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Kaolinitic clay protects against *Flavobacterium columnare* infection in channel catfish *Ictalurus punctatus* (Rafinesque)

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Abstract

Columnaris disease, caused by the bacterial pathogen *Flavobacterium columnare*, continues to be a major problem worldwide in both wild and cultured freshwater finfish. Despite the far-reaching negative impacts of columnaris disease, safe and efficacious preventatives and curatives for this disease remain limited. In this study, we evaluated the potential of kaolin ($Al_2Si_20_5(OH)_4$), a type of clay, for the prevention of columnaris disease. Channel catfish, *Ictalurus punctatus* (Rafinesque), fingerlings were experimentally challenged with *Flavobacterium columnare* in untreated water or with water containing kaolin (1 g L^{-1}). Over the 7-day course of study, kaolin treatment led to significantly ($P < 0.001$) improved survival (96%) as compared to untreated fish (78% survival). Histological examination of the gills revealed that kaolin-treated fish had substantially less gill damage than untreated controls. Quantitative PCR analysis of gill tissue revealed that kaolin significantly reduced *F. columnare* adhesion (measured at 1 h post-challenge) and colonization (24 h post-challenge). Incubation of kaolin with *F. columnare in vitro* demonstrated that kaolin reduced the number of *F. columnare* cells in culture supernatants, presumably through the formation of physical complexes through adsorption. In summary, kaolin can improve survival, reduce gill pathologies and reduce bacterial attachment to key tissues associated with columnaris disease in channel catfish by binding to *F. columnare*.

Keywords: catfish, columnaris disease, disease prevention, *Flavobacterium columnare*, kaolin.

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Introduction

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, is an opportunistic pathogen which causes substantial mortality globally in freshwater ornamental and farmed fish species. In particular, members of the family Ictaluridae, principally the economically valuable food fish channel catfish *Ictalurus punctatus* (Rafinesque), are highly susceptible to this pathogen (Arias *et al.* 2004; Shoemaker *et al.* 2008; USDA 2010).

Despite the importance of columnaris disease, preventatives or treatments remain limited. While antimicrobial drugs such as antibiotics exhibit some efficacy against columnaris disease, the use of antibiotics in aquaculture is under increasing scrutiny. In some geographical areas, the use of prophylactic antibiotics is widespread and without regulation, and antibiotics are inappropriately utilized to prevent or mitigate bacterial infections resulting from sanitary shortcomings in fish rearing (Cabello 2006). The extensive use of antibiotics in aquaculture in recent years has resulted in

Journal of Fish Diseases

the acquisition of drug resistance by some aquaculture pathogens (Defoirdt, Sorgeloos & Bossier 2011). Moreover, potential impacts on human health stemming from the emergence of drug-resistant bacteria and the transfer of these determinants of resistance to human-associated bacteria is a major concern (Declercq *et al.* 2013). Indeed, antibiotic resistance to several clinically important antibiotics such as quinolones and tetracyclines has been documented in *Flavobacterium columnare* isolates originating from ornamental fish (Declercq *et al.* 2013). Clearly, alternative preventatives and curatives are desperately needed for columnaris disease and other bacterial pathogens affecting cultured fish.

In contrast to many of the bacterial pathogens that plague freshwater fish, *F. columnare* primarily affects the external mucosal surfaces of fish such as the skin, fins and gills (Beck *et al.* 2012; Peatman *et al.* 2013). Accordingly, the preferential ectopic pathogenesis of this organism makes it highly amenable to prophylactic or treatment intervention with surface-acting compounds. One such potential compound is kaolin ($Al_2Si_20_5(OH)_4$), an inert clay which has a long history of medicinal use, where it has been principally exploited to adsorb pathogenic bacteria, particularly in the context of gastrointestinal disease (Hektoen & Rappaport 1915; Gunnison & Marshall 1937). Uses for kaolin in aquaculture have been previously explored as bulking agents in pelleted feeds (Grove, Loizides & Nott 1978; Jobling 1981) and to reduce egg adhesiveness and clumping in hatchery operations (Mizuno *et al.* 2004). Interestingly, various *Flavobacterium* species have been shown to be highly susceptible to adsorption by kaolin (Esterman & McLaren 1959; Soda, Ike & Fujita 1999). Therefore, based on the well-known properties of kaolin to adsorb bacteria, in the present study, we evaluated the potential of kaolin as a prophylactic agent to protect channel catfish from columnaris disease in an experimental challenge.

