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Influence of kaolin clay on *Aeromonas hydrophila* growth, chemotaxis, and virulence to channel catfish, *Ictalurus punctatus*

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Abstract

Aeromonas hydrophila is one of the most widespread bacterial pathogens affecting freshwater fish and an emerging pathotype of *A. hydrophila* has severely impacted the catfish industry over the last decade. In this study, we evaluated the effect of treatment with kaolin ($Al_2Si_2O_5(OH)_4$), an inert clay, on the chemotaxis and adhesion (two important steps of the infective process) of *A. hydrophila* to catfish mucus and the potential use of kaolin for controlling *A. hydrophila* outbreaks. Chemotaxis assays revealed kaolin clay significantly blocked the chemotaxis and adherence of *A. hydrophila* to catfish mucus. Kaolin treatment at a level of 0.1% led to a significant improvement in survival (66.7%) of experimentally challenged catfish as compared to untreated fish (28.9%). Kaolin treatment did not alter the growth of *A. hydrophila*, but bacterial concentrations in the upper phase of treated cultures were significantly reduced by kaolin treatment within 15 min, and significantly increased in the pellet by 45 min of treatment, indicating the rapid formation of physical complexes through adsorption followed by gravitational settling.

Introduction

Aeromonas hydrophila is a ubiquitous Gram-negative, motile, rod-shaped bacterium that is widely distributed in aquatic environments. Indeed, *A. hydrophila* and other motile Aeromonads such as *A. caviae* and *A. veronii* biovar sobria are among the most common bacteria inhabiting freshwater settings throughout the world, and these bacteria frequently cause a disease termed motile *Aeromonas* septicemia (MAS) in cultured and wild fishes (Cipriano 2001). Motile Aeromonads have also garnered considerable attention from a public health perspective due to infections in humans, which typically occur from consumption of contaminated food or drinking water. Historically, in the finfish host, *A. hydrophila* has been branded as a secondary pathogen, usually trailing the initial colonization of microbial invaders such as *Flavobacterium columnare* or ectopic protozoans (Xu 2012). However, since 2009, severe outbreaks of MAS have devastated segments of the US catfish industry, causing pronounced annual losses of channel

catfish (*Ictalurus punctatus*) and hybrid catfish (*Ictalurus punctatus x I. furcatus*) that have threatened the stability of the industry (Hemstreet et al. 2010, Gresham 2014). A new distinct and hypervirulent strain of *A. hydrophila* (vAh) was etiologically determined to be responsible for the MAS outbreaks (Hossain et al. 2013, Tekedar et al. 2013). Affected fish present with hallmark MAS lesions without the involvement of concomitant pathogens; however, the obvious field conditions governing vAh outbreaks are largely unknown (Hanson et al. 2014). Recent evidence revealed factors related to both the microbe and the Ictalurid host; which pointed to iron accessibility for vAh, and feeding status of catfish, as factors that heavily influence disease outcomes (Peatman et al. 2018).

The global distribution and importance of *A. hydrophila* in freshwater, coupled with the emergence of the clonal vAh pathotype, has prompted the search for effective management strategies to prevent or control outbreaks. Currently, culture species notwithstanding, the most effective means of disease treatment available to the aquaculture industry are antibiotics. Consequently, antibiotic use is under intense scrutiny from concerns over selection for antibiotic resistant strains along with evolving consumer demands. Moreover, antibiotic regimens escalate production costs and diminish marketing advantages as sustainable, environmentally-friendly products. Novel approaches, particularly those that are preventative rather than reactionary, are desperately needed. One compound potentially suitable in this regard is kaolin ($Al_2Si_2O_5(OH)_4$), an inert clay found throughout the globe. Kaolin has a long history of clinical use to adsorb pathogenic bacteria and their toxins (Hektoen & Rappaport 1915; Gunnison & Marshall 1937) and more recently was shown to bind to and subsequently protect against infections from *F. columnare*, the etiological microbe of columnaris disease (Beck et al. 2015).

In the present study, we utilized commercially available kaolin called AkuaPro, a product that is optimized for flocculating *F. columnare* bacteria. We sought to determine the extent to which this kaolin could modulate *A. hydrophila* chemotaxis and adhesion to host mucus, and flocculate *A. hydrophila* in aqueous suspensions. Following these initial studies that modeled the prerequisite steps of colonization, we utilized a challenge model to evaluate the prophylactic potential of kaolin in protecting channel catfish from an otherwise lethal dose of *A. hydrophila*.

Materials and Methods

Effect of Kaolin clay on A. hydrophila growth

To determine whether the presence of kaolin affects *A. hydrophila*, bacteria were plated and incubated at 28 ºC on agar containing various concentrations of kaolin. The kaolin used for all studies was the commercially available AkuaPro™ brand (Imerys, Roswell, GA). Tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) and agar (TSA) were dissolved in distilled water by mixing and heating. Prior to autoclaving, kaolin was added to yield experimental concentrations of 0%, 0.1%, 0.2%, 0.4% and 0.8%. The agar medium (17 mL) containing the various levels of kaolin was poured into Petri dishes and allowed to solidify at room temperature. A virulent vAh isolate ML-10-51K was retrieved from a glycerol stock preserved at -80 °C and streaked on TSA. After 24 h, a single colony was inoculated into 30 mL of TSB. This suspension was incubated at 28 °C for 18 h. The optical density (OD) of the bacterial culture was measured at 540 nm and adjusted to an OD of 1.0. Serial dilutions were then prepared in TSB and 10 µL was immediately spread-plated onto three separate TSA plates. After overnight growth, the colonies of *A. hydrophila* were counted and calculated based on the dilution factor.

