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Research Article

Antimicrobial Peptides in Housefly Larvae *(Musca domestica)* Affect Intestinal *Lactobacillus acidophilus* and Mucosal Epithelial Cells in *Salmonella pullorum*-infected Chickens

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Abstract

Pullorum disease, which is one the most serious intestinal diseases in poultry production, is generally treated by adding antibiotics to the feed of infected chickens. Although antibiotics are generally quite effective against the disease, they can harm small intestinal flora and mucosa. The objective of this experiment was to determine if antimicrobial peptides (AMPs) from housefly (*Musca domestica*) larvae can be used to treat pullorum disease. The study included AMPs extracted from *Salmonella enteric* serovar Pullorum-infected larvae as well as non-infected ones (referred to as induced-AMPs and non-induced AMPs, respectively). Tests were then conducted to determine (i) the activity of these AMPs against *S. pullorum* and (ii) the effects of the AMPs on intestinal *Lactobacillus acidophilus* and mucosa epithelial cells in *S.* pullorum-infected chicks. The results showed that *S.* pullorum-induced AMPs and non-induced AMPs both exhibited antimicrobial activity against *S. pullorum*. Small intestinal *L. acidophilus* populations in convalescent chicks that had been treated with induced AMPs showed similar patterns to those in healthy chicks. Induced AMPs also had relatively little effect on the number of mast cells, lymphocyte cells, and goblet cells in the small intestine of convalescent chicks compared with healthy chicks. In contrast, treatment with antibiotics generally reduced the number of all three cell types, especially in the duodenum. In conclusion, AMPs from housefly larvae offer potential for effective treatment of *S.* pullorum-infected chickens without the harmful side effects of antibiotics.

Keywords: *Larvae, Antimicrobial peptide, Salmonella Pullorum, Intestinal, Epithelial cells*

Karasinekteki *(Musca domestica)* Antimikrobiyal Peptidler *Salmonella pullorum* ile Enfekte Tavuklarda Bağırsak *Lactobacillus acidophilus* ve Mukozal Epitel Hücrelerini Etkiler

Özet

Kanatlı üretiminde en ciddi bağırsak hastalıklarından birine neden olan Pullorum hastalığı genellikle enfekte tavukların yemlerine antibiyotik ilavesi ile tedavi edilir. Antibiyotikler genellikle hastalığa karşı oldukça etkili olmakla birlikte ince bağırsak florasına ve mukozaya zarar vermektedir. Bu çalışmanın amacı; karasinek *(Musca domestica)* larvasındaki antimikrobiyal peptidlerin (AMP) pullorum hastalığının tedavisinde kullanılıp kullanılmayacağının belirlenmesidir. Çalışmada *Salmonella enteric* serovar Pullorum-enfekte (indüklenmiş AMP) ve enfekte olmayan (indüklenmemiş AMP) larvalardan ekstrakte edilen AMP kullanıldı. Çalışmada; (i) *S. pullorum*'a karşı AMP aktivitesi ve (ii) *S. pullorum*-enfekte civcivlerde bağırsak Lactobacillus acidophilus ve mukoza epitel hücrelerinde AMP etkileri araştırıldı. Elde edilen sonuçlar *S. pullorum* indüklenmiş AMP ve indüklenmemiş AMP'in her ikisinin de *S. pullorum*'a karşı antimikrobiyal aktivite gösterdiğini ortaya koymuştur. İndüklenmiş AMP uygulanarak tedavi edilen civcivlerin ince bağırsak *L. acidophilus* popülasyonu sağlıklı civcivlerinki ile benzerlik göstermekteydi. İndüklenmiş AMP; tedavi edilen civcivlerin ince bağırsak mast hücre, lenfosit ve goblet hücre sayılarında sağlıklı civcivler ile karşılaştırıldığında göreceli olarak az miktarda etkiye neden oldu. Aksine antibiyotik uygulaması özellikle duodenumda olmak üzere her üç hücre tipi sayısında genellikle düşmeye neden oldu. Sonuç olarak, karasinekten elde edilen AMP *S. pullorum* ile enfekte tavukların tedavisinde zararlı yan etkileri olmaksızın kullanılabilecek potansiyele sahiptir.

