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Evaluation of Haematology and Challenge Test of *Enterococcus Faecum* on *Clarias Gariepinus* at Different Inclusion Levels

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Abstract

A 70 day feeding trial was conducted to evaluate the effect of *Enterococcus faecium*, commercially known as "Cylactin" on the haematological profile and immune system of *Clariasgariepinus* fingerlings (9.13±0.55g). One hundred and fifty (150) fingerlings of *Clarias gariepinus* were randomly selected and distributed into five different treatments of three replicates each to give a total of fifteen (15) experimental units. Five isonitrogenus diets (40% C.P) and isocaloric diets (calculated G.E., 485.79Kcal/g) were formulated to give Probiotic inclusion levels of 0g (control) 5.0, 7.5, 10.0 and 12.5 X 10^9g cfu of 100/g feed, represented as T_o, T_1,T_2, T_3 and T_4 respectively, where To serves as the control. Fish were fed at 3% body weight for 70 days between 8 - 9hrs and 16 - 17hrs respectively on a static water medium. Result of the blood indices showed that PCV range from 10.2% in T_o to 14.0% in T_2, Hb from 7.50 in T_o to 9.25 in T_3 while the value of WBC ranged from 2.65 in T_o to 8.50 in T_2. RBC values ranged from 1.20 in T_0 to 2.10 in T_2, Thrombocytes from 124.5 in T_0 to 149.5 in T_3. Equally, the Lymphocytes and the neutrophils in the control (T_0) were not similar ($P< 0.05$) to all the probiotic diets. MCHC and MCH in the control diet (T_0) were significantly different ($P< 0.05$) with all the probiotic supplemented diets except in the MCV where the control (T_0) differed only ($P<0.05$) with the T_2 probiotic diet. Finally the use of Pseudomonas fluorescencs to challenge fish fed with diets containing the E. faecium, diet showed that the control diet T_o differed significantly with all the probiotic diets it was found that the control (T_0) differed significantly in mortality with all the probiotic diets. This suggests that the probiotic E. faecium can be added to fish feed to increase immunity and control the spread of disease, which invariably will enhance its health status.

Keywords: Evaluation, clarias gariepinus, enterococcus faecium, haematology challenge.

1. Introduction

Aquaculture is the world's fastest growing food industry, with a total of 59.7 million metric tons (FAO, 2012). Aquaculture's contribution to world food production and aquatic organisms for stocking has increased immensely in recent time. The world aquaculture report of 2012 discovered that the global production from aquaculture fisheries grew from 47.3 million tons to 63.6 million tons between 2006 and 2011 (FAO, 2012). But challenges are still facing the aquaculture industry these days which is not only the high cost of feeds but also the prevalence of diseases in both the hatchery and culture systems. This has prompted nutritionists and pathologists to search vigorously for alternative sources to some anti-

microbial agents such as antibiotics in order to maximize growth and increase fish production within the shortest possible time (Gonzalez, Encineas, Garcia-Lopez & Otero, 2000). Antibiotics were used for a long time in aquaculture to prevent diseases in fish. This caused various problems such as the presence of antibiotic residues in animal tissues, generation of bacterial resistance mechanisms, as well as imbalance in the gastrointestinal microbiota of aquatic organisms (Nakano, 2007). Again, the European Union (EU) has regulated the use of antibiotics in organisms for human consumption. Today, consumers demand natural products free from additives such as probiotics. These probiotics are friendly bacteria which is seen as the best option to antibiotics substitution in aqua-feed industry and the aquatic environment generally, (Gonzalez *et al*., 2000).

Blood volumes in fish comprises of 2-4% body weight, compared to values of 5-8% for other vertebrates (Brown, 1993). Blood analysis is crucial in many fields of Ichthyologic research and fishery management as they are used as possible indicators for physiological or pathological changes in fish (Adedeji, Taiwo & Agbede, 2000). The application of haematological techniques has proved valuable for fishery biologists in assessing the health of fish and monitoring stress responses (Ayoola, Ajani & Fashae, 2013). According to Babatunde, Oludinuji and Balogun (2001) any changes in the constituent component of any blood sample when compared to the normal values could be an indication of the metabolic state of the animal in relation to the state of health.

