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Effect of EDTA and Lysozyme on the Antimicrobial Activity of Ovotransferrin against *Listeria monocytogenes*

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Summary and Implications

This study evaluated the effect of EDTA and lysozyme on the antibacterial activities of activated ovotransferrin against 5 strains of *L. monocytogenes*. First, a disc test was performed to screen the concentrations of EDTA or lysozyme that showed antibacterial activities in ovotransferrin (O) or ovotransferrin in 100 mM NaHCO₃ (OS) solution. Turbidity and viability test were conducted using O or OS solution combined with either lysozyme (OL and OSL) or EDTA (OE and OSE). Also, OS combined with 2 mg/ml lysozyme (OSL) or/and 1 mg/ml EDTA (OSLE) were applied on commercial hams to determine if the solutions show antibacterial activities on meat products. The effect of initial cell population on the antibacterial activities of ovotransferrin combined with either EDTA or lysozyme was also determined. *L. monocytogenes* started to grow after 1 day of incubation in the presence of > 2.0 mg/ml lysozyme. OL groups showed weak antibacterial activities against *L. monocytogenes* in BHI broth culture and their activities were bacteriostatic. OSL groups were bactericidal against *L. monocytogenes*, resulting in 1 log reduction from initial cell population. Even though OSL showed stronger antibacterial activity than OS, lysozyme had no significant effect on antibacterial activity of OS against *L. monocytogenes*. Also, EDTA itself at 1.0 and 2.0 mg/ml were bacteriostatic against 5 strains of *L. monocytogenes*. They were more susceptible to EDTA than lysozyme, and OSE1 and OSE2 had bactericidal activity against *L. monocytogenes*. There was a significant difference in the survivor cell populations between OS and OSE groups ($p < 0.05$). Therefore, EDTA enhanced the antibacterial activity of OS against *L. monocytogenes*. However, ovotransferrin plus either lysozyme or/and EDTA did not show any antibacterial effect in commercial hams during storage at 10°C. In addition, the initial population of *L. monocytogenes* cells influenced the antibacterial activity of OSL or OSE.

Introduction

Natural antimicrobial agents originated from plant, animal, and bacterial sources are expected to provide great satisfaction to consumers who are sensitive to health and safety issues. Ovotransferrin, the second major egg white protein, is an iron-binding glycoprotein and transports and scavenges Fe (III) in eggs of poultry.

Antibacterial properties of ovotransferrin against a variety of microorganisms have been reported. However, the antimicrobial mechanisms of ovotransferrin are not fully defined yet. The iron binding capability of ovotransferrin was initially believed to be the major antimicrobial action of ovotransferrin. However, recent studies demonstrated that direct interactions of ovotransferrin with bacterial surface were the major cause of antimicrobial action of ovotransferrin.

L. monocytogenes can proliferate in the presence of curing salt at refrigerated temperatures, and colonize, multiply and persist on processing equipment or plant substances. The strains are sensitive to heat treatment and can be easily inactivated by cooking. However, ready-to-eat (RTE) meat products such as frankfurters, and deli meats have high incidence rates of listeriosis due to contamination during multiple handling and processing steps performed after cooking. Center for Disease Control and Prevention reported that 2,500 people suffer from listeriosis annually in the United States and one in five die from the disease (FSIS). Due to this risk, the U.S. Department of Agriculture currently established a “zero tolerance” policy for *L. monocytogenes* in RTE meat products. Therefore, developing potent antimicrobials against *L. monocytogenes* for RTE meat is necessary.

According to a previous study, apo-ovotransferrin alone was not effective in controlling *L. monocytogenes* in BHI broth. To improve the antimicrobial activities of ovotransferrin, therefore, combinations of several antimicrobial agents are necessary. The combinations of materials with different antimicrobial mechanisms are expected to increase antibacterial effectiveness since a simultaneous attack on different targets is likely to make the microorganism more difficult to overcome the environment. The use of ovotransferrin combined with EDTA or/and lysozyme as antimicrobials on the surface of meat or meat products is appealing to the meat industry because all the components are generally regarded as safe (GRAS). Lysozyme catalyzes the hydrolysis of (1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of cell wall peptidoglycan. Therefore, the antimicrobial activity of lysozyme is ascribed primarily to the enzymatic lyses of peptidoglycan in the cell wall of microorganisms. The application of lysozyme in foods has been limited because gram-negative bacteria that are protected by an outer membrane, which is relatively resistant to antimicrobial activities of lysozyme. However, lysozyme gained considerable interest for use in food systems because it is a natural enzyme produced by animals and its activity targets a specific cellular structure of microorganisms.

