High Concentration of Red Clay as an Alternative for Antibiotics in Aquaculture

Jaejoon Jung1, Seung Cheol Jee2, Jung-Suk Sung2, and Woojun Park1*

1Laboratory of Molecular Environmental Microbiology, Department of Environmental Science and Ecological Engineering, Korea University, Seoul 02841, Republic of Korea
2Department of Life Science, Dongguk University, Seoul 04620, Republic of Korea

The use of antibiotics in aquaculture raises environmental and food safety concerns because chronic exposure of an aquatic ecosystem to antibiotics can result in the spread of antibiotic resistance, bioaccumulation of antibiotics in the organisms, and transfer of antibiotics to humans. In an attempt to overcome these problems, high-concentration red clay was applied as an alternative antibiotic against the following common fish pathogens: *Aeromonas salmonicida*, *Vibrio alginolyticus*, and *Streptococcus equinus*. The growth of *A. salmonicida* and *V. alginolyticus* was retarded by red clay, whereas that of *S. equinus* was promoted. Phase contrast and scanning electron microscopy analyses confirmed the attachment of red clay on cell surfaces, resulting in rapid gravitational removal and cell surface damage in both *A. salmonicida* and *V. alginolyticus*, but not in *S. equinus*. Different cell wall properties of gram-positive species may explain the unharmed cell surface of *S. equinus*. Significant levels of oxidative stress were generated in only the former two species, whereas significant changes in membrane permeability were found only in *S. equinus*, probably because of its physiological adaptation. The bacterial communities in water samples from *Oncorhynchus mykiss* aquacultures supplemented with red clay showed similar structure and diversity as those from oxytetracycline-treated water. Taken together, the antibiotic effects of high concentrations of red clay in aquaculture can be attributed to gravitational removal, cell surface damage, and oxidative stress production, and suggest that red clay may be used as an alternative for antibiotics in aquaculture.

Keywords: Biosafety, cell membrane, community analysis, oxidative stress, pyrosequencing

Introduction

Antibiotics are frequently used in aquaculture to reduce economic loss caused by fish diseases, including those due to parasitic infestations and bacterial infections [15, 19]. Fish are cultured at high densities in fish farms to maximize profit, and the densities are maintained by the use of antibiotics to control the spread of pathogens [4]. Antibiotics used in aquaculture are a source of antibiotics that are prevalent in the natural environment. The presence of antibiotics in the environments has not drawn public attention, and attempts to assess the effect of their presence have only recently begun [16]. A survey performed in a stream near fish farms using antibiotics showed that the concentration of antibiotics in the stream was lower than the minimum inhibitory concentration and the predicted no effect concentration [21]. However, depending on the environment, microbial communities and their activities could be negatively affected by antibiotics at concentrations relevant to aquaculture [9].

Chronic effects of antibiotics in the natural environment have not been evaluated. Leaked antibiotics might contribute to enrichment of antibiotic resistance in pathogenic bacteria [30]. Accumulated antibiotics in sea animals such as *Octopus vulgaris* could have caused multidrug resistance of *Vibrio* strains isolated from *O. vulgaris* [34]. The use of antibiotics...
bacterial species were selected owing to the significant economic losses resulted from their infections in cultured *Oncorhynchus mykiss*. *A. salmonicida*, *V. alginolyticus*, and *S. equinus* are the causative agents of furunculosis, vibriosis with gastroenteritis, and streptococcosis, respectively [5-7]. This is the first report on the use of red clay in aquaculture as an alternative for antibiotics.

**Materials and Methods**

### Bacterial Strains and Growth Test

*Aeromonas salmonicida* subsp. *salmonicida* KCCM 41055, *Vibrio alginolyticus* KCCM 40513, and *Streptococcus equinus* KCCM 11930 were purchased from Korean Culture Center of Microorganisms (KCCM). *A. salmonicida*, *V. alginolyticus*, and *S. equinus* were grown in nutrient broth (NB), marine broth (MB), and tryptic soy broth (TSB) at 25°C, 30°C, and 37°C, respectively, unless otherwise described. Growth curves were determined by protein concentrations using a Bradford assay, because the turbidity of the red clay was an obstacle to measuring optical density. Approximately $10^6$ CFU/ml from overnight cultures of each bacterial species was supplemented with red clay in preparation for the assay.