Low haematological indices are indications of anaemic conditions of the fish (Harima & Adikwu, 2001), whereas the high values, especially with the WBC and Hb reflects stress in the fish (Asuwaju, Ojutke, Kolo, Obande & Agbede, 2012). Carnevali, Vivo, Suleizio, Gioacchim Olivoho, Sivis & Cresci. (2006) also reported that when fish was fed a diet supplemented with a probiotic helped reduce the cortisol levels that also tend to increase stress.

Probiotics are inherited microflora of the intestinal system with non- pathogenic nature and have features which are important for the host health and well-being (Thirumurugan & Viguesh, 2015) they are also living microbial cells that are administered as dietary supplements with the aim of improving health (Tannock, 1997). They are equally safe, effective, potent and are able to stay for a very long time (Senok, Ismeccl & Botta, 2005).

2. Materials and Methods

2.1. Study Area

The study was carried out at the fish farm complex of the Federal University of Technology, Owerri, Imo State. FUTO lies between Latitudes $5^0$20” and $5^0$28”N and longitudes 6^0 58”E $7^0$03”E. It is 90 meters above sea level, with an annual rainfall of between 192-194cm and mean temperature of 32^{o}C (FMA, 2011). It is located in the rain forest zone of Nigeria with seasonal variations spread between two seasons of dry and rainy seasons. The dry season occurs between November to March while rainy season spreads over April to October every year.

2.2. Sample Collection and Acclimatization

A total of one hundred and eighty (180) *Clarias gariepinus* fingerlings were obtained from DARUGO fish hatchery, Owerri, Imo State, Nigeria. The fish fingerlings were transported to the Fisheries Research farm of the Federal University of Technology, Owerri (FUTO) in white plastic aquaria (15 litre capacity) filled with borehole water. The collected fish were observed to be active after acclimatization for seven (7) days prior to the commencement of the experiment, and was fed with commercial 2mm vital feed.

2.3. Diet Preparation and Formulation

Table 1: Gross Composition of Experimental Diets Fortified with Enterococcus faecium Diets

	Level of Treatments				
Ingredients	T_0	T_1	T_2	T_3	T_4
Fish meal	43.35	43.35	43.35	43.35	43.35
Soybean mean	21.67	21.67	21.67	21.67	21.67
Yellow maize	15.73	15.73	15.73	15.73	15.73
Wheat bran	6.00	5.00	3.00	2.00	1.00
Bone meal	3.00	2.00	2.00	1.50	1.00
Vit. mineral premix	3.00	2.50	2.00	1.50	0.50
Vit. C	1.00	0.50	0.50	0.50	0.50
Methionine	0.50	0.25	0.25	0.25	0.25
Lysine	0.50	0.25	0.25	0.25	0.25
Palm oil	1.00	1.00	1.00	1.00	1.00
Codliver oil	1.50	1.00	1.00	1.00	1.00
Common salt	0.25	0.25	0.25	0.25	0.25
Starch	2.00	1.50	1.50	1.00	1.00
Probiotic (100/feed)	-	5.00	7.50	10.00	12.50

The commercial probiotic “Cylactin” was imported from the biological division of cerbios-pharma SA, Switzerland, the owners of the patent probiotic while the dry ingredients (consisting of fish meal, soybean meal, yellow maize, wheat bran and other additives) were purchased from fidelity, agro-services ltd, Egbu road Owerri, were computed according to Pearson Square Method. The ingredients were measured and mixed together with the different levels of the probiotic and made into paste by the addition of boiled water. The paste was then passed through a commercial pelleting machine to form into pellets. (see table 1). They were sun-dried and stored in air-tight black polythene bags to prevent rancidity and spoilage.

2.4. Experimental Design

One hundred and fifty (150) of the acclimatized fish were sorted out according to size at a mean weight of 9.13±0.55g to create treatments T_0(0g), T_1(5.0g), T_2(7.5g), T_3(10.0g) and T_4(12.5g). The experimental fish were divided into five treatments of 30 fish each, with each group subdivided into 3 replicates of 10 fish each. Each treatment group were randomly assigned to an experimental diet in a complete randomized design (CRD).

2.5. Haematological Evaluation/Examination

Blood samples (1.5ml) were collected from both the treated and control fish from the fish caudal peduncle using the 2.5ml heparinized syringe. The blood samples collected were immediately transferred into the sample bottles with the 0.5 anti-coagulant (EDTA) with gentle agitation of the sample bottles.

