the growth of E. coli O157:H7.

MATERIALS AND METHODS

Ovotransferrin

The apo-ovotransferrin (iron-free) used in this study was prepared by the method of Ko and Ahn (2008). After iron saturation of a 2×-diluted egg white solution, holo-ovotransferrin (iron-saturated) was separated by using ethanol. Iron was removed from the holo-ovotransferrin by using AG 1-X2 resin (chloride form, Bio-Rad Laboratories Inc., Hercules, CA) and then freeze-dried. The dried apo-ovotransferrin was dissolved in distilled water, its pH was adjusted to 7.4, and 0.15 M NaCl was added. The purity of the ovotransferrin used in this study was approximately 80%, and the residual iron in the prepared apo-ovotransferrin solution was less than 0.5 ppm. Before microbial study, the ovotransferrin solution was sterilized by filtering through a 0.22-µm syringe filter (Whatman Inc., Florham Park, NJ).

Bacterial Strains

Five different strains of E. coli O157:H7 (ATCC 43890, C467, FRIK 125, ATCC 43895, and 93-062) were used. Before inoculation, each strain was individually cultured in brain heart infusion (BHI) broth (Remel Inc., Lenexa, KS) for 24 h at 35°C and harvested to activate the strain. An aliquot (5 mL) of each culture solution was transferred to a sterilized centrifuge bottle and centrifuged at 10,000 × g for 10 min at 4°C. The pellet was collected, washed with saline, and then resuspended in 25 mL of saline. An inoculation cocktail was prepared by mixing equal volumes of each cell suspension to have approximately the same population of 5 strains of E. coli O157:H7.

Turbidity Test

After adding 2 mL of ovotransferrin solution (40 mg/mL) or OS to 2 mL of 2x-strength BHI broth, stock solutions of EDTA or lysozyme (Sigma-Aldrich Inc., St. Louis, MO) were added to the media. In the broth media, the final concentration of EDTA was 2.0 or 2.5 mg/mL, whereas the final concentration of lysozyme was 1.0, 1.5, or 2.0 mg/mL. In addition, 40 µL of cell suspension containing approximately 108 to 109 cells of activated E. coli O157:H7 cocktail was inoculated into the BHI broth to make the initial population of E. coli O157:H7 approximately 104 to 105 cfu/mL. During a 36-h incubation period at 35°C, samples were taken at approximately 3- to 6-h intervals. The antimicrobial capacities against E. coli O157:H7 of ovotransferrin solutions combined with EDTA or lysozyme were determined by measuring the turbidity with a spectrophotometer at 620 nm.

Viability Test

The antibacterial activity of ovotransferrin plus either EDTA or lysozyme was investigated by using a viability test. First, 2 mL of ovotransferrin solution (40 mg/mL) was added to 2 mL of 2x-concentrated BHI broth media containing 100 mM NaHCO3 (OS). A stock solution of either EDTA or lysozyme was added to the OS solution so that the solutions contained OS + 2 mg/mL of EDTA (OSE2) or 1 mg/mL lysozyme (OSL1). In addition, 40 µL of cell suspension containing approximately 108 to 109 cells of actively growing E. coli O157:H7 was inoculated into the prepared solution to make approximately 104 to 105 cfu/mL in the initial solution. Ethylenediaminetetraacetate or lysozyme alone was prepared as a control group. After inoculation, the prepared culture solutions were incubated for
36 h at 35°C. The number of viable cells was analyzed by spread plating each culture solution (0.1 mL) after diluting (1:10) with 0.1% sterile peptone water (Remel Inc., Lenexa, KS). The samples were incubated for 36 h at 35°C. The number of survivors on BHI agar plates was counted as colony-forming units per milliliter of sample.

