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Efficacy of vaccine from whole killed *Vibrio alginolyticus* cells on the immune response of white shrimp (*Litopenaeus vannamei*)

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Abstract

Vibrio alginolyticus causes high mortality in white shrimp, leading to significant worldwide losses to the aquaculture industries. Vaccine development has become a priority to prevent the spread of disease by activating the immune responses of aquatic organisms. This study aims to compare the efficacy of two types of whole killed *Vibrio alginolyticus* cells vaccines; formalin killed cells (FKC) and heat-killed cells (HKC), on the immune responses of white shrimp (*Litopenaeus vannamei*) via oral administration. The shrimp immunization for seven days, the vaccine provided with food twice per day. All shrimps challenged by injection with 0.1 mL of culture contain 1.5×10^6 CFU mL^{-1}; of *V. alginolyticus* and monitored for ten days. Total haemocytes count (THC) and the relative percent of survival (RPS) recorded. The shrimp immunized with HKC showed a significant increase in THC than shrimp immunized with FKC and the shrimp in control groups. In addition, the RPS values show significantly higher survival rates, 82.14% and 60.71%, in the immunized groups with HKC and FKC, respectively, compared to the control group 6.68%. This study found that the HKC vaccine offered a good immunity in shrimp against the infection of virulent *V. alginolyticus*.

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Introduction

Shrimp farming is one of the most crucial aquaculture products globally, with a global production of 4.88 million metric tons with an economic value of 39 billion USD (1). White shrimp (*Litopenaeus vannamei*) is the world's most cultured crustacean species, occupying 52.9% of crustacean aquaculture productions (2). The first introduction of *L. vannamei* to Asia was as a trail in the late 1970s. Still, it was only commercially cultured in Mainland China and Taiwan province of China at the beginning of 1996, followed by most Southeast Asian countries (3). Currently, *L. vannamei* is the most significant contributor to brackish water shrimp aquaculture in Malaysia (4). Like other invertebrates, shrimps depend on innate immunity as the primary defense mechanism against microbes (5). Hem lymph is the central part of shrimp nonspecific immunity. It contains different hemolytic types, such as hyalinocytes, granulocytes, and semi-granulocytes (6). Their function act as a part of acute immune response (7). It is responsible for phagocytosis (8). Likewise, semi-granulocytes have many small granules similar to those found in vertebrate granulocytes. These semi-granulocytes assist with phagocytosis and clotting (9). Furthermore, during the encapsulation process, semi-granulocytes release proteins that activate the identification of foreign microorganisms (10). Antimicrobial peptides (AMPs) are also another vital part of innate shrimp immunity. The shrimp granulocytes are secreted ubiquitously after stimulation of pathogens invasion (11). The AMPs form pores in the cell membranes of the microbes, causing instability of energy and ions and consequently bacterial death (12). Lysosomal enzymes participate in the degradation of the polysaccharide of the Gram-negative bacteria (13). Shrimps lack adaptive

