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Probiotic bacteria isolated from saline tilapia green water culture system inhibit gut colonization and prevent infection of *Aeromonas hydrophila* in the juvenile Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Aeromonas hydrophila is a major pathogen associated with serious production losses in the culture of *Oreochromis niloticus*. The present study was conducted to find potential probiotics from saline tilapia green water that could prevent *A. hydrophila* infection in juvenile tilapia. Several presumptive bacterial probiotics were isolated from saline tilapia green water and tested for inhibitory activities against *A. hydrophila*. Bacterial isolate exhibiting inhibitory activity against *A. hydrophila* was further tested for pathogenicity against the host fish. Gut colonization activity and optimum probiotic dose to inhibit *A. hydrophila* colonization in tilapia gut were conducted. Infection challenge test was addressed to evaluate the protective effects of the isolated probiotics against *A. hydrophila* infection in juvenile tilapia. Results showed that among the presumptive probiotic isolates, *Staphylococcus aureus* showed the highest zone of inhibition and was used in the experiments. No mortalities or disease signs were found in juvenile tilapia after a 30-day challenge test with the isolated *S. aureus*. Dietary supplementation with a probiotic dose of 10^6 CFU/ml was found optimal for gut colonization and inhibition of *A. hydrophila* colonization. The infection challenge test indicates that fish receiving 10^6 CFU/ml *S. aureus* supplemented diets were protected against pathogenic *A. hydrophila* infection. Collectively, the application of 10^6 CFU/ml *S. aureus* as a probiotic is a practical approach to prevent *A. hydrophila* infection in tilapia aquaculture.

INTRODUCTION

Aquaculture of the tilapia fish is an important economic activity in the Philippines and other tropical countries worldwide. It is the second cultured fish next to the carp species. In 2018, tilapia contributed 4,525,400 metric tons (MT), with an estimated percentage of 8.3 of the total species cultured in the world **(FAO, 2020)**. Tilapia species is preferable in aquaculture since it is low in the trophic level and could efficiently utilize detritus and plankton as energy sources for metabolism and growth. Additionally, this

fish is sturdy, grows fast, and has a short life cycle, and its technology for culture is low-level, simple and managed with low cost.

Despite the high production of the tilapia, the expansion in production and sustainability is limited to the occurrence of diseases. Pathogens that affect the tilapia aquaculture industry include fungi **(Ramaiah, 2006)**, parasites **(Brooker *et al*., 2007)**, bacteria **(Frans *et al*., 2011)** and viruses **(Ransangan *et al*., 2011)**. Among these pathogens, bacteria have been linked to major losses in the tilapia aquaculture for these pathogens could exist in the aquatic environment even without the host. Among the bacterial pathogens of the tilapia, the aeromonad species, *Aeromonas hydrophila* causes serious disease for the tilapia, viz. motile aeromonas septicemia (hemorrhagic septicemia), red sore disease and ulcerative infections **(Pakingking *et al*., 2020)**. Globally, *A. hydrophila* is the pathogen commonly associated with mortalities in cultured tilapia, resulting in significant economic losses **(Janda & Abott, 2010; Pakingking *et al*., 2020)**.

Antibiotics have been used to control the aeromonad diseases in tilapia aquaculture in the past decades. However, the high antibiotic cost, its limited applicability in large-scale production systems, the negative environmental impacts, and the development of antibacterial resistance associated with the use of antibiotics have led to a global consensus to limit the use of antibiotics in aquaculture **(Ko *et al*., 2005; Manage, 2018)**. To address these issues, attempts were made to develop and identify antibiotic alternatives for aquaculture applications. At present, vaccine and immunostimulants are the emerging potential strategies to prevent *Aeromonas* infections in tilapia, but the manner and the cost of application to a large-scale tilapia production system limits the applicability of these technologies.

Another alternative approach to the problem is the use of probiotics. Probiotic use has been proposed as an efficient way to manage bacterial infections in farmed aquatic animals. Currently, probiotics have become widely used to prevent pathogenic bacteria proliferation in the livestock, poultry and shrimp aquaculture industry **(Khuntia & Chaudhary, 2002; Chauhan and Singh, 2018)**. In aquaculture, probiotics bacteria have been commonly isolated from aquatic ecosystems and aquaculture production systems including the saline tilapia green water culture system (TGW) **(Lio-po *et al*., 2005)**. Tilapia green water is a term referring to the culture water wherein saline tolerant tilapia is reared at a salinity of 15-35 ppt. This culture system supports the proliferation of green microalgae including *Chlorella* spp. and *Nanochloropsis* spp.. It is a mature microbial ecosystem inhibiting and controlling the growth of pathogenic *Vibrio* species **(Lio-po *et al*., 2005; Wibowo *et al*., 2015; Sampollo *et al*., 2018)**. The pathogenic bacteria inhibitory activity of the TGW system has been linked to the presence of inhibitory substances secreted by bacteria, fungi, and algae present in this culture system.