**Effect of Initial Number on Antimicrobial Activity**

This study was performed to determine whether the population of cells would affect the antibacterial activity of ovotransferrin solutions combined with various solutions. The BHI broth media containing ovotransferrin were prepared by using the same method as described above. The OS plus 2 mg/mL of EDTA (OSE) or 1 mg/mL of lysozyme (OSEL) was added to prepared BHI broth media. The BHI broth culture containing EDTA (2 mg/mL) and lysozyme (1 mg/mL) without added ovotransferrin was prepared to identify whether the solution alone had antibacterial activity against *E. coli* O157:H7. In addition, 40 µL of cell suspension containing 10^6, 10^5, or 10^4 cells of actively growing *E. coli* O157:H7 was inoculated into 4 mL of BHI broth media to make the number of *E. coli* O157:H7 10^3, 10^5, or 10^6 cfu/mL. After the prepared culture solutions were incubated for 24 h at 35°C, the viability of each treatment was measured by using the same method as described above.

**Application of Ovotransferrin on Pork Chops**

Fresh, boneless pork loin chops from different animals were purchased from the Meat Laboratory at Iowa State University and sliced into 1-cm-thick pieces (7 x 5 cm). The sliced pork chops were vacuum-packaged (−1 bar of vacuum with a 10-s dwell time) in low-oxygen-permeable bags (nylon-polyethylene, Koch, Kansas City, MO; 9.3 mL of O₂/m² per 24 h at 0°C). After vacuum-packaging, the sliced pork chops were irradiated at 5 kGy with a Linear Accelerator (Circe IIIR, Thomason CSF Linac, Saint-Aubin, France) to kill any bacteria that may have been present in the pork chops. After irradiation, the pork chops were frozen, and were completely defrosted before use for the microbial study. Each package was opened aseptically with an alcohol-sterilized scissors. A cocktail stock suspension (0.2 mL) of 10^6 cfu/mL of *E. coli* O157:H7 was inoculated onto the surface of each pork chop to make the initial number inoculated 10^4 cfu/cm², and the pork chop samples were then randomly divided into 5 groups. Inoculated strains were spread manually for 30 s to distribute the inocula evenly.

Lysozyme, EDTA, or their combination was added to the OS solution. The treatments were as follows: 20 mg/mL of ovotransferrin containing 100 mM NaHCO₃ (OSA) plus 2 mg/mL of EDTA (OSEA), OSA plus 2 mg/mL of EDTA and 1 mg/mL of lysozyme (OSEL), 30 mg/mL of ovotransferrin (OSB) plus 2 mg/mL of EDTA (OSEB), and OSB plus 2 mg/mL of EDTA and 1 mg/mL of lysozyme (OSELB). One milliliter of each ovotransferrin solution was distributed onto the same surface of the pork chops and spread manually for approximately 30 to 60 s to distribute the solution homogeneously. For the control group, *E. coli* O157:H7 alone was inoculated onto the pork chops. After adding ovotransferrin to the pork chops, the samples were stored in a 10°C incubator. Viable cells on the pork chops were analyzed after 0, 2, 4, 8, and 12 d of storage; approximately 30 to 50 mL of sterile 0.1% peptone water solution was added to each bag, followed by pummeling for 1 min in a Stomacher (model 80, Seward Medical Ltd, London, UK) at normal speed. After serial dilution of the sample with 0.1% peptone water, 0.1 mL of diluted sample was spread homogeneously on a MacConkey agar plate (Difco Laboratories, Detroit, MI) in duplicate. The survivors were enumerated as colony-forming units per gram by the same method as presented above.

**Application of Ovotransferrin on Ham**

Commercial hams were purchased from 3 local retail stores, and hams from each store were used as a replicate. Because hams provide several factors that restrict the growth of some microorganisms, including low storage temperature, nitrite, salt, and reduced water activity (Gill and Holley 2003), they were not irradiated, unlike the pork samples. Hams were sliced to 0.2-cm-thick pieces and vacuum-packaged (−1 bar of vacuum with a 10-s dwell time) in low-oxygen-permeable bags (nylon-polyethylene, Koch, Kansas City, MO; 9.3 mL of O₂/m² per 24 h at 0°C; Koch). Before inoculation, each package was opened aseptically with an alcohol-sterilized scissors. A 0.2-mL quantity of actively growing *E. coli* O157:H7 cocktail stock suspension (10^5 cfu/mL) was inoculated aseptically onto the surface of the sliced ham to make 10^4 cfu/cm². After inoculation, the ham samples were mixed manually for 30 s to distribute the inoculum evenly, and the packaged samples were randomly divided into 5 groups. In addition, 1 mL of the 4 different ovotransferrin solutions, prepared as in the pork chop study, was distributed evenly on the surface of the hams. Samples inoculated with *E. coli* O157:H7 alone without any solution added were used as controls. All samples were repackaged under vacuum and stored at 10°C in an incubator. After incubation for 0, 3, 8, and 13 d, the numbers of surviving bacteria were enumerated by using the same method as in the pork chop study.