Ethylenediaminetetraacetic acid (EDTA) has been used in a number of food products as a chelating agent to

prevent oxidation. Although the mechanism of antibacterial effectiveness by EDTA is not fully understood, its chelating property to divalent cations like Ca^{2+} or Mg^{2+} should be involved. EDTA inhibits the growth of microorganisms by depriving Mg^{2+} , Ca^{2+} , and Fe^{2+} , which are essential factors for microbial growth, from microorganisms. EDTA is reported to enhance the antimicrobial activity of lactoferrin by destabilizing the outer membrane of bacteria and increasing the permeability of divalent cations. Also, chelating agents facilitate the detachment of cells from biofilm and enhance the killing of biofilm-producing microorganisms by depriving Mg^{2+} associated with lipopolysaccharides.

The objective of this study was to enhance the antimicrobial activity of ovotransferrin solution supplemented with 100 mM-sodium bicarbonate against a cocktail of 5 strains of *L. monocytogenes* by combining with lysozyme or EDTA. Many studies related to antibacterial activity of ovotransferrin *in vitro* have been reported, but little work has been done to apply ovotransferrin as an antimicrobial agent in meat or meat products. Therefore, the present study applied OS plus lysozyme or/and EDTA on commercial hams to determine their effect on the growth of *L. monocytogenes* in meat products.

Material and Methods

Apo-ovotransferrin (iron-free) used in this study was prepared by the method of Ko and Ahn. Five different strains of *Listeria monocytogenes* (NADC 2045, H7962, H7969, H7762, and H7596) were used. Prior to analyzing the antibacterial activity through viability and turbidity tests, disc diffusion method was used to determine the concentration of EDTA or lysozyme capable of promoting antibacterial capability of ovotransferrin. *L. monocytogenes* cocktail containing equal amount of each strain was diluted in series and then 0.1 ml of cell suspension corresponding to 10^5 or 10^6 CFU/ml was spread homogeneously on the BHI agar media plates. The antimicrobial capacity of ovotransferrin solutions combined with EDTA or lysozyme against the growth of *L. monocytogenes* was analyzed by measuring the turbidity of solution after incubation in a BHI broth culture at 35°C. The antibacterial activities of ovotransferrin plus lysozyme (OSL) and ovotransferrin plus EDTA (OSE) were investigated using a viability test.

Commercial hams were sliced, inoculated on the surface with activated *L. monocytogenes* cocktail stock suspension and vacuum packaged in low oxygen-permeable bags. After manually mixing for 30 second to distribute the inocula evenly, the packaged samples were randomly divided into 7 groups. Lysozyme or/and EDTA were added to ovotransferrin solution included 100 mM NaHCO_3 and 0.15 M NaCl . Each prepared ovotransferrin solution (1 ml) was distributed evenly on the surface of a ham. Viable *L. monocytogenes* cells on the pork chops were analyzed after 0, 2, 5, 10, 15, 22, and 29 days of storage.

Results and Discussion

Lysozyme at 1.0 and 1.5 mg/ml did not form clear zones, but 2.0, 2.5 and 3.0 mg/ml lysozyme did. The strength of clear zone formed by lysozyme combined with ovotransferrin or OS did not show significant difference compared with that of lysozyme (Table 1). *L. monocytogenes* was susceptible to ≥ 2.0 mg/ml lysozyme. The antimicrobial activity of lysozyme has long been believed to be due to its bacteriolytic actions, which hydrolyze β -1.4-glycosidic bond in peptidoglycan of gram-positive bacteria that do not possess any outer membrane, but gram-negative bacteria with outer membrane is reported to be relatively resistant to lysozyme. EDTA at 1.5 and 2.0 mg/ml made clear zone, but no clear zone was formed at 1.0 mg/ml EDTA. When EDTA was combined with ovotransferrin or OS, the strength of clear zone decreased (Table 1). This study indicated that more than 1.5 mg/ml of EDTA inhibited the growth of *L. monocytogenes*. However, when EDTA was combined with ovotransferrin or OS, the strength of clear zone decreased. Based on the disc test, more than 2 mg/ml lysozyme or 1 mg/ml of EDTA were chosen in turbidity and viability tests, and tests were performed to identify EDTA and lysozyme effects on antibacterial activities of ovotransferrin.