Flocculation dynamics

Overnight cultures of *A. hydrophila* ML-10-51K were harvested by centrifugation for 10 min and resuspended in sterile dechlorinated municipal water to give an optical density (OD_{540nm}) of 1.0. Next, 5 ml of the bacterial cell preparation was mixed with various concentrations (0, 0.025, 0.05, 0.1, 0.2 and 0.4%) of kaolin clay. After mixing, the samples were allowed to settle. At 1 cm depth, 200 µL samples in duplicate were collected at the beginning of the experiment and 15, 30 and 45 min after the mixing and transferred to a sterile 96-well plate and subjected to MTS/PMS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt); MTS/phenazine methosulfate; PMS) (Celltiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI) for determining the number of viable cells. At the end of the 45 min experiment, to determine the amount of viable cells at the bottom of each tube, 200 µL of settled kaolin/bacteria mixture in duplicate was also determined by MTS/PMS assay. The plate was covered with an aluminum foil to protect from light and incubated for 2 h at 28 °C. The $A_{490\ nm}$ was recorded using Epoch-2 microplate reader (BioTek,

Winooski, TX, USA). The assays were performed in three independent trials, and each trial was performed in triplicate.

The rate of flocculation and settling of kaolin clay at a concentration of 0.1% was also estimated against two different concentrations of *A. hydrophila* (4×10^{10} and 8×10^{10} CFU/mL) as compared to samples containing no bacteria in sterile water. Bacteria were grown overnight in TSB at 28 °C, pelleted by centrifugation, and resuspended in sterile water at an optical density (OD_{540nm}) of 1.2. Flocculation was measured by mixing 0.1% kaolin clay with the two different concentrations of bacteria in 15 ml tubes (final total volume 10 ml). After mixing, the samples were allowed to settle. At 1cm depth, 200 µl samples in duplicate were collected at 0, 15, 30 and 45 min after mixing and transferred to a 96-well plate where turbidity was measured at 540 nm.

Preparation of channel catfish mucus samples

Healthy catfish (100-200 g) were anesthetized with 100 mg/L tricaine methanesulfonate (Argent Chemicals, Redmond, CA). The anesthetized fish were held vertically, and mucus was collected from the skin by gently stroking with a soft rubber spatula into Petri dishes. Special care was taken to prevent damage to the skin and avoid contamination with blood or other extraneous products. The mucus from individual fish was pooled together and centrifuged at 4500rpm for 45 min and the pellet (epithelium cells and cellular debris) was discarded. The mucus protein concentration was determined using the Micro BCA™ Protein assay (Pierce, Rockford, IL) and adjusted to 0.5 mg/ml. The pooled mucus samples were stored at −20 °C before use.

Chemotactic response of A. hydrophila to catfish mucus

Chemotaxis assays were performed using blind-well chambers (Corning Costar, Cambridge, MA, USA) as described previously (Klesius et al. 2010) with slight modifications. *A. hydrophila* isolate ML-10-51K was grown in TSB at 28 °C for 18 h. The bacteria were harvested by centrifugation for 10 min and resuspended in sterile water to an optical density (OD_{540nm}) of 1.0. Next, a total volume of 200 µL of prepared bacterial suspension was mixed with various concentrations (0, 0.05, 0.1, 0.2 and 0.4%) of kaolin and then added to the bottom well of the chamber. Polycarbonate membranes (Nucleopore, Pleasanton, CA, USA) with a diameter of

13 mm and a pore size of 8.0 μm were then carefully placed on top of the bottom wells. Following assembly of the chambers, 200 μL of mucus at concentration of 0.15 mg/mL was added to the wells of the top chambers. Following incubation at room temperature for 90 min, the mucus sample (140 μL) from each chamber was transferred to a 96-well plate. The number of viable cells in each well was determined with the MTS assay as described above. The absorbance values of the mucus samples alone were subtracted from the absorbance values of the mucus test samples to normalize the absorbance values. The assays were performed in three independent trials, and each trial was performed with five replicates.

Detection of bacterial adhesion with crystal violet

A modified crystal violet method (Vesterlund et al. 2005) was used to assess the effect of kaolin treatments on the ability of *A. hydrophila* to adhere to catfish mucus samples. Mucus (125μL) was loaded into 96-microtiter plate wells and incubated for 48 h at 4 ºC. To remove unbound mucus components, wells were washed twice with 300μL phosphate buffer saline (PBS). *A. hydrophila* isolate ML-10-51K was grown in TSB at 28 °C for 18 h. The bacteria were harvested by centrifugation for 10 min and re-suspended in sterile water to an OD_{540nm} of 1.0. The bacterial culture was then diluted at a 1:2 ratio with sterile water. Then, 100 μL of the bacterial cell preparation was mixed with various concentrations (0, 0.025, 0.05, 0.1, 0.2 and 0.4%) of clay in quadruplicate. To avoid binding of the bacteria with the polystyrene, the volume of bacterial suspension was less than the volume of mucus. Negative controls (containing only various concentrations of clay without bacteria) were also assayed in quadruplicate. Bacteria were allowed to adhere for 90 min at 28 °C and the non-adherent bacteria were removed by washing the wells three times with 300 μL of PBS. The adherent bacteria were stained with crystal violet (100 μL/well, 0.1% solution) for 15 min. Wells were subsequently washed five times with distilled water to remove excess stain and allowed to air dry. The stain bound to the cells was released by adding 10% acetic acid. After a 20 min incubation at room temperature, the absorbance at 590 nm was determined using a plate reader. Absorbance values of negative controls without cells were subtracted from the absorbance values recorded for all the samples.

