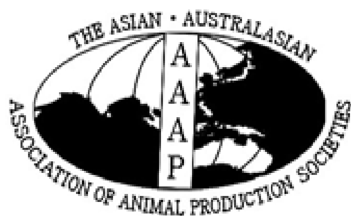

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Effects of Egg Yolk Antibodies Produced in Response to Different Antigenic Fractions of *E. coli* O157:H7 on *E. coli* Suppression

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ABSTRACT : The objective of this research was to provide the characterization and method for producing anti-*E. coli* O157:H7 antibodies in egg-laying hens and to determine if the antibody can restrain the proliferation of *E. coli* O157:H7 *in-vitro*. Selected antigenic fractions (whole cell, outer membrane protein and lipopolysaccharide (LPS)) from *E. coli* O157:H7 were injected to hens in order to produce anti-*E. coli* O157:H7 antibodies. The immune response and the egg yolk antibodies of laying hens against the whole cell, outer membrane protein and LPS antigens were monitored by ELISA. The level of antibodies against whole cell antigen monitored through ELISA sharply increased after the initial immunization, and it was found to be maximum on day 49 however, the level was maintained up to day 70. Antibodies (5 mg/ml) directed against the whole cell inhibited *E. coli* proliferation 10-13 times more than outer membrane protein or LPS. The antibody response against the whole cell antigens appeared to have higher activity in restraining the proliferation of *E. coli* O157:H7 than antibody against outer membrane protein or LPS. Results reflected that increasing the IgY's in the egg yolk could prevent greater economic losses due to human and animal health from pathogenic bacteria i.e. *E. coli* O157:H7. (**Key Words :** *E. coli* O157:H7, Lipopolysaccharide, Egg Yolk Antibody, Chicken)

INTRODUCTION

Chicken is the only avian species in which polyclonal antibodies, like IgG is transported from the hen to the egg yolk in a similar manner as the transport of mammalian IgG from the mother to the fetus (Rose and Orlans, 1981). Chicken antibodies, therefore, can be purified on a large scale from egg yolk. Antibodies from the avian species are highly defined, and the chicken is an easily accessible animal model. The antibody equivalent to chicken serum IgG, is termed IgY to denote that it is found in the yolk. Serum antibodies of hyperimmunized hens are efficiently transferred and accumulated in the egg yolk (Akita and Chan, 1998). Egg yolk, therefore, contain large amounts of IgY against pathogens, which can immobilize the existing or invading pathogens during embryo development and in day-old chicks. Egg yolk contains 8-20 mg of immunoglobulins (IgY) per ml of yolk (Benkirane, 1998; Al-Haddad, 1999). The production of anti-viral or bacterial antibodies (rotavirus, *E. coli*, echinococcoses and many others) and isolation of those egg yolk immunoglobulins has been extensively explored for the use of enhanced immunity by oral administration in humans.

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Some of the suggested uses of IgY in immunotherapy includes fortification of infant formulas (against *E. coli* or *salmonella*), prevention of dental caries (*S. mutans*, *Lactobacillus*) (Di, 2001), oral administration of antibodies against bacteria or virus to prevent gastrointestinal infection (Chang et al., 2002; Gast et al., 2002). *Eschericha coli* strains of serotype O157:H7 belongs to a family of pathogenic *E. coli* called enterohemorrhagic *E. coli* (EHEC) strains responsible for hemorrhagic colitis, bloody or non-bloody diarrhea, and hemolytic uremic syndrome in humans (Evelyn et al., 1997). “Hamburger Disease” caused by a special strain of *E. coli* O157:H7 is an emerging threat to world beef markets which is of economic importance to the beef producing countries. This strain of *E. coli* pathogenises by adhering to host intestinal epithelium and forming bacterial colonies. Antibiotic therapy is not recommended early in the infectious process, because disruption of the bacteria in the gut releases Shiga like toxins, which is highly dangerous. On the contrary, antibodies bind to the bacterial surface and inhibit the bacterial adhesion to host intestinal epithelium (Karmaill, 1989) and thereby prevent ulceration of the surface epithelium and bloody diarrhea. The complex formed between the antibody and bacteria are eliminated as a waste. The effectiveness of IgY in suppressing the activity of *E. coli* O157:H7 has been demonstrated in certain previous studies (Cipolla et al.,

2001). However, the antigenicity of different fractions of *E. coli* O157:H7 for IgY's production and E.coli suppression has not been explored extensively. The objective of the study was to produce antigen from *E. coli* O157:H7, develop specific polyclonal IgY and determine the potential of IgY in *E. coli* O157:H7 suppression.