L. monocytogenes entered log phase after 7 ~ 8 hr of incubation at 35°C in BHI broth culture. They seemed to face stationary phase after 24 hr incubation and then death. Lysozyme at 2 mg/ml delayed the growth of *L. monocytogenes* for 24 hrs, whereas lysozyme 2.5 mg/ml and 3 mg/ml delayed it for 30 hrs and 36 hr, respectively. Therefore, *L. monocytogenes* seemed to have a lag time to adapt to a new environment when lysozyme was added (Figure 1). Even though lysozyme alone delayed the growth of *L. monocytogenes* during initial period, *L. monocytogenes* started to grow after 24 ~ 36 hr incubation at 35°C. The turbidity values obtained from all OL or OSL groups were lower than 0.1 as in OS treatment (Figure 1). According to the turbidity test, *L. monocytogenes* were found to be controlled by OL or OSL groups even though they were resistant to lysozyme alone under the concentrations used.

In the viability test, which measures the number of viable *L. monocytogenes* cells after 48 hr incubation at 35°C, with 2.0, 2.5 or 3.0 mg/ml lysozyme was not different from that of the control (Figure 2). As BHI media used in this study is relatively nutrient-rich media, injured cells of *L. monocytogenes* by lysozyme treatment should have been repaired easily in the media. Thus they showed resistance to 2.0 ~ 3.0 mg/ml lysozyme. Another possible explanation for resistance of *L. monocytogenes* to lysozyme may be attributed to using a cocktail of 5 different strains of *L. monocytogenes* instead of using a specific strain.

The number of viable cells obtained when using OS slightly increased from 4.6 log CFU/ml, the number of the initial inoculation, to 4.9 log CFU/ml during 48 hrs incubation at 35°C. Therefore, OS seemed to be

bacteriostatic against *L. monocytogenes*. Also the number of viable *L. monocytogenes* cells in OL2, OL2.5, and OL3 increased from 4.6 log CFU/ml, the number of initial inoculated cell, to 5.1, 7.2, and 6.0 log CFU/ml, respectively (Figure 2). OL groups showed a weak antibacterial activity against *L. monocytogenes* compared with control. However, OSL groups showed a stronger antilisterial activity against *L. monocytogenes* than that of OL groups. In addition, OSL2, OSL2.5, and OSL3.0 were listericidal considering their changes from 4.6 log CFU/ml to 3.3, 3.0 or 2.9 log CFU/ml, respectively. Also there was a significant difference in antibacterial activity against *L. monocytogenes* between OL and OSL under lysozyme at 2.5 mg/ml and 3.0 mg/ml (Figure 2). Therefore, this study demonstrates that 100 mM sodium bicarbonate enhanced the antimicrobial activity of OL against *L. monocytogenes*, while lysozyme at 2.0, 2.5, or 3.0 mg/ml did not show a clear effect on antibacterial activity of OS toward *L. monocytogenes*.

L. monocytogenes were susceptible to EDTA as shown in the disc test. As EDTA alone showed a lower turbidity than OE or OSE at initial point, it was impossible to compare the antibacterial activity of OE or OSE with EDTA itself by turbidity analysis (Figure 3). As almost all treatments showed similar turbidity values of less than 0.1, it was difficult to determine if EDTA had a synergistic effect with ovotransferrin using turbidity test. However, according to the turbidity test, EDTA increased the antibacterial activity of OS (Figure 3). *L. monocytogenes* was found to be more susceptible to EDTA than lysozyme under the concentrations used in this study (Figure 4). EDTA increased the antibacterial activity of OS against *L. monocytogenes*. However, antimicrobial effects by EDTA itself seemed to be the major contributor to the listericidal activity of OSE.

OS treatment to BHI broth media inoculated with 10^4 and 10^5 CFU/ml occurred 1 log increase from the initial cell number during 35°C incubation for 48 hrs. However, the viable cells in BHI broth culture inoculated with 10^6 cells and treated with OS did not indicate any increase (Figure 5). BHI broth culture added with OSL2 and inoculated with 10^4 , 10^5 or 10^6 CFU/ml of *L. monocytogenes* resulted in 3.3, 3.7, or 4.5 log CFU/ml of viable cells, respectively, after 48 hrs incubation at 35°C. BHI broth inoculated with 10^4 , 10^5 or 10^6 CFU/ml of *L. monocytogenes* cells and OSE1 showed 3.1, 4.0, or 4.4

log CFU/ml of viable cells, respectively (Figure 5). These results demonstrate that OSL2 or OSE1 had listericidal activity against *L. monocytogenes* and the number of initial cells existing in the media affected the antibacterial activity of OSL2 and OSE1.