Bacterial challenge

Channel catfish, with an average weight of 60.4 ± 3.1 g, reared in 264 L vats supplied with flow-through pond water at the North Auburn Fisheries Station, Auburn University, Alabama USA was used in this challenge. Prior to challenge, 190 apparently healthy catfish were stocked in 50 L rectangular glass aquaria (15 fish per tank) for an acclimation period of 10 days. Upon arrival, 10 fish were randomly caught, bacteriologically examined following standard procedures, and confirmed negative for *A. hydrophila* infection. Glass aquaria were supplied with continuously aerated, dechlorinated, heated municipal water through a precise system that delivers a consistent flow rate of approximately 0.2 L/min desirable for disease challenge experiments (Mitchell and Farmer, 2010; Beck et al., 2012). Water quality parameters were measured daily and the averages during the study were as follows: dissolved oxygen 6.85 ± 0.30 mg/L, water temperature 28.7 ± 0.5 °C, ammonia concentration 0.31 ± 0.08 mg/L, and pH 7.6 ± 0.3. Fish were fed to apparent satiation with a commercial 32% protein catfish ration while maintaining a constant photoperiod (LD 12:12) throughout the study. The extensively characterized virulent strain of *A. hydrophila* (vAh), ML-10-51K, originally recovered from a moribund catfish with typical MAS symptoms in 2010 (Rasmussen-Ivey et al., 2016; Zhang et al., 2013, 2014, 2016a) was used to challenge fish. The bacterium was grown from a frozen glycerol stock at −80 °C in TSB supplemented with the iron chelator deferoxamine mesylate (DFO; Sigma, St. Louis, MO, USA) at a final concentration of 400 μM (Peatman et al. 2018) and was incubated at 28 °C with constant shaking at 150 rpm for ~ 20 h until the cell density reached approximately 1.5 ± 0.1 × 10^9 colony forming units per milliliter (CFU/mL) based on optical density at 600 nm (OD_{600}). Standard plate counts in triplicates were performed to confirm bacterial enumeration. At the conclusion of the acclimation period, fish in the glass aquaria were pooled and anaesthetized in a 20 L vessel filled with tank water (28 ± 1 °C) containing 100 mg/L of buffered tricaine methanesulfonate (MS-222). Once fish started showing signs of sedation as indicated by loss of equilibrium and slowing of opercular movement, the adipose fin was clipped at its base as described by Zhang et al. (2016a). The fin-clipped catfish were divided into four treatments: (I) unchallenged untreated control, (II) unchallenged and treated with Kaolin (kaolin control), (III) challenged untreated (challenged with vAh treated with no kaolin), and (IV) challenged treated (challenged with vAh and treated with kaolin). Each treatment group consisted of 3 replicates

(tanks), with 15 fish per tank. Tanks were assigned blindly to each treatment group. For the challenge, each tank was filled with 10 L of water (same water parameters as described above). In the kaolin-treated tanks, kaolin (1 g/L) was slowly added to the water (5 min prior to challenge) and mixed thoroughly as described previously (Beck et al., 2015). Subsequently, 100 mL of the bacterial culture was added (immediately after fin clipping) to each challenge tank resulting in a final concentration of ~$1.5 \pm 0.1 \times 10^7$ CFU/mL. Fish were kept immersed in the aerated challenge suspension for 1 h. At the end of the 1 h static challenge, the water flow (at 0.2 L/min) was restored. Unchallenged groups were treated identically except that they were exposed to 100 mL of sterile TSB medium (with 400 μM DFO). Fish mortality was monitored and recorded twice daily for 1-week post-challenge. Fish were not fed on the challenge day but were offered pelleted catfish feed on the next day post-challenge and throughout the remainder of the study. The challenge was terminated after 3 days without MAS mortality. Brain, liver, spleen, and kidney of freshly dead fish following challenge were sampled to confirm presence of vAh as the cause of death (Hossain et al., 2013). Studies involving animals were reviewed by the Auburn University Institutional Animal Care and Use Committee.

Statistical analysis

Data were analyzed with Prism statistical analysis software. Survival data were analyzed using the Kaplan-Meier survival analysis. All other data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test to determine significant differences between means of control and treatments. Treatment effects were considered significant at $P < 0.05$.

Results

Growth of A. hydrophila in the presence of kaolin

The overnight culture of *A. hydrophila* cells on TSA plates containing kaolin clay at concentrations ranging from 0% to 0.8% revealed that the presence of kaolin had no inhibitory effect on the growth of *A. hydrophila*. Colony counts for the control (0% kaolin) were 2.13×10^8 CFU/ml and at the highest dose (0.8% kaolin) were 2.53×10^8 CFU/ml. Following visual inspection, no apparent differences were observed between colony size or morphology.

