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# The impact of a mixture of biofloc fermentation medium and vinasse on attractability, palatability, and antibacterial properties against multi-antibiotic resistant *Aeromonas veronii*

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**Abstract** The diverse natural products have been developed to replace antibiotics and chemicals used in aquaculture. The discovered products mostly contribute to the enhancement of aquatic animal immunities and control bacterial pathogens. However, in-feed application of these substances has been noted for its negative effects on attractability and palatability. A mixture of biofloc fermentation medium after harvesting the biomass and vinasse, residues from bioethanol production, (PBFCM) was developed and tested for its potential. The results from the *in vivo* trial revealed that PBFCM was able to increase the attractability and palatability of soybean meal and herbivorous fish feed (25% protein) in juvenile Nile tilapia (*Oreochromis niloticus*). Moreover, PBFCM was capable of inhibiting the growth of the multi-antibiotic resistant *Aeromonas veronii*, a pathogenic bacterium which causes losses in freshwater aquaculture. The obtained results showed that PBFCM not only increased the consumption of feedstuff but also exhibited inhibitory activity. This finding may contribute to sustainable aquaculture by delivering an effective natural product sourced from valueless materials.

**Keywords:** Attractability, Biofloc fermentation medium, Multi-antibiotic resistant *Aeromonas veronii*, Palatability, Vinasse

## Introduction

Bacterial diseases have long been a problem causing devastating losses in aquaculture. Many species of bacteria belonging to the genus *Aeromonas*, gram-negative short rod shaped bacteria, are known to be pathogenic bacteria that cause serious problem worldwide. Recently, *A. veronii* has been reported as a major isolate of *Aeromonas* causing economic losses in several freshwater fishes (Zhang *et al*., 2018) including Nile tilapia (*Oreochromis niloticus*) in

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Thailand (Dong *et al*., 2015; 2017; Chirapongsatonkul *et al*., 2019). The tool commonly used to overcome bacterial diseases is the application of antibiotics as prophylactic and therapeutic agents; however, extensive use has contributed to the development, persistence, and spread of antibiotic resistant bacteria (Cabello *et al*., 2013). Alternative substances including natural products from potent herbal plants and effective microorganisms responsible for the control of pathogenic bacteria have therefore been investigated to replace antibiotics (Hai, 2015). Moreover, these products have been developed in order to use as immunostimulant, anti-stress, and growth promoting substances in aquatic animals (Chang, 2000; Citarasu, 2010).

Diverse routes are used to deliver the effective substances to aquatic animals, for instance, injection into the animals, mixed into rearing water, immersion for a short period, and supplementation of these products in an animal feed (Hai, 2015). The latter has been considered as the most practical procedure since it can deliver either a single substance or a combination or mixture of several components. However, most natural products, especially those from plants or plant extracts, have an unfavorable taste or smell. This may cause adverse effects on the reduction of intake in aquatic animals. As a result, this has led to a search for natural products containing effective antimicrobial activity which also promote animal intake. Other natural substances with the desired bacterial inhibitory activity and known as natural feed for aquatic animals have been highlighted.

Biofloc technology (BFT) is a concept of using excess nutrients accumulated in the aquaculture environment or culture pond as feed for microbial growth, and then these microorganisms are used as natural feed for aquatic animals. BFT was first developed in 1975 by AquaCop while the biofloc system was improved by Avnimelech in 1999 by adding carbohydrates as a carbon source (Liu *et al*., 2019). Biofloc is a heterogeneous complex composed of organic particles and beneficial microorganisms associated with extracellular polymeric substances (De Schryver *et al*., 2008; Ray *et al*., 2010). The microorganism community grown in this system is dynamic changing according to the nutrients and environmental conditions, however the underlying mechanisms that regulate BFT remain unclear. It has been reported that microbial biofloc is a quality feed for aquatic animals. Moreover, microorganisms and their components have been shown to be effective as probiotics and immunostimulants enhancing host immunity, antioxidant status, and disease resistance in aquatic animals (Smith *et al*., 2003; Vazquez *et al*., 2009). In addition, yeast products and yeast-containing feed ingredients are commonly used as an effective natural feed, protein source, and probiotic in the feed of animals and aquaculture animals (Shurson, 2018). Yeast biomass is

harvested from the fermentation process or is cultured on byproducts of certain industries such as vinasse from the biorefinery process and ethanol production (Nitayavardhana and Khanal, 2010; Reis *et al*., 2019).