The pathogenic *Vibrio* species inhibited by microbes in TGW shares and exhibits similar characteristics of *A. hydrophila*, a known pathogen of cultured freshwater fish. Nowadays, no reports were issued on the isolation and development of bacterial probiotics from tilapia green water that are effective against pathogens, specifically *A. hydrophila* of tilapia in freshwater culture system. The present study was conducted to isolate from the tilapia green waters species of bacteria with probiotics potential against the *A. hydrophila* pathogen of *Oreochromis niloticus* in freshwater culture system.

MATERIALS AND METHODS

1. Bacteria

Bacteria Isolates

For the current experiment, nine bacteria isolates were obtained from the aquaculture probiotic collection of the Institute of Aquaculture University of the Philippines Visayas, Miagao, Iloilo, Philippines. They were isolated from the saline tilapia green water culture system and were previously documented to be inhibitory to most *Vibrio* shrimp pathogens.

Antimicrobial properties of the presumptive probiotics from the tilapia green water were tested against *A. hydrophila*. Stock culture of *A. hydrophila* BIOTECH 10089, isolated from infected fish was obtained from the National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, College, Laguna 4031, Philippines and was used for the antagonistic assay. The bacterium was stored in a nutrient agar slant and refrigerated at 4ºC until further use.

Evaluation of anti-bacterial effects of presumptive probiotics

The experiment was conducted using the spot on the lawn method based on the method of **Cadirci and Citak (2005)** with some modifications. One hundred µl of the *A. hydrophila* cultured for 24h was spread in Luria-Bertani medium agar (LB agar). Twenty-four-hour culture of each probiotic isolates were then spotted on the surface of the pathogen lawn, and the test plate media was incubated for 24h at room temperature. Zone of inhibition was observed and was measured to determine antimicrobial property of the isolates against *A. hydrophila*. The best performing isolate exhibiting the largest zone of inhibition against *A. hydrophila* was identified, selected, and further analyzed for its gut colonization activity.

2. Tilapia subjected to probiotic pathogenicity test

To test the pathogenicity of the isolated probiotic on the host fish, three hundred juvenile tilapias (*O. niloticus*) were obtained from the Freshwater Aquaculture Station (FAS) of the Institute of Aquaculture CFOS, UPV. The fish were transported and acclimatized in laboratory culture conditions for seven days and were fed with

commercial diets. Water change was performed every two days, and constant aeration was provided throughout the acclimation period.

Before the experiment, ten fish with a mean bodyweight of 0.9 ± 0.2 g and total length of 38.87 ± 4.69 mm were randomly selected and subjected to bacterial and parasitic examinations and were found negative for bacterial and parasitic infections.

The best presumptive probiotic isolate determined from the previous experiment, based on their capacity to lyse the target pathogen exhibited as inhibitory zones, was cultured in tryptic soy agar (TSA) for 24h. The culture was then harvested and serially diluted to attain the concentrations of 10^3, 10^6 and 10^9 cfu/mL. Commercial feed was used as the basal diet for oral administration of the test probiotic. The probiotic supplemented diets were prepared by gently spraying bacterial suspensions containing 10^3, 10^6, and 10^9 cfu/mL to commercial feed and mixed thoroughly to achieve a dose of 10^3, 10^6 and 10^9 cfu/g. The experimental diets were air-dried and were labeled accordingly.

The experimental fish used in the test were divided into four experimental groups, of which three groups were administrated with commercial feed containing 10^3, 10^6, and 10^9 cfu/g probiotics, respectively, and the remaining group without bacterial supplementation served as the control. Each group had three replicates, and each replicate had 20 experimental fish. The fish in each replicate were maintained in 30L plastic containers containing 28L of dechlorinated freshwater. Aeration was provided throughout the experiment, and 50% of water was daily changed.

Pathogenicity of the test probiotic on *O. niloticus* was conducted by feeding the experimental fish with *S. aureus* supplemented diets at concentrations of 10^3, 10^6, and 10^9 cfu/g given *ad libitum* twice a day for 30 days. The control group was fed with the experimental diet sprayed with saline solution instead of the probiotics. Regular water changes and optimum water parameters were maintained throughout the experimental period. Fish were monitored daily to detect any adverse clinical signs and mortalities.

To evaluate the gut colonization activity of the isolated probiotics, three randomly selected fish were sacrificed every three days from each treatment until day nine. The gut of the sacrificed fish was homogenized in a microcentrifuge tube with 1.0 mL normal saline solution (NSS) as diluent. The homogenized solution was serially diluted to ten-fold dilutions and spread plated on nutrient agar according to the treatments. All the plates were incubated at room temperature. After 24h, the colonies were counted and recorded. The presence of the test probiotics in the gut was quantified by colony replica plating done in the lawn of *Aeromonas hydrophila* in solid media on days zero, three and six. The number of colonies exhibiting a zone of inhibition and resembling the colony morphology of the probiotics was quantified.

3. Evaluation of the protective effects of the selected probiotics against Aeromonas hydrophila infection

Four hundred pieces of tilapia (*O. niloticus*) were obtained from the Freshwater Aquaculture Station (FAS) of the Institute of Aquaculture. The specimens were transported and acclimatized in the laboratory culture conditions for seven days and were fed with commercial diets. Water change was conducted every two days, and constant aeration was provided throughout the acclimation period.