immunity, relying only on nonspecific immunity to fight pathogens (14). This hypothesis viewed vaccination as an ineffective route in controlling shrimps' disease (15). Recently, invertebrates' immune systems have shown a simplified immunological memory (16). Shrimp exhibit a specific immune response via antibody-independent mechanisms, making shrimp immune resistance temporarily higher when injected by antigens (17,18). The alternative adaptive immunity of invertebrates depends mainly on immunoglobulin (19). The previous findings have led to several immunization studies to protect shrimps from viral and bacterial infections (15). *Vibrio alginolyticus* has long been used in shrimp hatcheries as a probiotic. It can be isolated from shrimp culture water and is associated to healthy larval and juvenile shrimp, according to reports (20). It's also detected in healthy *Penaeus monodon* and *L. vannamei* hepatopancreas intestines (21,22). The shrimp immune response is effectively boosted by *V. alginolyticus*. (23). Furthermore, *V. alginolyticus* isolated from the gastrointestinal system of adult shrimp *L. vannamei* was found to be antagonistic to shrimp pathogenic *V. parahaemolyticus* PS-017 (24). The global shrimp aquaculture industry has suffered severe losses from disease outbreaks caused by a unique Vibrio in recent years. From 2009 to 2018, shrimp diseases have been costed the Asian shrimp industry about 4 billion USD annually (2). Flegel's research team estimated that bacterial pathogens, especially *Vibrio* spp. caused 20% of shrimp production losses (25). Vibriosis is a disease caused by *Vibrio* spp, considered the most critical threat to the aquaculture industry in estuarine and coastal environments (26,27). Vibriosis is the most prevalent disease in shrimp cultures resulting high mortality rate that reaches 86% from the infected population and records to be more chronic under stress conditions (28-30). *V. alginolyticus* is one of the most common reasons for Vibriosis in fish and shellfish worldwide (31,32). Antibiotics are currently the most commonly used solution in the aquaculture industry to protect farmed aquatic animals from bacterial infection. Still, the long-term antibiotic application has several negative consequences, including antibiotic residues in food and the raising of Multiple Drug Resistance bacteria (MDR), making antibiotic using no longer effective in disease control (33). Since vaccination is one of the most effective strategies of disease prevention, there is a growing interest in creating cross-immunity vaccines against Vibriosis (34). Inactivated bacteria vaccines are the most suitable immunostimulants. A safe and effective route prevents diseases before they occur (35). Inactivated bacteria vaccines are widely acknowledged to induce the shrimp immune system (15). The most potential inactivated cell vaccines are formalin-killed cells FKC and heat-killed cells HKC. These types of immunomodulatory are constituted against Vibriosis (36). Researches improved shrimp immunity after immunization with inactivated Vibrio. Oral administration of formalin killed Vibrio to banana shrimp *Fenneropenaeus mergences* at post larvae stage protected shrimps from *V. anguillarum* and *V. harveyi* infections (37). Another study by Lin and colleagues proves that vaccination by HKC and FKC against *V. alginolyticus* can enhance early immune responses in white shrimp *L. vannamei* (38). Also, some studies confirmed the effectiveness of heat-killed cells HKC against Vibriosis during the early stages of white shrimp (39). The composition of dead vibrio cell walls such as vibrio bacterin, β-glucan, and peptidoglycan can induce shrimp nonspecific immune system (15). Studies on heat and formalin killed Vibrio showed higher hemolytic counts and immune parameters in immunized shrimp than non-vaccinated (40). Researchers also recorded higher phagocytosis rates in different vaccinated shrimps (17). One of the most acceptable explanations of shrimp responding to immunization is that the immune system can develop immunological memory to prevent new infection (41). Oral vaccination can reduce the stress and harmful passive immunization to the cultured aquatic animals (42). The oral administration is the most suitable antigen delivery method to immune-stimulate many shrimps at any stage (4). Oral vaccination is preferred due to its low cost since it reduces labor tasks and equipment associated with the injection route (43). Previous advantages make a solid incentive to further the development of oral vaccines in aquaculture. Vibriosis outbreaks have recently emerged as the leading source of losses in the intensive aquaculture industry (44).

However, there is no specific vaccination for *V. alginolyticus*, and commercial vaccines (against other Vibrio spp.) do not appear to be successful in preventing Vibriosis in local fish farms. As a result, the primary goals of this research are developing cheap and successful forms of killed cells vaccines required to improve the shrimp aquaculture industry and evaluating the efficiency of two vaccines of killed *V. alginolyticus* cells in enhancing the immune system of white shrimp against *V. alginolyticus* infection.

Materials and methods

Ethics approval

This project includes studies that use shrimp such as experimental animals and include injection experimental shrimp with pathogenic bacteria for which Prof. Dr. Laith Abdulrazzak, the director of this research, obtain ethical authorization from Uuniversity Malaysia Terengganu Research Ethics Committee (UMT REC), the number of approval for this experimentation research is UMT/JKEPHMK/2022/67.

Shrimp maintenance

The research carries out at the University Malaysia Terengganu (UMT) at Fish Diseases Laboratory, Faculty of Fisheries and Food Science. Healthy juvenile shrimp, *L. vannamei* (n = 370) weight at 155±10.0 g collected from a shrimp farm in Balok, Pahang. The shrimp acclimatize in the

lab for ten days before the experiment beginning, every 30 shrimps housed in 60 L aerated aquariums. The seawater temperatures ranged 27-29°C, dissolved oxygen of 5.33-6.55 mg/L^{-1} and a pH at 7.8 to 8.0. The shrimps were fed a commercial diet at 3% of body weight twice per day. The rearing water replaces every two days, water quality parameters checked daily.