Accordingly, biofloc and its extracellular medium as well as the vinasse have good characteristics and could indeed be valuable substances for our aforementioned purpose. The present study was designed to test the efficacy of the developed product, the mixture of biofloc fermentation medium and vinasse (PBFCM), as an antibacterial substance against a multi-antibiotic resistant *A. veronii*. This bacterial strain was isolated from the motile *Aeromonas* septicemia (MAS) exhibiting in Nile tilapia in Southern Thailand and was previously tested for antibiotic susceptibility against various drugs of the tetracycline group. Since biofloc fermentation medium and vinasse, alone and in combination, likely have the smell of aquatic animal feed, the feasibility of using the combined mixture as a feed additive was tested using Nile tilapia as a model. The attractability was verified using soybean meal, a plant protein source which is typically used to replace fishmeal, while the palatability was determined using herbivorous fish feed (25% protein) that normally shows low intake levels.

## Materials and Methods

### *Preparation of PBFCM*

Biofloc was produced in an aerator fermentation system (AFS) using a mix of cassava starch and molasses in a ratio of 1:1 as carbon source following the procedure described by Wattitum *et al*. (2018). After 20 days, the biofloc biomass was separated by gravity sedimentation process for approximately 30 min followed by centrifugation at 8,000 × *g* for 15 min at 4 ℃. The supernatant representing the biofloc fermentation medium was collected at −20 ℃. Vinasse, residue from bioethanol production, was obtained from Yeast Master Co., Ltd. The vinasse was centrifuged at 8,000 × *g* for 15 min at 4 ℃ and collected at −20 ℃.

The biofloc fermentation medium was gently mixed with vinasse. To achieve complete dissolution of the mixture, 0.1% (v/v) of Triton X-100 was added. The mixture was aliqouted into a small volume prior to collection at −20 ℃ for further analyses.

### *Characterization of PBFCM*

The product PBFCM was analyzed for total protein, total carbohydrate, and total phenolic compounds. The protein amount was determined following

the Lowry method (Lowry *et al*., 1951) using BSA as standard and an absorbance was read at 640 nm. Total carbohydrate was determined by the phenol sulfuric acid method following Dubois *et al*. (1956) using glucose as standard and the absorbance was read at 490 nm. Total phenolic content was measured using Folin–Ciocalteu reagent following Gutfinger (1981) and Ziestin and Ben-Zaker (1993) and the absorbance read at 750 nm. The absorbance of all reactions was read in a microplate absorbance reader, EZ Read 2000 Microplate Reader (Biochrom, UK).

***Evaluation of antibacterial activity***

The pathogenic bacterium, *A. veronii* isolate I15, used in this study, was previously characterized for its species through biological and molecular aspects and its antibiotic resistance profile against drugs of the tetracycline group (Mueangkan *et al*., 2019). *A. veronii* isolate I15 was grown from stock culture frozen at −80 ℃ by culturing on tryptic soy agar (TSA, Difco) and incubation overnight at 35 ℃. Single colony was transferred into tryptic soy broth (TSB, Difco) with continuous shaking at 250 rpm at 35±2 ℃ for 18 h. The bacterium was transferred into the freshly prepared media containing TSB and 0.85% (w/v) NaCl at a ratio of 1:1 and adjusted to a final concentration of $2 \times 10^6$ CFU/ml.

Three independent sets of experiments were performed in order to establish the antibacterial activity of the mixture of biofloc fermentation medium and vinasse at the different concentrations, 2.5%, 5.0%, and 7.5%. Each set comprised 3 treatments: positive control which referred to the medium without the mixture; mock control representing the medium containing Triton X-100; and the test representing the medium with the mixture at the desired concentration. The product PBFCM was filtered through membrane filter, pore size 0.45 μm (Whatman), prior to being added into the medium. The growth of *A. veronii* isolate I15 in all treatments was observed in the controlled temperature incubator (RTS-1C Personal bioreactor, Biosan) under 32 ℃ for 24h.