Anahtar sözcükler: *Larva, Antimikrobiyal peptid, Salmonella Pullorum, Bağırsak, Epitel hücresi*

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INTRODUCTION

Salmonella infection is caused by a variety of *Salmonella* species [1]. More than 2.550 *Salmonella* serotypes have been reported, mostly belonging to *S. enterica* [2]. Pullorum disease caused by *S. enterica* serovar. Pullorum is one of the most serious poultry diseases in the world. *S.* Pullorum first infects the intestinal tract of chickens. The main clinical symptoms of pullorum disease in chickens are listlessness and white diarrhea [3]. Chickens can be infected at any age. Two to three weeks old chicks have the highest morbidity and mortality. Pullorum disease tends to be either chronic in adult chickens or latent without obvious symptoms [4-6]. The disease is extremely difficult to cure because the bacteria can be carried for long periods, resulting in persistent infection [7].

Intestinal floraisvital to chicken health [8]. *S.* Pullorum infection can cause lesions and damage villi in the small intestine. Antibiotics are the main means of controlling pullorum disease [9]. However, bacterial resistance to antibiotics has increased due to long-term use and overuse [10]. More than 2000 antibiotic resistant strains of *S.* Pullorum were identified worldwide between 1962 and 2007 [11].

The substitution of antimicrobial peptides (AMPs) for antibiotics is one way to prevent the development of resistant microbial strains [12-15]. However, several studies have shown that AMPs have no real advantage compared with traditional antibiotics [16-18]. In recent years, AMPs have been used as a feed additive to prevent *S.* Pullorum infection.

AMPs are polypeptides produced by an organism to protect it from infection by pathogenic microorganisms [19]. AMPs have broad spectrum antimicrobial activity and, furthermore, resistance to AMPs is not easily developed [12,20-22]. AMPs have been isolated from a variety of organisms including insects, plants, and vertebrates. Insects, which have the greatest number of species in the animal world, can secrete many kinds of AMPs [23]. The housefly *(Musca domestica)* is surrounded from the larval to adult stages by many different pathogens [24]. Some researchers have attributed the unique pathogen resistance of houseflies to AMPs which they secrete [25,26].

The purpose of this study was to determine the efficacy of AMPs from housefly larvae for treatment of chicks with pullorum disease. The specific objectives were (i) to determine the bacteriostatic activity and minimum inhibitory concentration (MIC) of the AMPs toward *S. pullorum* and (ii) to confirm that the AMPs have therapeutic effect by comparing intestinal *L. acidophilus* populations and mucosal epithelial cell numbers in healthy chicks with those in *S. pullorum*-infected chicks treated with AMPs. The effects of the AMPs were also compared with those of gentamycin sulfate, an antibiotic that is commonly used to treat pullorum disease. The results of this experiment should provide information about the potential use of AMPs from housefly larvae as a feed additive.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University. All chickens were housed and euthanized in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Bacteria

S. Pullorum (CVCC578) was purchased from the China Institute of Veterinary Drug Control. The standard strains were cultured in Luria broth (LB) at 37ºC until the logarithmic growth phase was reached. The bacterial cells were collected by centrifugation (8.000 g, 5 min) and the cell concentration was adjusted to 1×10^7 CFU/mL.

Crude Extractions of Housefly AMPs

The housefly larvae used in this study were obtained from the Insect Laboratory, College Agronomy, Shihezi University. To induce the production of *S.* Pullorum-specific AMPs, the larvae were pricked with a needle that had been dipped into the suspension of *S.* Pullorum cells described above. The AMPs from this group will be referred to as *S.* Pullorum-induced AMPs [27]. A second group of larvae were pricked with a needle that had been dipped into distilled water. The AMPs from this group will be referred to as non-induced AMPs. The larvae were then put into an incubator for 24 h at 25ºC and 60% relative humidity.