**Statistical Analysis**

All experiments were replicated 3 times, and data were analyzed by JMP software (version 5.1.1, SAS Institute, Cary, NC). The differences in mean values
were compared by Tukey’s honestly significant differences, and mean values and SEM are reported (Kuehl, 2000). Statistical significance for all comparisons was obtained at $P < 0.05$.

**RESULTS AND DISCUSSION**

**Antibacterial Activity of EDTA Alone or in Combination with Ovotransferrin**

*Escherichia coli* O157:H7 in BHI broth multiplied rapidly after 6 h of incubation and entered a stationary phase after 10 h of incubation at 35°C. *Escherichia coli* O157:H7 in the media containing 2 or 2.5 mg/mL of EDTA alone showed a lag time for 10 h and entered a stationary phase after 18 h of incubation (Figure 1), indicating that the *E. coli* O157:H7 strains were resistant to 2 or 2.5 mg/mL of EDTA. These results can be attributed to the harvest time of the *E. coli* O157:H7 strains for inoculation in this study. Gill and Holley (2003) reported that cells harvested from a log- or stationary-phase culture are a poor model for assessing antimicrobial activities because the repairability of bacteria at the log phase or the stationary phase is not equivalent to that of cells at the lag adaptation phase. Payne et al. (1994) reported that more than 1 mg/mL of EDTA inhibited *E. coli* O157:H7 in ultra-high temperature-processed milk. Generally, in gram-negative bacteria, the outer membrane lies outside the peptidoglycan, but it is protected by LPS molecules and stabilized by divalent cations, which act as salt bridges to neighboring LPS molecules and proteins (Marvin et al., 1989). Ethylenediaminetetraacetate could destabilize the outer membrane of the microorganism and increase its permeability by chelating divalent cations (Vaara, 1992; Boland et al., 2004). Jarvis et al. (2001) reported that 30 mM EDTA resulted in a complete loss of viability of *E. coli*.

The OS, OSE2, and OSE2.5 (OS + 2.5 mg/mL of EDTA) treatments had ≤0.1 optical density at 620 nm (Figure 1), indicating that these treatments inhibited the growth of *E. coli* O157:H7 during incubation at 35°C. However, all the turbidity values of OS, OSE2, and OSE2.5 were ≤0.1, so the effect of EDTA on the antibacterial activity of OS could not be distinguished by the turbidity test (Figure 1). Therefore, the viability test was performed to determine the influence of EDTA on the antibacterial activity of OS. The number of survivors in 2 mg/mL of EDTA alone was similar to that of the control. However, when 2 mg/mL of EDTA was added to OS, the antibacterial activity of OS increased significantly, indicating that it was bactericidal against *E. coli* O157:H7 (Figure 2). Yakandawala et al., (2006) reported that a combination of ovotransferrin, protamine sulfate, and EDTA reduced biofilm formation by catheter-associated bacteria, including

![Figure 1. Turbidity of brain heart infusion broth cultures inoculated with an *Escherichia coli* O157:H7 and ovotransferrin (20 mg/mL) solution combined with NaHCO3, EDTA, or both during a 35°C incubation. C = control, only 10⁴ cfu/mL of *E. coli* O157:H7; E2 = 2 mg/mL of EDTA; E2.5 = 2.5 mg/mL of EDTA; OS = 20 mg/mL of ovotransferrin + 100 mM NaHCO3; OSE2 = OS + 2 mg/mL of EDTA; OSE2.5 = OS + 2.5 mg/mL of EDTA.](image)
Klebsiella pneumoniae, Pseudomonas aeruginosa, and Staphylococcus epidermidis. In addition, Al-Nabulsi and Holley (2007) found that EDTA enhanced the antibacterial activity of lactoferrin against E. coli O157:H7. The improved antimicrobial activity by EDTA could be explained by its divalent cation-chelating attributes (Vaara, 1992; Boland et al., 2004).