***Fish used in the experiment***

Juvenile Nile tilapia (mean weight 2±0.2 g) were obtained from a commercial supplier in Nakhon Si Thammarat Province. Fish were acclimatized in a plastic aquarium tank containing 3,000 l of freshwater under controlled laboratory conditions in accordance with the institutional guidelines for the use of animals and the Ethical Principles and Guidelines for the Use of

Animals for Scientific Purposes (Ethical Principles) (National Research Council of Thailand, 1999). Fish were fed to apparent satiation 4 times a day. Fish with average body weight of 4.5±0.3 g were used for the attractability and palatability trials.

***Feed trial preparation***

The attractability and palatability test was determined using soybean meal and the herbivorous fish feed (25% protein) that normally show low intake levels in Nile tilapia, respectively. Four levels, 0%, 2.5%, 5.0%, and 7.5%, of the product PBFCM were sprayed onto either soybean meal or herbivorous fish feed, then air-dried at 40°C for 6 h and kept at −20 ℃.

***Assessment of attractability***

The attractability test was performed following Suresh *et al*. (2011) with moderate modification. The tank (60 x 130 x 30 cm in length, width, and height) contained an acclimatization chamber at one end with four feeding chambers at the other end. Acclimatization and feeding chambers were isolated by two movable acrylic shutters. Each feeding chamber had an opening hole to allow fish attempting to feed to hang in the chamber. The feed (1 g) was packed in a sterilized stainless steel mesh tea ball then fixed at 3 cm from the bottom, in the middle of chamber.

Four trials comprising soybean meal with different concentrations of the product PBFCM (0%, 2.5%, 5.0%, and 7.5%) were tested at a time. Thirty Nile tilapia were placed into the acclimatization chamber for 30 min and then the shutters were removed and all fish were allowed to approach the test feed at the end of the feeding chambers for 15 min. Data was collected by video throughout the experimental time of a total of 15 min. Each feed trial was randomly tested 4 times in 1 of the 4 assigned feeding chambers.

The attractability was present as

%Turn = (Number of fish approaching in each chamber/Number of fish approaching in all chambers) x 100

Time = The time of fish feeding in each feeding chamber

***Assessment of palatability***

The experiment set for the palatability test was conducted following Suresh *et al*. (2011) and Al-Souti *et al*. (2019) with moderate modification. Four trials including herbivorous fish feed with different concentrations of the

product PBFCM (0%, 2.5%, 5.0%, and 7.5%) were used for the palatability assessment. Four tanks (200-l plastic tank), containing 10 juvenile Nile tilapia, were used for each treatment. Fish were acclimatized in the experimental tank for 3 days and were fed 4 times a day (9.00 a.m., noon or 12.00 p.m., 5.00 p.m., and 8.00 p.m.). Rearing water was changed an average of 80% twice a day at 8.00 a.m. and 6.00 p.m.

The palatability test was performed on one meal per day (at 9.00 a.m.) for 3 days. At the desired time, 5 g of test feed was introduced into the tank and the fish were given 15 min to consume the feed. At the end of 15 min, the uneaten feed was collected, dried at 70℃ for 6 h, and weighed. Feed consumption was calculated as mg test feed/gram of fish biomass/meal.

### *Experimental design and statistical analysis*

The experiments to determine the attractability and palatability of the trial feeds with different levels of the product PBFCM were performed based on a completely randomized design (CRD) with 4 replications. All data were examined by one-way ANOVA via the SPSS Statistics software version 16.0 (SPSS Inc.). The significant differences were analyzed using Duncan's Multiple Range Test (DMRT). Significant differences were stated at $P<0.05$.

## Results

### *Characterization of PBFCM*

The product PBFCM was a brownish and slightly viscous solution. Its pH was around 4.74. The components of PBFCM are shown in Table 1. It comprises protein, carbohydrate, and phenolic compounds.

