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# Assessment of Immune Response to Two Immunostimulants as Alternatives to Antibiotics in Diets for Nile Tilapia (*Oreochromis niloticus*)

Tartiel El-Sayed Badawy [1]* and Diaa Al-Kenawy[2]

1. Central Laboratory for Aquaculture Research, Ecology and Biology Department Agricultural Research Center, Ministry of Agriculture, Cairo, Egypt.

The World Fish Center, Regional Center for Africa and West Asia, Abbassa, Abu-Hammad, Sharkia, Egypt.

*Corresponding Author

## ABSTRACT

An experiment was conducted with two different immunostimulants to detect changes in the activity of innate non-specific immune response, disease resistance and growth performance as alternatives to antibiotics in Nile Tilapia *Oreochromis niloticus*. In this experiment, fish fed diets supplemented with Immunoton*(amixture of vitamins E,C) and Lactoferrin* is a multifunctional iron-binding protein mainly present in the milk and other exocrine secretions of mammals. It belongs to the iron transporter family and plays an important role in the nonspecific immune response, antimicrobial and antioxidant actions. Seven treatments were applied with different diets containing 25% crude protein to obtain experimental treatments, three levels of Immunoton*IM (10, 20 and 30 g/kg diet), three levels of Lactoferrin LF* (200, 400 and 600 mg/kg diet ) in addition to control treatment, with three replicates each. The tested diets were applied in 21 glass aquaria each was stocked randomly with 35 Nile tilapia fingerlings with an average initial body weight 75±0.06 g/fish, each reared in aquarium (60×70×50 cm).The experiment lasted for 16 weeks. Aflatoxin AF* intoxicated fish showed off feed, sluggish swimming, dark skin and loss of scales. In addition 200µg/kg diet one time a day during three weeks for the end experimental period. The effects of AF*, IM* and LF* interactions on immunity, disease resistance and crowding stress of Nile tilapia were studied. There were significant differences ($P<0.05$) in AWG, DWG and SGR among treatments. Generally, growth performance , feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV%), apparent protein digestibility (APD) and energy utilization (EU%) were significantly ($P<0.05$) affected by the experimental treatments. Body dry matter (%) was not significantly ($P<0.05$) affected by the experimental treatments, while CP%, EE% and ash% significantly differed among treatments. The results showed that, the total leukocytic count and serum lysozyme were significantly increased with Lactoferrin* 600 mg/kg , Immunoton* 30g/kg ration for 4 weeks. On the other hand Tilapia fingerlings in the same groups produced lower value of Creatinine, Cholesterol, Glucose and Uric acid concentration while gave higher value of total protein, follicle stimulating hormone (FSH), gave best value of AST, ALT in plasma and increase in activity of total leukocyte count (TLC) compared with the control fish. This study showed that the feeding of IM* and LF* to healthy fish raised the alternatives to antibiotics and immune function of Nile tilapia *Oreochromis niloticus* immuno compromised with aflatoxin AF*and overcrowded.

**Keywords: Immunoton*, Lactoferrin*, Aflatoxin, Nile tilapia, growth performance, alternatives to antibiotics, non-specific immune response and crowding stress.**

## INTRODUCTION

The global aquaculture industry currently accounts for over 45% of all seafood consumed. That figure has been projected to increase to 75% over the next 20 years (FTU, 2007). In Egypt, the production of fish coming from aquaculture represented about 60% of total fish production sources (GAFRD, 20011). The use of antibiotics as routine feed additives has been banned in some countries because of public concern over possible antibiotic residual effects and the development of drug-resistant micro-organisms in humans (Tartiel, 2005). With the worldwide fish production and intensive cultivation system, fish are subjected to many diseases that lead to great losses and decrease in fish production. The lack of effective disease control has the potential of being chief limiting factor of the realization of highly stable fish production (Phillip, *et. al.*,2000). Aquatic animal diseases control in Egypt includes a limited number of Government-approved antibiotics and chemotherapeutics, beside limited vaccines that can be used to assist the environmental management(Tartiel *et.al.,* 2009). However, the approach which concentrates on treatment once disease develops using antibiotic are sometimes of little value or less successful due to emergence of antibiotic resistant micro-organisms (Tartiel, 2009).