Effect of Kaolin concentration and treatment period on flocculation and settling rate

The reduction of bacterial cells in the suspension treated with various concentrations of kaolin clay by gravity and treatment period was shown in Figure 1(a). Samples were taken from 1cm from the top of the tubes at different incubations times (0, 15, 30 and 45min) and the amount of cells were determined by MTS assay. The lowest concentration (0.025%) of kaolin tested led to a significant reduction of bacterial cells at 15 min and further reduced at 30 min of treatment. At the concentrations of kaolin from 0.05% to 0.4%, significant cell removal was completed by 15 min. At the end of the 45 min treatment, the amount of bacteria settled by flocculation was measured and shown in Figure 1(b). MTS assays showed that significant increases in settled bacterial concentrations at 45 min following the kaolin treatment ranging from 0.05-0.4%.

Bacterial concentration affects flocculation and settling rate of kaolin

The flocculation performance of kaolin clay at two different concentrations of bacterial cells was determined by residual turbidity measurements (Figure 2). Control experiment was also conducted using kaolin alone (without bacterial cells). In 15 min, turbidity of kaolin using the higher bacterial concentration (8×10^{10} CFU/ml) was 41% lower than that of the initial turbidity reading. These values for control (kaolin alone) and with the lower concentration of bacteria (4×10^{10}) were 20% and 23%, respectively. Clearance of turbidity was continued from 15 to 45 min with a higher rate in the samples containing kaolin-bacterial cell combinations as compared to that of the control.

Effect of pretreatment of A. hydrophila cells with kaolin clay on chemotaxis

The results in Figure 3 show that kaolin clay treatment significantly inhibited the chemotaxis response of *A. hydrophila* ML-10-51K at all the concentrations tested. A concentration of 0.5% was the lowest concentration tested that significantly inhibited chemotaxis. Increasing kaolin concentration up to 0.2% linearly decreased chemotaxis response of *A. hydrophila* to mucus samples from healthy channel catfish. Increasing kaolin concentration from 0.2% to 0.4%, however, showed no further significant effect.

Effect of pretreatment of A. hydrophila cells with kaolin clay on adhesion to catfish mucus

The results of the *in vitro* adhesion ability of *A. hydrophila* cells to mucus summarized in Figure 4. When kaolin was added to *A. hydrophila* cells at a concentration of 0.0125%, no significant effect was observed between untreated and kaolin treated cells. At concentrations of 0.05% or higher, kaolin significantly reduced the adhesion of *A. hydrophila* cells to catfish mucus, and at kaolin concentrations between 0.1% to 0.4%, the adhesion ability of *A. hydrophila* cells was significantly impaired.

Fish survival from bacterial challenge

Survival of catfish was significantly improved when kaolin was added to the water prior to challenge (Figure 5). Survival was 66.7% in kaolin-treated challenged fish (0.1% kaolin) and 28.9% in untreated challenged fish. Dead fish, irrespective of treatment, showed clinical appearances consistent with motile Aeromonad septicemia. There was no mortality, or any grossly visible abnormalities observed in unchallenged untreated negative control fish or unchallenged kaolin-treated fish.

Discussion

Diseases represent a significant barrier to intensification of aquaculture, accounting for as much as 45% of losses (Murray and Peeler, 2005). This is particularly true for the US catfish aquaculture industry where higher rearing densities in existing ponds or more recent innovations (split-ponds, in-pond raceways, etc.) are often accompanied with a significantly heightened risk of infectious disease as lower dissolved oxygen levels, high nitrogenous waste, and elevated stress can depress catfish immune responses. Motile *Aeromonas* Septicemia (MAS) is situated near the top of the priority list, not only for US producers but beyond, where MAS outbreaks within food fish and ornamental fish erode profit margins on a global scale (Rasmussen-Ivey et al. 2016). Broadly, most cases of MAS can be correlated to a predisposing factor such as physical injury, co-infection with another bacterial agent or parasite, or an immunosuppressive event such as environmental stress. More specifically, MAS is routinely a complicating factor in columnaris disease, saprolegniosis, and several diseases of viral etiology; and is commonplace following abiotic insults including nutritional stress, low dissolved oxygen, and handling stress (Shotts 1994).

The findings presented here suggest that the integration of kaolin-based prophylaxis or treatment into some production settings may be beneficial, particularly, in scenarios where the large-scale use of antibiotics is not appropriate or advisable, or when an *Aeromonas* outbreak is more likely to occur (i.e. higher stress periods such as after the grading, stocking, or transport of fish). Based on our results, it appears that the incorporation of kaolin into a solid/semi-solid matrix, in this case agar, had no inhibitory effects on the growth of *A. hydrophila.* In suspension, the lowest concentration of kaolin tested (0.025%) was found to significantly reduce the number of bacterial cells after only 15 min of incubation time. These findings are similar to Beck et al. (2015), which reported a two-log reduction in the number of *F. columnare* cells present in the supernatant of *in vitro* kaolin-treated cultures as compared to kaolin-free cultures and found that the plating of settled bacterial-kaolin complexes on appropriate media revealed that bacteria remained viable. The reduced amount of bacteria in the upper phase, but insignificant increase of bacterial cells at the sediment of the sample treated with 0.025% kaolin indicates that they are still in mid-phase and may be unable to form large enough flocs to settle out. Collectively, these

data reflect the ability of kaolin to form rapid physical associations in suspension with bacterial cells, presumably through adsorption.