MATERIALS AND METHODS

Antigen preparations

Three types of antigenic fractions of *E. coli* O157:H7 (ATCC; Manassas; VA; USA) used were whole cell, outer membrane protein and lipopolysaccharide.

Whole cell (WC) : Bacterial whole cell (WC) was prepared by inoculating single colony of *E. coli* O157:H7 in 10 ml of Brain Heart Infusion Broth (BHIB) and culturing for 18 h. Bacterias were pelleted by centrifugation at 4,000 g for 15 min at 4°C and washed once by suspension in 10 ml of 10 mM HEPES buffer, pH 7.4. Aliquots of 1 ml were microcentrifuged, the supernatants discarded, and the bacterial pellet resuspended in 1 ml sample buffer (10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.125 M Tris-HCL (pH 6.8) and 0.01% bromophenol blue (Thomas et al., 1997). The WC preparations were stored at -20°C.

Outer membrane proteins (OMP) : Outer membrane proteins (OMP) were prepared using a modification of the sarcosinate extraction procedure of Barekamp et al. (1981). *E. coli* O157:H7 were grown in BHIB for 18 h and harvested by centrifugation at 4,000 g for 10 min at 4°C. Pelleted bacteria were washed by suspension in 10 ml of 10 mM sodium N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid (PEPES) buffer, pH 7.4 and recentrifuged. Bacteria was resuspended in 15 ml HEPES buffer, placed in an ice water bath, and sonicated five times for 1 min with 30 seconds break between sonications (Model W-375, Ultrasonics Inc., Plainview, NY, USA). Intact cells were removed by centrifugation at 12,000 g for 15 min at 4°C. The supernatant was collected and centrifuged at 100,000 g for 1 h at 4°C. The resulting pellet was resuspended in 15 ml of a 1% sodium lauroyl sarcosinate solution and incubated for 30 min at 24°C on a laboratory rocker. The detergent insoluble fraction was collected by centrifugation at 100,000 g for 60 min at 4°C and resuspended in 250 μl distilled water. Outer membrane proteins fractions were stored at -20°C.

Lipopolysaccharide : Purified lipopolysaccharide (LPS) was obtained using Johnson and Perry's (1976) modification of the hot aqueous phenol extraction method of Westphal and Jann (1965). *E. coli* O157:H7 harvested from 10 L of BHIB by centrifugation at 4,000 g for 15 min at 4°C were resuspended in 50 ml of 0.01 M phosphate buffered saline (PBS), pH 7.0, containing 5 mM ethylenediamine-tetracetic acid (tetrasodium salt). A 100 mg aliquot of egg white lysozyme (American Research Company, Solon, OH, USA) was added and the suspension stirred at 37°C for 30 min. The volume was adjusted to 100 ml with 20 mM $MgCl_2$ and bovine pancreas ribonuclease and deoxyribonuclease were added at final concentrations of 1 mg. The suspension was incubated 10 min at 37°C and for an additional 10 min at 60°C in a water bath. An equal volume (100 ml) of preheated (70°C) 90% (v/v) aqueous phenol was added to the suspension and the resulting mixture was stirred for 15 min. The suspension was rapidly cooled to 15°C by stirring in an ice water bath. Solution was separated into aqueous and phenol layers after centrifugation at 18,000 g for 15 min. The aqueous phase was retained and dialyzed (12,000-14,000 molecular-weight cut off, Spectra/Por 4, Spectrum Medical Industries Inc.) against running tap water for 2 h at room temperature and against distilled water until no phenol odour was detected. The LPS was pelleted by centrifugation at 100,000 g for 2 h at 4 °C. The pellet was reconstituted in distilled water, lyophilized, and stored at -20°C.