The AMP was crudely extracted from the larvae using a modification of the method described by Gang et al.[28]. Briefly, the larvae were surface sterilized in 75% ethanol, washed with sterile water, and then dried. The larvae were homogenized in a mixture of 0.05 mol/L of ammonium acetate buffer (pH 5.0), 0.35 µg/mL PMSF, 0.2 mg/L EDTA, and 2% β-mercaptoethanol at a ratio of 1 mg larvae to 3 mL solution. The homogenate was centrifuged twice at 12000g for 30 min at 4ºC. The supernatant was decanted and then heated in a boiling water bath for 10 min. After rapid cooling, the samples were centrifuged at 12.000 g for 30 min at 4ºC in ultrafiltration tubes (molecular weight cut-off of 3 kDa). The supernatant was then stored at -80ºC.

Antibacterial Activity Assays

Antimicrobial activity was determined by the standard agar plate method [29]. Paper disks were soaked for 30 min in solutions containing either (i) *S.* Pullorum-induced AMPs, (ii) non-induced AMPs, or (iii) gentamycin sulfate antibiotic. *S.* Pullorum cells were spread evenly onto the surface of solid LB nutrient medium with a sterile glass-spreading

rod. The paper disks were placed on the surface of the medium after they were completely dry. The inhibition zones were measured after 24 h culture at 37°C. The areas of the inhibition zones were calculated to quantify the relative activity of each treatment against *S.* Pullorum. The interpretive criteria were as follows: low susceptible, inhibition zone diameter ≤10 mm; intermediate, 10 to 14 mm; susceptible, 14 to 19 mm; and highly susceptible ≥19 mm [28].

The minimum inhibitory concentration (MIC) of the *S.* Pullorum-induced AMPs was determined using the broth-double dilution method [30]. Normal saline solution (2.5 mL of 0.9% NaCl), and LB medium (2.5 mL) were added to sterile tubes containing 10^4 colony forming units (CFU) of *S.* Pullorum. *S.* Pullorum-induced AMPs were added to the tubes in 0.5 mg/L increments from 0 to 5.0 mg/L. In the control group, gentamycin sulfate was substituted for the AMPs. The tubes were incubated at 37°C for 48 h on a rotary shaker. The MIC was defined as the lowest peptide concentration causing the complete inhibition of *S.* Pullorum growth.

Artificial Infection Experiment

Specific pathogen-free (SPF) male chicks were purchased from a local hatchery. The chicks were raised in cages with *ad libitum* access to food and water. When they were 14-days-old, the chicks were randomly divided into five treatment groups of 50 chicks each. The chicks in four groups were injected with 2 mL of *S.* Pullorum suspension (1×10^7 CFU/mL) into the chest cavity [31]. All of the chicks presented symptoms of pullorum disease (i.e., diarrhea) 24 h after injection. The fifth group (referred to as the healthy group) was not injected with *S.* Pullorum, and *S.* Pullorum was replaced by injected the normal saline with the same dosage.

The *S.* Pullorum-infected chicks were treated in four different ways. One group was treated with *S.* Pullorum-induced AMP. Another group was treated with non-induced AMP. The chicks in these two groups were given 3 mL of the crude AMP extract (1 mg AMP/mL) daily. A third group was fed live housefly larvae. The AMP content of the housefly larvae was 0.5 μg AMP/g fresh weight. The AMP dosage was adjusted so that it was the same as that in the two AMP extract treatments. The fourth group was treated with 100 mg/L gentamycin sulfate antibiotic in the drinking water according to the manufacturer's instruction. These four treatments continued for 3-5 d until the disease symptoms disappeared. The healthy chicks (i.e., the fifth group) received normal food and water. The chicks were slaughtered 3, 5, and 7 d after the above treatments were started and their intestinal tracts were examined as described below.

Sample Collection

- *Isolation of Lactobacillus acidophilus: L. acidophilus* was isolated by washing the contents from the small intestine of each chick with normal saline solution under aseptic conditions. The samples were serially diluted 7 to 9 fold with saline solution and then plated onto De Man, Rogosave Sharpemrs(MRS) culture medium. The cultures were incubated for 24 h at 37°C.