Preparation of pathogen culture

Bacterial strain *V. alginolyticus* (GenBank accession no. MH879822.1) was previously isolated from *P. viridis* maintained and stored in Tryptic soy broth (supplemented with 2% NaCl (w/v), diluted to 15% glycerol final volume and stored at -80°C until use. The bacteria were thawed at 30°C using a heating block and streaked for 24 h on thiosulfate citrate bile salt sucrose agar, the viable counts of the *V. alginolyticus* cultures were estimated by a standard plate count method. The bacterial culture was washed three times with sterile saline and then resuspended in saline at the final concentration of 1.9×10^7 CFU mL^{-1} (27). Morphology of *V. alginolyticus* identified by Gram staining of bacterial cells from the green appearance colonies on the TCBS agar.

Preparation of formalin-killed *V. alginolyticus* vaccine

The formalin killed bacterial vaccine prepared according to Cao *et al.* (32). Briefly *V. alginolyticus* was inoculated in 500 ml of tryptic soy broth supplemented with 2% NaCl at 30°C for 24 hr. The Bacterial suspensions of *V. alginolyticus* 1.9×10^7 CFU mL^{-1} were centrifuged at 6000 rpm for 30 minutes at 4°C, bacterial pellets were washed twice with saline and centrifuged again to remove the remaining culture medium, pellets were resuspended in standard saline to achieve bacterial concentration at OD_{600} of 0.8 using a spectrophotometer. The formalin-killed whole-cell vaccine FKC prepared by adding formalin to the bacterial suspension achieving a final concentration of 0.5% (v/v) and then incubated overnight at 4°C, bacterial cells were collected by centrifugation at 6000 rpm for 30 min at 4°C and washed three times with sterile normal saline and centrifuged again to remove formalin residues.

Preparation of heat-killed *V. alginolyticus* vaccine

Heat-killed whole-cell vaccine HKC was prepared relying on Lin and his team procedure (38). The bacterial suspensions of *V. alginolyticus* was centrifuged at 6000 rpm for 30 minutes at 4°C, then bacterial pellets were washed twice with saline and centrifuged again to remove the remaining culture medium; pellets were resuspended in standard saline to achieve bacterial concentration 0.8 using a spectrophotometer at OD_{600}. The pellets suspension was incubated in a water bath at 65°C for 3 h to harvest bacterial pellets, and suspensions were centrifuged at 6000 rpm for 30 minutes at 4°C. Inactivation efficacy of FKC and HKC was determined by plating 100 µl of the above bacterial suspension on to tryptic soy agar supplemented with 2% NaCl at 37°C, presence of bacterial colonies was monitored for 3 days, finally prepared vaccines kept at 4°C until the further step.

Safety assessment

To evaluate the safety of the vaccine on shrimp's health, shrimps divided into two groups (n=10), individuals of the first group injected with 0.1 ml of 10^7 CFU ml^{-1} FKC, the other group injected with the same concentration of HKC, both groups were monitored for ten days to record any abnormality.

Determination of median lethal dose (LD_{50})

The LD_{50} value was determined to obtain a bacterial dose of *V. alginolyticus* that causes 50% mortality of the *L. vannamei* population. Shrimps was divided into 10 groups in 30 L aquariums (n=10) and supplied with adequate aeration, groups from 1 to 9 challenged by (IM) injection with 0.1ml of *V. alginolyticus* suspensions 1.5×10^1, 1.5×10^2, 1.5×10^3, 1.5×10^4, 1.5×10^5, 1.5×10^6, 1.5×10^7, 1.5×10^8, and 1.5×10^9 CFU mL^{-1} respectively, group number 10 served as control and injected with 0.1 ml of sterile phosphate-buffered saline, the mortality of shrimps was recorded every 24 h for five days, dead shrimps were removed from the aquarium every day to avoid water deterioration (27).

Food vaccine diet preparation

The oral vaccine of the FKC and HKC was prepared as feed top dressing (36). Briefly, the FKC pellets were diluted in sterile normal saline to obtain the final concentration of 10^{10} CFU kg^{-1} food. The suspension was mixed with 0.1% guar gum as a binder and applied uniformly on the shrimp commercial food 45%, the same technique performed with HKC while the commercial food without additions used for the control group. These vaccine formulations were kept at 4°C until they were utilised.

Experimental design

Shrimps divide into three groups (n=90). All groups feed for seven days with the following diets, the first group (G1) fed with commercial food mixed with FKC, the second group (G2) fed with commercial food mixed with HKC, and the third group (G3) provided with commercial food as a control. After seven days of feeding, shrimps were injected with 0.1 mL of *V. alginolyticus* suspension 1.5×10^6 CFU mL^{-1}. Total hemolytic count THC tested on the first, third, fifth, seventh, and ninth-day post-challenge (Figure 1). The mortality was monitored every 24 h for ten days, and dead shrimp were removed from the aquarium every day.