Before the experiment, ten fish with a mean bodyweight of 2.03 ± 0.73g and a total length of 48.83 ± 12.12 mm were randomly selected and subjected to bacterial and parasite examinations and were found negative for these pathogens.

Prior to the infection challenge test, *A. hydrophila* pathogen was activated to increase its virulence. In brief, a 10^{10} CFU/mL concentration of an overnight culture of *A. hydrophila* was prepared and introduced to the experimental animal by intraperitoneal injection. All moribund fish were sacrificed for the isolation of *A. hydrophila* from the kidneys, liver, or infected areas and plated on glutamate sucrose phenol red (GSP) agar. Highly virulent *A. hydrophila* recovered from GSP media was purified and re-isolated on nutrient agar and was used as the infective pathogen on the subsequent pathogenicity challenge tests. This was done 3 times to ensure that the pathogen virulence was activated.

Lethal dose determination of A. hydrophila, LD_{50} determination

Pathogenic *A. hydrophila* from the passage was evaluated to determine the LD_{50} of this bacterium to juvenile tilapia. The test was run using 12 plastic containers with a volume of 2 L. Each of the experimental containers contained 10 experimental fish, comprising the experiment run in five treatments with three replicates including the control. The experimental treatments were the bacterial dose of *A. hydrophila* at 10^6, 10^7, 10^8, 10^9 cfu/mL and a negative control that was used to infect the juvenile tilapia. Ten tilapia juveniles were placed on each container for an hour and were transferred into new 30-liter plastic containers with clean water and aeration. The experimental animals were under observation for 14 days. All moribund fish were recorded and evaluated for the presence of *A. hydrophila* by rubbing the kidneys, liver, or infected areas with an inoculating loop and plating it on GSP agar. Yellow colonies formed in the GSP agar indicated the presence of *A. hydrophila*. Mortalities were recorded. The LD_{50} was computed using the Probit Analysis following **Lieberman's (1983)** method.

Efficacy of the Probiotic to inhibit *A. hydrophila* infection in tilapia

To test the efficacy of the isolated probiotic to protect the juvenile tilapia against *A. hydrophila* infection, a trial was conducted using two hundred tilapia juveniles (*O. niloticus*), obtained from the Freshwater Aquaculture Station (FAS) of the Institute of Aquaculture. The fish were transported and acclimatized in the laboratory culture conditions for seven days and were fed with commercial diets. Water change was conducted every two days, and constant aeration was provided throughout the

acclimation period. Prior to the experiment, ten fish with a mean bodyweight of 1.34 ± 0.42 g and a total length of 47.67 ± 5.35 mm were randomly selected and subjected to bacterial and parasite examinations and were found negative for bacterial and parasitic infections. The experimental animals were divided into four experimental groups. Each experimental group had 3 replicates containing 10 experimental animals.

The experimental treatments included 4 groups as follows: the group that received the probiotic supplemented diet at 10^6 CFU/g diet and exposed to the pathogen (TPA), the group given probiotic supplemented diet but was not exposed to the pathogen (TP), group that was exposed only to the pathogen without the probiotic application (TA), and the control group that was neither exposed to the pathogen nor applied to the probiotic (TC). The probiotic dose in this test was based on the probiotic concentration that promotes efficient gut colonization as previously determined.

The feeding trial with probiotics lasted for a week, after which the animals at treatment groups of TA and TPA were exposed to a pathogenic dose of *A. hydrophila*, 10^7 CFU/mL, based on LD_{50} pathogenicity test. The animals were exposed to the pathogen for one hour, and were then transferred into a new rearing tank with clean and sterile water. The experimental animals were observed for 30 days and dead and moribund fish were recorded and collected daily. At the end of the experiment, the presence of *A. hydrophila* was evaluated in the moribund animals to satisfy the Koch postulate.

4. **Statistical analysis**

Data were analyzed by one-way analysis of variance (ANOVA). Tukey's tests as post-hoc were used to determine the significant differences among the different treatment means at $\alpha = 0.05$. All the statistical analyses were carried out using statistical analysis program 20 (SPSS).

RESULTS

1. Anti-bacterial effects of presumptive probiotics

The assessment of the antimicrobial property of the presumptive probiotics from tilapia green water indicated that, out of the nine isolates only two isolates showed positive antagonistic effects against *A. hydrophila.* Out of the two isolates, *S. aureus* (TG2) showed the biggest inhibitory zone against *A. hydrophila*. This isolate was selected and further used in the subsequent experiments (Fig. 1 & Table 1). The selected isolate was identified using the Biolog Bacterial Identification System (California, USA).