Materials and methods

Fish and experimental conditions

Fingerling channel catfish were reared at the Harry K. Dupree Stuttgart National Aquaculture Research Center, in Stuttgart, Arkansas, USA. Twenty fish (approximately 5 g each) were stocked per 18-L tank containing 10 L of filtered well water. Water was provided through the Ultra-Low-Flow water delivery system (Mitchell & Farmer 2010) at a rate of 29.1 ± 0.07 mL min^{-1}. This experimental columnaris disease challenge system has been demonstrated to allow for the natural progression of columnaris disease in a flow-through environment (Mitchell & Farmer 2010; Beck *et al.* 2012; Farmer, Beck & Straus 2012a; Farmer *et al.* 2012b, 2013). Temperature and dissolved oxygen were measured daily using an YSI Pro20 dissolved oxygen meter (Yellow Springs). Each tank received forced aeration from submerged air stones. Water temperature averaged 22.3 ± 0.03 °C and dissolved oxygen averaged 8.63 ± 0.03 mg L^{-1}. An Accumet Basic AB15 pH meter (Fisher Scientific) was used to measure pH (7.6–8.1) during the study. Standard titration methods (Eaton *et al.* 2005) were used to measure total alkalinity (218 mg L^{-1}) and total hardness (121 mg L^{-1}) of the well water. Fish were not fed the first day after challenge, but offered pelleted catfish feed (35% protein, 2.5% fat; Delta Western) on day 2 and throughout the rest of the study.

Experimental design

The study consisted of five replicate tanks of each of the following two treatments: (i) challenged untreated = *F. columnare* challenged fish treated with no kaolin, and (ii) challenged treated = fish challenged with *F. columnare* in the presence of kaolin (1 g L^{-1}) (obtained from KaMin, LLC; Macon), and two tanks of each of the following: (i) kaolin control = unchallenged and treated with 1 g L^{-1} kaolin, and (ii) unchallenged untreated fish (no kaolin). One tank from each of the challenged untreated and challenged treated groups was used for the time course sampling of gill for attachment/colonization (at 1 h and 24 h post-challenge) and for histopathology (24 h post-challenge). The remaining four tanks for these treatments were used to evaluate survival, and only dead fish were removed from these tanks during the study. Similarly, one tank from each of the kaolin control and unchallenged untreated fish was used to assess survival, while the additional tanks were dedicated exclusively to time course sampling. For kaolin treatments, kaolin (1 g L^{-1}) was slowly added to the water near the airstone to facilitate mixing within the tank. In kaolin-treated tanks, kaolin was added to water 5 min prior to

challenge to allow sufficient mixing time and the ultra-low flow was initiated. At dosing, the turbidity of kaolin-treated water was 921 formazin attenuation units (FAU) measured at a wavelength of 860 nm and the absorbance was 1.25 at 550 nm. At 4 h, the turbidity was 862 FAU and 0.85 at 550 nm, and at 24 h, the water had cleared substantially measuring 53 FAU and 0.11 at 550 nm. The concentration of kaolin was selected based on previous reports demonstrating that this dose was well tolerated in rainbow trout. In addition, preliminary studies at our laboratory determined that this concentration allowed for experimental fish to be monitored for clinical signs/mortality, while higher concentrations hindered observation (because of kaolin-mediated opacity/turbidity). The duration of the challenge experiment was 7 days. Animal care and experimental protocols were approved by the Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

Bacteriology

Fish were experimentally challenged with the virulent *F. columnare* isolate LSU-066-04 obtained from Dr. John Hawke (Louisiana State University). The isolate was retrieved from a glycerol stock preserved at −80 °C and streaked on Ordal's medium (Anacker & Ordal 1959). After 48 h, the isolate was dislodged from the agar using a sterile cotton swab and inoculated into 5 mL of *F. columnare* growth medium (FCGM; Farmer 2004). This suspension was incubated at 28 °C for 24 h and was used to inoculate 1 L of FCGM. The inoculated 1 L of broth was incubated for 24 h at 28 °C in an orbital shaker incubator set at 200 rpm; when the bacterial growth reached an absorbance of 0.75 at 550 nm, the flask was removed and placed on a stir plate at room temperature. Fish were challenged by adding 5 mL of the bacterial stock to each 10-L tank, with the exposed dose calculated to be 6.2×10^6 CFU mL^{-1}. Fish were observed twice daily to assess mortality.

