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The Effects of *Staphylococcus aureus* Cells Isolated from Albumen-Based Diet on the Growth and Survival of the Larvae of *Clarias gariepinus*

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Abstract: Live organisms serve as ideal food for the early stages of fish and they have been found to have appreciable levels of micronutrients. Feeding fish larvae with formulated diets result in high mortality; the use of microbial cells as first food which, benefit the fish larvae by improving their intestinal balance is now being employed in hatcheries and have been shown to be successful. In this study, live cells of *Staphylococcus aureus* isolated from enriched albumen diet was fed to frys of *Clarias gariepinus* for Ten days to examine the growth response, survival and to determine the density of the live cells of the microbe that will have deleterious effects on the fry. This study has also shown that feeding with live cells of *Staphylococcus aureus* resulted in 80% survival of the catfish (*Clarias* species) larvae. The density of bacteria cells fed to the fish increased from 0.23 to 9.86×10^6 for the 10 days feeding trial. Growth interms of length and weight increased progressively for both commercial larvae feed and for those that received bacterial cell. However, there was a slight improved ($P>0.05$) growth in larvae fed with *S. aureus*.

Key words: *Staphylococcus aureus*, *clarias gariepinus* larvae, enriched albumen

Introduction

Live foods are the primary basis for aquatic larviculture, but their inherent variability has brought about the search for a more controlled compound feed technology during the last 25 years (Jones *et al*., 1998). The larval stage of fish is defined by the metamorphosis of external and physiological characters from hatch until juvenile stage is attained. For practical purposes, larval fish can be divided into three groups according to alimentary tract morphology and the enzymes secreted in the gut (Dabrowski and Glogovski, 1977). Those with immature digestive system at first feeding are more difficult to feed and usually require live feeds as a part of their diet.

Acceptability of artificial diets by first feeding fish larvae varies enormously and this restricts the development of a nutritionally complete diet. Promising but limited success has been reported for fish larvae fed microencapsulated and microparticulated diets as partial or full replacement for live food as live food used for larval culture is superior to any artificial diets to date (Kanazawa *et al*., 1989).

The culture of larvae of many species of fish is largely dependent upon the availability of live microbial food, whether plant or animal and the value of formulated diets that serve as complete replacement for live food is obvious because of both cost and the lack of consistent nutrient quality of live food. Formulated diets that can achieve consistent and reliable production equivalent to that of live food still do not exist and have definitely been an impediment to the progress of fish culture throughout the world (D'Abramo, 2002).

Indigenous bacterial flora may contribute significantly to larval digestion at certain stages of metamorphosis (Pinn *et al*., 1997). Microflora may serve as a supplementary source of food and microbial activity in the gut may be a source of vitamins or essential amino acids (Dall and Moriarty, 1983) as the nutritional quality of live food varies significantly from batch to batch as a result of changes in the biochemical composition (Watanabe *et al*., 1983). Some selected organisms have been found to possess appreciable levels of micronutrient (Suminto and Hirayama, 1997; Nayar *et al*., 1998).

The bacteria found in feed consumed by fish are of great importance as they can be either hazardous or beneficial. High bacterial densities of some bacteria in culture of newly hatched fish are generally considered to be deleterious to the fry (Walne, 1958). Some bacteria strains are reportedly able to invade fry, some produce toxins, while others are able to do both (Guillard, 1959; Tubiash *et al*., 1965; DiSalvo, 1978; Nottage and Birkbeck, 1986). Some of the beneficial bacteria however help to restore the water quality of the culture system and creates a conducive natural environment (Nayar *et al*., 1998) in addition to their nutritional worth.

Thus, probiotics have been used successfully in aquaculture; this is the use of live microbial feed supplement which beneficially affects the fry by improving its intestinal balance (Fuller, 1989; Verschuere *et al*., 2000). Success on cultivation of many species of fish larvae is markedly improved when live a diet is provided (Rottmann *et al*., 1991).