**Table 1.** Chemical composition of PBFCM

| Component | PBFCM |
|---|---|
| Total protein | 5.71±0.12[1] |
| Total carbohydrate | 13.93±0.37[1] |
| Total phenolic compound | 2.87±0.01[1] |

[1/]: Expressed as Mean±SD in a unit of mg/ml.

### *Antimicrobial activity of PBFCM against the multi-antibiotic resistant A. veronii*

*A. veronii* isolate I15 was classified as multi-antibiotic resistant since it resisted oxytetracycline (OT), tigecycline (TGC), and tetracycline (TE). The

antimicrobial activity of PBFCM was evaluated based on the growth of the test bacterium in the media containing PBFCM. The growth curve and specific growth rate of *A. veronii* isolate I15 cultured in 2.5%, 5.0%, and 7.5% PBFCM are shown in Figure 1 and Figure 2, respectively. Mock control was done in all experiments to confirm that there was no effect of Triton X-100, the reagent used to enhance the solubility of PBFCM in the culture medium. The result showed that 2.5% PBFCM had no inhibitory effect since the patterns of the growth curve and specific growth rate were similar in the positive and mock control (Figures 1A and 2A). At 5.0% PBFCM, a weak inhibitory activity was observed; the lag phase was a bit delayed (Figure 1B) while the specific growth rate was still similar to those of the controls (Figure 2B). Strong antimicrobial activity was detected in 7.5% PBFCM; lag phase duration was obviously longer (Figure 1C), and the peak of the specific growth rate was lower and delayed compared to those of the controls (Figure 2C).

### *Attractability and palatability of PBFCM*

The attractability was evaluated by the behavior of juvenile Nile tilapia in approaching the feeding chambers with different feed trials (Figure 3). The results showed that the attractiveness level of the soybean meal with 2.5% of PBFCM was not statistically different ($P>0.05$) to that of the control soybean meal. %Turn of fish swimming in-out the feeding chambers of soybean meal with 2.5%, 5.0%, and 7.5% of PBFCM were 21.2±2.4%, 22.0±3.1%, 26.4±1.0%, and 30.5±1.7%, respectively while the time the fish spent in each chamber were 5.6±1.4 s, 7.8±2.3 s, 11.7±1.2 s, and 10.9±0.8 s, respectively. During the experimental period, fish responded and approached the feeding chambers of the control and 2.5% PBFCM with significantly lower %turn and spent less time there ($P<0.05$) compared to the trials of 5.0% and 7.5% PBFCM. However, there was no significant difference ($P>0.05$) between the soybean meal mixed with 5.0% and 7.5% PBFCM.

Feed consumption by the Nile tilapia in the test was used as representative for palatability. It could divide the feeding trials into 2 groups with a statistical measure comparable to the attractability (Figure 4). Feed consumption in the control and feed with 2.5% PBFCM did not significantly differ ($P>0.05$). Fish ate the feed trials with PBFCM 5.0% and 7.5% higher than those of the control and lower amount of PBFCM. Indeed, there was no significant ($P>0.05$) difference in feed consumption between the 5.0% and 7.5% PBFCM trials.

