Pathogenicity and immunogenicity of a mutant strain of *Listeria monocytogenes* in the chicken infection model

Running title: Infection and immunity of *L. monocytogenes* in chicken

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**Abstract:** *Listeria monocytogenes* (*L. monocytogenes*) has been exploited as a vaccine carrier based upon its ability to induce a strong cell-mediated immune response. At present, the safety of live-attenuated *L. monocytogenes* vaccines is being studied in clinical trials in patients. *L. monocytogenes* is also an attractive vaccine vector for use in poultry, however, the pathogenicity and immunogenicity of this organism in poultry remains to be fully elucidated. In this study, we investigated the pathogenicity and immunogenicity of an *actA* and *plcB*-deficient *L. monocytogenes* strain, yzuLMΔ*actA/plcB*, and its wild type parent strain, yzuLM4, in an avian infection model. The results showed that the wild type strain could infect ISA brown chickens causing serious tissue disruptions including various degrees of degeneration, necrotic lesions and inflammatory cell infiltration in liver, spleen, heart and kidney. However, the mutant strain showed reduced virulence (LD$_{50}$ was 3 logs lower) in embryonated eggs compared with the parent strain. The mutant strain also showed low virulence in chickens and was rapidly eliminated by the host. There were no obvious pathological changes in tissue sections, but the mutant strain still retained the ability to stimulate high levels of antibody against protein LLO. Booster immunization with the mutant strain led to rapid bacterial clearance from the liver and spleen of chickens challenged by the oral route. Collectively, our data suggest that the wild type serotype 1/2a *L. monocytogenes* strain can cause serious disease in chickens, but the mutant strain with a deletion of the *actA* and *plcB* genes is less virulent but induces a strong immune response. This mutant strain of *L. monocytogenes* is therefore a promising candidate as a safe and effective vector for the delivery of heterologous antigens to prevent zoonosis and infectious disease in poultry.

**Key words:** *Listeria monocytogenes*, chicken, pathogenicity, immunogenicity, mutant strain
1. Introduction

*Listeria monocytogenes* is an important foodborne intracellular pathogen which causes serious disease in humans with a very high mortality rate. The average case-fatality rate for *L. monocytogenes* infection is 20%–30% despite adequate antimicrobial treatment. Outbreaks of listeriosis have been reported in many areas of the world including North America, Europe and Japan (2, 6, 12, 13). As well as infecting humans, *L. monocytogenes* can also infect and cause listeriosis in animals including birds, goats, sheep, horses, dogs, cats and fish. In birds, more than 17 avian species have been verified to be susceptible to *L. monocytogenes*, with cases of listeriosis being reported in chickens, turkeys, pigeons and water fowl (3, 18). Birds can be infected via the airborne route by inhalation, uptake or debeaking. Young birds appear to be more susceptible to *Listeria* infection than older birds, the same is true in mammals, and listeriosis is most commonly manifested as septicemia (18).

Listeriosis in humans has only been recognized as an important foodborne disease since the 1980s, when a number of outbreaks were attributed to the contamination of poultry, particularly processed, ready-to-eat poultry products (19, 21). In recent years, dozens of recalls due to *Listeriae* in poultry products have been reported by the news media. For example, the outbreaks of listeriosis caused by infected delicatessen turkeys in multiple states of the United States of America in 2000 and 2002 were caused by *L. monocytogenes* serotypes 1/2a and 4b respectively (24). Like many animal species, poultry may excrete *Listeria* in feces and this bacterium has been detected in 4%–33% of the individual or pooled intestinal contents of broilers (4, 5, 22). Contact of poultry with faecal materials during slaughter processing has been suggested to be an important means of transmitting *L. monocytogenes* to poultry meat (22). Therefore, it is generally considered that *L. monocytogenes* is a bacterial pathogen of food-safety importance to both the poultry industry and public health.