The objectives of this study are to determine the bacterial agents present in formulated fish feed from

Table 1: Identification and characterization of Bacterial Isolate from Formulated Albumen based diets

1	2	3	4	5	6	7	8	9	10	11	12
A	CREAM	+	Cocci	+	+	+	--	+	+	+	*Staph. Aureus*
B	YELLOW	+	Cocci	+	-	+	--	-	+	+	*Staph. epidermidis*
Key:											
1 Colony			2 Colour						3 Gram Reaction		
4 Shape			5 Anaerobicgrowth						6 Coagulase		
7 Caralase			8 Oxidase						9 Mannitol		
10 Mannose			11 Maltose						12 Organism Identified		

poultry egg albumen and the effect of live bacterial cells (food) isolated from albumen based feed on the survival of *Clarias gariepinus* larvae.

Materials and Methods

Media Preparation: The Media used in this study are Nutrient Agar (NA), Mannitol Salt Agar and MacConkey Agar.

Serial Dilution: Test tubes (10) containing 9ml of sterile distilled water, each were set up into a test tube rack.
The sample (1ml) was transferred using a sterile pipette (1ml) into one of the test tubes labeled 10^{-1} dilution then the tube was shaken properly. Using another pipette, 1ml of the 10^{-1} dilution was transferred aseptically into another tube labeled 10^{-2} dilution. The sample was aseptically diluted into ten (10) folds (up to 10^{-9} serial dilutions) (Cheesborough, 1984).

Method of Inoculation: Two methods of inoculation were used; streak and spread plate technique.

Incubation: Agar plates inoculated with sample isolates were incubated in an inverted position and the side of the Petri-dish was sealed with paper tape and place in the incubator for a period between 24 hours. The plates were incubated at room temperatures.

Preparation of Albumen Meal: Thirty (30), one hour old eggs were separated from the yolk in the Microbiology laboratory, Lagos State University into a clean beaker.
1% of 1000ml of fish oil was added to the Albumen and blended to obtain a homogenous solution. The blended Albumen was poured into a transparent clean nylon and steamed for 10 minutes. After 10 minutes, the mixture was observed for coagulation and then oven-dried at 55°C for four days. The oven-dried mixture was milled into a powdery form. From the milled albumen sample, 10 grams was collected and placed in the oven so as to determine its dry weight.

Inoculation of sample on Nutrient agar: Fresh Albumen and Albumen based formulated feed were inoculated on nutrient media. The former was inoculated directly using spread plate method while the latter was first subjected to the serial dilution method described above then using a sterile graduate pipette, 0.1ml inoculum from 10^{-1} to 10^{-4} (feed) were plated on Nutrient Agar. Each inoculated plate was duplicated in order to consider the average count.
This analysis was carried out once in a week for three (3) consecutive weeks. The inoculated plates were then incubated using an incubator with temperature maintained at 25°C for 18-24 hours. Growth was observed after 24 hours and sub cultured onto Mannitol salt agar and Mac Conkey agar.

The above analysis was carried out for two categories of feed

1. The formulated Albumen-based feed prepared under aseptic condition.
2. The formulated Albumen-based powder prepared under aseptic conditions and exposed at the fish hatchery.

Analysis of bacterial agents present in the formulated diets

Identification of Test organisms: The test organisms were identified using a number of characteristics. Cultural and morphological characteristics were of vital importance in this process and were thus, observed.

Cultural characteristics: The test organism exhibited a number of colonial characteristics with respect to their shape, size, elevation, translucence and pigmentation. This provided clues to their probable identification.

Morphological characteristics: Gram staining techniques were involved in this case. The isolates were stained and mounted for microscopic observations. Their shape, Gram reaction, cell size and arrangement were the main parameters observed in morphological characterization of the test organism.

Gram staining: Using aseptic techniques, smears of fresh cultures of 18-24 hours of the isolates were made by placing a drop of water on a clean slide with sterile flamed loop.
The smear was air-dried, then the slides were flooded with crystal violet for 30-60 seconds and then iodine as mordant was added for one minute.
The smear was then decolorized rapidly (few seconds) with acetone-alcohol by adding the reagent drop wise until crystal violet failed to wash from the smears. The

Table 2: Numbers of Bacterial colonies isolated from formulated diet exposed at the hatchery for three weeks

Distinct Bacterial Colonies (X 10^6 cfu/ml)						
Sample	Week 1		Week 2		Week 3	
Formulated Diet	Isolate A	Isolate B	Isolate A	Isolate B	Isolate A	Isolate B
	2.02	1.72	3.47	2.76	4.80	4.70