### Microscopic Observation

To determine morphological changes of cells in the presence of red clay, cells in the exponential phase were observed using a phase contrast microscope (Imager A1, Zeiss) and scanning electron microscope (Quanta 250 FEG, FEI). Cells in the exponential growth phase were incubated with red clay for 15 min, and damage to cell surfaces was observed before the cells had recovered or undamaged cells began to proliferate. Cells were fixed by Karnovsky’s fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M phosphate buffer (pH 7.2) for 2 h and then dehydrated with increasing concentrations of ethanol (from 30% to 100%). Fixed cells were placed onto an aluminum stub and dried overnight.

### Removal of Bacterial Cells from Surface Water by Red Clay

To determine if red clay could gravitationally remove bacterial cells from bulk water by aggregation with cells, the number of cells in the supernatant was counted after centrifugation of the mixture of cells and red clay. Bacterial cells were harvested in the exponential growth phase. Cells and red clay (5% (v/v)) were vortexed together for 1 min to allow contact. After centrifugation at 500 $x_g$, the number of cells was measured from serially diluted supernatant spread on an agar plate.

### Fatty Acid Methyl Ester (FAME) Analysis

To investigate the permeability of bacterial cell membranes, the ratio of saturated and unsaturated fatty acids was determined by FAME analysis. Red clay was added to culture media in the exponential growth phase of bacterial cells. Cells were allowed to

in aquaculture often results in the bioaccumulation of antibiotics in the target cultured fish and in wild fish, which can be a serious problem because of the potential for these antibiotics to be transferred to humans [4, 37]. The biosafety of cultured fish grown in such conditions is not well defined. Some aquatic organisms have been subjected to the detrimental effects of antibiotics, suggesting that toxic effects in humans could also be possible [25]. In addition, chemicals such as disinfectants and pesticides have unfavorable effects on target fish, food quality, and biosafety [24].

To overcome the environmental and health risks of antibiotics used in aquaculture, trials of various treatments such as vaccination, phage therapy, and immune status-improving diet [17, 23, 29] have been conducted. As an alternative to antibiotics, organic compounds derived from plants have also been used [32]. Such phytotherapies in aquaculture are preferred over synthetic chemicals because there are low environmental risks and side effects on fish and human health. Inorganic minerals are another alternative that has low environmental risk. An application of red clay (also known as yellow loess) in rainbow trout (*Oncorhynchus mykiss*) culture was performed, and it showed that red clay promoted the growth rate, and lysozyme and superoxide dismutase activities of rainbow trout as much as those of rainbow trout grown treated with oxytetracycline [18]. Higher survival rates of rainbow trout infected with *Aeromonas salmonicida* support the effectiveness of red clay in aquaculture. However, the use of red clay to replace antibiotics in preventing bacterial infections has not been investigated. Our previous study identified concentration-dependent effects of red clay on bacterial growth. At low concentrations, red clay can promote the growth of soil bacterial species and affect the bacterial community structure [12]. In the case of the diesel-degrader *Acinetobacter oleivorans* DR1, the expression of genes related to alkane and fatty acid metabolism and oxidative stress defense was upregulated by low concentrations of red clay (0.1% (v/v)) [13]. Our unpublished data suggested that red clay at high concentrations had unfavorable effects on bacterial growth, the soil bacterial community, and soil enzyme activity.

The goal of this study was to provide detailed scientific evidence to support the use of red clay as an alternative to antibiotics in aquaculture. To achieve this goal, the toxic effects of high-concentration red clay (HCRC) on pathogenic fish bacteria such as *A. salmonicida*, *Vibrio alginolyticus*, and *Streptococcus equinus* were examined, and the aquatic bacteria community from water samples from *Oncorhynchus mykiss* culture was analyzed after the application of HCRC.
grow 1 h after the addition of red clay, allowing time for changes in fatty acid composition that reflected changes in cellular processes such as gene expression and fatty acid metabolism. FAME samples were prepared according to MIDI Technical Note No. 101. FAMEs were analyzed using gas chromatography equipped with a flame ionization detector (Agilent 7890) and the Sherlock Microbial Identification System. The permeability index was calculated as the ratio between unsaturated and saturated acids.