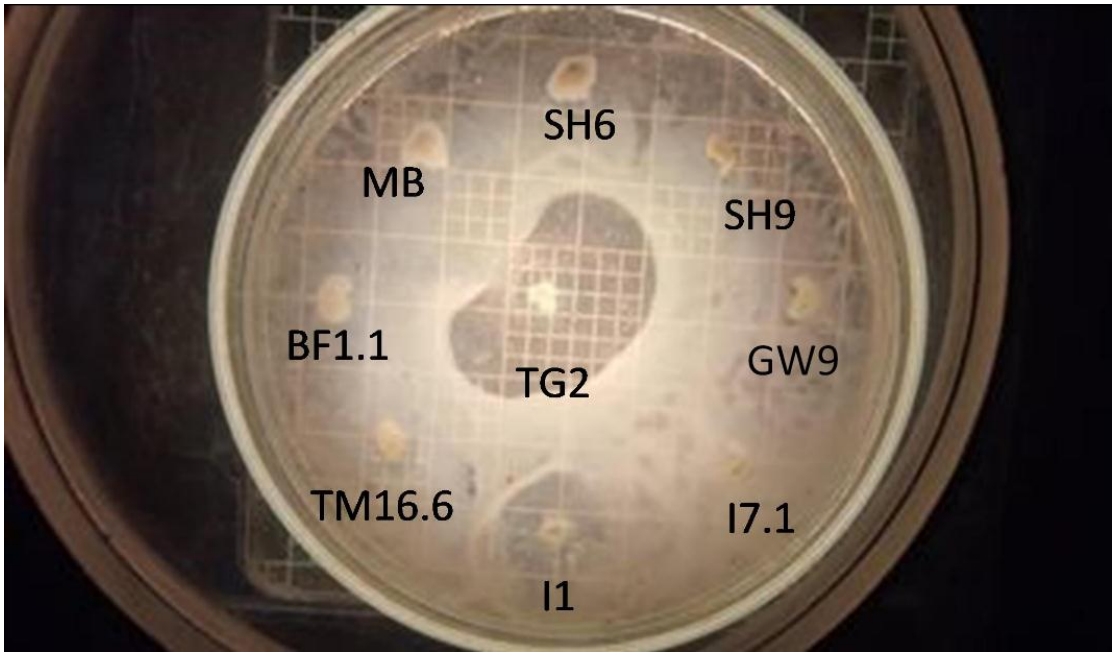

Fig. 1. Zone of inhibition of probiotics isolated from saline tilapia green water on *Aeromonas hydrophila* lawn

Table 1. Zone of inhibition of probiotics isolated from saline tilapia green water against *Aeromonas hydrophila* lawn

Isolate	**Zone of Inhibition (mm)**			**Average**
TM6.16	0	0	0	0
SH6	0	0	0	0
I7.1	0	0	0	0
SH9	0	0	0	0
BF1.1	0	0	0	0
GW9	0	0	0	0
MB	0	0	0	0
I1	10	9	11	10*
TG2 (*Staphylococcus aureus*)	33	31	33	32.33**

2. Assessment of *S. aureus* as a potential probiotic to juvenile tilapia

Probiotic Pathogenicity Test for *O. niloticus*

The isolated probiotic was further tested to assess its pathogenicity on juvenile *O. niloticus.* Results showed that after 30 days of challenge, disease signs and mortality were not observed in the experimental, indicating that the isolate is not pathogenic and is safe to use as a probiotic for the tilapia.

Total Gut Bacteria

Before the experiment, the initial count of total gut bacterial count of fish in all the treatment groups was found similar. However after feeding, the fish with commercial diets supplemented with different concentrations of the test probiotic, differences in bacterial gut count were significant starting with day three until day nine of culture. Regardless of the sampling period, the bacterial gut count followed a dose-response pattern that was increasing with increasing the probiotic dose applied. In every sampling period, the control group exhibited the lowest total bacterial gut count, while the 10^9 CFU/mL treatment group exhibited the highest bacterial count in all sampling dates. The 10^6 CFU/mL treatment group was significantly higher than the control treatment group and the group that received the lowest probiotic concentration at 10^3cfu/g feed. In addition, 10^6 treatment groups exhibited a peak of bacterial gut colonization on day six that remained until day nine. This gut bacterial count was similar to that of the group that received the highest probiotic dose on day nine (10^9 cfu/g feed) (Fig. 2).