Bacterial cell densities were shown to increase by the week for both organisms

Table 3: Body weight and length of test and control fry fed with Albumen based formulated diet

Feeding Re-gime	Test fry (B)		Control fry (A)	
	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)
Formulated	10.50	19.00	8.00	17.00
diet (A) or	10.50	17.00	10.00	15.00
Live cells of	11.00	31.00	11.00	19.00
S. aureus (B)	9.00	12.00	10.00	22.00
	8.00	13.00	13.50	19.00
	8.00	14.00	13.50	12.00
	11.00	14.00	10.00	14.00
	12.00	14.00	12.00	14.00
	10.00	13.00	11.00	26.00
	15.50	25.00	14.00	11.00
Mean	10.60	17.20	11.30	16.90

slides were washed with safranin as counter stain for 2 minutes. The stain is washed off with clean water. The slides were blot dried and the smear is examined microscopically, first with 40 X objective to check the staining and to see the distribution of material, and then with the oil immersion objective to look for bacteria and cells.

Biochemical Test

Coagulase Test: This test was used to detect the presence of coagulase as it is important in the differentiation of *Staphylococcus aureus* (+) from *Staphylococcus epidermidis* (-).

Slide Method for Coagulase Test: A slide was marked into two sections. A loopful of normal saline (0.85%) NaCl in aqueous solution was place on each marked section and a loopful of an 18-24 hours old culture was added to each drop. The suspension was homogenized by stirring with a wire loop for some seconds. Clumping within 5 seconds is indicative of a coagulase positive result.

Catalase Test (Slide Method): Most aerobic organisms are capable of producing the enzyme catalase. The rate of production varies from one organism to other and Superoxide Dimutase Enzyme is mixed with Hydrogen Peroxide (H_2O_2); gaseous oxygen is released.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

A fresh culture of 18-20 hour old of the test organism was prepared with the use of sterile distilled water on a clean glass slide. Few drops of Hydrogen Peroxide was added using a dropping pipette. Effervescence caused by the release of oxygen indicates the presence of Catalase Enzyme, hence the test is positive. This was used to differentiate *Streptococcus* (catalase negative) from *Staphylococcus* (catalase positive).

Oxidase Test: A filter paper was moistened with a few drops of 1% tetramethyl - p - phenylenediamine dihydrochloride. With a wire loop, growth from agar medium was smeared on the filter paper. A positive test which was the development of a purple colour within 10 seconds confirms the presence of oxidase enzyme. The oxidase test strips were impacted with 1% Tetramethyl-p-phenyldiamine solution.

Feeding Experiment with formulated powdered egg albumen: Fifty test fry were fed with the formulated albumen based diet while fifty control fry were fed with commercial fish feed (coppens). The feeding trial was carried out for 10 days to observe the effect of the dry homogenized powdered egg albumen on the survival of the fry and the difference in growth compared to the control fry (Table 3).

Bulk production of Live cells of isolate: After the series of biochemical test and proper identification of the isolates (the two pure cultures). Isolate A was grown in nutrient broth in large amount by incubating for 18-24 hours. After incubation, the suspension in the test tube was spun using a centrifuge so as to obtain more cells at the base of the test tube. The nutrient broth was poured and after decanting and dilution, live cells of isolate A were obtained.

Feeding Experiment with Live cells of Test Organism: Fifty test fry were fed with the live cells of isolate A while fifty controls were fed with a commercial fish feed (coppens) (fry were obtained from Lagos State University Hatchery). Feeding was done for ten days so as to determine the response of the fry to the live feed. The density of the test organism that was incorporated into the culture water containing the fry was increased daily during the experimental periods and their values for the ten day feeding trials were recorded (Table 4).

Larval fish measurement: Larvae were sampled from the test and control tanks so as to measure wet weights and fish length (50 larvae/tank). Wet weights (mg) of individual larvae were determine to the nearest milligram after blotting dry on a paper towel. Total fish

Table 4: Density of cells of *Staphylococcus aureus* fed to fifty fry for an experimental period of 10 days

Study period (day)	1	2	3	4	5	6	7	8	9	10
Density of isolate(X 106cfu/ml)	0.23	0.47	0.77	1.21	3.90	4.76	7.00	8.12	8.89	9.86

length (mm) was measured to the nearest millimeter using a using an optical micrometer. This was done for larvae fed with formulated diets and live cells of microbial isolates (Table 3).