Production of antibodies against *E. coli* O157:H7

ISA Brown hens (40 wks of age) kept in individual cages for immunization and egg productions were divided into three groups (total 30 layers, 10 layers per group). Each group received either the freeze-dried *E. coli* whole cell (WC), or outer membrane proteins (OMP) or lipopolysaccharide fractions (LPS). The cells were suspended in sterile PBS (1 mg/ml, pH 7.2) and emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich; Saint Louis; Missouri; USA). One ml of the prepared emulsion was injected intramuscularly per hen at four sites (at 0.25 ml per site). Two booster injections were given on 2nd and 6th wk of first injection with the same dose emulsified with Freund's incomplete adjuvant. Eggs were collected every day during the immunization period and stored at 4°C until used.

Separation of antibody

Water soluble fraction (WSF) containing IgY antibody was prepared from egg yolk according to the procedure of Akita and Nakai (1992) and physically separated from the egg white and poured into a graduated cylinder by puncturing yolk membrane. The egg yolk was diluted with 10 volumes of distilled water (acidified with 0.1 M HCl, pH 5.0) and the mixture was incubated at 4°C for 6 h. After centrifugation at 12,000×g at 4°C for 20 min, the WSF was filtered through Whatman No. 1 filter paper at 4°C and assayed for IgY.

Antibody concentration

Radial immunodiffusion (RID) was performed using modifications of methods described by Mancini et al. (1965). Solution A was prepared by mixing 0.3 ml rabbit

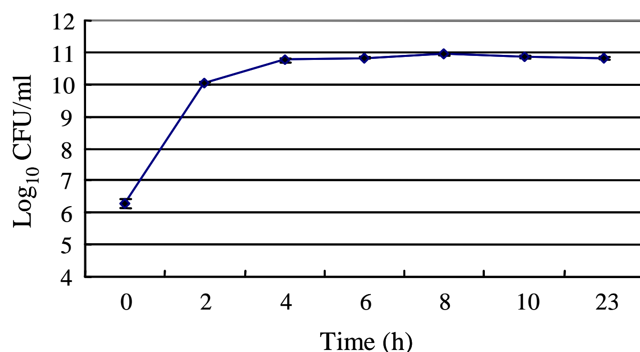

Figure 1. Survival curve for *E. coli* O157:H7 in brain heart infusion broth incubated at 38°C.

anti-chicken IgG with 1.7 ml barbital buffer (50 mM sodium barbital, 10 mM barbital, pH 8.6) and incubating in a 56°C water bath. Solution B was prepared by mixing 70 mg of agarose with 4.6 ml barbital buffer and 0.4 ml of 0.35% (wt/vol) sodium azide, and holding the mixture in a boiling water bath until the agarose dissolved. Solution A and B were then equilibrated at 56°C, mixed well, and poured into RID plates. Water soluble fraction (6 ml) and IgG standards (6 ml) containing 0.05 to 0.8 mg of chicken IgG were added to 2.5 mm diameter wells. A standard curve was obtained by plotting square values of diameter of the precipitation rings developed at room temperature for 2 to 3 days.

Antibody activity

Egg yolk antibodies were raised against WC, OMP and LPS fractions and assayed by an ELISA procedure. Determination of the antibody activity of IgY fractions was conducted as per the procedure described by Akita and Nakai (1993). Each well of the 96 well polystyrene plates was coated with 100 µl *E. coli* whole cell suspension, outer membrane protein fraction and LPS solution (100 µg/ml) in a 0.05 M carbonate buffer (pH 9.6) for 24 h at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tw) deionized water. After washing, 150 µl of 1% (wt/vol) solution of BSA in carbonate-bicarbonate buffer (0.05 M, pH 9.6) was added to each well and incubated at 37°C for 30 min. The BSA solution was then discarded and each well was washed three times with PBS-Tw. Diluted WSF was added to the plates and incubated at 37°C for 2 h. After washing with PBS-Tw, 150 µl of rabbit anti-chicken IgG conjugated with horseradish peroxidase (1:1,000 in PBS-Tw.) was added and incubated at 37°C for 2 h. After washing with PBS-Tw, 100 µl of freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) were added in 0.05 M phosphate citrate buffer (pH 5.0) contained for 30 min. Absorbency of chromophore produced in the reaction mixture was read at 405 nm using a Bio-Tek EL 309 microplate reader.