Consequently, even though OS was bacteriostatic against E. coli O157:H7, the OSE2 combination resulted in a reduction of E. coli O157:H7 of approximately 3 to 4 log. Therefore, OSE2 has potential as an antibacterial agent for controlling E. coli O157:H7.

**Antibacterial Activity of Lysozyme Alone or in Combination with Ovotransferrin**

In the turbidity test, lysozyme alone at 1.0, 1.5, and 2.0 mg/mL could not inhibit the growth of E. coli O157:H7. The OS, OSL1, OSL1.5 (OS + 1 mg/mL lysozyme), and OSL2 (OS + 2 mg/mL of lysozyme) had <0.1 optical density at 620 nm (Figure 3). Even though Park (1997) reported that approximately 0.1 to 0.4 g/L of lysozyme had antibacterial activity against E. coli in a nutrient broth and vegetable juice, the current study suggests that E. coli O157:H7 had resistance to lysozyme alone in the concentrations used. When either 1 or 2 mg/mL of lysozyme was combined with OS, however, the combinations (OSL group) had antibacterial activity against E. coli O157:H7 (Figure 3).

A viability test was performed to determine whether OSL was bactericidal or bacteriostatic against E. coli O157:H7. As shown in Figure 2, there was no significant difference in antibacterial activity between 1 mg/mL of lysozyme and the control. The population of viable E. coli O157:H7 cells after 36 h of incubation at 35°C (Figure 2) with the OS treatment was 4.5 log cfu/mL, whereas that of OSL1 was 3.2 log cfu/mL (the initial cell number was 4.0 log cfu/mL). The combination (OSL1) was bacteriostatic against E. coli O157:H7 (P < 0.05), indicating that the antimicrobial activity of OSL1 could be ascribed to OS. Therefore, results of this study suggest that 1 mg/mL of lysozyme did not significantly affect the antimicrobial activity of OS against E. coli O157:H7.

However, several reports show conflicting results regarding the effects lysozyme. Ellison and Giehl (1991) claimed that a combination of 2 mg/mL of lactoferrin and 0.5 mg/mL of lysozyme showed bactericidal capacity against E. coli. When lysozyme was combined with bovine lactoferrin, it showed bactericidal activity against E. coli even though the lactoferrin was bacteriostatic at most (Boesman-Finkelstein and Finkelstein, 1985; Yamauchi et al., 1993). Naidu et al. (2003) reported that some strains of E. coli with a low binding capacity to lactoferrin recovered easily from the antimicrobial actions of lysozyme, whereas other strains of E. coli with a high binding capacity failed to be repaired. Considering these suggestions, the use of several strains, as in this study, can be assumed to reduce the effect of
lysozyme on the antibacterial activity of ovotransferrin by inducing the rapid repair of some strains.