Understanding both the *L. monocytogenes* infection process and the host immune response against *L. monocytogenes* is important for the development of preventive and therapeutic strategies. *L. monocytogenes* has also been proposed as a potential carrier for the delivery of heterologous antigens. For this reason, it has been extensively studied in mice (1, 20, 23), however, few studies have been reported using the chicken infection model. There is a need for novel effective avian vaccines against *Listeria* infection and recombinant *Listeria* vaccines are an attractive alternative to traditional avian vaccines which are costly and carry the risk of toxin leakage. Hence, demonstrating its pathogenicity and immunogenicity is especially necessary in the way of development as a vector to deliver heterologous antigens of avian pathogens.
2. Materials and methods

2.1. Bacterial strains

The *L. monocytogenes* virulent strain yzuLM4, serotype 1/2a, was isolated and preserved by the Jiangsu Key Laboratory of Zoonosis (Yangzhou, China). The *L. monocytogenes* mutant strain yzuLM4ΔactA/plcB, containing in-frame deletions within the *actA* and *plcB* genes by allelic replacement, as described previously (26), was used. The bacteria were grown in 10 ml BactoTM brain heart infusion (BHI) medium (Becton Dickinson Co., Franklin Lakes, New Jersey, USA) per 100-ml flasks by shaking at 37°C for 14 hours, then they were inoculated (1:50) in another 10 ml BHI per 100-ml flasks and grown for 3 hours at the same culturing condition.

2.2. Animals

Specific pathogen-free (SPF) chicken embryos and female chickens were procured from the Shandong Institute of Poultry Science (Jinan, China). Animals were housed, handled and immunized following approval by the institutional animal experimental committee.

2.3 Preparation of *Listeriae* strains

For immunization and protection studies, fresh bacterial cultures, prepared from an overnight culture, were used. Briefly, the mutant strain yzuLM4ΔactA/plcB and the wild-type parent strain were grown in BHI media, harvested in the exponential growth phase (OD600 nm=1.0) and washed twice with phosphate buffered saline (PBS, pH7.2). The pellet was resuspended in PBS and the bacterial concentration was calibrated by optical absorption and CFU counting. Further dilutions were prepared in PBS to obtain the required number of bacteria for immunizations or challenge.

2.4 Virulence in chicken embryos

The LD<sub>50</sub> of the two strains was estimated using the trimmed Spearman-Karber method (11). In a separate experiment, 0.1 ml of an appropriate 10-fold dilution of the mutant or parent strain in PBS was injected via the chorilallantoic membrane into 14-day-old embryonated eggs using the technique described previously (14). Five eggs were used for each inoculum. Eggs receiving PBS were included as a control. The inoculum concentration was confirmed by enumeration of the viable count on BHI plates. Inoculated eggs were incubated in a horizontal position at 37.5°C, and embryo death was monitored daily over 8 days by transillumination for LD<sub>50</sub> calculation.

2.5 Infection and bacterial enumeration

Two groups of 9-day-old chickens were used. yzuLM4ΔactA/plcB and yzuLM4 at a dose of 10^7 CFU/mL per ml PBS.
1×10⁹ colony forming units (CFU) respectively were injected into the right pectoralis muscle of 25 chickens in each group using 1 ml syringe. On day 1, 2, 3, 4 and 6 post-inoculation, chickens were sacrificed, the spleens and livers were taken out and homogenized by glass homogenizers, after 10-fold serial dilutions, they were plated on BHI plates (37g BHI medium and 18g agar in 1 liter distilled water, pH7.4, sterilization at 121°C for 15min, pouring to the sterile plates). Colonies were counted and identified after 24 h of aerobic incubation at 37°C. The detection limit of this procedure was 10² CFU per organ.

2.6 Histopathology

Two groups of chickens were immunized with the same route and dose as in section 2.5, a third group was included which served as a control and received intramuscular inoculation with PBS. On day seven post-immunization, sections of liver, heart, spleen, duodenum and kidney were fixed in 13% neutral buffered formalin. Paraffin-embedded sections were cut at 5µm, stained with hematoxylin and eosin, and examined for histological lesions with a 400× microscope (Leica Microsystems, Wetzlar GmbH).

2.7 Affection to chicken body weight

Two groups of chickens were inoculated as in section 2.5. The chickens were then boosted at 23-days of age. On days 7 and 14 post-priming, and on days 7, 14, 21 and 28 post-boosting, the chickens were weighed.