While catfish were utilized in the present study, the rapid and robust physical interaction between kaolin and *A. hydrophila* suggests that these findings are independent of the host/culture species and should translate well to related settings where *A. hydrophila* may be a problem. However, whether kaolin can interact similarly with other *Aeromonas* species remains unclear, which should be a topic of exploration in the future. One area where kaolin is particularly well-suited is hatchery environments where it and closely related clays have been employed for a wide array of uses: to remove egg adhesiveness, as a turbidity agent to reduce negative social interactions (i.e., bullying, fin nipping) and minimize cannibalism, to enhance contrast to improve prey identification and consumption of live feeds, and to reduce the loads of organic matter and microbes (Attramadal et al. 2012; Mizuno et al. 2004; McEntire et al. 2015). Interestingly, and related to the latter point, the initial bacterial concentration had a significant effect on settling rate of kaolin as demonstrated here using residual turbidity measurements. In contrast to treatments featuring kaolin alone (without bacterial cells), in 15 min the turbidity of kaolin using the highest bacterial concentration (8×10^{10} CFU/ml) was 41% lower than that of its initial turbidity reading. By comparison, these values for the control (kaolin alone) and the lower concentration of bacteria (4×10^{10} CFU/ml) were 20% and 23%, respectively. Clearance of turbidity continued from 15 to 45 min with a higher rate in the samples containing kaolin-bacterial cell combinations as compared to that of the control.

The adhesion of bacteria to the host mucosal epithelium is an essential prerequisite to disease initiation (Benhamed et al. 2014). This initial attachment of bacterial cells to the surface mucus involves many factors including hydrophobicity, surface charge, surface roughness, surface micro-topography and water flow, components of the surface, pH of the milieu, viscosity, etc. (Cortes, Jessika, and Ruben 2011). The results in Figure 3 show that kaolin clay treatment significantly inhibited the chemotactic response of *A. hydrophila* to healthy catfish mucus at all concentrations tested, even at the lowest concentration of 0.05%. Peak inhibition of chemotaxis was achieved at a rate of 0.2% and no additive effect was observed by increasing the concentration to 0.4%. As demonstrated in Figure 4, at concentrations above 0.05% or higher,

kaolin also significantly reduced the adhesion ability of *A. hydrophila* cells to healthy catfish mucus, an effect that was even more evident at higher concentrations (between 0.1% to 0.4%).

Building upon these in vitro studies, a subsequent laboratory challenge with *A. hydrophila* (isolate ML-10-51K) revealed that the presence of kaolin in the water during the delivery of the challenge inoculum markedly improved the survival of catfish over the course of a 5-day study (Figure 5). Consistent with previous findings (Beck et al. 2015) unchallenged animals that were exposed to kaolin did not experience any mortality nor display any grossly observable lesions or alterations in behavior. However, the present study was not a comprehensive investigation of safety nor efficacy of the full complement of kaolin types, doses, and duration; therefore these and related parameters should be determined empirically for each species of interest.

The flocculation and aggregation capacity of kaolin and similar clays is not newly recognized, particularly when employed for the removal or binding of microorganisms, their polysaccharides, and extracellular polymeric substances (reviewed by Mueller 2015). Kaolin exists as small particles with an average size of approximately 500 nm and particles consist of three surfaces, one silica tetrahedral basal plane surface, one alumina octahedral basal plane surface, and one edge surface (Liu et al. 2014). While the precise mechanism of action governing kaolin-mediated protection in our challenge trials is not fully elucidated, the complex surface chemistry of kaolinite may explain its efficiency in bacterial adsorption and especially the presence of hydrophobic areas on the silica face (Yin et al. 2012). It has been reported that cell concentration, salinity, mineral content and pH could influence the interaction between clay and bacteria (Kurane et al. 1986, Labille et al 2005, Mueller 2015). To minimize the influence of these factors, this experiment was carried using the same water source throughout. However, these properties suggest that the effectiveness of kaolin at microbial binding may vary across disparate water chemistries and environmental conditions; thereby necessitating testing on a case-by-case basis before implementation in a culture setting.

While the properties mentioned above likely reduce pathogen adherence and entry; here, complete protection was not achieved as approximately 33% of treated fish succumbed to disease from this hypervirulent isolate of *A. hydrophila*. As immersion-based interventions with

kaolin are not suitable for all culture settings future studies should explore the effectiveness of kaolin in the context of other routes of administration. It is important to note here, that our study did not examine whether kaolin is curative for MAS as the kaolin was added just prior to the addition of the challenge inoculum (i.e., preventative). It stands to reason that if kaolin treatment were delayed to a timepoint after challenge that vAh would have gained unrestricted access to the circulatory system where it can rapidly induce mortality (Zhang et al 2016b; Peatman et al. 2018).

