BACTERIA ISOLATION FROM SKIN, GILLS AND WATER OF *Oreochromis* sp. (RED TILAPIA) WHEN TREATED WITH *Eisenia foetida* (OLIGOCHAETE), *Pleurotus sajor-caju* (OYSTER MUSHROOM), AND *Nepenthes gracilis* (PITCHER PLANT)

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ABSTRACT

Aquaculture farmers are focused on large intensive farming to fulfill the demand of protein source, thus increasing the probability of bacteria-related disease outbreak. Therefore, an alternative fish diet has to be introduced to reduce bacteria-related infection while increasing the production. The aim of this study was to determine the microbial community on *Oreochromis* sp. when treated with modified diets; commercial pellet added with oligochaete (*Eisenia foetida*), oyster mushroom (*Pleurotus sajor-caju*), pitcher plant (*Nepenthes gracilis*) and combination of all ingredients with proportion of (90:10) and (70:10:10:10). One hundred ninety bacterial colonies from 11 species were identified using biochemical tests: Gram stain, Oxidase test, Catalase test, KOH String test, Carbohydrate (Glucose, Lactose and Sucrose) test, Indole test, Methyl Red Test and Voges-Proskauer Test (MRVP test), Citrate test and bacteria growth tested on Mannitol salt agar. *Oreochromis* sp. also secretes mucus that consists of protein to protect fish from pathogens with various environment conditions. Therefore, this study can be used to increase a better understanding of modified organic diet with higher protein content of microbial community in lower vertebrates.

Key words: Bacteria identification, *Eisenia foetida*, feed utilization, *Nepenthes gracilis*, *Pleurotus sajor-caju*, protein content

INTRODUCTION

The overfishing activity of marine organisms provide vast source of nutrients and proteins. This situation has lead to the destruction of the marine ecosystem (Jackson *et al*., 2001). Therefore, aquaculture has been introduced to fulfill the protein demand and provide continuous supply without harming its' natural aquatic ecosystem (Diana, 2009). The problem with this system is that it is practiced on a large-scale, thus fish are exposed to stress conditions, diseases related to infection and worst environmental state (Balcazar *et al*., 2006); which later leads to vast economic loss due to low production caused by mortality of the fish.

Infection and mortality of fish is control by an immune system and this weakens by having non-nutritional diet (Landolt, 1989), thus bacteria and other parasite spread effectively and benefit from contaminated water (Schachte, 2002). The interaction between fish and environment is directly proportional to each other. Aquaculture waste affects fish condition by altering the first line (Hassanshahian *et al.*, 2014) and the second line of defense by invading the internal organs via the uptake of water through gills during respiration, therefore bacteria will gets deposited on that organ.

The immune system and the microbial community of fish are said to vary by having different diets. Hence, in this experiment, fish were treated with commercial pellet modified with an additional organic supplement. This study aims to evaluate the effect of different diets on microbial community from fish mucus, gills and water as well as protein content of *Oreochromis* species.

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MATERIALS AND METHODS

Experimental fish, maintenance and study design

Seventy five *Oreochromis* sp. weighing about 10g to 50 g were obtained from supplier in Wakaf Mesira, Tepoh, Terengganu and brought to freshwater hatchery, Universiti Malaysia Terengganu. The fish were kept in 150L fiberglass tanks with recirculating fresh water at a constant temperature of 24°C and not exceeding 30°C. The fish were fed *ad libitum* twice daily with commercial pellets and the wastewater was taken out every 7 days. Fish were divided into groups of 15 and divided over 5 tanks based on treatments, respectively. Aerator was used for oxygen supply, and the experiment was conducted after 14 days of acclimatization to the aquarium. Only healthy fish, as indicated by their activity and appearance were used.