- *Small Intestine Tissue Sections:* Four cm long sections of the duodenum, jejunum, and ileum were excised and then immediately put into 4% formalin and fixed for 72 h. The samples were then paraffin-embedded according to methods described by Watters et al.[32] and Alketa et al.[33]. Briefly, the tissue specimens on the surface of the formaldehyde were washed with tap water, dehydrated with graded alcohol, washed twice within xylene, and then embedded in paraffin. Xylene was used to remove wax and then the samples were rehydrated with graded alcohol. Tissue samples were cut into 5 μm thick sections using a histotome.

Five sections from each sample were dyed. The tissue sections were floated on distilled water, collected onto clean glass slides, dried in an oven, and then stained with hematoxylin and eosin (HE) and toluidine bluestain (0.8% toluidine blue, 0.6% potassium permanganate, dissolved in boil distilled water). The samples were decolorized and then sealed with neutral gum. The morphology of the small intestine sections was observed under an optical microscope. Five visual fields (1392 nm ×1040 nm) were randomly selected. The average positive cell number was regarded as the total cell number.

Statistical Analysis

Statistical analyses were performed using SPSS software version 17.0 (IBM, Armonk, NY). Independent *t*-tests and one-way ANOVA were used to analyze changes in the inhibition zone diameter, intestinal *L. acidophilus* populations, and the numbers of intestinal mucosal epithelial cells. Differences were considered to be significantly different when $P<0.05$.

RESULTS

Antibacterial Activity of AMP

The antibacterial activity of AMP against *S.* Pullorum was confirmed using the disc diffusion method. The inhibition zone diameters decreased significantly in the order antibiotic >*S.* Pullorum-induced AMPs>non-induced AMPs *(Table 1)*. The inhibition zone diameter of gentamycin sulfate was 11.76% greater than that of *S.* pullorum-induced AMP group ($P<0.05$) and 11.84% greater than that of non-induced AMP ($P<0.01$).

The MIC of AMPs against *S.* Pullorum was determined using liquid LB agar containing different concentrations of *S.* Pullorum-induced AMP and gentamycin sulfate *(Table 2)*. The MIC of induced AMP was 3.0 mg/L whereas that of gentamycin sulfate was 2.0 mg/L.

Changes in Intestinal L. acidophilus Populations

The populations of *L. acidophilus* in different parts of the small intestine are shown in *Fig. 1*. *L. acidophilus* numbers in the duodenum increased in the healthy, induced-AMP, and non-induced AMP groups between d 3 and 7. There was no significant difference between the healthy and non-induced groups on d 3. Moreover, the temporal changes in *L. acidophilus* numbers were similar in the induced AMP group and the healthy group. In contrast to the AMP groups, *L. acidophilus* numbers in the larvae-fed and antibiotic groups decreased with time. This meant that antibiotic reduced *L. acidophilus* numbers in the duodenum of convalescent chicks compared with healthy chicks, whereas AMP had little effect.

L. acidophilus numbers in the jejunum of the induced AMP group were similar to those in healthy chicks. *L. acidophilus* numbers remained steady or decreased with time in the other three treatments groups. In the ileum, *L. acidophilus* numbers increased with time in the induced-AMP, larvae, and healthy groups. The pattern of change in the induced-AMP was similar to that in healthy chicks. In conclusion, *S.* Pullorum induced AMP had relatively little effect on intestinal *L. acidophilus* in convalescent chicks ($P>0.05$).