Histopathology

Gill samples were collected and immediately fixed in 10% neutral-buffered formalin 24 h after the bacterial challenge. After 24–48 h of fixation, gills were briefly rinsed with water, transferred to 70% isopropanol and stored until routine paraffin embedding with a Leica TP1020 tissue processor. Tissues were sectioned with a Leica RM2135 microtome to 5–6 μm, mounted on slides and stained with haematoxylin and eosin. Gill tissues (obtained from three fish per experimental treatment) were evaluated in blinded fashion for pathological changes in kaolin-treated unchallenged fish, kaolin-treated challenged fish and untreated challenged fish.

Quantitative PCR for *F. columnare* quantification

At 1 h and 24 h after challenge, a section of the left second gill arch was collected (approximately 50 mg) from each fish ($n = 3$ fish per treatment group at each time point) for qPCR analysis to quantify bacteria on the gill as previously described (Farmer, Mitchell & Straus 2011; Beck *et al.* 2012; Farmer *et al.* 2012a,b). DNA extractions were performed according to the manufacturer's instructions using a DNeasy Blood and Tissue Kit (Qiagen). The extracted template DNA was used for pathogen detection, identity confirmation and quantification utilizing the primers of Panangala, Shoemaker & Klesius (2007) which were FcFp [5-CCTGTACCTAATTGGGGAAAAGAGG-3′], FcRp [5-CGGTTATGGCCTTGTTTATCATAGA-3′] and FAM labelled probe [5′-ACAACAATGATTTTGCAGGAGGAGTATCTGATGGG-3′]. This primer and fluorescent probe set targets a region of the chondroitin AC lyase gene of *F. columnare*. Primers and FAM labelled probe were obtained from Applied Biosystems Incorporated. Quantitative polymerase chain reaction (qPCR) assays were performed on a Lightcycler 480 Real Time PCR system (Roche Applied Science). All samples were run in duplicate. A standard (1.0×10^4 CFU mL^{-1}) and a control without extracted template (no-template control) were included on each plate; the standard was used as a positive control and to validate the internal standard curve within each run. Reactions included 500 nM of forward (FcFp) and reverse (FcRp) primers, a 250 nM labelled probe, 1 μL of template DNA, Bio-Rad 2X master mix (Bio-Rad Laboratories) and molecular grade water to give 20 μL total reaction volumes. The initial DNA denaturation step was 95 °C for 10 min, followed

by 45 cycles of 95 °C for 10 s and then 60 °C for 30 s. These data were calculated using a Roche Lightcycler 480 software macro for absolute quantification. A standard curve was applied which had been previously generated from bacterial samples grown in broth and then serially diluted and counted from 10^8 to 10^2. For normalization, the qPCR data for gills were divided by the amount of template DNA put into each reaction, and results are reported as CFU/ng of template DNA.

In vitro effects of kaolin on *F. columnare*

To examine the effects of kaolin on *F. columnare*, bacterial cells (10 uL of the bacterial challenge stock) were incubated in 2 mL of well water or in well water containing kaolin (1 g L^{-1}) for 1 h (four replicates per condition), similar to that previously described by Gunnison and Marshall (1937). After incubation, the preparations were gently centrifuged (47 ***g*** for 5 min) to pellet the kaolin, but not freely suspended bacterial cells. The supernatants were collected and subjected to quantitative PCR for bacterial enumeration and plated on Ordal's medium to examine culturability.

Statistical analysis

Survival data were analysed with SigmaPlot 11 using Kaplan–Meier log-rank survival analysis, and all pairwise multiple comparisons used the Holm–Sidak method with adjusted P values. Quantitative PCR data were analysed with SigmaPlot 11 using one-way ANOVA. Treatment effects were considered significant at $P \leq 0.05$.

Results

Survival

When kaolin was present in the water of challenged fish, survival was significantly improved ($P < 0.001$) over the 7-day duration of the study (Fig. 1). Survival was 96.3% in kaolin-treated challenged fish and 78.8% in untreated challenged fish. There was no mortality or any grossly visible abnormalities observed in unchallenged untreated negative control fish or unchallenged kaolin-treated fish. Moribund or freshly dead fish, irrespective of treatment, showed clinical appearances consistent with columnaris disease with focal to widespread depigmentation of the skin, frayed fins (mainly the caudal fin) and infrequent ocular lesions.

