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Evaluation of striped catfish, *Pangasius hypophthalmus* (Sauvage, 1878) dried viscera and its intestinal putative probiont on juvenile African catfish, *Clarias gariepinus* (Burchell, 1822) growth performance

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Abstract
The growth performance of juvenile African catfish, *Clarias gariepinus* were evaluated after feeding with striped catfish, *Pangasius hypophthalmus* dried viscera and its intestinal putative probiont. The chosen isolated putative probiont were identified as *Weissella* sp. through 16S rDNA sequence analysis. Utilization of dried striped catfish viscera as well as *Weissella* sp. as supplementation in African catfish diet show better growth performance in compare to the unsupplemented treatment. At the end of experiment, fish fed with *Weissella* sp. (T3) showed significantly high growth performance (P<0.05) in terms of its body weight, body weight gain, average daily gain and specific growth rate when compared to control (T1), but exhibited no significant difference (P>0.05) with T2. Furthermore, histological assessment of intestinal villi height of T3 shows significantly longer (P<0.05) than other two treatments. Hence, African catfish diet composed of probiont, *Weissella* sp. suspension at dosage of 10^9 cfu/ml diet (T3) was recommended to promote *C. gariepinus* growth performance.

Keywords: Post-harvest, viscera, probiont, catfish, lactic acid bacteria

Introduction
Pangasius hypophthalmus is one of the widely cultured freshwater fish in Southeast Asian region especially in Vietnam and Malaysia. It is commonly sold fresh locally or processed as frozen fillets for domestic and international consumption due to it good taste with desirable qualities such as white flesh, low fat content and easily digestible protein content [1]. Many processing companies have been established to meet the demands for the products developments. The weight of whole fish is comprised of 40 to 60% of flesh used for human consumption [2]. Inevitably, the post-harvest processing of *P. hypophthalmus* has resulted large volume of waste by-products such as skin, viscera, head and bone. The utilization of waste by-product may increase the economic value of the fish [4]. The unwanted viscera usually was discarded away since it was not consumed by human. Thus, in the current study, the normally unutilized viscera were evaluated for potential use in aquaculture sector. Furthermore, striped catfish post-processing by-product were known to contain many beneficial effect that could be well utilized in cosmestic industry [5] such as extracting collagen from the swim bladder [3] and production of hand cream from its skin [6]. Fish gastrointestinal contain many microflora bacteria and is reported to be a potential probiotic as reported by Muthukumar and Kandeepan [7] whom isolated them from fish intestines in several fish species such as in *Catla catla, Labeo rohita, Cirrhinus mirigala* and *Cyprinus carpio*. The most important part in sustaining high fish health and growth is through feeding. The inclusion of probiont and dried viscera might able to maximize catfish production safely and might able to reduce production cost by limiting the used of using expensive and limited availability of fish meal [8]. African catfish, *C. gariepinus* has been considered as one the most cultured freshwater fish and represented as widely produced food fish in the world particularly in Malaysian region [34, 35]. Furthermore, it has high tolerance to environmental stress, fast growth rate as well as great economic interest which make it very suitable target for current study [36]. Therefore, present study would like to evaluate and determine the efficacy of using feed enriched with *P. hypophthalmus* dried viscera as well as its intestinal probiont in feeding African catfish juvenile.

Materials and Methods

Striped catfish, *P. hypophthalmus* dried viscera nutrient composition and its intestinal probiont characteriistic

Collection of Samples and Nutrient Identification

The discarded *Pangasius hypophthalmus* viscera were obtained from the farmer in Sg. Temerloh Pahang, Malaysia who used to sell the fish routinely to restaurants. The discarded part obtained were stored at 5 ºC and transferred promptly to laboratory. A total of 12 samples of discarded parts comprised of liver, heart, swim bladder, stomach, intestines, gonadal tissue and fats were cleaned thoroughly with deionised water and grinded by using a blender. The grinded samples then were oven dried at 105 ºC for 24 hour until completely dried. The proximate analysis was carried out on the homogenized sample according to AOAC method [9].

Serial Diluting and Bacterial Screening of the Isolated Putative Probiont

One gram of midgut part of intestines were separated and were homogenised in normal saline. The homogenates were serially diluted and streaked onto Man de Rose agar (MRSA) in triplicates and incubated for 48 hours at room temperature. The average number of colonies formed on the agar plate were calculated and expressed as cfu/ml. Colonies with different morphological properties were re-streaked onto MRS agar to obtain pure cultures. Preliminary biochemical tests such as gram staining, catalase and oxidase were carried out for the isolated bacteria.