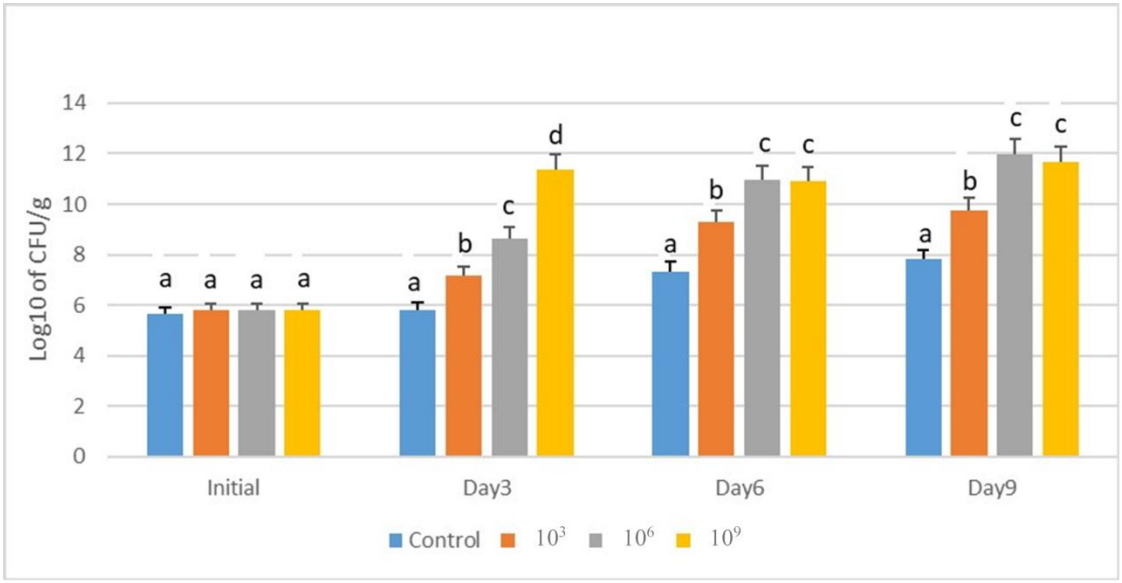

Fig. 2. Changes in the microbial gut count of juvenile tilapia fed with different concentrations of probiotic *Staphylococcus aureus*.
Bars bearing similar letter superscripts are not significantly different.

A. hydrophila-inhibiting bacteria found in the gut of probiotic fed fish

Results on the gut colonizing activity of the test probiotics, *S. aureus* are presented in Fig. (3). Initial tests showed that *A. hydrophila* inhibiting bacteria were not present in the gut of the experimental fish. Results showed that on day three of the experiment, treatment with 10^9 cfu/g showed the highest number of gut bacteria inhibiting *A. hydrophila* growth. Treatments 10^6, 10^3 and the control followed a decreasing trend. All

treatments showed significant differences in terms of the gut probiotic counts on day three. On day six, treatment 10^6 exhibited the numerically highest gut probiotic bacterial content, compared to all the other treatments. It was then followed by treatments 10^9, 10^3, and the control with zero counts of the test probiotics. Treatments 10^6 and 10^9 were not significantly different, but were higher than treatment 10^3 and the control. Treatment 3 was significantly higher than the control. Furthermore, the results showed that 40%~77% of the total isolated colonies from the gut of probiotic-treated fish were antagonistic to *A. hydrophila*, indicating efficient gut colonization of the probiotic *A. aureus* to the host fish.

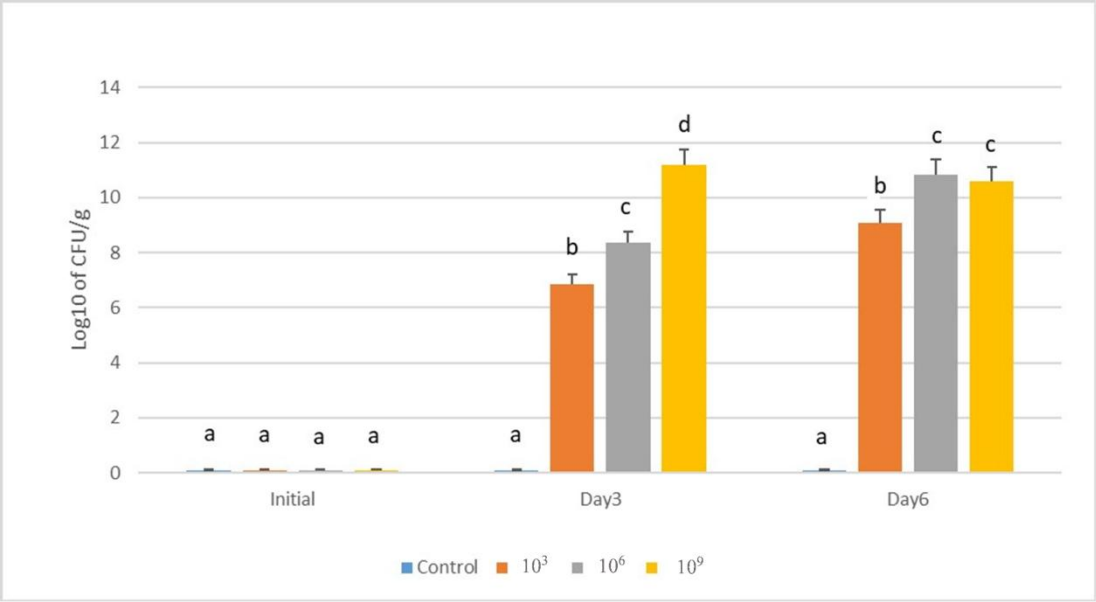

Fig. 3. Bacterial count from the gut of juvenile tilapia exhibiting a zone of inhibition against *Aeromonas hydrophila* when fed different concentrations of probiotic *Staphylococcus Aureus*

- Bars bearing similar letter superscripts are not significantly different.