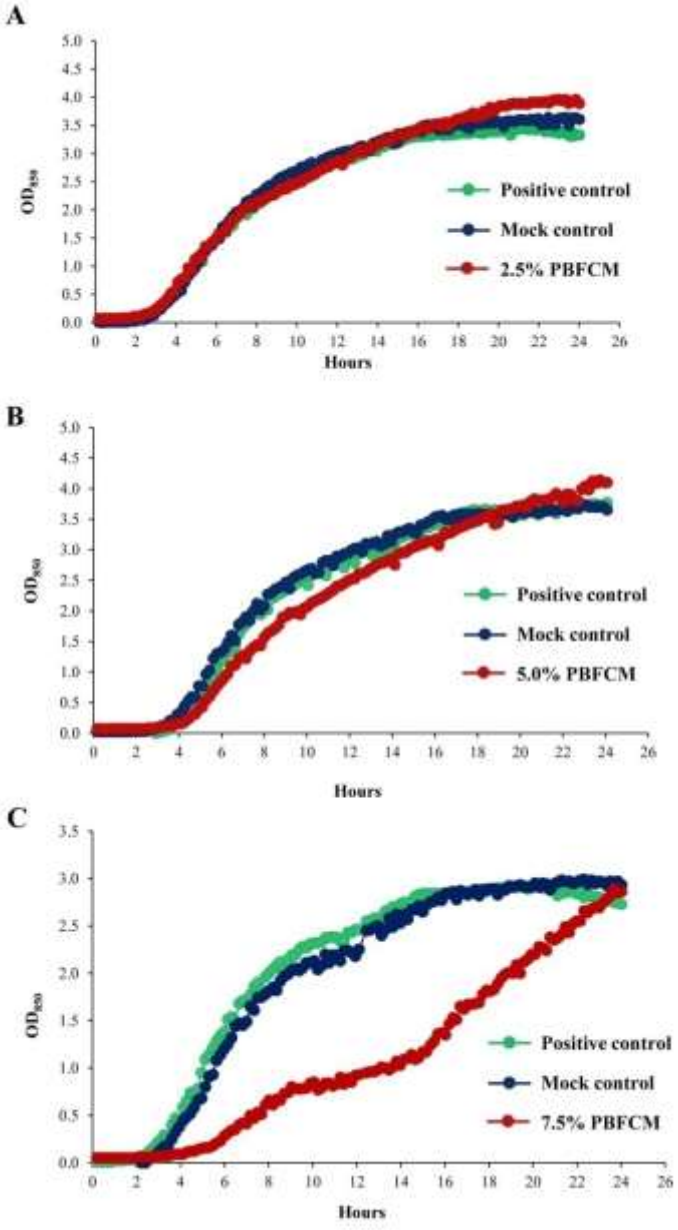


**Figure 1.** Growth curve of the multi-antibiotic resistant *A. veronii* cultured in different medium trials; positive control, mock control containing Triton X-100, and the medium containing the mixture of biofloc fermentation medium and vinasse (PBFCM), (A) 2.5% PBFCM, (B) 5.0% PBFCM, and (C) 7.5% PBFCM