Pellet preparation

Five types of pellet were prepared; commercial pellet, commercial pellet with *Eisenia foetida*, commercial pellet with *Pleuratus sajor-caju*, commercial pellet with *Nepenthes gracilis*, and combination of all of the ingredients. The proportion of each additional nutrient was 10% respectively. The ingredients were mixed together by using stand mixer machine. Small amount of distilled water was added to ensure the even distribution of the ingredients, thus softening the mixture. The speed of stand mixer machine was increased for approximately 5 minutes. Once the mixture reached the dough-like texture, it was shaped like a ball and put in mince machine (pellet machine) with the hole of 0.1mm diameter. The products were crushed into smaller particles by using a knife and let to dry for 24 hours at 50°C to 60°C in an electrical oven.

Samples collection

Fish were starved for 24 hours before harvested and anesthetized using a few drops of 0.1g/L Benzocaine. Mucus was removed by using blunt scrapper from dorso-lateral surface of fish (Guardiola *et al*., 2016) and bacterial samples were cultured by using sterile cotton swabs and striked on Nutrient Agar (NA). Next, gills were collected by evisceration by using surgical scissors and knife. Ten millilitre of water samples were collected using Falcon tube and cultured by using sterile cotton swabs and striked on NA agar plate.

Bacteria isolation and identification

A pure colony was selected from petri dish and sub-cultured to a fresh NA agar by using streak plate techniques, labeled accordingly and plate was incubated at 37°C for 24 hours before stained and tested for biochemical tests.

Gram staining

Pure cultures were selected and placed onto slide and heated for fixation followed by few drops of Crystal violet for 1 minute and heat-fixed smear. Iodine was added to wash off the purple dye and act as a mordant. Next, acetone was placed to decolorize remaining stain following an addition of safranin for 1 minute before washed with tap water. The slides were blotted using tissue paper and observed under compound light microscope.

Oxidase test

Impregnated oxidase strip method was used for this experiment. The bacteria were selected from pure colony by using sterile inoculation loop then, smeared onto clean oxidase paper and colour observation was recorded in 10 seconds to 30 seconds.

Catalase test

Bacteria was selected from pure colony by using sterile inoculation loop and placed onto clean slide. Two or three drops of hydrogen peroxide (H_2O_2) was placed onto the bacteria.

*Potassium hydroxide test (*KOH) String Test

A drop of 3% of KOH solution was placed on a microscope slide. A piece of pure culture bacteria were transferred to KOH using toothpick and later the mixture was stirred.

Carbohydrate fermentation test: glucose, lactose and sucrose

The bacterial samples were inoculated into each test tube containing phenol red with; glucose, lactose and sucrose. Cultures were incubated at 37°C for 24 hours. The carbohydrate broths were examined for colour changes, and presence of gas bubbles were examined in the Durham tube.

Indole test

Each bacterial sample was inoculated (stab inoculation) into Sulfide, Indole, Motility (SIM) media and incubated for 24 hours at 37°C. After incubation, 10 drops of Kovac's reagent was added to all tubes and agitated gently.

MR-VP test (Methyl Red Test and Voges-Proskauer Test)

Bacteria samples were inoculated into 2 tubes containing MR-VP broth and incubated at 37°C for 18 hours to 24 hours. After incubation, the bacterial cultures were divided into two test tubes (A and B). Five drops of methyl red was added in tube A for

Methyl Red test; 10 drops of Barrit's reagent A and B was added in tube B for Voges-Proskauer test. The tubes were shaken at every 3 to 4 minutes and colour changes were recorded 15 minutes after the addition of reagents.

Citrate test

A single isolated colony was picked and streaked on the surface of the Simmons Citrate slant agar. The screw caps were placed loosely as citrate utilization needs oxygen. The cultures were incubated for 18 to 24 hours at 37°C and the presence of growth on the surface of the slant was observed, accompanied by changes in colour.

Bacteria growth characteristics on mannitol salt agar

Bacterial cultures were streaked on MSA agar and incubated at 37°C for 24 hours to 48 hours and colour of bacterial colonies were observed.