Total haemocytes count

Three Shrimps were selected randomly from each group. Approximately 300 µL of haemolymph drawn from the third walking leg via 1 ml syringe contains ice-cold anticoagulant solution (AS, glucose 20.5 g L^{-1}, sodium citrate 8 g L^{-1},

sodium chloride 4.2 g L^{-1}, pH 7.5). The THC technique proceed according (45). 10 µl trypan blue stain and 1 µl of rose bengal stain added to 10µl of hem lymph in eppendorf tube. The tube was held on ice to avoid coagulation, and then the mixture was removed to Neubauer hemocytometer to examine THC under the light microscope (46).

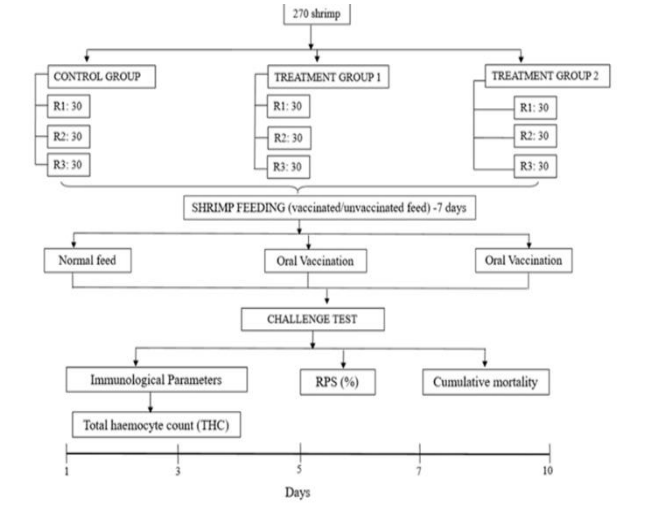

Figure 1: Flow chart diagram of the experimental designs.

Relative percent of survival

The survival rates and the protection of shrimps immunized with HKC and FKC vaccines are expressed as a relative percentage of survival (RPS). Shrimp mortality for all groups was recorded for ten days post-challenge test. The RPS determined the following formula RPS= (1-(% mortality/% control mortality)×100 (47).

Statistical analysis

Analysis of variance by one-way ANOVA with Duncan test using SPSS Statistics software used to analyse results. The variation of all data presents as mean, standard deviation (mean ± S.D.) from 3 replicates for each experiment, and the significance level determines $P<0.05$.

Results

Safety assessment

Intraperitoneal injection in shrimp was used to test the safety of both vaccination formulations. During the 10 days following the vaccination, all of the injected shrimp survived, and no aberrant behavior was observed.

Median lethal dose (LD_{50})

The lowest dose of live *V. alginolyticus* that cause 50% cumulative mortality of white shrimp after five days post challenging was 1.5×10^6 CFU mL^{-1}; this concentration adopts as a lethal dose (LD_{50}) for the next experimental step.

Relative percent of survival

The cumulative mortality rates after ten days post-challenge in control, FKC, and HKC treated groups were 92.32%, 36.67%, and 16.67%, respectively. Control group deaths stopped after seven days post-challenge, while mortality of treated shrimps with HKC and FKC vaccines were prevented from the fifth- and sixth-days post-challenge, respectively (Figure 2). Significant mortality rates differ between the control group, HKC and FKC immune stimulated groups.

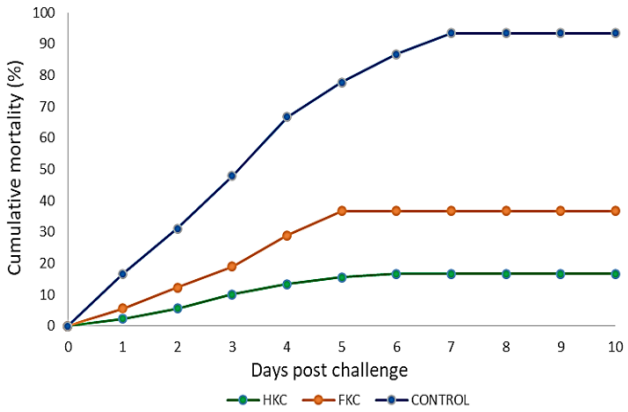

Figure 2: Cumulative mortality of control and vaccinated groups of white shrimps challenged with *V. alginolyticus* for FKC (formalin killed cells vaccine) and HKC (heat killed cells vaccine) treated groups.