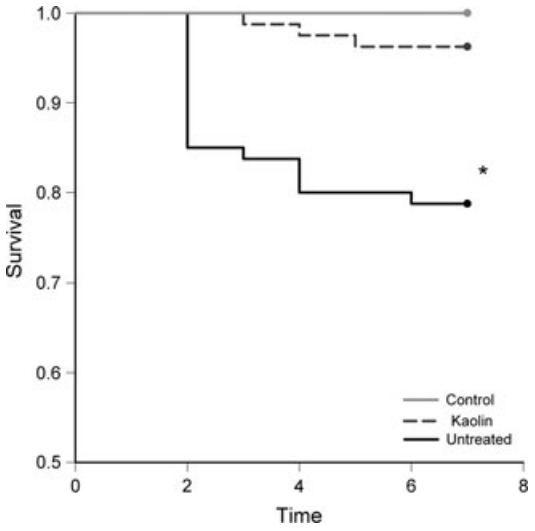

Figure 1 Kaplan–Meier survival analysis of channel catfish challenged with a lethal dose of *Flavobacterium columnare* and not treated (Untreated) or treated with 1 g L^{-1} kaolin (Kaolin). Control fish (grey line) were not challenged but were treated with kaolin. The asterisk indicates a significant difference between the untreated and kaolin-treated challenged group ($P < 0.001$).

Histopathology

Channel catfish that were challenged and not exposed to kaolin showed extensive gill damage at 24 h (Fig. 2). Lesions were characterized by marked hyperplasia that occasionally resulted in the complete fusion of adjacent lamellae and by the sporadic occurrence of focal oedema. The gills of kaolin-treated challenged fish were near normal in structure and infrequently showed evidence of mild hyperplasia. There were no detectable lesions in the gills of unchallenged kaolin-treated fish. No parasites were observed in any gill section.

F. columnare adhesion/colonization

The results from the qPCR analysis indicated that at both time points examined (1 h and 24 h), untreated challenged fish had significantly higher numbers of *F. columnare* cells (colony forming units/nanogram of template DNA) associated with the gill as compared to kaolin-treated fish (Fig. 3). At 1 h, challenged-untreated fish had significantly more *F. columnare*, $3.17 \times 10^4 \pm 1.2 \times 10^4$

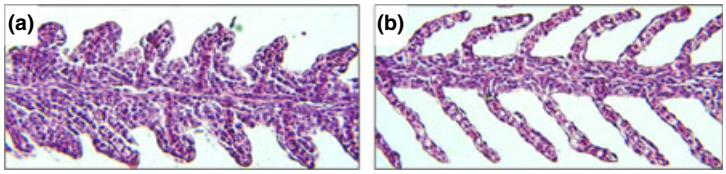

Figure 2 Representative photomicrographs of gill from channel catfish 24 h after experimental challenge with *Flavobacterium columnare* in (a) untreated water and in (b) water containing 1 g L^{-1} of kaolin. 200× magnification.

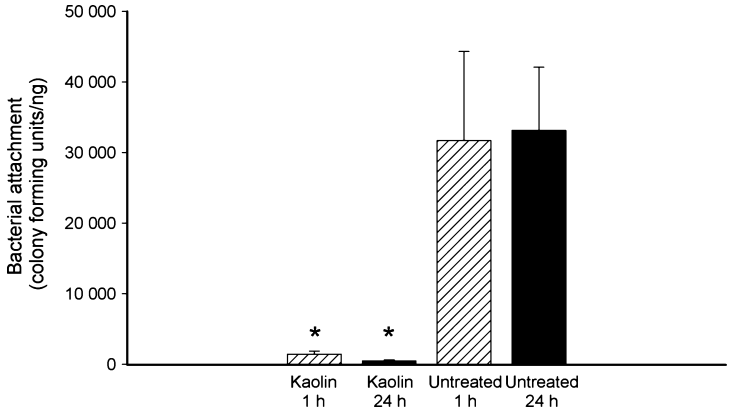

Figure 3 Kaolin may exert its protective effects by blocking adhesion of *Flavobacterium columnare* to the gill. Quantitative PCR (specific for the chondroitin AC lyase gene) results showing mean bacterial adhesion (colony forming units; CFU) on gill tissue at 1 h and 24 h after challenge in kaolin-treated and untreated fish. Asterisks denote statistically significant differences between kaolin-treated and untreated values at the respective time points indicated ($P \leq 0.05$).