**Effect of Initial Cell Number on Antimicrobial Activity**

This study was performed to determine whether the initial cell population would influence the antibacterial activity of OSE or OS combined with 2 mg/mL of EDTA and 1 mg/mL of lysozyme (OSEL). After 24 h of incubation at 35°C, OS did not completely restrain the growth of *E. coli* O157:H7. In addition, the antibacterial action of OS against *E. coli* O157:H7 was more effective when initial cell numbers were $10^4$ cfu/mL than when they were $10^6$ cfu/mL. However, the antibacterial activities of OSE and OSEL, which were bactericidal against *E. coli* O157:H7, were not affected by the number of initial bacteria (Figure 4). In addition, combinations consisting of EDTA (2 mg/mL) and lysozyme (1 mg/mL) could not inhibit the growth of *E. coli* O157:H7 in BHI broth media when inoculated with $10^5$ and $10^6$ cfu/mL of cells initially. However, when EDTA (2 mg/mL) and lysozyme (1 mg/mL) were combined with OS, the combination (OSEL), exhibited a strong bactericidal activity against *E. coli* O157:H7 in BHI broth, approximately 1 log cfu/mL. Many researchers have reported that the antibacterial activities of lysozyme against gram-negative and gram-positive microorganisms are related to membrane disruption (Kalchayanand et al., 1992). Ethylenediaminetetraacetate treatment increased the permeability of the outer membrane by releasing LPS, and it enhanced the antibacterial action of lysozyme. Branen and Davidson (2004) claimed that when EDTA and lysozyme are combined, bactericidal activity against *E. coli* increases. However, this study revealed that 2 mg/mL of EDTA plus 1 mg/mL of lysozyme did not inhibit the growth of *E. coli* O157:H7. Gill and Holley (2003) reported that even though lysozyme and EDTA enhanced antibacterial activity against gram-positive organisms, their combination did not show antimicrobial activity against some gram-negative organisms. The susceptibility of *E. coli* O157:H7 to EDTA plus lysozyme may be dependent on the state of strains at inoculation. Arnold et al. (1981) demonstrated that cultures at the early exponential phase were more susceptible to the antimicrobial action of lactoferrin than those at the early stationary phase, which were more resistant. Based on this suggestion, the current study used *E. coli* O157:H7 at the stationary phase, and the susceptibility of *E. coli* O157:H7 to EDTA plus lysozyme was much lower than the susceptibilities obtained from other studies.

![Figure 3. Turbidity of brain heart infusion broth cultures inoculated with an *Escherichia coli* O157:H7 and ovotransferrin (20 mg/mL) solution combined with NaHCO₃, lysozyme, or both during a 35°C incubation. L1 = 1 mg/mL of lysozyme; L1.5 = 1.5 mg/mL of lysozyme; L2 = 2 mg/mL of lysozyme; OS = 20 mg/mL of ovotransferrin + 100 mM NaHCO₃; OSL1 = OS + 1 mg/mL of lysozyme; OSL1.5 = OS + 1.5 mg/mL of lysozyme; OSL2 = OS + 2 mg/mL of lysozyme; C = control, only $10^4$ cfu/mL of *E. coli* O157:H7.](image-url)
Ovotransferrin Applications on Pork Chops and Commercial Hams

*Escherichia coli* O157:H7 inoculated at $10^4$ cfu/cm$^2$ grew on the pork chops and increased to $10^7$ cfu/mL after 12 d of storage at 10°C. In addition, OSEA, OSELA, OSEB, and OSELB showed no antimicrobial activity (Table 1). Unlike on the pork chop samples, the viable *E. coli* O157:H7 cells inoculated on commercial hams decreased slowly during 13 d of storage at 10°C (Table 2). *Escherichia coli* O157:H7 grew better on pork chops, which contained more drip on defrosting than commercial hams, whereas the viable *E. coli* O157:H7 cells inoculated on hams seemed to decrease slowly, unlike those on pork chops. In addition, OSEA, OSELA, OSEB, and OSELB treated on hams showed no antibacterial activity against *E. coli* O157:H7, and there was no significant difference in the number of survivors between the control and other treatments in both pork chops and commercial hams ($P < 0.05$; Table 2).

The antibacterial activities of some antimicrobials can be modulated by the nutrient or laboratory medium. Many divalent cations, such as Ca$^{2+}$ and Mg$^{2+}$, can block the biological activity of lactoferrin by forming tetramers (Bennett et al., 1981) or by inhibiting the direct interaction of lactoferrin with LPS (Ellison and Giehl, 1991; Yamauchi et al., 1993; Rossi et al., 2002).

![Figure 4](image_url)

**Figure 4.** Effect of initial cell population on the antibacterial activity of ovotransferrin (20 mg/mL) containing 100 mM NaHCO$_3$ and EDTA (2 mg/mL) or lysozyme (1 mg/mL) against *Escherichia coli* O157:H7 in brain heart infusion (BHI) broth culture during a 24-h incubation at 35°C. $10^4 = 10^4$ cfu/mL of BHI broth; $10^5 = 10^5$ cfu/mL of BHI broth; $10^6 = 10^6$ cfu/mL of BHI broth; OS = 20 mg/mL of ovotransferrin + 100 mM NaHCO$_3$; OSE = OS + 2 mg/mL of EDTA; OSEL = OS + 2 mg/mL of EDTA + 1 mg/mL of lysozyme; EL = 2 mg/mL of EDTA + 1 mg/mL of lysozyme. **Different letters shows that the means are significantly different ($P < 0.05$; n = 3).