Pathogenicity Test of the Isolated Putative Probiont

The isolated putative probionts were screened and chosen based on its ability to inhibit the growth of a pathogenic bacteria, *Aeromonas hydrophila*. Antibacterial activity of the isolates was tested through spot method by using cell-free cultured broth of the individual selected colonies. A 5 µl concentrated culture broth was dropped onto Mueller Hinton agar which was earlier evenly spread with 100 µl of *A. hydrophila* inoculum (final concentration of 10^6cfu/ml). Observations recorded after 24-48 hour of incubation period at 37 ºC. The probiont were chosen based on the highest inhibition zone formed on the agar.

Haemolysis Test

The haemolysis test was carried out to the isolated selected probiont by using blood agar. Pure cultures of theisolated probiont were streaked on the blood agar plates and incubated at 37 ºC for 24 hour. Strains Isolates which did not shows anying no clear halos zones (i.e. γ-hemolytic or non-hemolytic) were selected as potential probiotics probionts while those having a clear hemolysis zones (β-hemolytic or completely haemolytic) or a greenish halo (α-hemolytic or partially haemolytic) were discarded.

Assays for Temperature, $NaCl_2$, pH and Bile salt tolerances

MRS broth were prepared and distributed equally in 50 ml bottles according to respective assays viz. different concentrations of $NaCl_2$ ranging from 1.5%, 2.5%, 3.5%, 4.5%, 5.5%, 6.5% and 7.5%, different pH levels i.e. 2, 4, 6 and 8 and two concentration of bile salt i.e. 0.15% and 0.3%. The broth media and the control bottles were autoclaved at 121 ºC for 15 minutes for sterilization. After cooling, all of the bottles were inoculated with an overnight culture (30 µL, approximately bacterial density 1.5 x 10^9 cfu/ml) of the selected strain in the MRS broth followed by incubation at different properties tested. The incubation process were carried out according to different temperatures (5 ºC, 15 ºC, 25 ºC, 35 ºC, 35 ºC, 45 ºC and 55 ºC); different concentration $NaCl_2$ concentrations (1.5%, 2.5%, 3.5%, 4.5%, 5.5%, 6.5% and 7.5%); different pH levels (2, 4, 6, 8 and 10) and different concentration of bile salts (0.15% and 0.3%). Then an overnight culture (30 µl) of isolated probiont were inoculated in different tested MRS broth and incubated for 24 hour respectively. The spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the optical density at 600 nm (OD_{600}) of respective incubated bacteria and growths were compared at the end of the test.

Antibiotic Sensitivity Test

Mueller Hinton agars were used to test the bacterial culture sensitivity test towards certain common antibiotic medicines used in aquaculture field. The antibiotic sensitivity tests were carried out for the selected isolates against the most common antibiotics in aquaculture using disc diffusion technique [10]. Its included Streptomycin (10 µg), Ampicilin (10 µg), Choloroamphenicol (10 µg), Vancomycin (5 & 30 µg) and Amoxilin (10 µg). The 24-hour broth culture of the isolates was spread on Mueller Hinton agar and antibiotic Bio-discs (BioMerieux, France) were subsequently placed on plates by Oxoid Disc Dispenser System. All plates were then incubated at 37 ºC for 24-48 hour to observe the inhibition zone respectively [11, 12].

DNA Extraction and Identification of selected probiont by Molecular Techniques

Briefly, genomic DNA of the isolates was extracted by using DNA extraction kit (Genomic DNA Mini kit, Genaid; BioMeriux). Isolated probionts bacterial DNA were amplified by PCR through partial bacterial 16S rDNA genes by using the universal bacterial primers 27F_2730935 (AGAGTTTGATC(A,C)TGGCTCAG) as forward primers and 1492R_2730938 (TACGG(C,T)TACCTTGTTACGACTT) as reverse primers. The purified products were sequenced by First Base Laboratories Sdn Bhd. Selangor, Seri Kembangan, Malaysia. The 16S rRNA gene sequencing, approximately 1500 bp, was analyzed by NCBI software and then compared with BLAST data search in Gen Bank from the National Center for Biotechnology Information (NCBI) for identification.

2. Efficacy of striped catfish, *P. hypophthalmus* viscera and its intestinal probionts as feed supplement for juvenile African catfish, *C. gariepinus* growth performance

Fish Diet Preparation

Commercial African catfish pellet which contained crude protein 33%, moisture 67% and lipid 6% was used as the basal diet of the feed. Treatment #1 act as control for the experiment since no additives were added. As for Treatment #2, repellet commercial feed were added with 20% of dried *P. hypophthalmus* viscera while Treatment #3 were added with probionts bacterial suspension at dosage of 10^9 cfu/ml by spraying and mixing into the repelleted feed. All of the prepared diet were air dried and stored in a cool and dry place before used. The proximate analysis of the experimental diet were measured according to AOAC [9].