L. monocytogenes with 10^4 initial cells grew slowly in commercial hams to 10^5 CFU/ml during storage at 10°C for 29 days. In contrast to *in vitro* test, 1OSL, 1OSLE, 2OSL, and 2OSLE did not show any antimicrobial activity against *L. monocytogenes* (Table 2). Therefore, it was suggested that even though OSL2 and OSE1 showed clear bactericidal effect against *L. monocytogenes* in model systems, they did not have antibacterial activities against *L. monocytogenes* in commercial hams during storage.

EDTA plus lysozyme did not show any antimicrobial activity against *L. monocytogenes* in hams in this study. Such a difference may be due partially to media compositions. As food products provide a nutrient-rich environment, injured microorganisms by antimicrobials may recover more easily in food systems than those under cell starvation or limited media conditions. Another possible explanation about no antibacterial activities by OSL and OSE in commercial hams could be distribution problem or dilution effect of ovotransferrin to the products, state of strains in inocula, or stability of ovotransferrin on the surface of meat products. Generally, antimicrobial activity of ovotransferrin is dependent upon the dose of ovotransferrin. In the present study, 1 ml of 20~30 mg/ml ovotransferrin was distributed on the surface of hams, and the concentration was likely to be diluted in certain parts of the product so that low antibacterial effect against *L. monocytogenes* might be shown in the area. Also, ovotransferrin was spread manually, and thus it is difficult to distribute ovotransferrin uniformly or homogeneously on whole products. Also, ovotransferrin incorporated could have been penetrated or absorbed inside the product so that it could not be involved in antimicrobial activities. Even though antimicrobials such as bacteriocins are effective in inhibiting pathogens including *L. monocytogenes* and other spoilage microorganisms in model systems, high population of survivors or recovering features is often observed in food systems. This study showed that there are many limitations in applying ovotransferrin as antimicrobial agent in food systems.

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Table 1. Effect of ovotransferrin combined with NaHCO₃ and EDTA or NaHCO₃ and lysozyme on clear zone formation on BHI agar media plates inoculated with *L. monocytogenes* during 35° C incubation for 24 ~ 48 hrs.

Treatment	Conc. (mg/ml)	Control	OTF ^a	OTF + NaHCO ₃ ^b
	0	-*	-	-/+
	1.0	-	-	-
	1.5	-	+/-	-
Lysozyme	2.0	++	++	+/-
	2.5	++	++	++
	3.0	++	+++	++
EDTA	1.0		-	-
	1.5	++	+**	+
	2.0	++	+	++

^a20 mg/ml ovotransferrin

^b100 mM NaHCO₃ was used at this study.

*No clear zone formed on BHI agar media plate.

**Formation of clear zone: the strength of clear zone was described as + (weak), ++ (middle), and +++ (strong) by its size or clarity on the BHI agar. (n = 3)

Table 2. Viable cells of *L. monocytogenes* on commercial hams treated with or without ovotransferrin in 100 mM NaHCO₃ (OS) plus or/and lysozyme (2 mg/ml) and/or EDTA (1 mg/ml) during 10°C storage.

Treatments	Storage period (days)						
	0	2	5	10	15	22	29
	----- Number of viable cells (log 10 CFU/ml) -----						
Control	4.5 ^a ± 0.0*	4.6 ^a ± 0.2	5.0 ^a ± 0.0	5.3 ^a ± 0.4	5.8 ^a ± 0.0	5.4 ^a ± 0.2	5.3 ^a ± 0.4
1OSL	4.3 ^a ± 0.1	4.7 ^a ± 0.1	5.1 ^a ± 0.1	5.5 ^a ± 0.1	5.6 ^a ± 0.4	5.5 ^a ± 0.9	5.2 ^a ± 0.3
1OSLE	4.4 ^a ± 0.1	4.6 ^a ± 0.0	5.0 ^a ± 0.1	5.1 ^a ± 0.2	5.9 ^a ± 0.3	5.6 ^a ± 0.2	6.0 ^a ± 0.4
2OSL	4.4 ^a ± 0.0	4.6 ^a ± 0.0	5.0 ^a ± 0.1	5.2 ^a ± 0.2	5.2 ^a ± 0.1	5.5 ^a ± 0.0	5.4 ^a ± 0.6
2OSLE [†]	4.3 ^a ± 0.1	4.6 ^a ± 0.1	4.9 ^a ± 0.2	5.1 ^a ± 0.1	5.6 ^a ± 0.2	5.7 ^a ± 0.3	5.4 ^a ± 0.2
L	4.3 ^a ± 0.0	4.5 ^a ± 0.1	4.9 ^a ± 0.1	4.9 ^a ± 0.3	5.4 ^a ± 0.3	5.0 ^a ± 1.0	5.4 ^a ± 0.5
LE	4.4 ^a ± 0.2	4.5 ^a ± 0.1	5.0 ^a ± 0.1	5.1 ^a ± 0.1	5.5 ^a ± 0.5	5.6 ^a ± 0.3	6.2 ^a ± 0.6