Nitro Blue Tetrazolium (NBT) Assay
Superoxide anion radicals produced in the presence of red clay were measured using an NBT assay [1]. Red clay was added to bacterial cells in the exponential growth phase and incubated for 15 min only. Prolonged incubation after the addition of red clay could allow the cells to express oxidative stress defenses and the red clay-induced oxidative stress would be inaccurately measured. Cells were harvested by centrifugation, and the cell pellet was resuspended in 0.5 ml of 1 mg/ml NBT for 30 min. Then, 0.1 ml of 0.1 M HCl was added, and the cells were centrifuged at 15,000 × g for 1 min. Dimethyl sulfoxide (0.4 ml) was added to the cell pellet to solubilize the reduced NBT. The absorbance was measured at 575 nm and normalized by the protein concentration measured from the same number of cells on the same sample.

Water Sampling, DNA Extraction, and Bacterial Community Analysis by 454 Pyrosequencing
To investigate the bacterial community structure in rainbow trout (Oncorhynchus mykiss) aquaculture, water samples were obtained from a local fish farm (E-hwajung Aquaculture, Sangju, Republic of Korea). Aquaria (300 L each) had semi re-circulating systems receiving freshwater at a flow rate of 2 l/min. The oxygen concentration was maintained at almost saturation by an aeration system. The water temperature and pH were maintained at 15 ± 1°C and 7.5 ± 0.3, respectively. Water was treated with red clay at a concentration of 2%, and was sampled after 12 weeks of culture.

To isolate DNA from water samples, water (4 L) was filtered through a 0.22 µm nitrocellulose membrane (Millipore, USA). The membrane was cut into small pieces and subjected to DNA isolation using a NucleoSpin Soil kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions. For the bacterial community analysis, I6S rRNA genes were amplified using the 27F and 516R universal primers with barcode sequences targeting the V1–V3 region of bacterial rRNA. Sequencing library construction and all sequencing procedures were conducted by Macrogen (Republic of Korea). Low quality and short sequencing reads were omitted from the raw data file produced using the Initial Process function in RDPipeline of the Ribosomal Database Project (RDP) [33]. Taxonomic classification was performed using the Classifier function in RDPipeline. Shannon and Chao1 diversity indices were calculated from a complete linkage cluster file using RDPipeline. Finally, a rarefaction curve was calculated from RDPipeline and visualized using Microsoft Excel.

Results

Adverse Effects of HCRC on the Growth of A. salmonicida and V. alginolyticus
Our previous study determined that low concentrations of red clay (0.1% (v/v)) facilitated growth of well-known soil bacteria, including Pseudomonas putida, Cupriavidus necator, and Acinetobacter oleivorans DR1 [12]. However, HCRC appears not to be beneficial for the growth of two bacterial species used in this study. To confirm the adverse
effects of HCRC, growth curves were determined for the fish pathogens *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio alginolyticus*, and *Streptococcus equinus* in the presence of various concentrations of red clay (Fig. 1). The growth of *A. salmonicida* and *V. alginolyticus* was not significantly affected by 1% (v/v) red clay. Although growth was not totally inhibited at this concentration, it was retarded by 5% and 10% red clay. On the contrary, the growth of *S. equinus* was enhanced, and the maximum protein concentration was higher with the addition of red clay at all concentrations, suggesting that different bacterial species can be affected differently by HCRC.

**Attachment of Red Clay Particle to Cells and the Gravitational Precipitation of Bacteria**

To identify morphological changes or damage to cell surfaces, cell morphologies were observed under phase contrast and scanning electron microscopes (Figs. 2 and 3). Apparent attachment of red clay particles to *A. salmonicida* cells was observed with 5% and 10% red clay, and *A. salmonicida* was elongated with 1% red clay. Red clay also attached to *V. alginolyticus* cells; however, they did not experience cell elongation. On the contrary, *S. equinus* did not attach to red clay particles and showed no morphological changes. As seen in Fig. 2, the size of a red clay particle is much smaller than that of a cell. Cell surface damage was suspected as the cause for retarded growth in the presence of red clay particles. To confirm this hypothesis, cell surfaces were observed via scanning electron microscopy (SEM). SEM images indicated agglomerated red clay particles around cells. The size of agglomerates varied from approximately 150 to 400 nm; hence, an individual particle would be much smaller (Fig. 3). The cell surfaces of *A. salmonicida* and *V. alginolyticus* were smooth in the exponential growth phase with no red clay added. However, with red clay, the cell surfaces became rough. The rough surfaces are believed to reflect damage from red clay particles rather than morphological changes, because the size of an individual clay particle is comparable to previously described nanosize particles that resulted in physical damage on cells surfaces [11]. The cell surface of *S. equinus* was not affected.