Lactoferrin* is a family of an iron-binding glycoprotein that is closely related to the plasma iron-transport protein transferring and consists of a single chain peptide with a molecular, Lactoferrin has a wide range of effects on the immune system, both in vivo and in vitro (Esteban *et. al.*,2005). Immunoton* is an approach that has been actively investigated in recent years as a method for disease prevention. It does not involve recognition of a specific antigen the immune response towards a specific pathogen, but causes an overall immune response that hastens recognition of foreign proteins (Mulero, *et. al.,* 1998 ). So the use of immunostimulants for prevention of diseases in fish is considered an alternative and promising area (Sakai, 2009). Aflatoxins are a family of extremely toxic, mutagenic, and carcinogenic compounds produced by *Aspergillus flavus.* A practical definition of a mycotoxin is a fungal metabolite that causes an undesirable effect when animals or humans are exposed. Mycotoxicoses are diseases caused by exposure to foods or feeds contaminated with mycotoxins (Sahoo and Mukherjee, 2011 ).

The immune system is an important defensive mechanism against invading organisms, impaired immune function will decrease resistance to infectious diseases. Suppression of immune responses and cause immunomodulation by aflatoxin has been demonstrated in domestic animal and fishes. The feasibility of studying the disease and infection is facilitated by various stressors, handling, intensive culture and overcrowding biomass (Aly, *et. al.,* 2008). It is, therefore, important to enhance the tolerance against stress for cultured fish.

Nile Tilapia *Oreochromis niloticus* is among the most important cultured species in Egypt and allover the world. The use of immunostimulants has been suggested to be an effective mean to control fish mortality in increased biomass in aquaculture. The efficacy of the dietary LF on the immune function and disease resistance is not well established (Gahr, *et. al.,* 1991). The aim of the present work is to study the effect of dietary intake of Lactoferrin and Immunoton on innate immune response and growth performance as alternatives to antibiotics, also, investigate the adverse effect of aflatoxin and evaluate the protective effects of dietary against mycotoxin contaminated rations in Nile tilapia *Oreochromis niloticus* under the over crowding stress.

## MATERIALS AND METHODS

### *Experimental design and feeding regime*

The present work was carried out in Abbassa, Abo-Hammad, Sharkia, Egypt, to study the effect of using graded levels of

Immunoton* and Lactoferrin* on immune response and growth performance, body composition, efficiency of feed and protein utilization Nile tilapia fingerlings. Seven hundred and thirty fife fish fingerlings were stocked in 21 glass aquaria representing the seven treatments in triplicates, each aquarium (50X70X60 cm) supplied with tap water through a closed recycling system at constant level of 195 liters were used. Each aquarium was supplied with compressed air through a central air compressor. Water temperature and dissolved oxygen were recoded daily, temperature was thermostatically controlled using automatic electrical heaters and maintained at 25°C where the average of dissolved oxygen was above 6.8 mg/l. Other water quality parameters including pH and ammonia were measured every two days, where the average range of total ammonia was 0.12 – 0.23 mg/l and pH was in range of 7.2 ± 0.5 during the experiment. Fish were exposed to 12 hr light illumination/ 12 hr darkness photoperiod using 40-watt fluorescent lamps. Fish were daily fed at a rate 2% of their biomass and weekly weighed for adjusting food amounts according to the new weights. The calculated amount of food was daily offered by hand to fish, in equal portions at 8.00 am and 2.00 pm and fed three times daily. Fish in each aquarium was biweekly weighed in order to adjust the daily feed rate which was 3% of live body weight three times daily, 6 days a week for 16 weeks. Fish were fed frequently a diet containing 25% crude protein. Seven treatments were applied with different diets, three levels of Immunoton* (10, 20 and 30 g/kg diet fed) and three levels of Lactoferrin* (200, 400 and 600 mg/kg diet fed) in addition to control treatment, with three replicates each. Faeces and feed residues were removed by siphoning from each aquarium, and a half of aquariums water was replaced with de-chlorinated tap water. Two thirds of the water in each aquarium was daily replaced after removing accumulated excreta and all water in the aquaria were totally replaced every week. At the end of the experiment, fish were collected, counted and weighed.

### *Experimental fish*

A total number of 735 apparently healthy Nile tilapia (*Oreochromis niloticus*) fingerlings with an average body weight (75±0.06 g/fish) were obtained from Abbassa Fish Hatchery, Abbassa, Abo-Hammad, Sharkia, Egypt. Fish were acclimated indoor tank (600 L fiberglass) for 2 weeks to laboratory conditions for adaptation to the new environment before being randomly divided into seven equal experimental treatments. The fish were distributed randomly at a rate of 35 fish per glass aquarium (35 fish each treatment, three replicate/glass aquaria) representing seven nutritional groups. One group served as control and six groups represented the feed additives tested, it's a commercial product available in the market manufactured by El Safa Pharm for manufacturing, Al Nubaria, Egypt. Each aquarium was supplied with compressed air via air-stones from air compressor. The fish groups fed diets containing different levels of Immunoton* and Lactoferrin*. Fish in each aquarium was biweekly weighed and subsequently the amount of given feed was calculated. Dead fish were removed and kept in a deep freezer at -18°C until the chemical analysis at the end of the experiment.