Experimental Condition and Feeding Trial

The feeding trial was conducted at Aquatic Animal Health Unit of Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Malaysia for the duration of 45 days. African catfish juvenile having size of 22.71±0.14 gm body weight were stocked in 35 L glass aquarium with a stocking density 10 tails of fish per tank. Nine glass tanks were used to represent triplicate of three experimental treatments, including the control groups. The environmental conditions such as temperature, dissolved oxygen and pH were maintained at optimum condition which were at 30 ºC, 6.5 mg/L and 7.5 respectively. All fish were fed to satiation twice daily at 0900 and 1600 hrs. The growth performance and survival of fishes were monitored at every 15 days by measuring the total length and body weight for each treatments. The important parameters used to measure the growth performance of the fishes were based on survival, body weight, total length, body weight gain, specific growth rate, feed conversion ratio and total length gain.

Histological Assessment

At the end of experiment, assessment on thickness of intestinal villi height of experimental fish were done through histology [13]. The microstructures of the fish gut epithelia were observed under compound light microscope (Olympus, Taiwan). The height of the intestinal villi height was captured and measured through camera connected to the microscope (Dino Eye, ANMO).

Statistical Analysis

All data recorded and measured were analysed through one-way ANOVA by statistical software to determine the significant difference among all treatments.

Results and Discussions

***P. hypophthalmus* Viscera Nutrient Determination**

The proximate analysis of *Pangasius hypophthalmus* dried viscera showed that its contained 16.37±0.81% crude protein, 48.1±0.01% crude lipid, 67.53±1.86% moisture and 0.2±0.06% of ash. Present findings showed the *P. hypophthalmus* viscera used has higher protein content value in comparison with study reported by Alam [14] whereby viscera from various kind of freshwater fish viscera shows only about 14.01% crude protein. This might due to the differences in the diets of the fish. Viscera organs was one of the fish body which contained significantly higher amount of fats than other parts in fish body [15] which caused high lipid level in the samples. Furthermore, the composition of viscera nutrient varies greatly among individual depending on age, sex, environment and season [16].

Properties and Molecular Identification of Putative Probiont

The average total aerobic bacterial count on MRSA indicated $9.5x10^7$ cfu/ml with 12 different colonies. The first criterion used to select the best probiont from mixed colonies of bacteria are through the ability of the bacterial strain to inhibit the growth of pathogenic bacteria [17]. Therefore, amongst 12 different colonies screened, only one colony showed marked inhibition zone when undergone pathogenicity test with *A. hydrophila*. *A. hydrophila* is one of the most pathogenic bacteria for aquatic organism, thus the isolates which showed inhibition to its growth were chosen for further identification. Similar results were showed by Butprom *et al.* [18] whereby only one isolates out of 25 random colonies able to inhibit growth of *A. hydrophila*. The primary steps which involved in the selection of probiotic bacterial to be used in aquaculture system are through the collection of background information and acquisition of potential probiont [19]. The isolate was identified as gram positive and catalase-negative bacteria. The haemolysis test done on blood agar also showed that the isolate was a non-pathogenic bacteria since it showed no clear halos (γ-hemolytic or non-hemolytic). Table 1 shows the optical density reading of isolated probiont after 24-hour incubation at different temperatures, $NaCl_2$, pH and bile salts. Based on the results obtained, isolated probiont was found able to grow in temperature ranging from 25 ºC to 35 ºC, salinity 1.5% to 6.5%, viable at pH 4, 6, 8 and 10, and tolerable to 0.15% and 0.30% bile salts. Current findings are in agreement with results reported by Nguyen *et al.* [20] whom reported the viability of *Lactobacillus plantarum* at pH 4-10. Antibiotic susceptibility profiles showed that the isolated was resistant to Chloroamphenicol (10 µg), Vancomycin (30 µg) and Amoxilin (10 µg). The isolated probiont was found out to be susceptible to only Streptomycin (10 µg), Vancomycin (5 µg) and Ampicilin (10 µg). Results analysed from NCBI website from PCR 16s criteria showed that the isolate was identified as *Weissella* sp., with 96% identification. As reported by Shukla and Goyal [21] *Weissella* sp. showed characteristics as non-motile, short rod, non-spore forming, gram positive, as well as heterofermentative bacteria which was grouped under lactic acid bacteria, thus were in line with current findings. Previously, *Weissella* sp. has been classified as *Leuconostoc* and *Lactobacillus* species and was firstly proposed in 1993 [22] due to the similarity in shape possessed between them. The use of *Weisella* sp. as probiotic in aquaculture fields which just discovered has received significant attention in recent years due to it ability to produce high dextran in comparision to other probiotic [23].