3. Protective effects of the probiotics against *A. hydrophila* infection on juvenile tilapia in a pathogen challenge test

Prior to the infection challenge test, LD50 of the pathogen *A. hydrophila* to juvenile tilapia was identified. Results showed that the immersion exposure of juvenile *O. niloticus* to different bacterial concentrations that had undergone three pathogenic passages resulted in a mortality curve that followed a dose-response pattern (Fig. 4). The first mortalities were observed on day one on treatments 2 (10^7), 3 (10^8) and 4 (10^9). The first mortality on treatment 1 (10^6) was observed on day three. Mortalities continued to appear on treatment 4 until day eight, on which all the fish were found dead. Mortalities continued to appear on treatments 2 and 3 until day 12, where no more mortalities were recorded. Mortalities on treatment 3 continued until day 11 on which all fish died. No more mortalities were recorded on treatments 2 or 3, starting from day 11 until the

experiment was terminated. No deaths were recorded in the control that was not exposed to the pathogen.

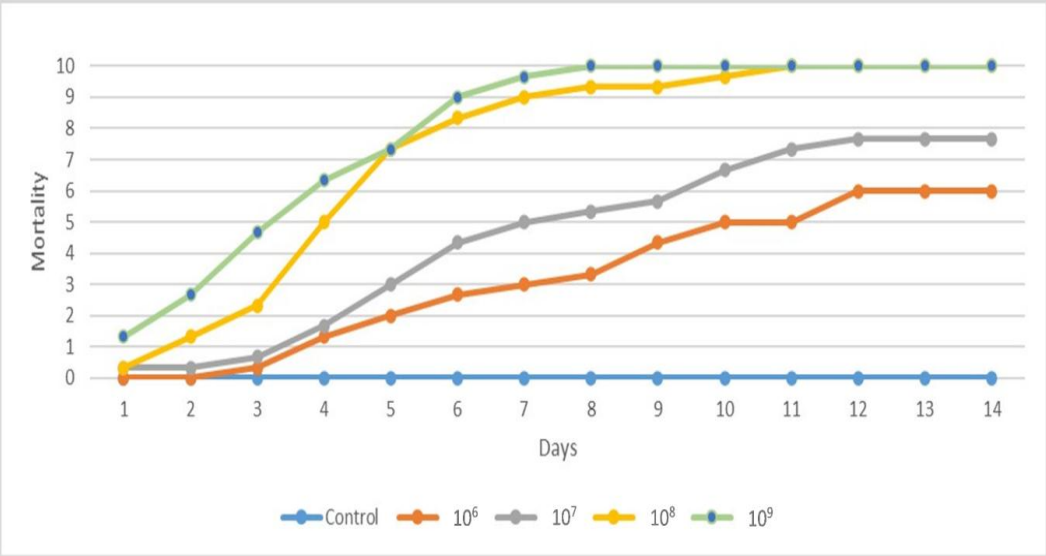

Fig. 4. Cumulative mortalities of juvenile tilapia (*Oreochromis niloticus)*, when challenged with different concentrations of *A. hydrophila*

A. hydrophila was recovered using a specific bacterial media (GSP) from the kidney of all moribund fish and fish exhibiting disease signs from all treatments except the control, indicating that the death was caused by this pathogen. The LD50 of the pathogen in the juvenile tilapia was determined to be 1×10^7cfu/mL when calculated using the Probit method **(Lieberman, 1983)**. This bacterial dose was used in the subsequent test to determine the protective effects of the probiotics.

The pathogen infection challenge results indicate that application of *Staphylococcus* probiotics through dietary supplementation at a dose of 10^6 cfu/g feed improved the survival rate of the tilapia juveniles when exposed to pathogenic *A. hydrophila* challenge (treatment TPA). No mortalities were observed on treatment TC and treatment TP. Nevertheless, significant mortalities were observed in treatment TA, which received the pathogen without the probiotics (Fig. 5). Infected fish during the challenge test exhibited disease signs typical of *A. hydrophila* infection that included skin ulcerations, fin rot, loss of scales and exophthalmia. As confirmatory tests and to satisfy the Kochs' postulate, pure colonies of *A. hydrophila* were isolated from the kidney of moribund infected fish.