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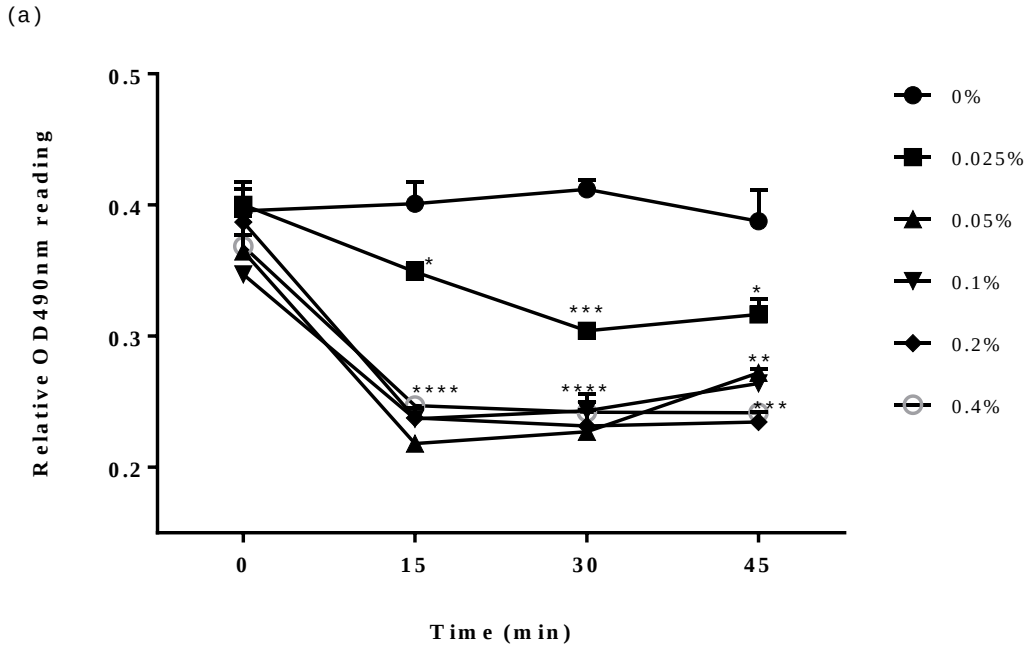

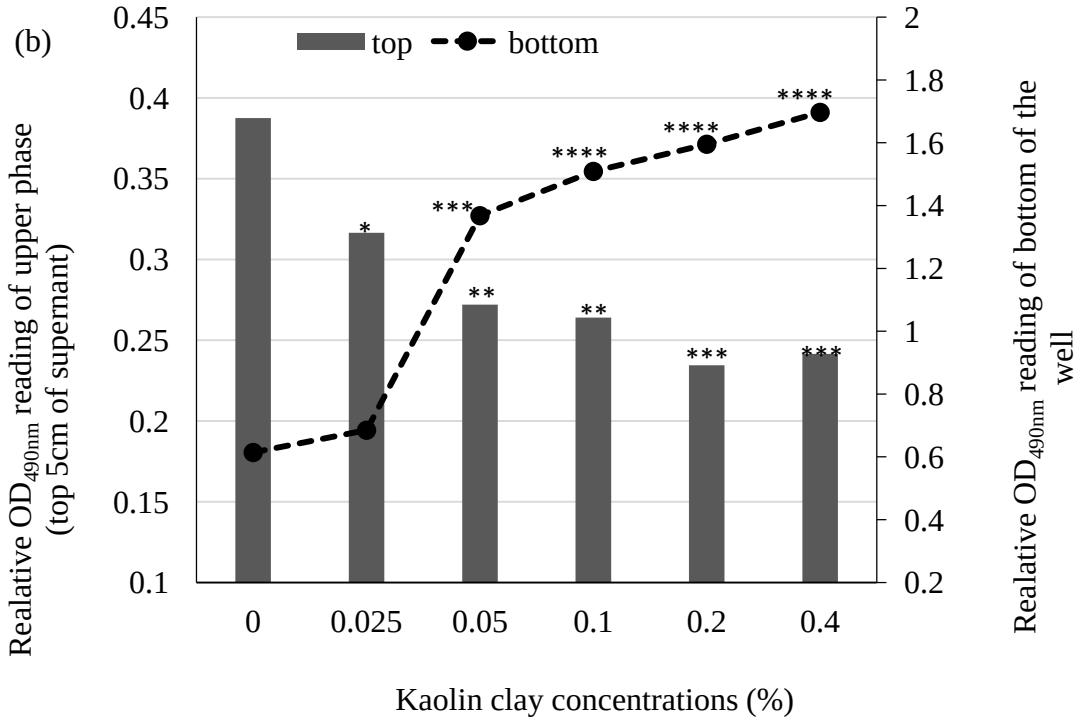

Figure 1. *Aeromonas hydrophila* ML-10-51K cells were treated with various concentrations of kaolin clay (0, 0.025, 0.05, 0.1, 0.2 and 0.4%) and samples were taken at different time periods (0, 15, 30 and 45 min). The amount of bacteria remaining in suspension at 1cm in depth from the top of the tubes at each time period (a) and the precipitated bacteria out of suspension was collected from the bottom of the tubes at 45min (b) were determined using MTS assay. Data are presented as mean ± standard error of mean (SEM) from three experiments. The asterisks indicate significant differences between the control and kaolin-treated groups. Number of asterisks represent degree of statistical significant difference from the control; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