The blood samples collected were used to conduct laboratory examination for both the Erythrocyte and Leucocyte counts as stated by Herman and Conroy (1970) and modified by Svobodova *et al*. (1991), Haemoglobin concentration by the method of Wedemeyer and Yasutake (1977) and then Kelly (1979), the Haematocrit (PCV) by Blaxhall and Daisley (1973) and modified later by Svobodova *et al*. (1991) while those of white blood differential was confirmed by the method of Dacie and Lewis (1995). The platelets (thrombocytes) were determined as was stated by Blaxhall and Daisley (1973).

The red blood cell (Erythrocytes) count was first diluted with the Hayem's fluid (1g NaCl + 5g Na_2SO_4, 0.5g $HgCl_2$ in 200ml distilled water) before being introduced into the improved Neubaur counting chamber (haemocytometer) for counting under the microscope as described by Conroy and Herman (1970) and Svobodova *et al*. (1991), thus;

Number of cells in 80 small square	=	n	
Number of cells in one small square	=	$\frac{n}{80}$	1
	=	$\frac{1}{400mm^2}$	2
Area of one small square	=	$\frac{1}{10mm}$	3
Depth of the chamber	=	$\frac{1}{400}$ x $\frac{1}{10}$	4
	=	$\frac{1}{4000mmm^3}$	5
Volume of 1 small square	=	$\frac{n}{80}$cells	6
Number of cells in 1/4000mm^3 of diluted blood	=	$\frac{1}{200}$	7
Number of cells in 1mm^3 of diluted blood	=	$\frac{n}{80}$x $\frac{4000}{1}$x $\frac{200}{1}$	8
The dilution of blood	=	n x 50 x 200	
Number of cells in 1mm^3 blood	=	n x 10,000 cells	

Therefore, the number of cells counted (n) was multiplied by diluting factor of 200 and volume factor of 50 (Svobodova,*et al*., 1991).

White blood cell or Leucocyte count.

The blood for the Leucocyte count was first diluted with the Turks fluid (1% glacial acetic acid + 100ml of distilled water with pinch of crystal violet) before also introduced into the Neubaur counting chamber (haemocytometer), and mounted under the microscope as described by Conroy and Herman (1970) before finally multiplied by both the dilution factor of 20 and volume factor of 2 as stated by Svobodova,*et al*. (1991) thus,

Number of cells counted in $5mm^2$ = N

Depth of chamber = $\frac{1}{10}$ 9

Number of cells counted in $\frac{5}{10}$ = $\frac{1}{2}mm^3$ of diluted blood 10

Number of cells in $1mm^3$ of diluted blood = N x 2 cells

Since blood was diluted 1/20 then

Number of cells in $1mm^3$ blood = $N \times \frac{2}{1} \times \frac{20}{1}$ 11

= N x 40

The dilution factor was multiplied by the volume factor to estimate the population of the Leucocyte where DF = 20 and VF = 2 (Svobodova *et al*., 1991).

Haemoglobin Concentration

The concentration was determined with the cyanmethanoglobin method that makes use of a sphectrophotometer, where blood samples were mixed with certain reagents before the transmittance was read on a sphectrophotometer at a wavelength of 540nm as stated by Wedemeyer and Yasutake (1977) and then by Kelly (1979).

Haematocrit Count

This was determined by the wintrobe and westergreen method as described by Blaxhall and Daisley (1973) and later by Svobodova *et al*. (1991), using the hawkley microhaematocrit reader with commercially available heparinized capillary tubes of 25mm diameter that was filled two-thirds with blood samples of which one end was sealed with plasticine plug sealant before being centrifuged at 10,000rpm for 10 minutes. The haematocrit was estimated thus;

Haematocrit, % = $a \times \frac{100}{b}$ 12

where, a = length of red blood cell column

and b = total length of the blood column.

The blood differential was determined from a blood smear made on a clean greese, free glass slide that was quickly dried in the open air. Lieshmans stain was then poured on the slide and allowed for 4 minutes. This was mixed with phosphate buffer (6.0) solution and then with tap water. The slide was allowed to dry before being examined with oil immersion under a microscope of magnification X 100 (Dacil & Lewis, 1995).