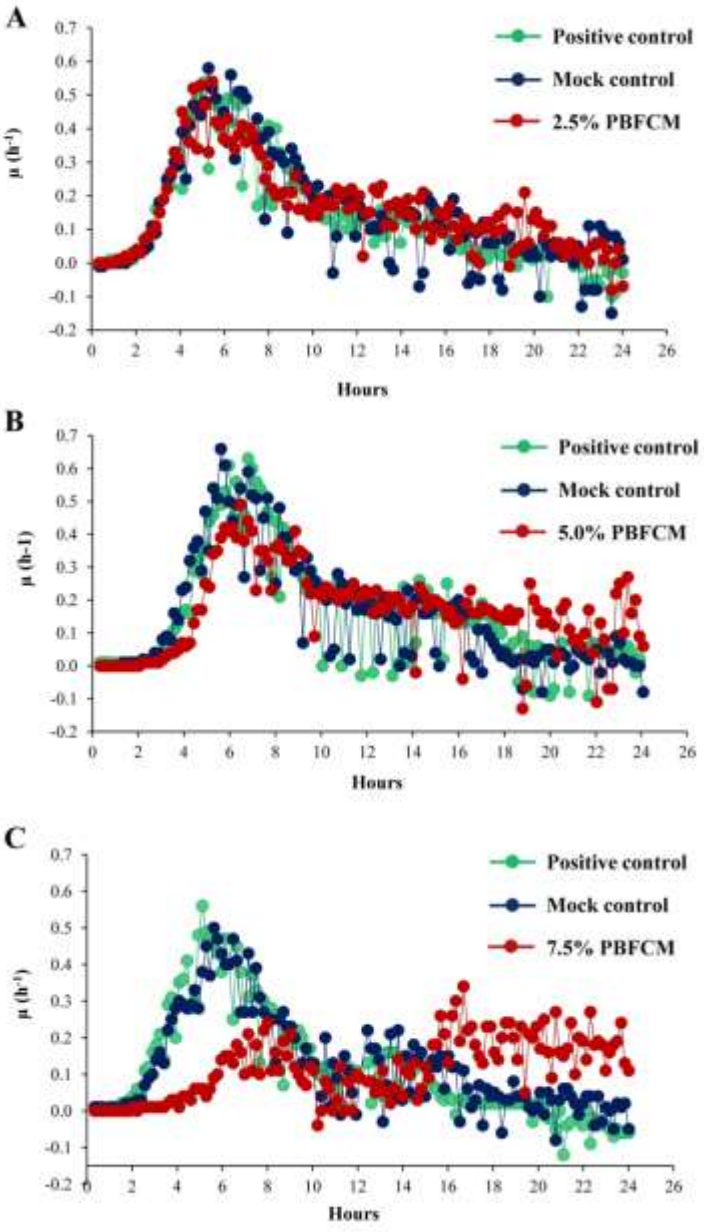


**Figure 2.** Specific growth rate of the multi-antibiotic resistant *A. veronii* cultured in different medium trials; positive control, mock control containing Triton X-100, and the medium containing the mixture of biofloc fermentation medium and vinasse (PBFCM), (A) 2.5% PBFCM, (B) 5.0% PBFCM, and (C) 7.5% PBFCM

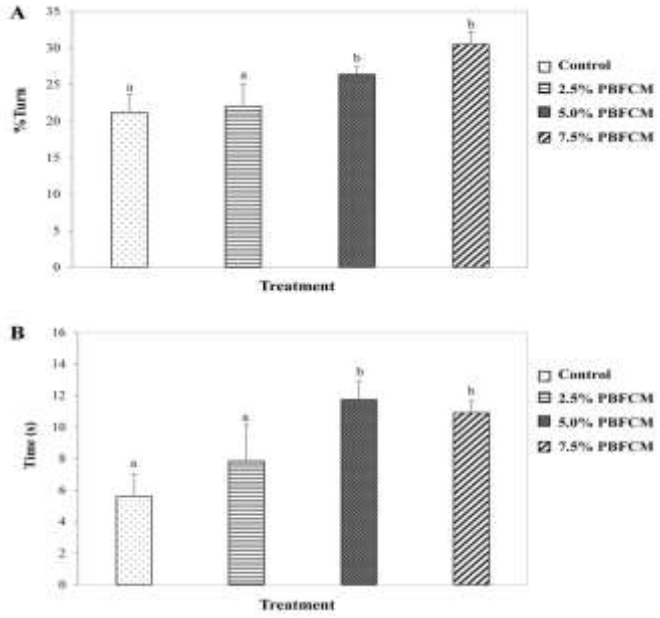


**Figure 3.** Attractability in terms of (A) %Turn and (B) Time (s). Juvenile Nile tilapia were treated with different feed trials: soybean meal with 0% of the mixture of biofloc fermentation medium and vinasse (PBFCM) as control, and soybean meal mixed with 2.5%, 5.0%, and 7.5% PBFCM. The results were expressed as mean±SD. The different letters stand for statistically significant differences ($P<0.05$) between feeding trials

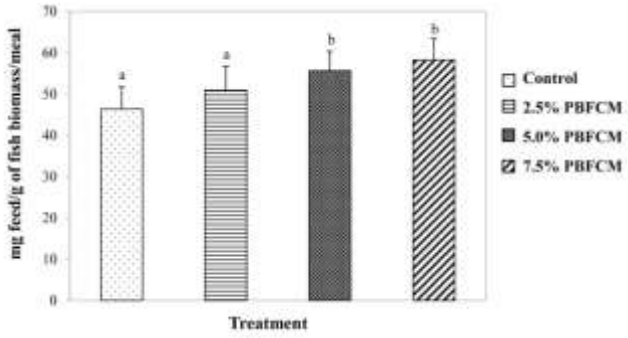


**Figure 4.** Palatability of the herbivorous fish feed supplemented with different levels of the mixture of biofloc fermentation medium and vinasse (PBFCM). Juvenile Nile tilapia were treated with different feed trials consisting of the herbivorous fish feed with 0% PBFCM as control and with 2.5%, 5.0%, and 7.5% PBFCM. The results were expressed as mean±SD. The different letters stand for statistically significant differences ($P<0.05$) between feeding trials