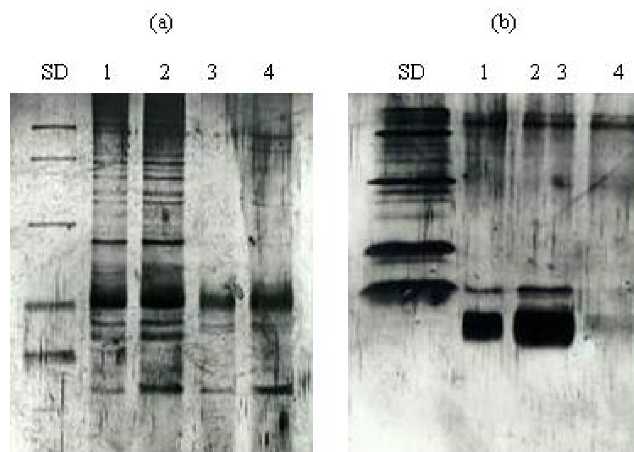

Figure 2. Silver staining prolife of SDS-PAGE (40 m A) run for 4 h (a) and 3 h (b). (a) 1-2 WC (loading volume 5, 10 µl), 3-4 OMP (loading volume 5, 10 µl) and LPS of *E. coli* O157:H7. And (b) 1-3 LPS (loading volume 5, 10, 2 µl) (SD: Standard marker (66, 48.5, 29, 18.4, 14.2 kDa). Running of SDS-PAGE was at sample (a) was run at 40 m A for 4 h and sample (b) for 3 h.

Suppression of *E. coli* 0157 with IgY's in broth

Dried anti-*E. coli* O157:H7 IgY's obtained from WC, OMP and LPS were serially diluted in BIBH. Serially diluted IgY's at the concentration of 0, 0.5, 1.0, 2.5, 5.0, 10 mg/ml were mixed with *E. coli* O157:H7 cell suspension (initial inoculation cell count: 2×10^6 cfu/ml). The mixture was incubated at 38°C for 6 h and examined for bacterial agglutination.

Suppression of *E. coli* 0157 against whole cell (WC) antibody in broth

Whole cell (WC) antibody at the concentration of 0, 5, 10, 15 mg/ml was serially mixed with *E. coli* O157:H7 cell suspension (initial inoculation cell count: 2×10^2 cfu/ml). The mixture was incubated at 38°C for 11 h and examined for bacterial agglutination.

Statistical analysis

The values were expressed as means±SE. Statistical analysis was performed by one-way analysis of variation (ANOVA), and significant differences were detected ($p<0.05$) by Duncan's multiple range tests using a PC statistical package (SAS, 2002).

RESULTS AND DISCUSSION

Growth of *E. coli* O157:H7

Growth of *E. coli* O157:H7 in brain heart infusion broth (initial inoculation cell count: 6.3 $\log_{10}$ cfu/ml) increased rapidly (Thomas et al., 1997) at 38°C until 4 h (Figure 1). Cell counts at 4 h were 10.7 $\log_{10}$ cfu/ml. Maximum cell counts were recorded at 8 h (10.9 $\log_{10}$ cfu/ml) after initial

Table 1. IgY concentrations in egg yolk and water soluble fraction

Items	WC	OMP	LPS
Yolk weight (g)	16.72±0.43[1]	17.15±0.35	16.66±0.40
WSF(mg/ml)	8.36±0.42	8.29±0.35	8.52±0.25
Egg yolk (mg/ml)	10.41±0.52	9.87±0.54	10.30±0.31

* Four samples were randomly tested for the IgY concentration.
[1] Mean±standard error.

inoculation. The cell count of *E. coli* O157:H7 remained relatively constant till 10 h and decreased slowly thereafter.

Preparation and separation of antigen from *E. coli* O157:H7

Separated WC, OMP and LPS fractions from *E. coli* O157:H7 were examined by silver stained SDS-PAGE (Figure 2). The WC fraction was apparent in the molecular mass range of 97 to 12 kDa in silver stained gel. The six bands stained with moderate intensity, while the remaining 15 bands were stained light. OMP fraction showed two major bands with molecular masses of 18 and 12 kDa on silver stained gel. Silver stained LPS fraction had one major protein with molecular mass of 12 kDa. The findings of the present study is in corroboration with the findings of Baldwin et al. (1997) who also determined a major protein in LPS fraction with molecular mass range of 12-14 kDaA on silver stained 12.5% polyacrylamide gel. Penny and Hitchcock (1984) reported similar molecular mass range of 94-10 kDa for whole cell fraction on silver stained gel. However, the range provided was observed to be slightly wider as compared to the results of the present study.