### Table 1. Changes in the number of *Escherichia coli* O157:H7 survivors (number of viable cells, log$_{10}$ cfu/mL) on e-beam-irradiated pork chops not treated or treated with ovotransferrin solutions contained 100 mM NaHCO$_3$ plus either EDTA (2 mg/mL) or lysozyme (1 mg/mL) during storage at 10°C$^6$

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$^1$All values within a column are significantly different ($P < 0.05$; n = 3).

$^2$Control = only *E. coli* O157:H7; OSA = ovotransferrin (20 mg/mL) + 100 mM NaHCO$_3$; OSEA = OSA + 2 mg/mL of EDTA; OSELA = OSA + 2 mg/mL of EDTA + 1 mg/mL of lysozyme; OSB = ovotransferrin (30 mg/mL) + 100 mM NaHCO$_3$; OSEB = OSB + 2 mg/mL of EDTA; OSELB = OSB + 2 mg/mL of EDTA + 1 mg/mL of lysozyme.
activity of ovotransferrin in pork chops and commercial hams may have been due mainly to divalent ions such as Ca$^{2+}$ and Mg$^{2+}$, which were assumed to exist on hams and pork chops.

Cutter and Siragusa (1995a,b) demonstrated that the combination of nisin with chelators showed antibacterial activity against *E. coli* and *Salmonella* spp. in buffer, but this combination showed no antibacterial effectiveness against the same organisms on beef. Branen and Davidson (2004) reported that the combination of EDTA and nisin, which exhibited antimicrobial activity against *E. coli* O157:H7, showed no antimicrobial activity in 2% fat ultra-high temperature-processed milk. In addition, even though a combination consisting of lysozyme and nisin enhanced bactericidal activity in de Man, Rogosa, Sharpe media, it did not have such an effect in pork juice (Gill and Holley, 2003). According to these reports, the results obtained from the application of antimicrobials to food products were significantly different from those of the model systems. Generally, food products provide a nutrient-rich environment, so microorganisms injured by certain antimicrobials are considered to recover more easily in these systems than under conditions of cell starvation or in laboratory media.

In addition, the distribution or dilution effect of ovotransferrin on pork chops and hams could explain the low antibacterial activity of ovotransferrin in these products. Ellison and Giehl (1991) claimed that the bactericidal effect is affected by the concentration of lactoferrin as well as by the degree of iron saturation in the lactoferrin. Even though in the present study approximately 20 to 30 mg/mL of ovotransferrin was distributed on the surface of the pork chops and hams, the concentration was likely diluted on the surface of these products. In addition, because the ovotransferrin was spread manually, a homogeneous distribution of ovotransferrin on whole products might be impossible. Moreover, the large amount of drip formed during the defrosting process for pork chops might have prevented the complete incorporation of ovotransferrin on the surface of the pork chops. Therefore, the antimicrobial action of ovotransferrin in pork chops and hams seemed to be modulated because of the reasons described above. In addition, it is necessary to overcome some of these limitations and the limitations of the approaches used in various studies before ovotransferrin can be applied in meat or meat products.

### Conclusions

Ethylendiaminetetraacetate at 2 mg/mL and lysozyme at 1 mg/mL enhanced the antibacterial activity of OS against *E. coli* O157:H7 in BHI broth. Ethylendiaminetetraacetate at 2 mg/mL plus OS resulted in a reduction of approximately 3 to 4 log, whereas 1 mg/mL of lysozyme plus OS resulted in a reduction of approximately 0.5 to 1 log during incubation at 35°C. In addition, the initial cell numbers in media did not affect the antibacterial activity of OSE or OSEL against *E. coli* O157:H7. Moreover, OSE or OSEL showed no antimicrobial activity on pork chops and commercial hams. The antimicrobial activity of OSE or OSEL against this strain was significantly different from the results of the model system using BHI broth. Therefore, we suggest that it is necessary to perform further studies on the application of ovotransferrin to provide solutions to these possible limitations.

### REFERENCES