In addition to morphological changes and cell surface damage, attachment of red clay may affect the physical behavior of cells in liquid. For example, cells can be easily removed from surface water by gravity. To test this possibility, the number of cells in the supernatant was counted after centrifugation at 500 × *g*, which did not rapidly

![Fig. 2. Phase contrast microscopy images of *A. salmonicida*, *V. alginolyticus*, and *S. equinus* grown in various concentrations of red clay.](image)
remove cells from the supernatant within a short time (Fig. S1). Seventy-nine percent of *A. salmonicida* and 75% of *V. alginolyticus* cells remained in the supernatant after 1 min of centrifugation. However, when the red clay was added, only 10.0% and 22.8% of *A. salmonicida* and *V. alginolyticus* cells, respectively, remained in the supernatant after 1 min of centrifugation. There was no difference in *S. equinus* cells in suspension after the addition of red clay. These results imply that the addition of red clay may reduce the chance of contact between bacterial pathogens and fish by aiding in gravitational precipitation through the formation of red clay-cell aggregates.

**Oxidative Stress Induced by HCRC in *A. salmonicida* and *V. alginolyticus***

After SEM images of *A. salmonicida* and *V. alginolyticus* confirmed membrane damage by red clay, and following a report that membrane perturbation is a causative factor in oxidative stress, we investigated the generation of oxidative stress in microorganisms when red clay was treated [28]. An NBT assay performed after the exposure of bacterial cells to red clay revealed markedly increased oxidative stress in *A. salmonicida* and *V. alginolyticus*, whereas the response of *S. equinus* was not significantly different (Fig. 4). The highest absorbance was seen with 5% red clay. This result showed that cells were experiencing oxidative stress after the contact with red clay.

**Increased Membrane Fluidity in *S. equinus* as an Adaptive Physiology**

Bacteria can modify the biophysical properties of their membranes by altering their fatty acid composition [36]. Alteration of the fatty acid structures in bacterial membrane results in different permeability, allowing them to cope with abrupt environmental changes that cause various stresses. The fatty acid compositions of bacterial membranes were analyzed to determine changes in permeability with the addition of red clay (Fig. 5). The membrane permeability, calculated as the ratio of unsaturated to saturated fatty acids, was not significantly changed by red clay in *A. salmonicida* and *V. alginolyticus*. *S. equinus* showed an increase in permeability with the addition of >1% red clay. Increases in membrane permeability are attributable to a decrease in the major saturated fatty acids, such as C14:0, C16:0, and C18:0, or an increase in the major unsaturated fatty acids.
fatty acid, C14:1 ω5c (data not shown).

Bacterial Community of Aquaculture Samples Supplemented with HCRC

Based on our data, we hypothesized that red clay would cause stress and affect bacterial physiology in a species-dependent manner; hence, we expected that the bacterial community structure would also be influenced by red clay in an aquaculture system. To evaluate this possibility, water samples were collected from *Oncorhynchus mykiss* aquacultures supplemented with red clay instead of antibiotics. Barcoded pyrosequencing data showed that Proteobacteria, Actinobacteria, and Bacteroidetes were the major phyla, representing 78.3% of total sequences (Fig. 6). A large portion of bacteria (20.7%–25.2%) was not classified into any phylum, suggesting that the bacterial ecology in aquaculture has not yet been fully described. *Limnohabitans* was the predominant genus (34.5%), followed by *Solimonas*, *Perlucidibaca*, and *Acinetobacter* in a control sample. The relative abundance of *Limnohabitans* was increased to 64.3% and 54.1% in red clay-added and oxytetracycline-added water samples, respectively. *Solimonas*, the second-most abundant genus in the control, disappeared with the addition of red clay and oxytetracycline. Rarefaction curves of the three communities (control, red clay, and oxytetracycline) showed a reduction in bacterial diversity with red clay and oxytetracycline treatment (Fig. S2). In accordance with the rarefaction curves, the Shannon and Chao1 diversity indices were reduced in red clay- and oxytetracycline-treated samples (Table S1). Subsequently, the community analyses and diversity measurements showed similar results.