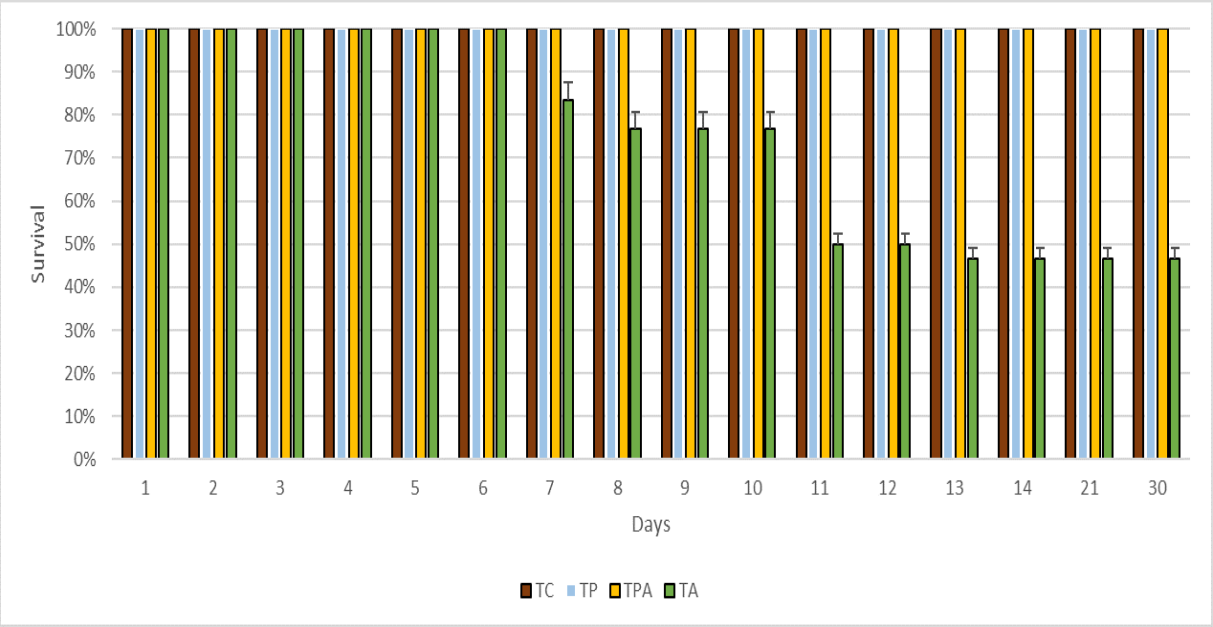

Fig. 5. Survival of juvenile tilapia (*Oreochromis niloticus*) when exposed to pathogenic *Aeromonas hydrophila* where treatment TC (received only the probiotic), treatment TPA (received both the probiotic and the pathogen) received *Staphylococcus aureus* supplemented diet at a dose of 10^6 CFU/g diet for a week, and treatments TC (control) and TA (received only the pathogen) served as the negative and the positive control group and were fed diets with no probiotics prior to the challenge. After a week of feeding, treatment groups TA and TPA were challenged by immersing the experimental groups to a pathogenic dose of *A. hydrophila* at 10^7 CFU/mL for an hour. The challenge lasted for 30 days.

DISCUSSION

Tilapia green water has been known to inhibit the growth of pathogens such as *Vibrio parahemolyticus* and *Vibrio harveyi* **(Cadiz *et al.*, 2016)**. In the study of **Huervana *et al.* (2006)**, green water from tilapia tanks was shown to inhibit the growth of luminous bacteria (*V. harveyi*). Furthermore, it has been well-documented that tilapia green water could prevent the growth of pathogenic *Vibrio* in marine animals, but the efficacy of this system in inhibiting bacterial pathogens of freshwater aquatic organisms remained under-investigation. The present study evaluated the presence of potential bacteria from TGW with inhibitory properties against *A. hydrophila*, a pathogen of cultured tilapia. In this context, the saline tilapia green water was evaluated for the presence of bacteria that are inhibitory against *A. hydrophila.* Multiple bacterial isolates were found to exhibit antagonism (zone of inhibition) on *A. hydrohila* with one isolate exhibited significant inhibitory activity and was identified as *S. aureus* (TG2). Similar results were recorded by **Hamza *et al.* (2018)**, revealing that *Staphylococcus lentus* has inhibitory effects against *V. harveyi* and reported its ability to produce biofilm. The study showed that *S. lentus* supernatant enhanced the anti-biofilm production, increase

biosurfactants, inhibited the growth of *V. harveyi*, and protected *Artemia salina* from *V. harveyi* infection. Another species of *Staphylococcus* was found to inhibit the pathogenic activity of White spot syndrome virus (WSSV) on white leg shrimp *Litopenaeus vannamei*. Active proteolytic activity was suggested as the mechanism responsible for the inhibitory activity of this probiotic against WSSV infection in shrimp **(Leyva-Madrigal *et al.*, 2011)**. These earlier works indicated that the genus *Staphylococcus* had strong inhibitory activity against viral and bacterial pathogens and concur with the present findings that *S. aureus* has a strong inhibitory activity against the gram-negative bacterial pathogen, *A. hydrophila*.

In addition, the present results suggest that *S. aureus* is not pathogenic to tilapia juveniles. Other studies also showed that some *Staphylococcus* species are non-pathogenic to vertebrates. **Borah *et al.* (2016)** showed that some species of *Staphylococcus* sp. were negative for hemolytic compounds and were not pathogenic. Idenical results were recorded in the study of **Zhang *et al.* (2003)** who deduced that, a strain of *Staphylococcus epidermidis* was found negative for hemolysins and other bacterial-associated toxin genes. Likewise, **Khusro *et al.* (2018a)** reported that *Staphylococcus hominis* was negative to hemolytic compounds, DNase, and gelatinase activities and was documented non-pathogenic. Moreover, some *Staphylococcus* species, such as *S. carnosus* and some strains of *S. xylosus* and *S. equorum* are non-pathogenic and are used in meat and cheese fermentations **(Rosenstein & Götz, 2012)**.

In contrast, **Shah and Tyagi's (1986)** reported that an isolate of *S. aureus* caused an eye disease in silver carp (*Hypophthalmichthys molitrix*) and was considered pathogenic. This indicates that in bacteria, similar or related species may exhibit different activities on the host organisms. In the present study, the *S. aureus* isolate was categorized non-pathogenic to tilapia.