Protein content analysis (Kjeldahl Protocol)

This protocol can be categorised into three processes; digestion, neutralization and titration. The percentage of crude protein (percentage protein content) was calculated by the following formula:

Percentage of Protein, % = Percentage of Nitrogen x F (Protein factor: 6.25)

$$\text{Percentage of Nitrogen} = \frac{(T-B) \times N \times 14.007 \times 100}{\textit{Weight of sample in mg}}$$

T = Volume of titration of sample
B = Volume of titration for blank (0)
N = Normality of HCl (0.1)

Data collection and statistical analysis

The number of bacterial colonies identified and percentage average protein content were analysed using SPSS 20.0 (SPSS Inc.). One-way ANOVA was used to test the relationship between both of the data with p<0.05 between treatments and control group considered significant. Whilst, for bacteria colony isolated and percentage average protein content comparison, a Pearson Correlation Coefficient, r, was applied where 0 > r indicates the positive and 0 < r indicates the negative relationship.

RESULTS

One hundred ninety bacterial colonies were isolated from skin, gills and water as in Figure 1. The highest bacterial colonies isolated were from gills at day 21 and the least isolated at day 0 from water. The

highest bacteria colonies observed from *N. gracilis* treatment, while the least from mixture of *P. sajor-caju* (Figure 2). Eleven species of Gram positive and Gram negative bacteria were isolated from skin, gills and water as recorded in Table 1.

Figure 3 shows the relationship between number of bacteria isolated and the average protein content. Higher average protein content lead in low bacterial number and the phenomenon was vice versa from day 0 to day 21. However, different trend was observed at day 28 as the average protein content and number of bacteria isolated were both decreased and showed negative association (r<0) when tested using Pearson Correlation Coefficient. Figure 4,

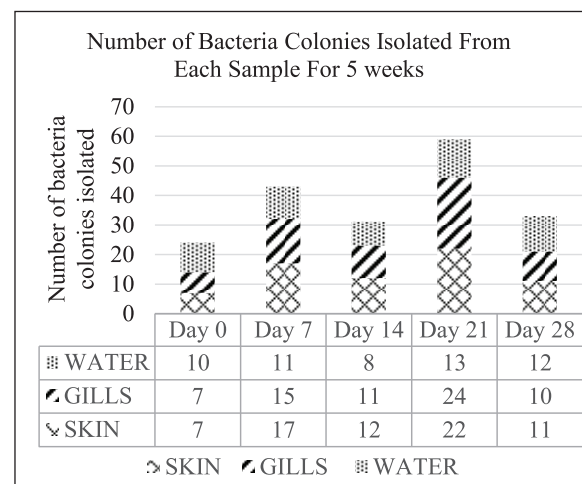| Number of Bacteria Colonies Isolated From Each Sample For 5 weeks				
	Day 0	Day 7	Day 14	Day 21	Day 28
WATER	10	11	8	13	12
GILLS	7	15	11	24	10
SKIN	7	17	12	22	11

SKIN   GILLS   WATER

Fig. 1. Number of bacteria colonies isolated from skin, gills and water of *Oreochromis* sp. for 5 weeks (Day 0, Day 7, Day 14, Day 21, and Day 28.
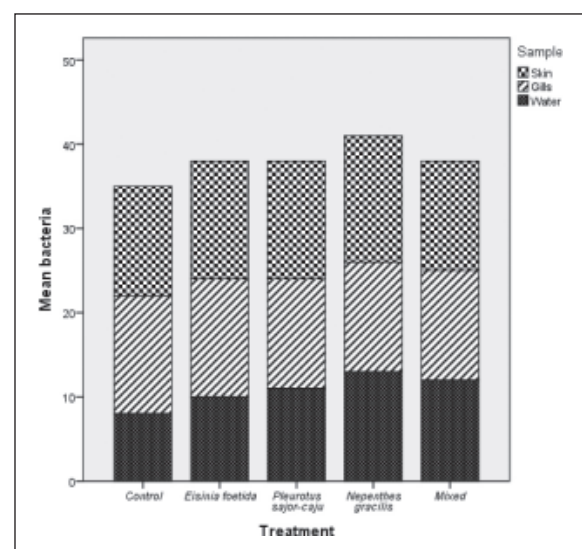

Fig. 2. The mean number of bacteria colony isolated from three different organs (Skin, Gills and Water) of *Oreochromis* sp. throughout the weeks based on different treatments.