Thrombocyte (platelets) count

Blood samples were thoroughly mixed and drawn into 2 red blood cell pipettes diluted with sodium citrate to 0.2ml mark. Haemocytometer was used where the first few drops from the red cell pipette was discarded while one side of the haemocytometer was filled with blood from the pipette. The moist chamber was placed on the haemocytometer and allowed to stand for 15 minutes for the thrombocytes to settle. This was viewed under the microscope at a low power magnification of X10. The thrombocytes in the two large chambers and both sides of the machine were counted and recorded as specified by Blaxhall and Daisley (1973).

2.6. Challenge Test

Challenge test is normally performed to determine the ability of probiotics to confer immunity to host organism when challenged with pathogens. At the end of the 12^{th} week and 4 days after the administration of the diets 30 fish from each of the treatment diets (10 fish replicate) were subjected to immune competence test (disease resistance) using 2.0ml/litre of 24-hour broth culture of pathogenic strain of pseudomonas flourescens (1.5 X 10^9 cells/ml) obtained from the Microbiology Department, Federal Medical Centre (FMC) Owerri, Imo State.

The organism was subcultured in Pseudomonas nutrient agar and incubated at 37^oC for 24 hours. Transparent flourescent-like colonies resulting after incubation were purified and identified using the criteria of Holt *et al*. (1994). During the challenge test experiment, fifteen (15) *Clarias gariepinus* from each treatment group were challenged interperitonally (I/P) with 0.2ml sterile saline containing (1.5 X 10^9/ml) pathogenic strain of *Pseudomonas flourescens.* Clinical signs, post mortem lesions and mortalities were monitored for 7 days post challenge and the rate estimated according to El-Attar and Moustafa (1996).

2.7. Statistical Analysis

Data obtained above were computed and analysed using the one-ay analysis of variance (ANOVA) and differences between means separated with the Duncan's multiple range test (Duncan, 1955). Test of significance was done at X=0.05 using the statistical computer package, SPSS version 15, windows 7.

3. Results and Discussion

Table 2: Some Haematological Values of Clarias gariepinus fed diets with graded levels of E. faecium for 70 days.

Parametters	Treatments				
	T_0	T_1	T_2	T_3	T_4
PCV(%)	10.20^{b}	13.20^{a}	14.00^{a}	13.60^{a}	12.80^{ab}
Hb(g/dl)	7.50^{b}	8.00^{a}	8.50^{a}	9.25^{a}	7.80^{b}
WBC	2.65^{c}	4.20^{b}	8.50^{a}	7.35^{a}	3.80^{b}
RBC	1.20^{c}	1.52^{b}	2.10^{a}	1.93^{a}	1.46^{b}
THRO	124.5^{b}	146.0^{a}	145.5^{a}	149.5^{a}	136.5^{b}
Neutro	36^{a}	34 ab	32b	31b	30b
Lympho	58^{b}	60^{a}	65^{a}	62^{a}	61^{a}
Monocytes	03^{b}	03^{b}	02^{c}	05^{a}	04^{a}
Eusophils	02^{b}	02^{b}	01^{c}	02^{b}	03^{a}
Basophils	01^{b}	01^{b}	0^{c}	0^{c}	02^{a}
MCHC	73.5^{a}	60.6^{b}	60.7^{b}	68.0^{a}	60.0^{b}
MCH	62.5^{a}	52.6^{b}	40.5^{c}	47.9^{c}	53.4^{b}
MCV	85.0^{a}	86.8^{a}	66.6^{b}	70.4^{ab}	87.6^{a}

Means on the same row, with same superscripts are not significantly different (P>0.05)

Table 3: Pathogenic Pseudomonas flourescens Challenged with LAB (Enterococcus faecium) on Clarias gariepinus fingerlings over 7 days period.

	T_0	T_1	T_2	T_3	T_4
Mortality rate(%)	100^{a}	80^{b}	50^{bc}	40^{cc}	40^{c}
Survival rate (%)	00	20	50	60	60
No of fish injected	15	15	15	15	15
Route of Injection	I/P	I/P	I/P	I/P	I/P
Dosage of bacteria	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml
(cfu/ml)	of 10^{9}	of 10^{9}	of 10^{9}	of 10^{9}	of 10^{9}

I/P = Interperitoneally

Means on the same row, with same superscripts are not significantly different (P>0.05)