Antibody concentration

The WSF from egg yolks were collected during the immunization period (42 to 56 d) were analyzed for antibody concentration (Table 1). IgY concentrations in WSF were similar (p>0.05) in the groups, and they were averaged as 8.36 (WC), 8.29 (OMP) and 8.52 (LPS) mg/ml respectively. Similar results have also been reported by Sunwoo et al. (1996) who observed an average of 82-84% of WSF IgY from an egg yolk. IgY concentrations in egg yolk were not significantly different (p>0.05) between the three groups, and averaged 10.41 (WC), 9.87 (OMP) and 10.30 mg/ml (LPS) respectively. A similar trend has been reported previously in egg yolk IgY following immunization of chickens (Shimizu et al., 1988).

Antibody activity in egg yolk

The immune response of laying hens against the WC, OMP and LPS antigens were monitored by measuring antibody activities in egg yolk by ELISA. The level of activity of anti-*E. coli* WC (Figure 3) sharply increased after the initial immunization and reached peak on day 49

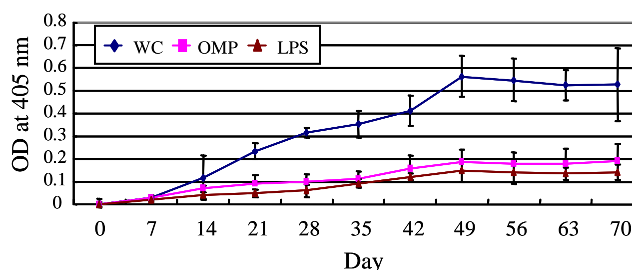

Figure 3. Changes in antibody activity in egg yolk during the immunization period. 10 birds each were injected with the antigenic fractions of WC, OMP and LPS on day zero and the booster was given on day 14 and day 42. Four egg yolks were randomly collected for each antigenic fractions every week for antibody analysis. * Arrows indicate the time of booster injection of antigens.

and was maintained till day 70. On the other hand, the levels of antibodies activity of OMP and LPS antigens slowly increased till day 42 after the initial immunization. This suggest that the elevated activity of anti-*E. coli* IgY could be maintained much higher in the WC rather than the OMP or LPS. The level of specific activities of IgY increased a week after the initial immunization and thereafter, increased constantly. The lag time of 1 week could be attributed that it takes time for specific antibodies to be produced in chicken serum, transferred and gets accumulated in egg yolk as reported by Li et al. (1998). Shimizu et al. (1988) immunized chickens against the whole cell of *E. coli* and observed elevated levels of anti-whole cell activity, suggested that the whole cell proteins were potent antigens in chickens. Gast et al. (1991) reported highest optical density of IgY at 3-5 weeks post inoculation against *salmonella enteritidis* and *pullorum* with two antigenic injections at an interval of 2 weeks. Level of specific antibodies of WC IgY showed increased optical density of 0.6 at 7 weeks in the current study with 3 antigenic injections given at an interval of 0, 2 and 4 weeks. Finding of the present study was in corroboration with Lee et al. (2002) who reported the highest optical density of IgY against *S. typhimurium* at 7 weeks. Author opines that the two antigens i.e. salmonella typhimurium or *E. coli* O157:H7 might have similar and strong inductive potential in boosting initial immunization.