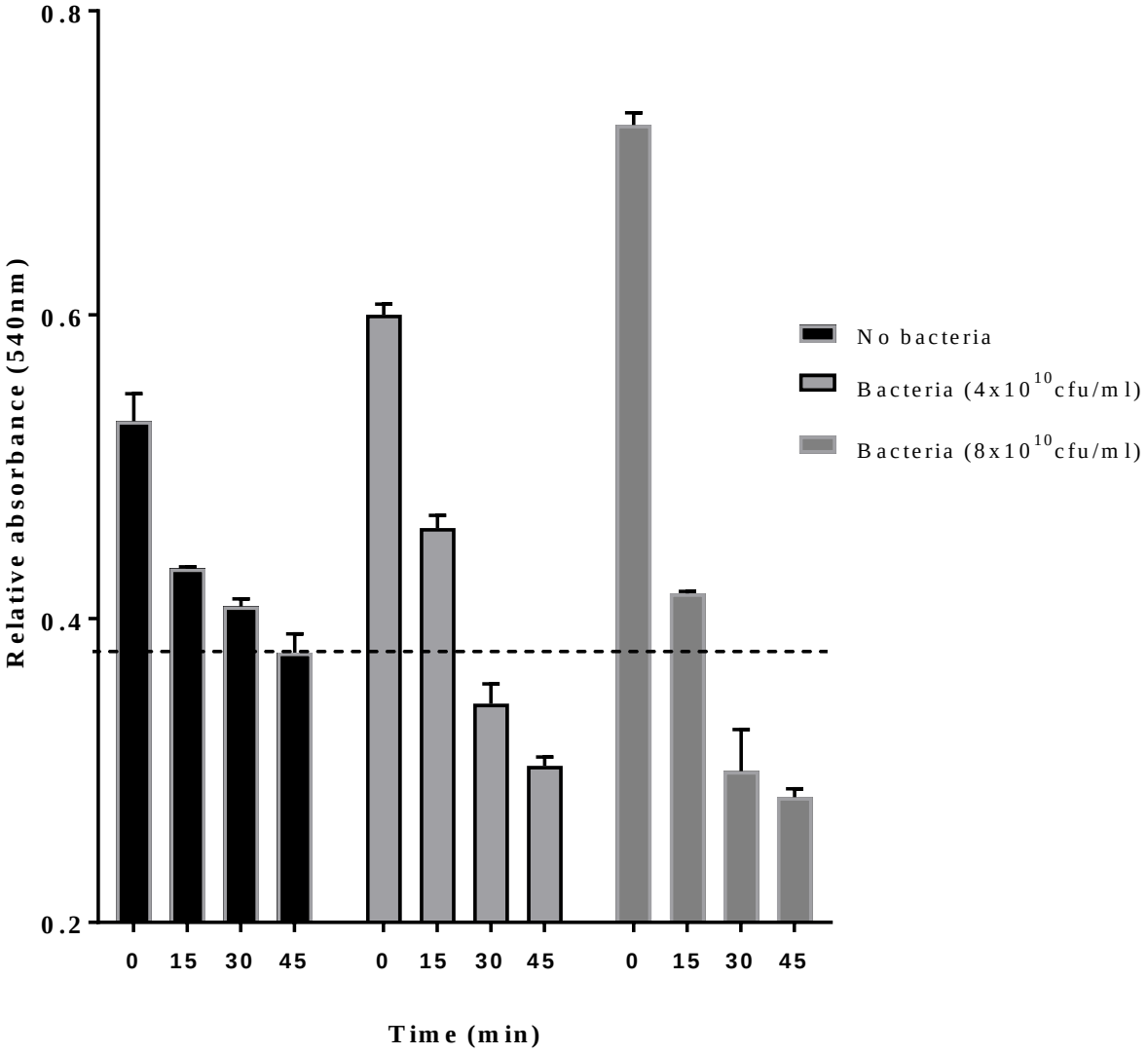

Figure 2. *In vitro* effect of two different concentrations of *Aeromonas hydrophila* ML-10-51K ($4x10^{10}$ CFU/ml and $8x10^{10}$ CFU/ml) on flocculation/precipitation rate of kaolin clay (0.1%) in aquarium water compare to absence of bacteria by measuring the turbidity at optical density (OD_{540nm}) at the different time periods (0, 15, 30 and 45min).