Table 1. *Inhibition of S. Pullorum by antimicrobial peptides (AMPs) and gentamycin sulfate antibiotic*

Group	Diameters of Inhibition Zone
Non-induced AMPs (control)	22.8 ± 1.47^{Aa}
S. Pullorum-induced AMPs	25.5 ± 0.87^{b}
Gentamycin sulfate	28.5 ± 0.96^{Ba}

$^{A-B}$ *Values with different superscripts are significantly different at P<0.05*

Table 2. *The colony forming ability of S. Pullorum as affected by antimicrobial peptides (AMPs) and gentamycin sulfate antibiotic*

Concentration (mg/L)	*S.* Pullorum
S. Pullorum-induced AMPs	
5.0	-
4.5	-
4.0	-
3.5	+
3.0	+ +
2.5	+ +
2.0	+ +
1.5	+ +
1.0	+ +
0.5	+ +
0	+ +
Gentamycin sulfate	
5.0	-
4.5	-
4.0	-
3.5	-
3.0	-
2.5	+
2.0	+ +
1.5	+ +
1.0	+ +
0.5	+ +
0	+ +

Note:"-":no colony; "+":microcolony; "+ +":normal colony

Intestinal Morphology

Mast Cells: The number of mucosal mast cells increased from the duodenum to the jejunum to the ileum on all sample dates *(Fig. 2)*. In the duodenum, the healthy group had the most mucosal mast cells in the duodenum among all treatments. The induced-AMP group had the second most mast cells on d 3 and 5 (10.14%-11.59% less than the healthy chicks). The larvae group had the fewest mast cells on d 3 and 5. There was no significant difference in mast cell number among the four groups of *S.* Pullorum-infected chicks (i.e, gentamycin sulfate, larvae, non-induced AMPs, and induced-AMPs) on d 7. In the jejunum, there was no significant difference in mast cell number between the induced-AMP group and the healthy chicks on any date. The induced-AMP group had significantly more mast cells than (i) the non-induced AMP group on d 3 (5.15% more) and (ii) the larvae group on d 3 and 5 (13.97%-18.38% more). In the ileum, the gentamycin sulfate group and the induced-AMP group had as many or significantly more mast cells than the healthy group. There was no significant difference in mast cell number between the non-induced AMP group and the healthy chicks on d 5 and 7.

Lymphocyte Cells: The number of intestinal lymphocyte cells increased from the duodenum to the jejunum to the ileum on all sample dates *(Fig. 3)*. There was no significant difference in lymphocyte cell numbers between the induced-AMP group and the healthy chicks in any section of the small intestine on any sampling date. Lymphocyte cell numbers in the non-induced AMP group were as great as or greater than those in the induced-AMP and healthy groups. The exception was in the duodenum on d 3. Lymphocyte cell numbers in the duodenum and the jejunum were lowest in the larvae group on all sampling dates. Lymphocyte cell numbers in the gentamycin sulfate group were intermediate between the AMP groups and the larvae groups in the duodenum and the jejunum.

Goblet Cells: The number of goblet cells increased from the duodenum to the jejunum to the ileum on all sample dates *(Fig. 4)*. In the duodenum, goblet cell numbers in the induced-AMP group were significantly less (1.10%-15.38% less) than those in the healthy group on all sampling dates.