Suppression of *E. coli* O157:H7 with antibodies

The effects of the antibody concentrations (0-10 mg/ml) obtained from WC, OMP and LPS on the proliferation of the bacteria were investigated by mixing the broth (brain heart infusion) with *E. coli* O157:H7 (2×10^6 cfu/ml) and incubated at 38°C for 6 h. The decline in bacterial population with WC directed antibodies (5 mg/ml) was highly significant (p<0.05) compared to OMP or LPS supplemented broth. Therefore, the WC fraction directed

Table 2. Changes in *E. coli* O157:H7 population with different concentrations of antibodies obtained from WC, OMP and LPS immunization (unit: cfu/ml)

Antibody concentration (mg/ml)	WC	OMP	LPS
0.0	$6.0\times10^{8\ a}$	$6.3\times10^{8\ a}$	$7.9\times10^{8\ a}$
0.5	$2.3\times10^{8\ b}$	$3.6\times10^{8\ ab}$	$5.9\times10^{8\ a}$
1.0	$1.4\times10^{8\ b}$	$3.2\times10^{8\ a}$	$3.6\times10^{8\ a}$
2.5	$1.2\times10^{8\ a}$	$2.7\times10^{8\ a}$	$2.7\times10^{8\ a}$
5.0	$2.0\times10^{7\ b}$	$2.6\times10^{8\ a}$	$2.0\times10^{8\ a}$
10.0	$1.0\times10^{7\ b}$	$1.1\times10^{8\ a}$	$1.4\times10^{8\ a}$

[a, b] values with different superscripts in the same row differ significantly ($p<0.05$).
* Initial inoculation cell count (*E. coli* O157: H7): 2×10^6 cfu/ml.
* Incubation temperature and time: 38°C for 6 h.

antibody produced in the egg of immunized chickens were more effective in restraining the proliferation of the bacteria than OMP or LPS directed antibodies (Table 2).

Suppression of *E. coli* O157:H7 with the antibody of whole cell

Whole cell fraction antibodies was found to be better in comparison to the other fractions. It was further tested to determine if the WC antibodies at different concentrations could restrain the proliferation of E.coli O157:H7 at the initial inoculation of 2×10^2 cfu/ml. 2×10^2 of *E. coli* O157:H7 was initially inoculated in broth (brain heart infusion) containing antibody at the concentrations of 0, 5, 10 and 15 mg/ml and incubated at 38°C for 11 h. The results indicate (Table 3), that the antibody concentration was effective in restraining the proliferation of the pathogenic bacteria. The bacteria grew prolifically to 6.4×10^7 cfu/ml in the media without antibody whereas the bacterial growth was inhibited to 1.5×10^4 cfu/ml in the media containing WC antibody at 10 mg/ml. Shimizu et al. (1988) reported that IgY (10 mg/ml) of whole cell of *E. coli* strain (ATCC 23985) restrained their proliferation in media containing IgY mixtures at 37°C. Gurtler et al. (2004) experimentally infected laying hens with S. *enteritidis* at an infection dose of 2×10^8 cfu/hen and obtained eggs with *S. enteritidis* specific antibodies. He reported that salmonella contaminated eggs of laying hens could be reduced by oral administration of whole egg powder containing S. *enteritidis* specific antibodies. Similarly, specific binding activity of IgY leading to the inhibition of bacterial growth using immunoelectron microscopy was also been demonstrated by Sim and Sunwoo (2003). The effectiveness of IgY in suppressing the activity of *E. coli* O157:H7 has been demonstrated in our study (Cipolla et al., 2001).

CONCLUSION

Anti-*E. coli* O157:H7 antibodies were isolated from eggs laid by hens immunized with antigens from WC, OMP or LPS. Antibodies against WC appeared to have higher activity than those against OMP or LPS at the same antibody concentrations (5 mg/ml). Growth of *E. coli* O157:H7 was inhibited 10-13 fold more with WC directed antibodies than OMP or LPS. This could be inferred from the results of the current study that increasing the IgY's in the egg yolk could prevent greater economic losses due to human and animal health from pathogenic bacteria i.e. *E. coli* O157:H7.

Table 3. Changes in *E. coli* O157:H7 population with different concentrations of antibodies obtained from WC immunization (unit: cfu/ml)

Antibody concentration (mg/ml)	0	5	10	15
E.coli O157: H7(cfu/ml)	$6.4\times10^{7\ a}$	$6.5\times10^{4\ b}$	$1.5\times10^{4\ b}$	$9.5\times10^{4\ b}$

[a, b] values with different superscripts in the same row differ significantly ($p<0.05$).
* Initial inoculation cell count (*E. coli* O157:H7): 2×10^2 cfu/ml.
* Incubation temperature and time: 38°C for 11 h.

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