2.8 Serum antibody titer to LLO protein, as measured by enzyme-linked immunosorbent assay (ELISA)

Two groups of chickens were immunized as in section 2.5. Chickens were boosted at 30-days of age. On day 21 post-priming and post-boosting, blood from the vein of the chicken wings was taken, the sample was incubated at 4°C for 30 mins, then the serum was harvested by centrifugation at 1000×g for 5 min. Serum antibody against LLO protein was determined by indirect-ELISA. The LLO protein expressed by recombinant E. coli was purified and used as a coating antigen in the ELISA plate (0.34 µg per well). All serum samples were serially diluted for analysis. The secondary antibody HRP-conjugated rabbit anti-chicken IgG (Pierce Biotechnology, Rockford, Illinois, USA) was used at a concentration of 1:10,000. Serum from non-immunized SPF chickens and the monoclonal antibody against LLO were used as the negative and positive controls, respectively, for validation of the ELISA assay. The A490 data from the negative control serum were used to calculate the P/N values. The experiment was carried out in triplicate.

2.9 Protection efficacy
Two groups of 10 9-day-old chickens were intramuscularly injected with yzuLM4∆actA/plcB at a dose of 1×10^9 CFU, another two groups served as controls and received intramuscular inoculation with PBS. Chickens were boosted at 23 days of age. On day 14 post-boosting, one group of immunized chickens and control group were orally gavaged with 500µl of 4×10^10 CFU inoculum of yzuLM4 in PBS through gavage needle on a 1 ml syringe. Another group of immunized chickens and control group were challenged via intramuscular injection route with yzuLM4 at dose of 1×10^9 CFU. The birds were euthanized by carbon dioxide asphyxiation 48 h post challenge, the liver and spleen were removed immediately. Each tissue was weighed, homogenized by glass homogenizers and suspended (1:10) in PBS, ten-fold dilutions were prepared for cell-counting on BHI agar plates. Then the plates were incubated at 37°C for 24 h. Round, smooth and milky colonies typical of Listeria were counted.

2.9 Statistical analysis

Differences between groups were analyzed using the Statistical Package for the Social Sciences (SPSS version 15.0; SPSS, Chicago, IL, USA). P values less than 0.05 were considered to be significant, and those less than 0.01 were considered to be highly significant.

3. Results

3.1 Reduced virulence of the L.monocytogenes mutant strain in embryonated eggs

The mutant strain showed reduced virulence (LD₅₀ 3 logs lower) compared to its parent strain, in the chick embryo model test (Table 1). More than 80% of chicken embryos infected with the wild type strain died at 72 h post-inoculation, and all embryos died at 7 days post-inoculation. However, none of the chicken embryos had died after inoculation with the mutant strain at 72 h post-inoculation, and only 25% of inoculated embryos died at 7 days post-inoculation. These results showed that the virulence of the mutant strain was significantly reduced.

3.2 In vivo survival and persistence of the mutant strain and its wild type L.monocytogenes

Because all of chicken were survivor over some amounts of days with infection, they were sacrificed before recovering bacteria from their organs. The first and crucial step in the in vivo characterization of the mutant and wild type strains was the determination of their growth kinetics in chick spleen and liver. As indicated in Figure 1, for the mutant strain group, no bacteria were isolated from the liver and the number of bacteria in spleen dropped rapidly from day 2 post-inoculation, leveling off and persisting.
at a low number up to day 5 post-infection. However, for the wild type strain infection group, the number of bacteria in the liver was high (>4 logs) on day 1, reached a peak on day 2, then gradually decreased and the bacteria were cleared by day 8. The number of *L. monocytogenes* in the spleen was highest on day 1, then gradually decreased and the bacteria were finally cleared on day 8. This result showed that there were fewer bacteria in the spleen and liver after infection with the mutant strain and that the mutant strain cleared quicker than the wild type strain. The bacterial growth kinetics in vivo therefore indicated that the translocation ability of the mutant strain was reduced, along with attenuated virulence.

### 3.3 Effect of infection on chicken body weight

As shown in Table 2, the body weight of chickens infected with the wild type strain was significantly decreased in the intramuscular challenge group as compared with the unchallenged controls and the mutant strain (P<0.05). However, the body weight of chickens infected with the mutant strain showed no significant difference compared with the unchallenged controls, except at week 1 post-boosting (P>0.05; Table 2). There was a significant association between infection with the wild type strain and body weight, while the mutant strain did not affect body weight, suggesting that the mutant had highly attenuated virulence.

### 3.4 Gross pathology and histopathological studies of *L. monocytogenes*-infected chickens

**Gross pathology.** After intramuscular infection of chickens with the *L. monocytogenes* virulent strain yzuLM4, the gross pathological symptoms included loss of appetite, reduced intake, flagging spirit and diarrhea. Systemic abnormalities such as splenomegaly, mild hepatomegaly, myocarditis and white milliary spot necrosis at the site of injection were observed in these chickens. No gross symptoms were observed in either the mutant inoculated group or the control group (Fig. 2).