Results

In this experiment, two bacterial colonies were isolated from the formulated feed sample exposed at the hatchery. The two bacterial colonies were *Staphylococcus aureus* and *Staphylococcus epidermidis* as shown in Table 1.

The colony forming unit (cfu) of each distinct colony varied for the three week experimental periods during which the feed was exposed at the hatchery as shown on Table 2. The colony forming unit of isolate A (*Staphylococcus aureus*) per ml of the formulated feed sample for the first week was $2.02x10^6$ cfu/ml, while that for the second and third weeks were $3.47x10^6$ cfu/ml and $4.80x10^6$ cfu/ml respectively. The colony forming units of isolate B (*Staphylococcus epidermidis*) for the three experimental weeks were $1.72x10^6$ cfu/ml, $2.76x10^6$ cfu/ml, and $4.70x10^6$ for the first, second and third week respectively.

During the feeding experiment with the albumen based formulated feed, the test fry responded positively to the formulated diet just as the control. Ten fry (of the fifty) from the test and control sample tanks which were randomly weighed and their length measured respectively showed the difference in their average weight and average length as 0.3mg and 0.7mm respectively. The average weight of the test fry was 17.2mg and length 10.6mm while that of the control was 16.9mg and 11.3mm respectively.

The feeding trials using the live cells of *Staphylococcus aureus* did not have any adverse effect on the fry as no death was recorded and the fry were still active at the end of the feeding trial. As shown on Table 4, the colony forming units of *Staphylococcus aureus* was $0.23x10^6$ cfu/ml on the first day of the feeding trial; it was increased to $3.90x10^6$ cfu/ml on the fifth day and finally $9.86x10^6$ cfu/ml. After feeding the fry with increased number of cells of the test organism, the fry were discovered to have responded well to the live cells.

After being fed with increasing density of the live cells of the test organism, the fry were able to survive on the live cells that they were fed with after the feeding experiment. Two fry died on the second day, mortality increased to nine on the tenth day of the feeding trial. Thus a forty one fry survived at the end of the experiment; a survival of 80% was recorded.

Discussion

Formulation of compound, adequate diets for fish larvae at the early life stage is not easy to be achieved because the estimation of nutritional requirement of fish larvae cannot be carried out by traditional nutritional approach. Moreover, live diets ingested by larvae contain exogenous substances such as enzymes and nutritional growth factors that contribute to the digestion of prey, frequently omitted in formulated diets (Rosenlund *et al.*, 1997).

The feed, composed of dried homogenized egg albumen and fish oil was examined for bacteria growth. Two bacterial species (*Staphylococcus aureus* and *Staphylococcus epidermidis* colonies) were isolated from the albumen based feed sample. These species were isolated from both hygienically prepared fish diet and that exposed at the hatchery.

The presence of small number of *Staphylococcus* in food is common. It occurs naturally in poultry and other raw products such as egg and poultry meats. Studies in the United State and United Kingdom have found that poultry products and cold cooked meats are the most common vehicle of *Staphylococcus* species (Mossel *et al.*, 1995).

This study has also shown that feeding with live cells of *Staphylococcus aureus* promoted growth and resulted in 80% survival of the catfish (*Clarias* species) larvae.

It has been suggested that previous focus on procedures to limit or remove the presence of bacteria from the culture water of a system for production of fish larvae may, in fact, have been a bias that led to culture failures. Properly conditioned water with active bacterial populations rather than “sterile” culture water may be contributory to successful larval culture using organisms from formulated diets.

While recognizing the need to follow practices that will not induce abnormally high levels of bacteria, reduction to a level less than that found in the natural environment may reduce the probability of satisfactorily conditioning the gut for digestion of the microdiet.

The success with the feeding of some live foods may partly arise from the contribution of associated bacteria that is exposed to the gut epithelium of larval fish. The prevailing thought that bacterial level must be all but eliminated because of potentially adverse effects on culture needs to be reexamined.

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