CFU ng^{-1} vs. $1.43 \times 10^3 \pm 4.2 \times 10^2$ CFU ng^{-1} in kaolin-treated challenged fish ($P = 0.03$), while at 24 h, challenged-untreated fish also had significantly more *F. columnare* $3.3 \times 10^4 \pm 8.9 \times 10^3$ vs. $4.7 \times 10^2 \pm 1.3 \times 10^2$ as compared to kaolin-treated challenged fish ($P = 0.001$).

In vitro adsorption experiment

After gentle centrifugation, fewer *F. columnare* cells were present in the supernatant of kaolin-treated cultures vs. cultures incubated in kaolin-free conditions (Fig. 4). Kaolin treatment showed a two-log reduction in the number of bacteria in supernatant with $2.63 \times 10^5 \pm 4.28 \times 10^4$ CFU mL^{-1}, vs. untreated well water containing $3.04 \times 10^7 \pm 2.6 \times 10^6$ CFU mL^{-1}. Plating of the cultures onto Ordal's medium immediately after the 1 h incubation revealed that both untreated and kaolin-treated bacterial cultures remained viable as evidenced by extensive colony growth morphologically consistent with *F. columnare*.

Discussion

Various surface-acting compounds or disinfectants including copper sulphate ($CuSO_4$), potassium permanganate ($KMnO_4$), diquat (6,7-dihydrodipyrido[1,2-a:2′,1′-c]pyrazinediium dibromide), hydrogen peroxide (H_20_2) and chloramine-T have demonstrated mixed efficacies against columnaris disease in both prophylactic or therapeutic situations (Wakabayashi 1991; Thomas-Jinu & Goodwin 2004; Darwish, Mitchell & Hobbs 2008; Darwish & Mitchell 2009; Farmer *et al.* 2012a,b; Farmer *et al.* 2013; Bowker *et al.* 2013). Limitations such as regulatory issues, water quality/chemistry, safety/toxicity and the challenge models and target species by which these compounds were tested for effectiveness may impede their use in production settings.

For example, in the context of disease prevention for channel catfish, $CuSO_4$ treatments to fingerling channel catfish have resulted in improved survival when administered prophylactically at precise time points before a columnaris disease challenge, but exacerbate mortality when a columnaris challenge is performed too soon after the prophylaxis, which was linked to $CuSO_4$ damaging the respiratory epithelium (Farmer *et al.* 2013). Moreover, both the effectiveness and toxicity of $CuSO_4$ are strongly limited by water chemistry. Copper ions can bind rapidly to organic and inorganic materials, thereby reducing the longevity of copper

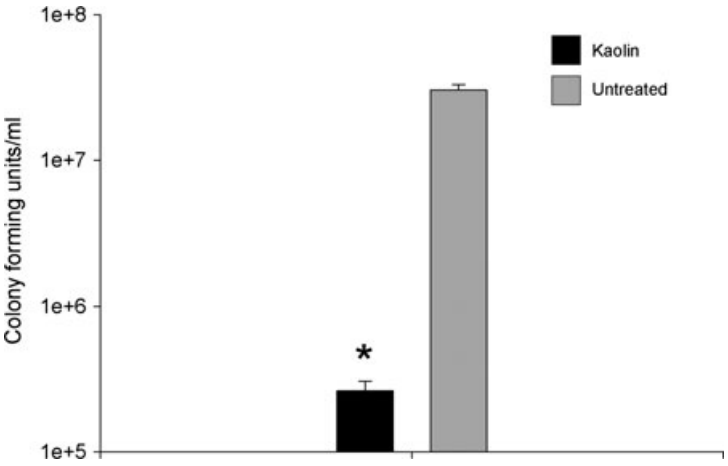

Figure 4 *Flavobacterium columnare* cells were incubated in the absence (untreated) or presence of 1 g L^{-1} kaolin for 1 h. After which, the preparations were centrifuged at 500 rpm for 5 min. The number of bacterial cells remaining in supernatants after centrifugation was determined using a quantitative PCR assay for the *F. columnare* chondroitin AC lyase gene. The asterisk denotes a significant difference between untreated and kaolin-treated cultures ($P \leq 0.05$).

in the environment and concomitantly its ability to exert therapeutic effects (Farmer *et al.* 2013). Furthermore, copper toxicity is closely linked to water chemistry parameters, specifically hardness and alkalinity, with toxicity becoming more problematic in softer waters. Much like $CuSO_4$, water quality metrics, such as the level of organic matter, need to be carefully considered before using compounds such as $KMnO_4$, which is a potent oxidizer that has shown mixed results against columnaris disease. Much like $CuSO_4$, the timing of a treatment is imperative with $KMnO_4$ as evidenced by studies where exposing fish to $KMnO_4$ simultaneously with a challenge dose of bacteria showed a clear benefit, but when $KMnO_4$ treatments were administered at later time points, no therapeutic benefit was evident (Darwish *et al.* 2008; Darwish, Mitchell & Straus 2009; Farmer *et al.* 2012a,b).