The Relative Percent of Survival was documented as an alternative way to display vaccine efficacy (Figure 3). RPS was only 6.68% in the control group. Meanwhile, treated groups show significantly higher survival rates, 82.14%, and 60.71%, for groups vaccinated with HKC and FKC, respectively.

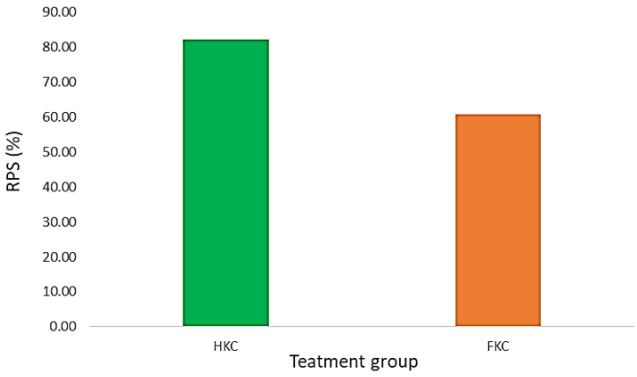

Figure 3: RPS of FKC (formalin killed cells vaccine) and HKC (heat killed cells vaccine) treated groups of white shrimps challenged with *V. alginolyticus.*

Total haemocyte count

The total haemocytes count was obtained after one-day post-challenge and observed every two days. The rates of THC increased gradually from the first-day post-challenge and reached the highest levels at the seventh-day post-challenge before declining, recorded at the ninth-day post-challenge (Figure 4). In the first sample (1-day post-challenge), there are slight variations in THC values among representatives of all three groups. THC elevated significantly for the three groups in the second samples (3 days post-challenge). Still, the highest count was for HKC treated group, followed by FKC and control groups. THC values continue to rise in the fifth- and seventh days post-challenge recording the highest rates before dropping for all groups on the ninth day. The error bar that indicates the standard error in the control and the HKC group shows that there is no error bar were no vast differences among haemolymph collected samples. At the same time, representatives from the HKC group have the highest value of the standard error.

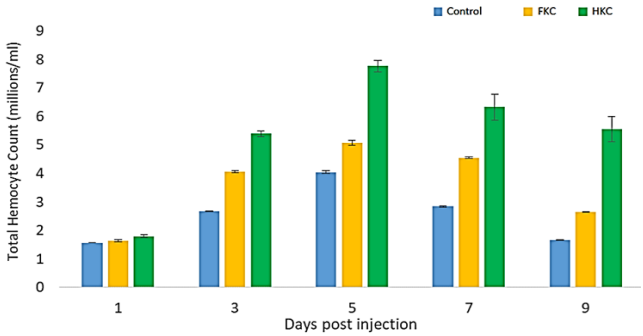

Figure 4: THC, FKC and HKC groups of white shrimps challenged with *V. alginolyticus*, FKC and HKC treated groups.

Discussion

Our findings are in accordance with previous research on Asian tiger shrimp *P. monodon* vaccinated with whole-cell Vibrio harveyi vaccine HKC and FKC which showed a high protection rate, 81.6, and 80.2%, respectively, in contrast to the control group that exhibits only 35.6% protection after challenging with *V. harveyi* (48). Similar to the recent findings on the survival rate of white shrimp *L. vannamei*, given a combination of HKC and FKC Vibrio bacterial cells, then challenged with Vibriosis infection five weeks later, was considerably more significant than that of shrimp given marine saline (38). Administered a Vibrio bacterin as feed top-dressing at 10^8 CFU/kg feed to the shrimp (37,38). Other experiments were carried out by Roy *et al.* (49) on *P. monodon* post larvae shrimp immunized with formalin-killed *V. anguillarum* cells vaccine which administered through feed top-dressing too.

Although oral vaccination was not as successful as injection in protecting white shrimp *L. vannamei* against pathogenic *V. alginolyticus* (50), our data show that an oral immunization practice might be an excellent way to protect shrimp from Vibriosis, particularly in agricultural situations, according to the fact of rarely the sickness is as severe as the experimental challenge (51). Previous research suggests that the vaccine was degraded in the digestive tract before being absorbed by enterocytes. As a result, reduced levels of antigen reaching the immune system of animals may be the fundamental explanation for oral vaccination's poorer efficiency (50). In the current investigation, a substantial dose of bacterin 10^{10} CFU/Kg diet was utilized in oral vaccination; consequently, even after degradation in the digestive tract, enough antigens will reach the hindgut (52).