**Histopathological lesions.** Analysis of histopathological lesions in chickens infected by wild type *L. monocytogenes* revealed serious disruption (Fig. 3). The pathological characteristics of the visceral organs were as follows. The liver sections showed inflammatory cell infiltration in the hepatic lobule. In the spleen section, hyalinosis and fibrinous exudates were observed in the reticular fiber of the spleen and homogeneous red staining material had accumulated among lymphocytes. The kidney section revealed that not only had the connective tissue of the kidney hyperplasia proliferated and the mesenchyme broadened, but also numerous inflammatory cells had appeared in the venule. In the heart section, unequal sized focal necrotic lesions had appeared in the disintegrated muscle fibers. In the intestine section, cast off intestinal epithelial cells were evident. However, it is clear from Figure 4...
that no obvious pathological changes appeared in the sections taken from chickens inoculated with the mutant strain. The mutant strain induced only a mild inflammatory reaction as evidenced by infiltration of inflammatory cells into the peripheral and central veins of the liver and the tissue of the kidney.

3.5 _L.monocytogenes*-specific serum antibody response

To analyze the humoral immune response induced by _L.monocytogenes_, we chose the highly antigenic LLO protein as an indicator and determined the level of LLO antibody in chicken sera. Although the level of antibody induced by the mutant strain was significantly reduced compared with the wild type strain (Fig. 4A and 4B), the attenuated mutant still retained the ability to stimulate a high level of antibody against LLO. Combined with the results of kinetic studies of _L.monocytogenes_ infection in chickens, these findings suggested that the level of antibody induced by _Listeria_ correlated with the number of bacteria entering the host.

3.6 Protection acquired after immunization with the mutant strain against wild type strain challenge

To further analyze the ability of the mutant strain to induce protective immunity, chickens were immunized and boosted with _L.monocytogenes_ mutant strain yzuLM∆actA/plcB. Because the wild type strain yzuLM4 could infect ISA brown chickens and cause disease, but it was not fatal to chickens at 1×10^9 CFU per chicken. Based upon the fact that the wild type strain yzuLM4 was not fatal to chicken at 1×10^9 CFU by intramuscular route, we evaluate the protection efficacy by comparing the CFU counts of _L.monocytogenes_ in the organs. CFU numbers in spleens and livers of chickens were determined at day 2 after challenge infection by intramuscular route or oral route (Fig. 5A and Fig. 5B). The results showed that the immunized chickens cleared _L.monocytogenes_ bacteria quicker than the un-immunized group at day 2 either postchallenge by the oral route or intramuscular route. This difference was statistically significant (p<0.01). The vaccination regime resulting in quicker clearance suggested that immunization of chickens with _L.monocytogenes_ strain yzuLM∆actA/plcB could induce the protective immune responses.

4. Discussion

Recombinant _L.monocytogenes_ strains have been designed and verified as effective delivery systems for a variety of heterologous antigens inducing profound and effective anti-pathogen or antitumor immunity in a mouse model (9, 10, 17). However, the pathogenic mechanism and the host immune response to _L.monocytogenes_ still remain to be systematically investigated in an avian model. _L.monocytogenes_ serotypes 1/2a and 4b are responsible for the majority of cases of human listeriosis.
worldwide, and serotype 1/2a belong to lineages II, lineages II strains are common in foods, seems to be widespread in the natural and farm environments, and also isolated from animal listeriosis cases and sporadic human clinical cases (15). Furthermore, many countries have seen a shift in the L. monocytogenes serotypes causing human infections from predominantly serotype 4b to 1/2a and 1/2b (2, 16). Taking this into consideration, we used the serovar 1/2a mutant strain yzuLM4ΔactA/plcB and its parent strain yzuLM4 in this study to infect chickens via muscular injection and investigated pathogenicity, immunogenicity and the efficacy of immunization.