The ability to colonize the gut is a major criterion for defining the efficacy of a potential probiotic **(Fuller, 1987; Standen *et al.*, 2016)**. The present study showed that *S. aureus* probiotic isolate exhibited active colonization of the tilapia gut. The results showed that the best application dose of the probiotics was 10^6 CFU/mL. At this dose, the maximum colonization was observed on day six and persisted up until day nine. The abundance of surface-located proteins is the *Staphylococcus*' primary way of attachment to the host's plasma proteins or extracellular matrix (ECM) components **(Rosenstein & Götz, 2012)**. In addition, *Staphylococcus* species could attach to fibril-forming collagens of types I, II, and III, laminin, elastin, fibronectin, vitronectin, fibrinogen, von Willebrand factor, and thrombospondin, which can be found in the intestines **(Rosenstein & Götz, 2012)**. Moreover, **Khusro *et al.* (2018a)** showed that *S. hominis* can withstand low pH levels and is resistant to bile salts suggesting that these bacteria could survive and colonize the gastrointestinal tract. Additionally, this bacterium exhibits a strong auto-

aggregation capability, adherence capability and is highly resistant to phenols and lysozymes.

Moreover **Khusro *et al*. (2018b)** elucidated that, *Staphylococcus succinus* exhibited strong gut attachment capability and can evade and survive gut-associated immunological activities of the host animal. Similarly, **Borah *et al*. (2016)** showed that the high viability of *Staphylococcus* sp., when exposed to lysozymes, bile salts, and a wide range of pH could be attributed to its high auto-aggregation and high hydrophobicity properties. The retrieval of *Staphylococcus* probiotics was reported by **Abarike *et al*. (2018)**, indicating that the probiotics were thriving in the gut of the experimental animal and were able to compete and exclude the pathogenic bacteria. All these earlier reports on the gut colonizing activity of the genus *Staphylococcus* support the present finding, confirming the efficient colonizing property of *S. aureus* on juvenile tilapia gut.

Results of the present study postulates strong protective effects of *S. aureus* as a probiotic against *A. hydrophila* infection. Only the treatment group receiving no probiotics exhibited mortalities. The high survival observed on the probiotic treated groups could be attributed to the efficient gut colonization of the probiotic and the strong antibacterial activity of this probiotic against *A. hydrophila*. This result ties well with previous studies on the application of probiotics before the onset of infection **(Irianto & Austin, 2002)**. Application of probiotics serves as a preventive measure to the colonization of the infectious bacteria in gut epithelial cells. The probiotics may have a significant advantage to be the first to adhere in the intestinal mucous, thus restricting the surface area for the pathogenic bacteria to adhere. *A. hydrophila* manifests its pathogenicity by first colonizing the gastrointestinal tract then production of biofilm, and eventually secretion of enterotoxin molecules that kills the host **(Janda & Abbott, 2010)**. The high survival of the group receiving *S. aureus* during the challenge test could be attributed to the strong gut colonizing activity and the capacity of this probiotic to secrete compounds that are inhibitory to *A. hydrophila*. High colony counts of *S. aureus* in the gut of the experimental fish and the nonexistent *A. hydrophila* as observed in the present study further confirms the notion of competitive exclusion coupled with direct killing activity as the probiotic effect that accounts for the high survival during the challenge test of the treatment group receiving the probiotics supplementation.

The present results are consistent with earlier findings elucidating the preventive and inhibitory activities of probiotics, including lactic acid bacteria **(Chabrillon *et al*., 2005; Sugimura *et al*., 2011)** and *Brevibacillus brevis* **(Mahdhi, 2012)** against *V. harveyi* infection in aquatic animals. Efficacy of these probiotics to prevent infection was associated with the decreased capability of *V. harveyi* to adhere in the intestine due to the presence of probiotics colonizing the gut epithelium before the exposure and the contact of the experimental animals with the *Vibrio* pathogen. Similarly, earlier works have shown the capability of probiotics to inhibit pathogens by exclusion and direct bacterial

killing activity **(Taoka *et al*., 2006)**. *B. brevis* was documented to secrete compounds that were inhibitory to *Vibrio* species **(Mahdhi, 2012)**. Pseudomonas M174 was also reported to suppress the growth of the pathogen *Flavobacterium psychrophilum* **(Korkea-Aho *et al*., 2011)**, and *Vibrio fluvialis* was shown to exclude and inhibit the growth of the fish pathogen *Vibrio anguillarum* **(Sorroza *et al*., 2012)**. All these earlier works support the present findings elucidating that *S. aureus* as a probiotic that could protect juvenile tilapia against *A. hydrophila* infection. The protective effect of this probiotic is associated with its high gut colonizing property and its direct killing activity on *A. hydrophila*.

CONCLUSION

Collectively, the present results indicate that *S. aureus* can be used as a probiotic applied as a dietary supplement at a dose of 10^6 CFU/g feed to prevent *A. hydrophila* infection in tilapia juveniles (*O. niloticus*). The inhibitory activity of this probiotic against *A. hydrophila* is attributed to its strong gut colonizing activity and ability to secrete bacteriostatic compounds against this pathogen. Application of this probiotic is a practical approach in the prevention of *A. hydrophila* infection in the tilapia fish farming.

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