Table 1. The physical appearance of suspected bacteria colonies isolated

Suspected bacteria	Colour	Shape	Elevation	Margin	Other appearances (Size, Smell)
Bacillus subtilis	Pale yellow	Circular	Convex	Entire	Punctiform, translucentodor
Bacillus megaterium	Pale yellow	Circular	Convex	Entire	Punctiform, opaque,
Bacillus cereus	Whittish	Circular	Raised	Entire	Medium-sized, opaque, odor
Bacillus faecalis	Pale yellow	Irregular	Flat	Undulate	Fluffy-like, rough-edgelarge in size
Lysteria monocytogenes	Whittish	Irregular	Raised	Undulate	Cloudy, opaque, medium-sized
Mycobacterium phlei	Pale yellow	Filamentous	Flat	Filiform	Large in size
Erysipelothrix rhusiopathiae	Pale yellow	Circular	Convex	Entire	Medium-sized, opaque
Staphylococcus aureus	Bright yellow	Circular	Convex	Entire	Punctiform
Staphylococcus epidermidis	Bright yellow	Circular	Convex	Entire	Punctiform
Pseudomonas aeruginosa	Greenish	Circular	Flat	Entire	Medium-sized, translucent
Methylobacterium extorquens	Pink	Circular	Convex	Entire	Punctiform, opaque
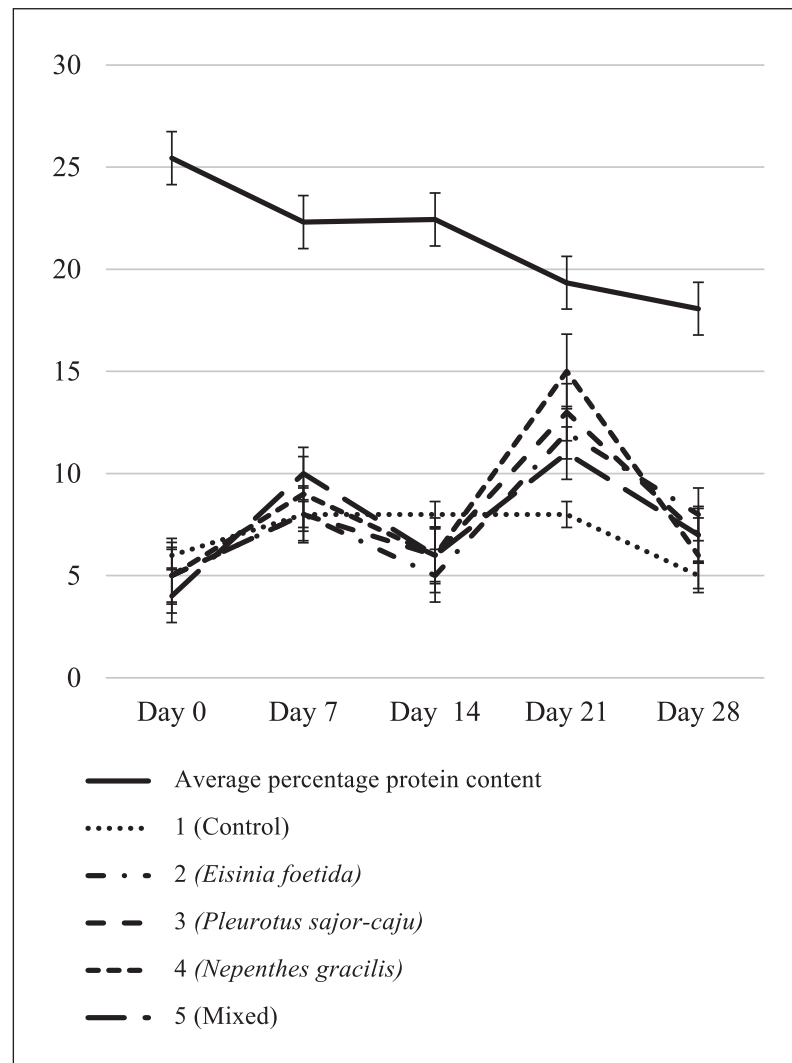
Fig. 3. The relationship between bacteria colony isolated and average protein content throughout the week. There were a significant different $p<0.05$ when data analyzed using one way ANOVA by SPSS 20.0 (SPSS Inc.).
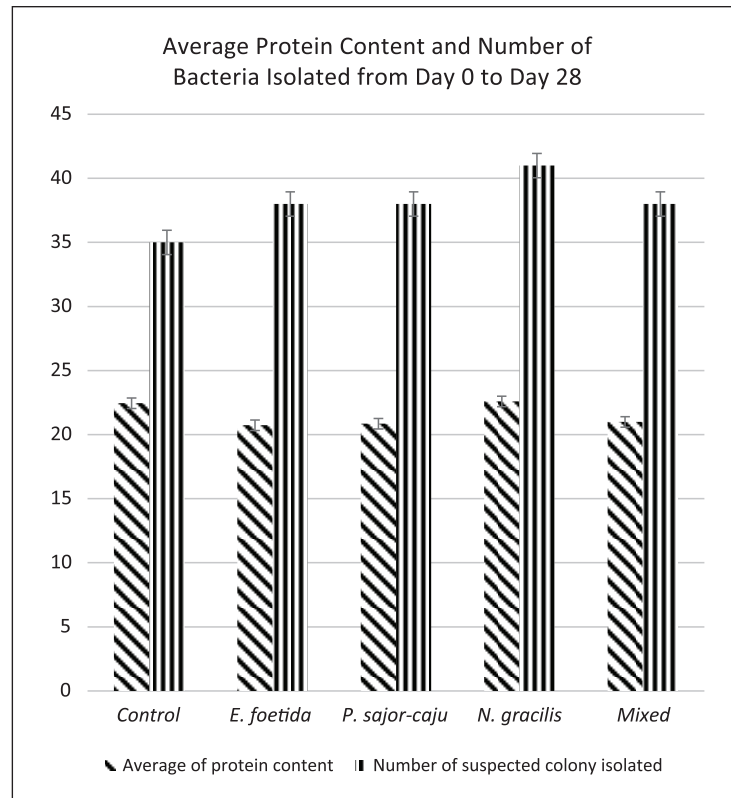
Fig. 4. The relationship between number of bacteria colony isolated and average protein content in the view of treatments applied. There were no significant different p>0.05 when data were analyzed using one way ANOVA by SPSS 20.0 (SPSS Inc.).