L. monocytogenes is an important food-borne pathogen and listeriosis is a zoonotic disease. L. monocytogenes is highly effective at crossing the intestinal barrier, the blood-brain barrier and the fetoplacental barrier, causing meningoencephalitis, mastitis, abortion, metritis and septicemia. In this study, we found that the serotype 1/2a wild type strain yzuLM4 which is highly virulent in mice (LD₅₀ of 1.47×10⁴ in BALB/c mice), is also highly pathogenic in SPF chicken embryos (LD₅₀ of 2.65×10⁵) and ISA brown hens. This bacteria could cross the natural chicken carrier and reach the liver and spleen with about 10⁴ and 10⁷ CFU respectively, 24 h after infection via the intramuscular route. The bacteria were finally cleared by day 8. Analysis of tissue sections showed that serious pathological changes in the liver and spleen were caused by the virulent strain. The pathology seen in this study is consistent with the findings of Huff et al (8). The high virulence of the wild type strain resulted in significantly decreased body weight compared with chickens infected with the attenuated strain, indicating that the wild type strain seriously affected the growth of chickens. However, the invasive ability of the attenuated strain yzuM4ΔactA/plcB was clearly reduced. L. monocytogenes was not isolated from the liver and the number of bacteria isolated from the spleen was reduced about 10³-fold on day 1 and the bacteria were cleared three days earlier than with the parent strain. Pathological analysis of tissue sections also showed only minimal inflammation in the visceral organs due to the immune response caused by the attenuated strain. This mutant strain did not affect body weight, with no difference in body weight detectable between the mutant and the control group. Hence these results suggest that the ability of the mutant strain of L. monocytogenes to diffuse and translocate is reduced, thereby significantly reducing the virulence.

To evaluate the ability of L. monocytogenes to elicit an immune response in ISA brown hens, we tested for antibody being produced against protein LLO in an indirect-ELISA. Although the results indicated that the antibody titer showed a statistically significant difference between the attenuated and virulent strains, the attenuated strain could still induce a high anti-LLO titer after inoculation via
injection. LLO protein is a major immunodominant listerial antigen playing an important role in the presentation of passenger antigens to the class I pathway of the major histocompatibility complex (MHC) and therefore elicits strong CD8+ T-cell-mediated immune responses (7, 25). Therefore the central role of LLO in the listerial infection cycle, coupled to the significant antigenicity of this protein, suggested that the mutant strain retains the ability to induce strong cellular immunity. In this study, we found that chickens vaccinated with the attenuated strain acquired sufficient immune protection against the wild type strain yzuLM4 to clear *L. monocytogenes* infection more rapidly than un-vaccinated chickens. This result confirmed that the attenuated strain retains the ability to induce an immune response against *L. monocytogenes*. It also indicated that the attenuated strain yzuLM4ΔactA/plcB is effective at delivering pathogenic antigens and inducing an immune response, a property that could be applied to the prevention of infection by pathogens.

In this study, we found that the wild type strain yzuLM4 could infect ISA brown chickens and cause disease, but it was not fatal to chickens at 1×10⁹ CFU per chicken via the intramuscular infection route. However, in another study we showed that serotype 4b strain yzuF36 was fatal to chickens by the same infection route and dosage (data not published), indicating that the virulence of strain yzuLM4 is lower than that of strain yzuF36 in chicken infection model. Furthermore, the wild type strain yzuLM4 is virulent in mice (LD50= 1.47×10⁴), but less virulent in chickens. These results confirmed that differences exist in the pathogenic tropism between *L. monocytogenes* strains. Our results are concordant with Martin Wiedmann’s opinions, his study suggested that four *L. monocytogenes* lineages (I, II, III, IV) were identified so far represent distinct ecologic, genetic, and phenotypic characteristics, which appear to affect their ability to be transmitted through foods and to cause human disease (15).

In summary, we investigated the characteristics of *L. monocytogenes* infection and immunogenicity, and analyzed the safety and efficacy of *L. monocytogenes* immunization. The results confirmed that chickens were susceptible to the strains used in these experiments. The mutant strain yzuLM4ΔactA/plcB showed decreased invasion and virulence, but was able to elicit a strong immune response and provide immune protection against the wild type homologous strain. Further studies are now required to determine the most effective dosage and vaccination procedure. Taken together, the attenuated strain of *L. monocytogenes* described in this study is a promising vaccine candidate for preventing listeriosis, and this *L. monocytogenes* strain may be further exploited as a potential candidate to express heterologous antigens for the prevention of avian infectious diseases.
Acknowledgments

This work was supported by National Basic Research Program of China (2006CB504400), National Natural Science Foundation of China (30425031), National Key Technology R&D Program (2009BADB9B01).