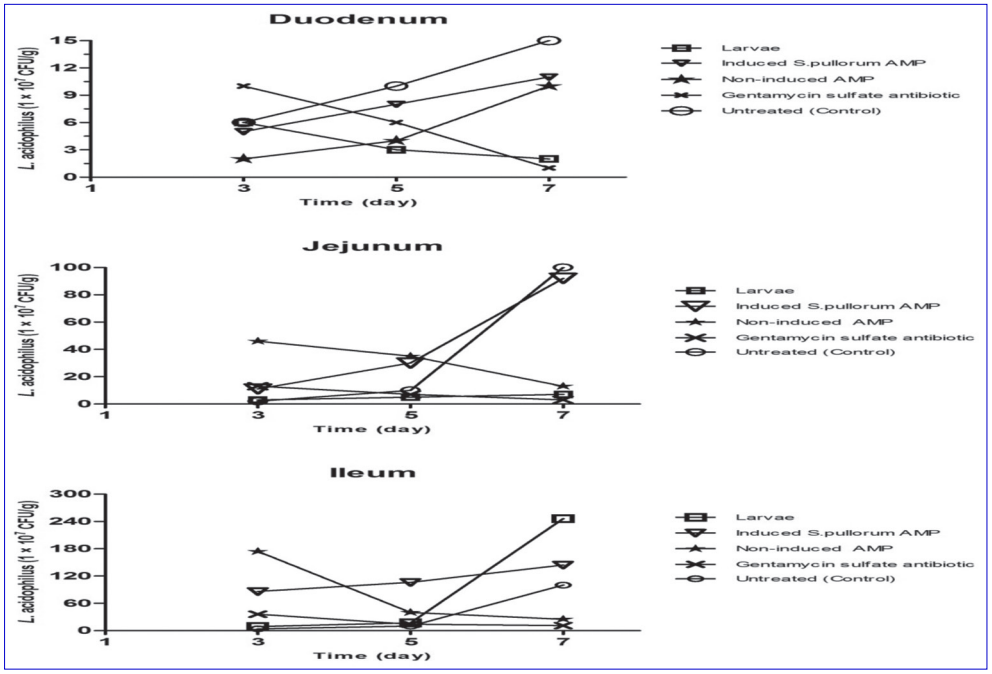

Fig 1. Intestinal *L. acidophilus* among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

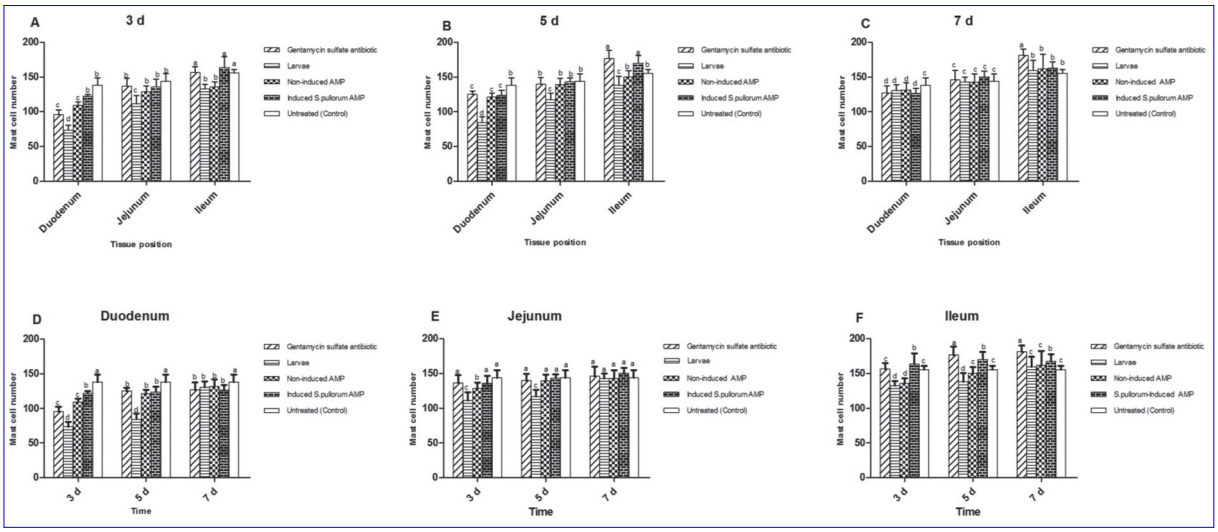

Fig 2. Number of intestinal mucosal mast cells among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

The non-induced AMP group had 3.33% fewer goblet cells than the induced-AMP group on d 3; however there was no significant difference between the two groups on d 5 and 7. The larvae group had the fewest goblet cells in the duodenum on d 3 and 5. There was no consistent pattern to the differences among the treatments in the jejunum and the ileum.

DISCUSSION

AMPs are small, biologically active molecular polypeptides produced by biological organisms after induction by pathogenic microorganisms [12]. AMPs have broad-spectrum antimicrobial activity against a vast variety of foreign pathogens including bacteria, fungi, and viruses. These pathogens are not inclined to develop resistance to AMPs [34]. In contrast, widespread antibiotic use in recent years has led to the development of antibiotic-resistant bacteria. Because of their potential for preventing and treating infections by drug-resistant bacteria, AMPs have received a great deal of research interest in recent years [35].