## Discussion

Recently, the use of natural products, especially herbs and herb extracts, exhibiting antimicrobial activity as well as immunostimulants, has been developed as alternative disease control to replace antibiotics in aquaculture. This may be due to their eco-friendliness, low risk of residues in aquatic animals, and the reduction in the frequency of antibiotic-resistance (Syahidah *et al*., 2015). The practical procedure for administering these effective substances to aquatic animals is oral administration by feed supplementation. However, there have been concerns about the attractability and palatability of the supplemented diet that may cause a reduction in feed intake leading to negative effects on animal growth. Considering that herbs and herb extracts often have an unpleasant smell and taste, has led to the discovery of other natural substances which also express effective characteristics in antimicrobial activity and immune improvement.

In view of the above, this present work therefore aimed to discover an alternative substance which has inhibitory efficacy against the pathogenic bacterium *A. veronii* and also expresses feed attractability and a pleasant sense for the test subject Nile tilapia. Since biofloc and yeast are considered as effective components typically used as feed or feed supplements/additives as well as being probiotic in aquaculture, the mixture of biofloc fermentation medium and vinasse or PBFCM was then developed and tested for both the mentioned important aspects. The multi-antibiotic resistant strain of *A. veronii* previously isolated and characterized in our laboratory was the candidate bacterium used to verify the effectiveness of our developed product. PBFCM showed antimicrobial activity and bacterial growth regulatory activity against the test *A. veronii* in a dose-dependent manner. This may be due to the contributing function of molecules or substances within the product. Bassler (2002) has reported that there are control effectors through quorum sensing (QS), a cell-to-cell communication process occurring among microorganisms within a species or between inter-species, in biofloc. These molecules have been increasingly attracted the attention of scientists since they provide a suitable ecological niche and stabilize the bacterial community in the microbial aggregation as well as control many bacterial behaviors (Fuqua and Greenberg, 2002; Dandekar *et al*., 2012; Sun *et al*., 2018; Zhang *et al*., 2019). In addition, the bacteria are able to secrete extracellular substances that are able to restrict other unwanted bacterial species. Similarly, these might be some molecules or substances secreted by yeast into the vinasse, during sugar-based ethanol fermentation, since the dominant microorganism in the vinasse is yeast. Indeed, quorum quenching (QQ) of the yeast has been demonstrated to inactivate the

bacterial QS and also regulate the growth of other microorganisms (Leguina *et al*., 2018; Christwardana *et al*., 2019). Given our obtained result, PBFCM was able to restrict bacterial growth either through the QQ signal or the extracellular substances produced by the yeast and bacteria within the biofloc, respectively.

PBFCM could also serve the second expected aspect of attractability and palatability of the desired product. PBFCM, at the concentration of 5.0% and 7.5%, was able to significantly increase the attractability of soybean meal and the palatability of the herbivorous fish feed. This phenomenon may be due to the presence of molecules or substances within the product. It has been reported that there are various bioactive compounds in bioflocs including carotenoids, chlorophylls, polysaccharides, phytosterols, taurine, and fat-soluble vitamins (Ju *et al*., 2008). Correspondingly, Tantikitti (2014) has reported that the characteristics of attractants or stimulants eliciting strong feeding behavior responses in crustacean are low molecular weight, water/ethanol soluble, and amphoteric or basic compounds, for instance free amino acids (taurine, hydroxyproline, glutamic acid, alanine, and glycine), organic acids, nucleotides, or small peptides. Moreover, yeast cells and their derivatives are known for their β-glucan, mannooligosaccharide, and nucleic acids to increase growth performance and induce immunostimulant effects in aquatic animals.

In conclusion, this work demonstrated for the first time the capability of PBFCM, the developed product mixed from the by-products of biofloc production and ethanol fermentation, as a growth inhibitor of the multi-antibiotic resistant *A. veronii* and a potent feed attractant. Based on the results obtained, PBFCM could indeed be a valuable substance supporting a more environmentally sustainable form of aquaculture.

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