Blood parameters are essential tools for studying the effects of diets on organ functions by providing vital information for health assessment and management of cultured fish, especially as a stress response. Raizani-Paiva, ishikwa, Das Eiras and Felizardo, (2000) reported that the study of the physiological and haematological characteristics of fish is an important tool in the development of aquaculture particularly in regard to the use in the detection of healthy from diseased or stressed animals. The difference between the experimental and control mean values showed that there was an increment in the mean values of PCV, WBC and Hb of *Clarias gariepinus* fed E. faecium supplemented diets, especially at diet T_2 when compared to the control, T_0. This observation is in line with the finding of Aliyu-Paiko (2009) who observed similar results while working with Lactobacillus acidophilus probiotic on *C. gariepinus*. The result also correlated with the work of Adedeji *et*

al. (2000) who reported that there is a correlation between haemoglobin concentration and fish activities as he observed that the fish fed *L. acidophilus* were very active throughout the duration of study than the fish in the control diet. The same was observed in the present study where the fish in most probiotic diets were very active throughout the period of the study. The results obtained from this study showed higher values in the blood parameters in Clarias gariepinus fed diets with Enterococcus faecium supplementation. This could be attributed to the higher immune responses as reported by panidrahia, *et al.,* (2005). Douglas and Jane (2010) also reported increase amount of WBC and Lymphocytes, attributing it to immune responses and ability of the animal to fight infection more than the non-probiotic treated animals. Lower levels of the blood was discovered in this study to be less than what was obtained from *C. gariepinus* (14.63-20.69X10^{-3}mm) by Osuigwe, Obiekezie and Onuoha. (2005) and Akinwande, Moody, Sogbesan and Ugwumba. (2005) for *H. longifulus* (4.96-9.11X$10^{4}$$mm^{-3}$). Again, the increase in the Erythrocyte count and hemoglobin in contents in this study were similar to the works of Irianto and Austin (2002), Choudbury, pal, Sabu, Kumar, Das and Mukheryee (2005) Khattab, Shababy and Abdel-Rhman (2006). Moreover, Ayoola *et al*., (2013). also stressed that haematological parameters of fish is also a function of size, species, protein source, quality and quantity of feed diets, physiological state of fish and environmental conditions of the water medium, including the probiotics. Therefore the result of this study showed that the *E. faecium* had a positive effect on the RBC, Hemoglobin content and Haematocrit values. Other parameters evaluated in this study such as the Thrombocytes, MCH and MCV indicated support that the fish fed probiotic diets were healthier than the control. (Adewoye, Olayinka & Olaniyi, 2013). This could be attributed to decreased Cortisol levels in the blood plasma as reported by Carnevali *et al*., (2006) in the sea bass (*Dcentracas lalmax*). In this study the increase in blood parameters was not with increasing inclusion levels of the probiotic. This result did not agree with the report of Omotoyin (2006) where there was gradual increase in blood parameters of fish as the inclusion level of poultry litter in the diet of *C. gariepinus* increases. Pseudomonas flourescens used as a challenge to fish fed diets supplemental with *E. faecium* showed that *E*. *faecium* reallyboosted the immune system of fish. Reduced cumulative mortality was found in Atlantic Cod, *Gadus morhua* fed probiotic diets and challenged with pathogenic bacteria, *Vibrio angullarium.* Similarly,Nwanna, Ajana and Bamidele. (2014) and Marzouk, Moustafa and Mohammed (2008) both reported the efficacy of probiotics over pathogenic microbes, *Pseudomonas aeruginosa* and *Pseudomonas flourescens* over 14 and 7 days challenge respectively. In this study, *E. faecium* treated diets, especially at treatments 3 and 4 were lower in mortality when compared to the non-probiotic treatment, T_0.

4. Conclusion and Recommendation

The present study has revealed that the probiotic *Enterococcus faecium* is a friendly bacteria that is found naturally in fish. Results of the study showed that the probiotic is a suitable replacement for antibiotic administration to fish because of its efficacy in boosting the immunity of fish. Again since most of the antibiotics are no longer reliable because of the resistant nature of most microbes to them, this revelation has also shown that the probiotic can be used as a preventive measure in the spread of diseases in fish culture. This assertion was based on the challenge test conducted on the probiotic against a pathogenic bacteria, *Pseudomonas flourescens,* which showed higher mortality in the control diet than the probiotic treated diets.

This suggests that the probiotic, *Enterococcus faecium* can be added in the feed of fish, especially *Clarias gariepinus* so that it will increase its immunity and control the spread of disease, which in the long run increase fish yield and then profit to fish farmers.

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