^aMeans within a column with no common superscript differ (P < 0.05, n = 4).

Control: only *L. monocytogenes* inoculated. *Mean ± standard deviation.

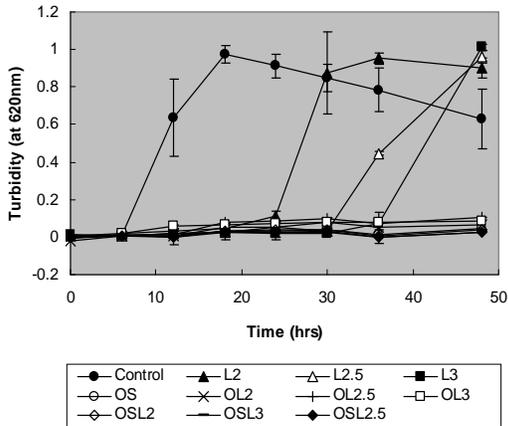
1OSL: 20 mg/ml ovotransferrin + 100 mM NaHCO₃ + 2 mg/ml lysozyme

1OSLE: 1OSL + 1 mg/ml EDTA, 2OSL: 30 mg/ml ovotransferrin + 100 mM NaHCO₃ + 2 mg/ml lysozyme

1OSLE: 2OSL + 1 mg/ml EDTA, L: 2 mg/ml lysozyme

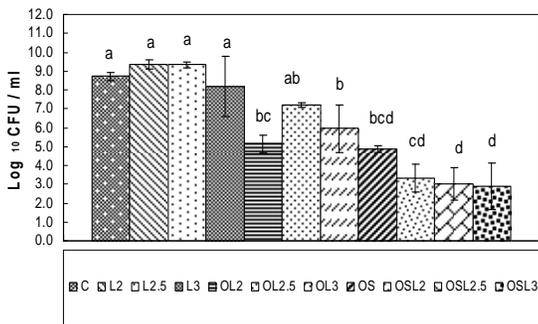
LE: 2 mg/ml lysozyme + 1 mg/ml EDTA

Figure 1. Turbidity of BHI broth cultures inoculated *L. monocytogenes* and ovotransferrin (20 mg/ml) combined with NaHCO₃ or/and EDTA during 35°C incubation.



C: control, 10⁴ CFU/ml of *L. monocytogenes*; L2: 2 mg/ml Lysozyme; L2.5: 2.5 mg/ml Lysozyme; L3: 3.0 mg/ml Lysozyme; OS: 20 mg/ml ovotransferrin + 100 mM-NaHCO₃; OL2: 20 mg/ml ovotransferrin + L2; OL2.5: 20 mg/ml ovotransferrin + L 2.5; OL3: 20 mg/ml ovotransferrin + L 3.0; OSL2: OS + 2 mg/ml lysozyme; OSL2.5: OS + 2.5 mg/ml lysozyme; OSL3: OS+ 3.0 mg/ml lysozyme

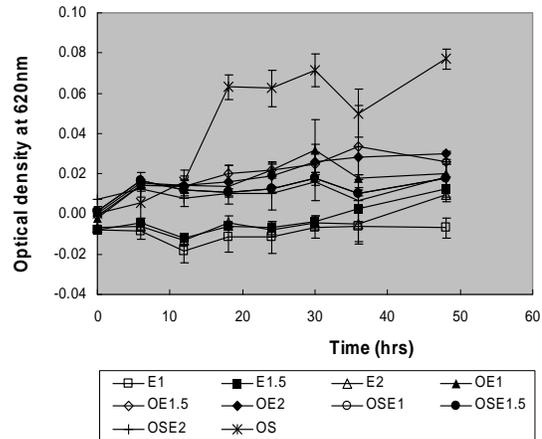
Figure 2. Antibacterial activity of ovotransferrin (20 mg/ml) combined with 100 mM NaHCO₃ and/or lysozyme against the growth of *L. monocytogenes* on BHI broth culture during 35°C incubation for 24 ~ 36 hr.