The activity of housefly AMPs against *S.* Pullorum was tested using the agar plate method *(Table 1)*. *S.* Pullorum-induced and non-induced AMPs both inhibited *S.* Pullorum. The inhibition zone diameter of *S.* Pullorum-induced AMPs was greater than that of non-induced AMPs and

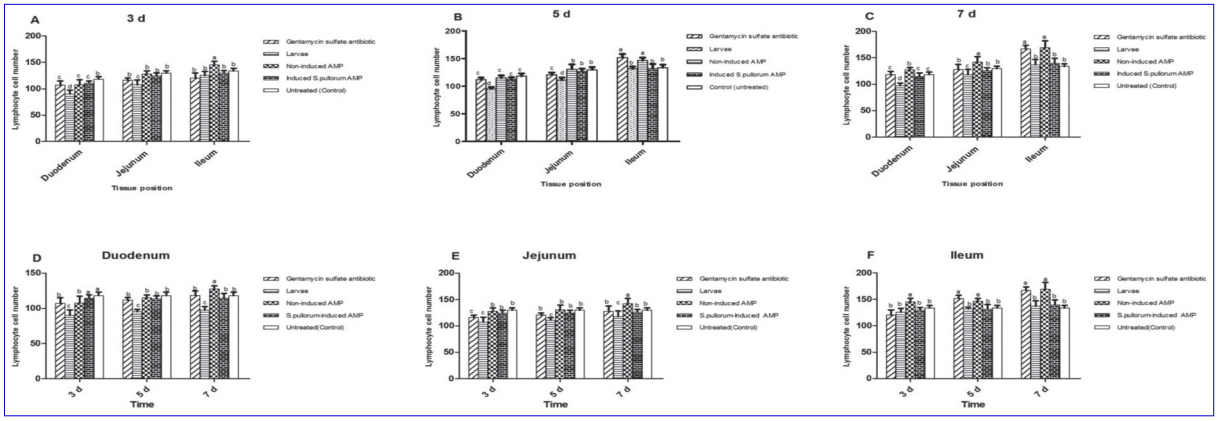

Fig 3. Number of intestinal lymphocyte cells among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