Table 1: Optical density of probiont growth in tolerance test for different temperatures, salinities, pH and bile salt concentrations after 24 hour incubation. Different superscript shows present of significant differences among respective treatments (P<0.05).

Parameter	Values	Optical density at 600 nm after 24 hour incubation (OD_{600})
Temperature	5 ºC	0.025±0.01 ^{f}
	15 ºC	0.062±0.00^{e}
	25 ºC	2.193±0.01^{b}
	35 ºC	2.246±0.00^{a}
	45 ºC	0.141±0.01^{c}
	55 ºC	0.129±0.00^{d}
$NaCl_2$ Tolerance	0%	2.235±0.01 ^{a}
	1.5%	2.199±0.01^{ab}
	2.5%	2.116±0.16^{bc}
	3.5%	2.112±0.01^{abc}
	4.5%	2.005±0.01^{c}
	5.5%	1.81±0.02^{d}
	6.5%	1.45±0.01^{e}
	7.5%	0.147±0.16^{f}
pH	2	No growth
	4	0.1673±0.01^{e}
	6	1.6507±0.01^{b}
	8	2.049±0.00^{a}
	10	1.523±0.01^{c}
Bile Salt Concentration	0%	1.85±0.00 ^{a}
	0.15%	0.137±0.00^{b}
	0.30%	0.134±0.00^{b}

Efficacy of *P. hypophthalmus* Viscera and its Intestinal Probiont on Juvenile *C. gariepinus* Growth Performance

The efficacy of *P. hypophthalmus* viscera and its intestinal probiont on juvenile *C. gariepinus* growth performance is presented in Table 2. Results showed that T3 which was the inclusion of putative probiont showed significantly high (P>0.05) body weight, body weight gain, average daily gain and specific growth rate in comparison to T1, but not with T2. On the other hand, feed intake and feed conversion ratio there were no significant difference (*P*>0.05) amongst all treatments although numerical value presented showed high feed intake in group of fish fed putative probiont, T3. The numerical value of feed conversion ratio presented a low value in fish fed with probiont (T3) and dried *P. hypophthalmus* viscera.

As shown in Table 2, the high growth performance in juvenile *C. gariepinus* could be seen on the weight increase (P<0.05) and best performance was shown in fish group fed with probiont and 20% *P. hypophthalmus* dried viscera. These results proven the efficacy of *P. hypopthalmus* which contained high lipid and intestinal probiont promote the growth of *C. gariepinus* juvenile within short period i.e. 45 days. Previous researchers had reported that probiotic were proven to improve growth and survival of some fish species such as *Labeo rohita* fingerlings [24], juvenile *Lates calcarifer* [25] and *Channa striatus* fish [26] due to its function to inhibit the colonization and growth of pathogenic bacteria in fish gut through the production of organic acids and antimicrobial compounds [27, 28, 29]. Thus farmers can utilize local intestinal probionts and include them in juvenile fish diet to improve the fish growth performance.

Table 2: Growth performance of juvenile *C. gariepinus* fed with *P. hypophthalmus* viscera and putative probiont and proximate analysis of feed used

Growth Parameters	Treatment 1	Treatment 2	Treatment 3	P-value
Initial Weight (g/f)	2.71±0.14^{a}	2.71±0.14^{a}	2.71±0.14^{a}	.....
Final Weight (g/f)	16.49±4.36^{a}	18.91±4.87^{ab}	27.73±7.37^{b}	0.12
Weight Gain (g/f)	13.79±4.23^{a}	16.22±4.91^{ab}	25.03±7.47^{b}	0.12
Average Daily Gain (g/f/d)	1.00±0.30^{a}	1.13±0.28^{ab}	1.65±0.37^{b}	0.11
Specific Growth Rate (%/d)	3.97±0.53^{a}	4.27±0.66^{ab}	5.13±0.65^{b}	0.16
Initial Total Length (cm)	7.65±0.24^{a}	7.82±0.07^{a}	7.70±0.30^{a}	0.80
Final Total Length (cm)	14.15±1.76^{a}	14.46±1.43^{a}	16.17±1.76^{a}	0.42
Total Length Increase (cm)	6.50±1.53^{a}	6.64±1.47^{a}	8.47±2.06^{a}	0.43
Feeding Intake (g/f)	2.76±0.63^{a}	2.61±0.35^{a}	3.58±0.71^{a}	0.14
Feed Conversion Ratio (FCR)	2.86±0.60^{a}	2.38±0.51^{a}	2.18±0.05^{a}	0.18
Survival Rate (%)	80.00±10.00^{a}	90.00±10.00^{a}	86.67±11.55^{a}	0.14
Moisture (%)	0.59±0.45	0.19±0.17	0.19±0.20	0.22
Crude Protein (%)	28.18±0.49	28.14±0.62	28.73±1.00	0.62
Crude Lipid (%)	7.70±3.32 ^{a}	18.62±0.88 ^{b}	3.69±0.07 ^{a}	0.01
Ash (%)	7.55±1.53	7.52±0.306	8.20±0.256	0.32