^{a-d} Bars with different letters indicate significantly different values (P < 0.05, n=4).

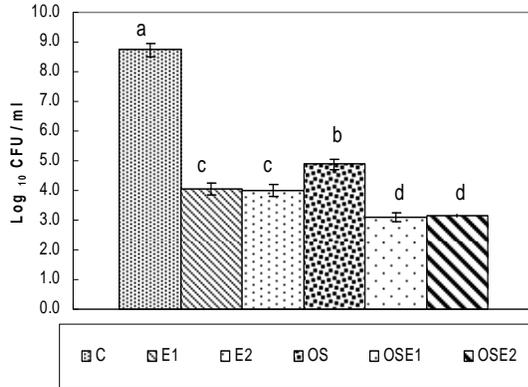
C: control, only 10⁴ CFU of *L. monocytogenes*; L2: 2 mg/ml Lysozyme; L2.5: 2.5 mg/ml Lysozyme; L3: 3.0 mg/ml Lysozyme; OL2: 20mg/ml ovotransferrin + L2; OL2.5: 20mg/ml ovotransferrin + L2.5; OL3: 20mg/ml ovotransferrin + L 3.0; OS: 20 mg/ml ovotransferrin + 100 mM-NaHCO₃; OSL2: OS + 2 mg/ml lysozyme; OSL2.5: OS + 2.5 mg/ml lysozyme; OSL3: OS+ 3.0 mg/ml lysozyme.

Figure 3. Turbidity of BHI broth cultures inoculated *L. monocytogenes* and ovotransferrin (20 mg/ml) combined with NaHCO₃ or/and EDTA during 35°C incubation.



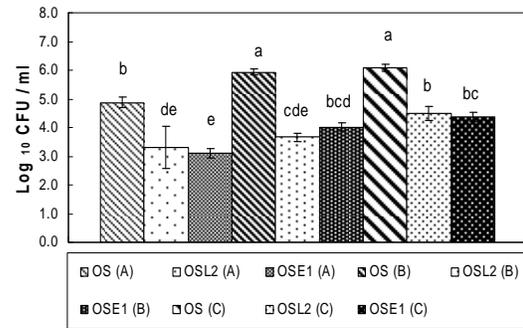
C: control, only 10⁴ CFU/ml of *L. monocytogenes*
 E1: 1 mg/ml EDTA; E1.5: 1.5 mg/ml EDTA; E2: 2.0 mg/ml EDTA; OS: 20 mg/ml ovotransferrin + 100 mM NaHCO₃; OE1: 20 mg/ml ovotransferrin + E1; OE1.5: 20 mg/ml ovotransferrin + E2; OE2: 20 mg/ml ovotransferrin + E3; OSE1: OS + E1; OSE1.5: OS + E1.5; OSE2: OS+ E2

Figure 4. Antibacterial activity of ovotransferrin (20 mg/ml) combined with 100 mM NaHCO₃ and/or EDTA (1 mg/ml) against the growth of *L. monocytogenes* in BHI broth culture during 35°C incubation for 48 hr.



^{a-d} Bars with different letters indicate significantly different values ($P < 0.05$, $n=4$).
 C: control, only 10^4 cells of *L. monocytogenes*
 E1: 1 mg/ml EDTA
 E2: 2.0 mg/ml EDTA
 OS: 20 mg/ml ovotransferrin + 100 mM NaHCO₃
 OSE1: OS + E1
 OSE2: OS + E2

Figure 5. Effect of initial cell population on the antibacterial activity of ovotransferrin (20 mg/ml) solution combined with 100 mM NaHCO₃ and EDTA (2 mg/ml) or lysozyme (1 mg/ml) against *L. monocytogenes* in BHI broth culture during 35°C incubation for 48 hr.



^{a-d} Bars with different letters indicate significantly different values ($P < 0.05$, $n=4$).
 A: 10^4 CFU/ml BHI broth
 B: 10^5 CFU/ml BHI broth
 C: 10^6 CFU/ml BHI broth
 OS: 20 mg/ml ovotransferrin + 100 mM NaHCO₃
 OSL2: OS + 2 mg/ml lysozyme
 OSE1: OS + 2 mg/ml lysozyme + 1 mg/ml of EDTA