The total hemolytic count in vaccinated shrimp was considerably higher than in uninfected shrimp, which is consistent with earlier findings (37-49). The hemlymph hemolytic reflects the strength of the immune response of white shrimp against *V. alginolyticus*. These cells' primary function is to recognize foreign cells and eventually bind, engulf and eliminate pathogens by phagocytosis (53,54). We reasoned those differences in activity between our results and those of other studies to the different bacterial cell inactivation and administration routes. Bacteria binding, serving as a putative antigen receptor, and binding with Haemocytes aid the shrimp immune system in bacterial clearance via phagocytosis (55). From the present study data, HKC is more immunogenic for white shrimp than FKC, similar to the previous result on killed bacteria vaccine which found a higher immune response of heat-killed cells *Aeromonas hydrophila* vaccine comparing with formalin killed cells vaccine (56). The superior efficacy of HKC could be attributed to the higher antigenic content of this type of vaccine. This could be due to the cell wall of *V. alginolyticus*, have heat-stable molecules that can resist the strict conditions used in heat-killed vaccine preparation (57). FKC on the other hand, has a lower antigenicity due to the cross-linking capabilities of formalin, which resulted in lower antigenicity in formalin-based vaccines (58). As a result, there is little discrepancy in identifying two vaccines, resulting in a variable in immunological response. As a result, entire cells of *V. alginolyticus* have a high potential for use as a biocontrol agent in shrimp aquaculture (59,60). Furthermore, studies are needed to distinguish the efficacy of booster, condition of vaccination, administration route, and the period of immune memory that white shrimp obtained against Vibriosis.

Conclusion

In conclusion, these data demonstrate that oral immunization with heat-killed *V. alginolyticus* vaccine induced a proactive response characterized by increased survival rate and immune responses against pathogenic *V.*

alginolyticus. However, more large-scale field experiments are needed to thoroughly assess the efficiency of the *V. alginolyticus* vaccine.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication and or funding of this manuscript

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فعالية اللقاح المأخوذ من خلايا ضمة نظيرة حالة للدم المقتولة بالكامل في الاستجابة المناعية للروبيان الأبيض

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الخلاصة

تسبب ضمة نظيرة حالة للدم معدلات نفوق عالية في الروبيان الأبيض، مما يؤدي إلى خسائر كبيرة لصناعات الاستزراع المائي في جميع أنحاء العالم، ولذلك أصبح تطوير اللقاحات أولوية لمنع انتشار المرض عن طريق تنشيط الاستجابات المناعية للكائنات المائية. تهدف هذه الدراسة إلى مقارنة فعالية نوعين من لقاحات خلايا الضمة نظيرة جالة للدم، الأول للخلايا المقتولة بالفورمالين والأخر لخلايا مقتولة بالحرارة في تمنيع الروبيان لمدة سبعة أيام والذي جرع فمويا مرتين في اليوم. في هذا البحث تم حقن الجمبري بمقدار ٠,١ مل من المحلول بتركيز ١,٥*١٠^٦ الوحدات المولدة للمستعمرة/ مليلتر من خلايا الضمة نظيرة الحالة للدم ومتابعتها لمدة عشرة أيام، تم خلالها تسجيل إجمالي عدد خلايا الدم والنسبة المئوية النسبية للبقاء على قيد الحياة. الروبيان الذي تم تحصينه بالخلايا المقتولة بالفورمالين اظهر زيادة معنوية عد خلايا الدم من الروبيان المحصن باللقاح المقتول بالفورمالين والروبيان في مجموعات المراقبة. بالإضافة إلى ذلك، فإن الدراسة تظهر قيم معدلات البقاء أعلى بشكل ملحوظ، ٦٠,٧ و ٨٢,١٤% في المجموعات المحصنة مع اللقاح المقتول بالفورمالين والنسبة المئوية للبقاء، على التوالي، مقارنة بمجموعة السيطرة ٦,٦٨%. هذه الدراسة استخلصت أن اللقاح المقتول بالفورمالين اعطى مناعة جيدة في الروبيان الأبيض ضد العدوى الخبيثة بالضمة نظيرة الحالة للدم.