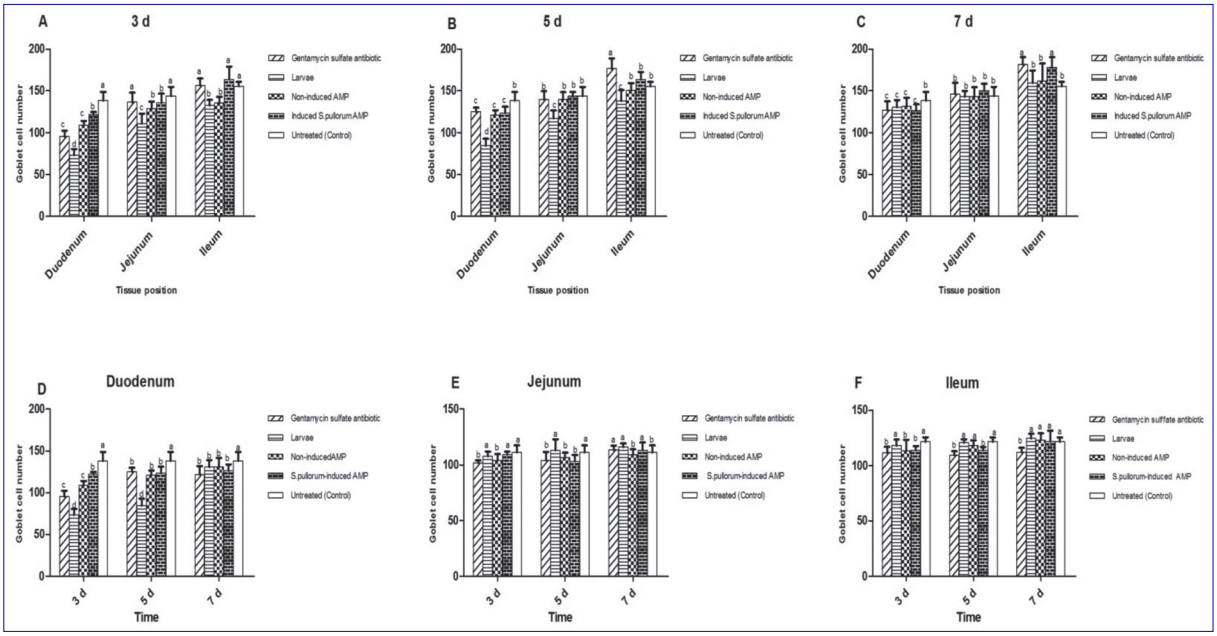

Fig 4. Number of intestinal goblet cells among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

close to that of gentamycin antibiotic. This indicated that *S.* Pullorum infection induces housefly larvae to produce AMPs with increased bioactivity against *S.* Pullorum. This agrees with a previous report that bacterial infection and injury induced AMP secretion in *Calliphora vicina* larvae [36].

L. acidophilus is an important intestinal bacteriumin healthy chickens [37]. This probiotic bacterium improves and adjusts the balance among intestinal microflora, thereby enhancing immunity, preventing infection, and preventing inflammation in small intestinal mucosa [38]. The present study showed that feeding chicks with *S.* Pullorum-induced AMP not only cured *S.* Pullorum-infected chickens but also had no significant effect on intestinal *L. acidophilus* populations. In contrast, the antibiotic, larvae, and non-induced AMP treatments reduced *L.acidophilus* in the small intestine, perhaps by damaging small intestinal mucosa. Additional study needs to be done to confirm this hypothesis.

The intestinal mucosal barrier includes both a mechanical barrier and an immunological barrier. Mucosal immune cells include mast cells, lymphocytes, and goblet cells. Mast cells originate from hematopoietic stem cells in bone marrow [39]. Mast cells can modulate the host's innate immune response for phagocytesis of Gram-negative bacteria. Mast cells may alter intestinal homeostasis and enhance intestinal permeability during parasite infections of the gastrointestinal tract [40]. In this test, AMPs, antibiotics, and larvae were administered orally to *S.* Pullorum-infected chickens and then changes in mast cell numbers were

observed across time. The results showed no significant difference in mast cell numbers between convalescent chicks after treatment with AMPs and healthy chicks ($P>0.05$). In contrast, the antibiotic and larvae treatments significantly reduced mast cells numbers in convalescent chicks ($P<0.01$). Overall, the results indicate that the AMPs had no significant effect on intestinal mucosal mast cells after treatment.

Lymphocyte cells protect intestinal mucosal immunity. Many autoimmune diseases as well as intestinal diseases are related to either declines in the number of lymphocyte cells or to their dysfunction [41-43]. In our study, the number of lymphocyte cells increased from the duodenum to ileum in all treatments on all sampling dates. Furthermore, the number of lymphocyte cells in the convalescent chicks increased from d 3 to 7. This indicated that *S.* Pullorum infection increased lymphocyte cell numbers in the small intestine, enhancing the immunity of the chicks. There was no significant difference in the number of lymphocyte cells between the induced-AMP group and the healthy group on d 7.

Goblet cells are glands which secrete glycoprotein. Goblet cells protect the intestinal epithelium and play an important role in the gut immunity of neonatal animals before passive immunization [44]. Goblet cells are sentinel cells which help to expel bacteria by stimulating mucus secretion from adjacent crypt cells [45]. In this study, the number of goblet cells increased from the duodenum to the ileum in all treatments. Furthermore, the number of goblet cells in each section of the small intestine increased slightly between d 3 and 7 in convalescent chicks. Goblet cell numbers were much less in convalescent chicks in the antibiotic and larvae groups than in healthy chicks. In contrast, goblet cell numbers in the AMP groups were similar to those in healthy chicks.

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