Reference


Figure legends

Fig. 1 The CFU counts of L.monocytogenes in the organs of commercial ISA brown chickens after intramuscular infection. (A: liver; B: spleen). Twenty-five chickens in each group were intramuscularly injected with yzuLM4∆actA/plcB or yzuLM4 at a dose of 1×10^9 CFU respectively. On day 1, 2, 3, 4 and 6 after the infection, chickens were sacrificed and the number of viable bacteria in the organs enumerated as described.

Fig. 2 The gross lesions in the visceral organs of chickens. (A: control group; B: yzuLM4; C: yzuLM4∆actA/plcB)

Fig. 3 Histopathology of chickens via intramuscular routes of infection with L.monocytogenes. (A: The liver of chicken infected with the wild type strain (400×); B: The liver of chicken infected with mutant strain yzuLM4∆actA/plcB (400×); C: Normal control liver of chicken (400×); D: The spleen of chicken infected with wild type strain yzuLM4 (400×); E: The spleen of chicken infected with mutant strain yzuLM4∆actA/plcB (400×); F: Normal control spleen of chicken (400×).

Fig. 4 LLO antibody levels in sera after inoculation of chickens with L. monocytogenes. (A: On day 21 post-priming; B: On day 21 post-boosting). Data presented are representative of three independent experiments.

Fig. 5 The CFU counts of L.monocytogenes in the organs of commercial ISA brown chickens immunized with yzuLM4∆actA/plcB and challenged with virulent yzuLM4 at 2w post-boosting. Chickens were sacrificed on day 2 postchallenge by intramuscular route or oral route, the number of viable bacteria in spleens and livers enumerated as described (Fig. 5A and Fig. 5B). Data presented are
representative of three independent experiments. **P < 0.01, compared to the corresponding control group.

Table 1. LD$_{50}$ of *L. monocytogenes* for embryonated chicken eggs

Table 2. Effect of injection with *L. monocytogenes* on chicken weight
Fig. 1

A. Liver

B. Spleen

Fig. 2

A

B

C
Fig. 3

LLO antibodies level in chicken sera 3-wks post boosting

OD_{490}

control group
yzuLM4 ∆ actA/plcB
yzuLM-4

Fig. 4

LLO antibodies level in chicken sera 3-wks post priming

A

control group
yzuLM4 actA/plcB
yzuLM-4

B

control group
yzuLM4 actA/plcB
yzuLM-4
Fig. 5

A) Challenge by intramuscular route

B) Challenge by oral route

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### Table 1 LD50 of *L.monocytogenes* for embryonated chicken eggs

<table>
<thead>
<tr>
<th>Group</th>
<th>Infection dose(CFU)</th>
<th>lgLD50&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.33x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.33x10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>zyuLM4</td>
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<td></td>
</tr>
<tr>
<td>△actA/△plcB</td>
<td>3/5&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3/5</td>
</tr>
<tr>
<td>yzuLM4</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>PBS</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>yzuLM4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>The logarithm of median lethal dose to base 10
<sup>2</sup>Number of dead /total number

### Table 2 Effect of injection with *L.monocytogenes* on chicken weight ( grams)

<table>
<thead>
<tr>
<th>Time</th>
<th>Control group</th>
<th>yzuLM4-actA/plcB</th>
<th>yzuLM4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1w post-priming</td>
<td>176.47±11.58a</td>
<td>170.45±14.73a</td>
<td>143.90±14.21b</td>
</tr>
<tr>
<td>2w post-priming</td>
<td>260.95±22.93a</td>
<td>250.72±21.13a</td>
<td>217.91±19.36c</td>
</tr>
<tr>
<td>1w post-boosting</td>
<td>357.93±25.12a</td>
<td>338.77±23.42b</td>
<td>305.53±24.84c</td>
</tr>
<tr>
<td>2w post-boosting</td>
<td>508.12±25.66a</td>
<td>497.43±21.08a</td>
<td>458.22±23.34b</td>
</tr>
<tr>
<td>3w post-boosting</td>
<td>578.45±27.44a</td>
<td>563.24±24.27a</td>
<td>521.46±28.50b</td>
</tr>
<tr>
<td>4w post-boosting</td>
<td>645.62±27.44a</td>
<td>630.81±24.27a</td>
<td>588.70±28.49b</td>
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</tbody>
</table>

1) Within a line, data followed by the same lowercase letters represents no significance at 0.05 level by comparison of different treatment.