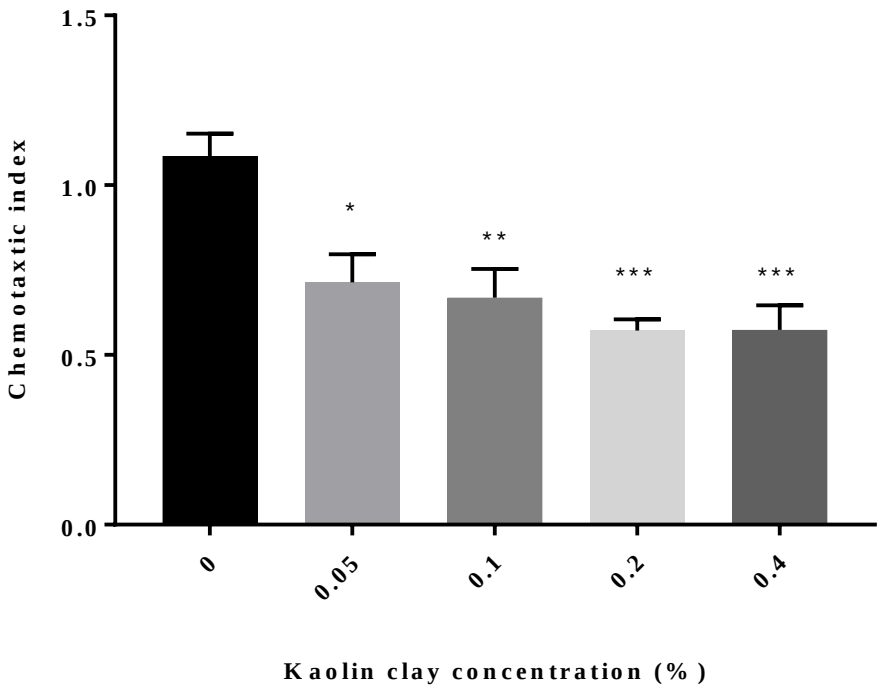

Figure 3. Effect of kaolin clay presence at various concentrations (0%, 0.05%, 0.1%, 0.2% and 0.4%) on chemotactic response of *Aeromonas hydrophila* ML-10-51K on hybrid catfish mucus as determined by MTS assay. Data are presented as mean ± standard error of mean (SEM) from five replicates. The asterisks indicate significant differences between the control and kaolin-treated groups. Number of asterisks represent degree of statistical significant difference from the control; *, P≤ 0.05; **, P≤ 0.01; ***, P≤ 0.001; ****, P≤ 0.0001.

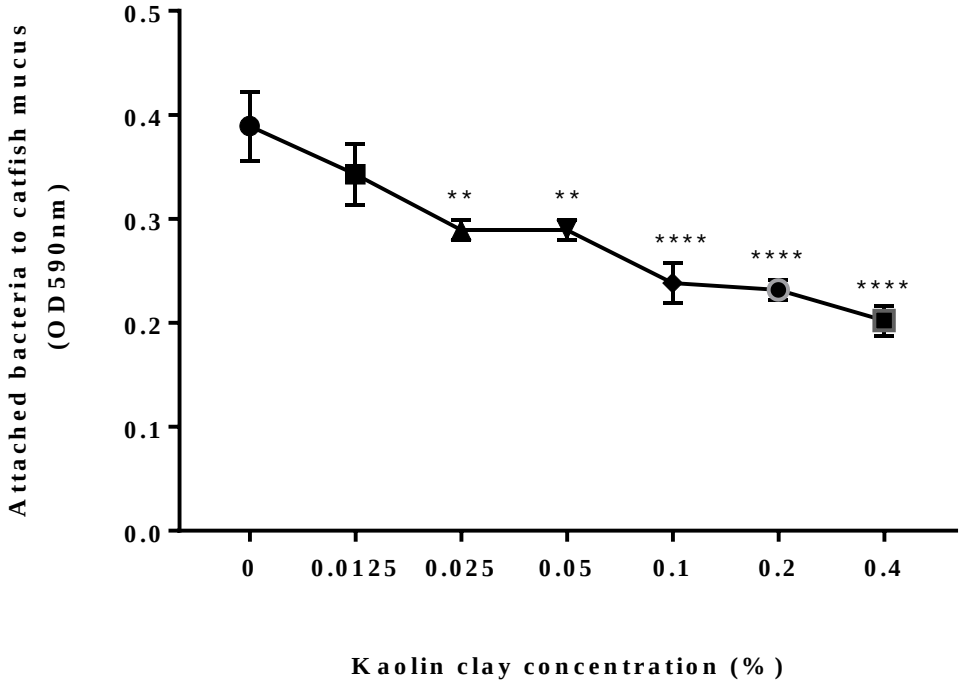

Figure 4. Amount of *Aeromonas hydrophila* ML-10-51K attached to hybrid catfish mucus absence or presence of kaolin clay at various concentrations (0%, 0.025%, 0.05%, 0.1%, 0.2% and 0.4%) on as determined by crystal violet. The asterisks indicate significant differences between the control and kaolin-treated groups. Number of asterisks represent degree of statistical significant difference from the control; *, P≤ 0.05; **, P≤ 0.01; ***, P≤ 0.001; ****, P≤ 0.0001.

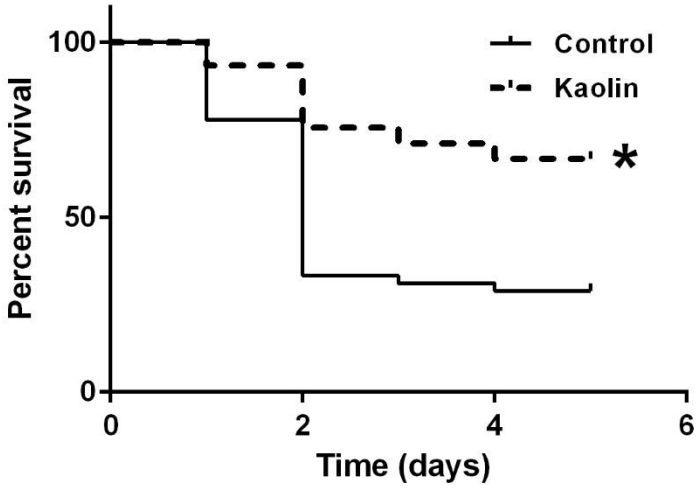

Figure 5. Kaplan-Meier survival analysis of channel catfish challenged with a lethal dose of *Aeromonas hydrophila* ML-10-51K without (control) or with (0.1% kaolin) treatment. The asterisk indicates a significant difference between the control and kaolin-treated challenged group ($P<0.001$).

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