N.B.: Means with different superscripts within rows are significantly different (P<0.05)

On the other hand, the dried viscera from *P. hypophthalmus* which showed to contain high lipid was able to provide high energy source about twice the energy as proteins and carbohydrates for promoting catfish growth performance as earlier reported by Craig [30]. The high energy in the diet was found able to promote catfish growth performance [31]. Better growth performance shown by *C. gariepinus* weight increment, feed conversion ratio, average daily gain and specific growth rate as shown in Table 2 also proven the efficacy of inclusion of both product from viscera of *P. hypophthalmus* in fish feed was better as compared to unsupplemented diet.

Histological assessment of *C. gariepinus* Intestinal Villi Height

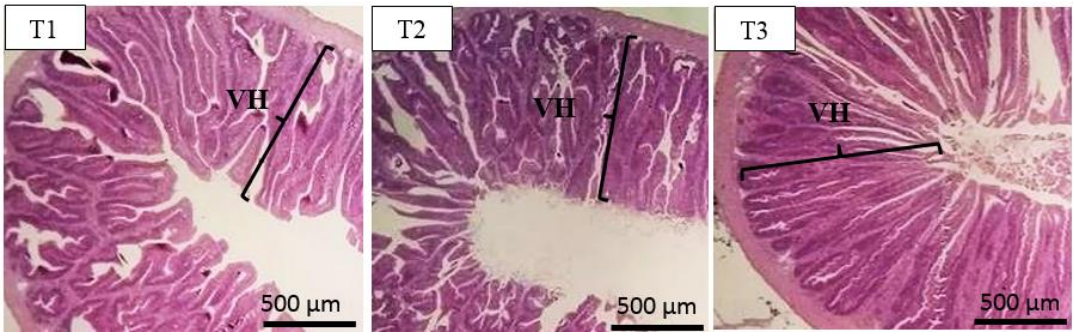

Fig 1: Histological section of small intestine of *C. gariepinus* juvenile at end of feeding trial, showing differences in intestinal villi height (VH) (T1: control fish, T2; viscera supplemented fish, T3; probiont-supplemented fish). H&E, Mag. 400X

Based on observation and measurement made on histological section of intestinal villi height of fish in Table 3 and Figure 1, it could be clearly observed that the intestinal villi of T3 showed a longer length as compare to T1 control and T2 viscera supplemented fish. Statistical analysis indicated that T2 has a significantly shorter (P<0.05) of intestinal villi height as compared to T1 and T3. However, there was no significant differences (P>0.05) could be detected between T1 and T3.

Table 3: Measurement of intestinal villi height of *C. gariepinus* after 45 days of feeding trial

Treatment	Intestinal Villi Height (µm)
T1	642.0±29.9[b]
T2	557.6± 7.9[c]
T3	762.3±9.1[a]

Intestinal villi height is one of the most important part which could determine the efficacy of the nutrient absorption of fishes. Based on measurement made, probiont-supplemented fish in T3 shows highest intestinal villi height followed by fish in T1 and T2. This could explained that more efficient nutrient absorption has occurred when fish fed with probiont supplemented feed which resulted to a better growth performance of fish physically. Intestine is one of the most important part in fish body which could clearly relates to changes in nutrient absorption of fish [32]. Reduction of mucosal surface area, thickness and intestinal villi height or length could be observed in damselfish after starving for 13 days [33]. Thus, the highest intestinal villi height shown by fish fed with probiont-supplemented feed (T3) proved that high nutrient absorption occurred in the fish intestine which was reflected by better growth performance as compared to other treatments.

Conclusion

The probiont-supplemented feed, T3 has shown better growth performances of catfish *C. gariepinus* physically and histologically as compared to viscera-supplemented feed, T2 and control fish, T1. Therefore, suspension of probiont isolated from *P. hypophthalmus* intestine at dosage of 10^9 cfu/ml diet was recommended in diet for African catfish (*C. gariepinus*).

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