In contrast to the powerful oxidizers discussed above, the chemically and biologically inert clay kaolin may offer fewer negative effects on fish health. Similar to the present study, other studies have documented an absence of microscopic lesions in the gill of kaolin-exposed fish. Histological evaluation of the gills of juvenile rainbow trout exposed to ~1 g L^{-1} kaolin for 64 days was normal in architecture (Goldes *et al.* 1988), a much longer duration than the 7-day period used in the present study. Only when the fish were exposed to >4 g L^{-1} for long duration were any branchial lesions observed, yet these findings were attributed to the presence of an *Ichthyobodo necator* outbreak during the study, and not directly linked to kaolin (Goldes *et al.* 1988). An additional benefit of kaolin is that there is no risk of antibiotic resistance, or harmful chemical residues being introduced into the environment or the eventual consumer product. Furthermore, the United States Food and Drug administration classifies kaolin as generally recognized as safe for use as an indirect human food ingredient (Code of Federal Regulations, 21CFR186.1256).

In the present study, kaolin treatment markedly reduced the number of *F. columnare* cells initially adhering to (1 h post-challenge) and colonizing the gill (24 h post-challenge). Importantly, the adhesion of the *F. columnare* to host cells is considered to be an essential prerequisite to disease initiation (Decostere *et al.* 1999a,b). Firm bacterial adhesion enables the pathogen to withstand cleansing mechanisms operating on the surfaces of the gill, such as the resistance to water flow, mucus secretion and the constant cellular turnover of the respiratory epithelium (Decostere *et al.* 1999a,b). Additionally, in comparison with the aqueous environment, the gill surface may serve as a nutrient-rich environment, offering growth advantages to the pathogen (Decostere *et al.* 1999a,b). By forming physical associations with the bacterial cells, presumably through adsorption, kaolin effectively reduced adhesion to respiratory surfaces which may have contributed to the improved survival. Such adsorptive characteristics could be exploited in a way to reduce the number of bacterial pathogens in culture systems, particularly if methods could be developed to filter or siphon bacterial–kaolin complexes from culture systems or to use kaolin in concert with other compounds with bacteriostatic/bacteriocidal activities that could induce cytotoxicity to kaolin-bound bacteria. In addition, while the present study was

conducted with a single species of fish, kaolin could afford protection against columnaris disease across a range of fish species because of its direct adsorptive interactions that bind and prevent bacterial attachment, a mechanism that is likely independent of host fish species. As mentioned previously, other *Flavobacteria,* such as those native to soil, have been shown to be strongly adsorbed by kaolin. Therefore, kaolin may bind to and protect host fish from other aquatic *Flavobacteria* of concern, such as *F. psychrophilum* or *F. branchiophilum*, pathogens of global importance, particularly in the culture of Salmonids. Future studies should explore the utility of kaolin towards other *Flavobacteria*, as well as other problematic bacterial pathogens of fish.

There could be some shortcomings to kaolin based on our results demonstrating that kaolin precipitated bacteria out of suspension, yet they remained viable. This viability could potentially lead to an outbreak or recurrence of the disease at a later time point. In addition, kaolin-treated water showed appreciable turbidity, which could be problematic by making it difficult to visualize dead or moribund fish and recognize an outbreak. Such turbidity may also limit the application of kaolin to pond settings as it could block photic penetration and negatively affect algal blooms.

The present investigation was designed as a proof-of-concept study and not an exhaustive analysis on different kaolin formulations, doses and durations. Future studies should compare such experimental permutations which may further improve the efficacy of kaolin and also evaluate the potential of kaolin in other fish species. Nevertheless, the findings presented here suggest that the application of kaolin to some production settings may be beneficial. Particularly, in scenarios where columnaris outbreaks have been shown to occur, such as after the grading, stocking or transport of fish, prophylactic kaolin treatments may improve disease-free survival in these situations.

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