**Discussion**

The toxicity of nanoparticles, and particularly metal nanoparticles such as ZnO, in bacterial cells has been previously investigated, and the extent of cellular damage was related to the size, shape, and surface properties of these particles [22]. Many types of particles that cause biocidal effects are nanoscale; however, microparticles can also have toxic effects [20]. SEM images showed that red clay contained particles of nanometer size and they could form aggregations of micrometer scale. Therefore, cell damage on the membrane surface was the first expected detrimental effect of red clay. However, not all bacterial species suffer from cell surface damage, as shown by *S. equinus* in this study. Evasion of cell damage by *S. equinus* could be due to the rigidity of its peptidoglycan layer, consisting of cross-linked polysaccharide chains [27]. In contrast, the exterior lipopolysaccharide layers of gram-negative bacteria do not provide this strength and rigidity.
[2]. Consistent with this difference, less toxic effects of silver nanoparticles on gram-positive than gram-negative bacteria have been reported previously [8].

It was worth noting that S. equinus showed faster growth and reached a higher maximum protein concentration in the presence of red clay (Fig. 1), suggesting a beneficial effect of red clay on this species. Our previous investigation showed that red clay could promote the growth of soil bacteria and diesel biodegradation [12]. Transcriptomic analysis of Acinetobacter aleivorans DR1 indicated that genes related to fatty acid metabolism and oxidative stress defense were highly upregulated in hexadecane-degrading conditions with red clay [13]. Therefore, S. equinus could experience a positive effect on growth, metabolism, and stress defense from red clay.

The oxidative stress generated in membrane-damaged A. salmonicida and V. alginolyticus is consistent with previous reports showing that nanoparticles and cell membrane damage caused oxidative stress in bacterial cells [3, 31]. The oxidative stress induced by cell damage could be one reason for the retarded growth of A. salmonicida and V. alginolyticus with the addition of red clay (Fig. 1). Transcriptomic analysis of A. oleivorans DR1 in the presence of red clay showed the upregulation of cyclopropane fatty acyl phospholipid synthase, the increase in membrane permeability, and a concomitant promotion of bacterial growth [13]. Not only the membrane fatty acid composition, but also the expression of various transporters could change in response to red clay, suggesting that adjustments between the extracellular and intracellular environments may have been needed. Therefore, increased membrane permeability, inferred from the ratio of unsaturated and saturated fatty acids in S. equinus, with the addition of red clay can be considered an adaptive physiological change of the organism.

The bacterial community structure and diversity in red clay- and oxytetracycline-supplemented water samples were similar, even though the modes of action of the two compounds in bacterial cells are very different: red clay causes cell membrane damage followed by oxidative stress, whereas oxytetracycline interferes with translation by binding to the 30S ribosomal subunit. The disappearance of Solimonas, which was the second most dominant genus in the control sample, was the most apparent difference between the control samples and the red clay and oxytetracycline samples. This could be explained by the susceptibility of Solimonas to the unfavorable condition created by red clay and oxytetracycline, causing oxidative stress and antibacterial activity, respectively. Solimonas is believed to be sensitive to oxidative stress caused by red clay and tetracycline treatments, because previous Solimonas studies have reported that catalase-negative strains such as Solimonas terrae and its catalase and oxidase activities might fluctuate depending upon the experimental conditions [14, 26]. Susceptibility to tetracycline in Solimonas has been reported [26]. On the contrary, Limnohabitans could survive antimicrobial activity owing to its resistance [10, 35].

In conclusion, this study suggested that red clay could be a possible alternative to antibiotics in aquaculture. Cell surface damage and oxidative stress appeared to be the major detrimental effects of red clay on bacterial cells, although the extent of stress may vary by species. These effects on individual cells may lead to a change in bacterial community structure, similar to that seen in the oxytetracycline-supplemented water sample. More detailed investigations will be needed to determine acceptable replacements for antibiotics that do not compromise food safety.

Acknowledgments

The authors declare that there are no conflicts of interest. This research was supported by a grant (15162MFD669) from the Ministry of Food and Drug Safety in 2015.

References