shows the relationship between bacterial colony isolated and average protein content from all treatments. The average protein content was statistically not significant (P>0.05) between treatments by One-Way ANOVA.

DISCUSSION

The presence of microbes indicates fish health as they can be found on all tissues at all ages reflecting to their surrounding environment and food consumption. Fish diets strongly support hypothesis that it can influence the microbiota community in fish (Ibrahem *et al.,* 2010). Microbes on skin, gills and water migh possess the ability to produce immune response, thus preventing diseases. Cipriano (2011) reported the changes from opportunistic pathogens that may exist on skin and mucus by low number of bacteria. *N. gracilis* used as dietary supplement, most likely to be in high number on skin due to nutrient-rich in mucus and in the gills (Landeira-Dabarca *et al.*, 2013; Pakingking *et al.*, 2015). Therefore, it can be

concluded that diets applied in this study does affect the bacterial community (Larsen, 2014), thus influencing the microbiota on fish tissues with the unusual occurrences of *Mycobacterium phlei, Erysipelothrix rhusiopathiae,* and *Methylobacterium extorquens.* Interestingly, these bacteria were not transmitted via water medium, but might have come from pellets as also reported by Di Maiuta *et al.* (2013), on loricariid catfish which symbionts with other organisms, and not by environmental changes.

Peptide is a source of protein in mucus where it has antimicrobial peptide, which enables to trap microorganisms. In this study, the number of bacteria isolated was statistically related to protein content over time, thus higher potential to protect fish from any pathogens. This gives an idea that the immune system starts to respond to the presence of bacteria for counter attack in a suitable situation. Nevertheless, it can be concluded that mucus and protein contents react in time as a response of change in microbial community. The protein content was increased in order to protect the skin from bacterial infection and other opportunistic bacteria.

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