### *Experimental diets*

Chemical proximate analyses of feed ingredients used in the presents study are presented in Table (1) where the control basal diet was without feed additives and the other diets were supplemented by 10, 20, and 30 g Immunoton*/Kg for diets 2, 3 and 4 & Lactoferrin* 200, 400, and 600 mg/Kg for diets 5, 6 and 7, respectively. Seven equal groups, Group (Gp.1) was the control, Groups (2-4) were fed on ration containing Immunoton* and Groups (5-7) were fed on ration containing Lactoferrin*, experimental diets were formulated to contain 25% crude protein and almost 400 Kcal gross energy/100g. The

***Table (1): Composition and proximate chemical analyses (on DM bases) of the experimental diet used.***

| Ingredients (%) | Diet 25 % CP |
|---|---|
| Fish meal (60% CP) | 10 |
| Corn gluten (60%CP) | 12 |
| Soybean meal (44% CP) | 22 |
| Wheat bran | 20 |
| Yellow corn | 30 |
| Sunflower oil | 3.5 |
| Di-calcium phosphate | 0.5 |
| Vitamin & Mineral [1] | 1.5 |
| Immunoton* | - |
| Lactoferrin* | - |
| $Cr_2O_3$ [2] | 0.5 |
| Total | 100 |
| Chemical composition (%) | Proximate analysis |
| Dry matter DM | 90.78 |
| Crude protein CP | 25.20 |
| Ether extract EE | 6.70 |
| Crude fiber CF | 6.90 |
| Ash | 7.69 |
| Nitrogen free extract NFE[3] | 53.70 |
| Gross Energy Kcal/100g[4] | 426.09 |

***1- Each Kg vitamin & mineral mixture premix contained Vitamin A, 4.8 million IU, $D_3$, 0.8 million IU; E, 4 g; K, 0.8 g; $B_1$, 0.4g; Riboflavin, 1.6 g; $B_6$, 0.6 g, $B_{12}$, 4 mg; Pantothenic acid, 4 g; Nicotinic acid, 8 g; Folic acid, 0.4g; Biotin, 20 mg; Mn, 22 g; Zn, 22 g; Fe, 12 g; Cu. 4g; I, 0.4 g; Selenium, 0.4 g and Co, 4.8 mg.***
***2- $Cr_2O_3$ : Chromic Oxide***
***3- NFE (nitrogen free extract) = 100 – (protein + lipid + ash + crude fiber).***
***4- Gross Energy (GE) : Calculated according to NRC (1993) as 5.64, 9.44 and 4.11 kcal / g for protein, lipid and NFE, respectively.***

***Table (2):- Essential amino acid analysis of the bases diet.***

| Amino acid | Basal diet | Requirements* |
|---|---|---|
| Therionine | 3.76 | 3.75 |
| Cysteine | 1.25 | 1.18 |
| Methionine | 2.72 | 2.68 |
| Isoleucine | 3.92 | 3.11 |
| Leucine | 7.68 | 3.39 |
| Phenylalanine | 5.21 | 3.75 |
| Valine | 4.24 | 2.80 |
| Lysine | 5.12 | 5.12 |
| Histdine | 2.23 | 1.72 |
| Arginine | 4.22 | 4.20 |

****Santiago and Lovell (1988), FAO (2004).***

***Table(3):- Chemical analysis of the ingredients used in the experimental diets on DM basis.***

| Proximate analysis (%) | Fish meal | Soybean meal | Yellow corn | Corn gluten | Rice bran |
|---|---|---|---|---|---|
| Dry matter | 92.21 | 90.57 | 87.30 | 91.26 | 91.18 |
| Crude protein | 72.00 | 44.00 | 7.7 | 60.00 | 12.8 |
| Ether extract | 8.8 | 2.1 | 4.1 | 2.9 | 14.00 |
| Crude fiber | 0.6 | 7.4 | 2.5 | 1.6 | 11.0 |
| Ash | 10.2 | 6.5 | 1.3 | 2.0 | 11.3 |
| NFE* | 8.4 | 40 | 84.4 | 33.5 | 50.9 |
| GE** | 525.9 | 458.0 | 429.8 | 506.8 | 452.2 |

NFE* (nitrogen free extract) = 100 – (protein + lipid + ash + crude fiber).
GE** (Gross Energy Kcal/100g ): Calculated according to (NRC, 2004)

experimental diets were formulated from available ingredients in the local market to contain 25% crude protein according to recommended requirements needed for this stage of feeding (Jauncey,2000). The experimental diets were prepared by mixing the dry ingredients in few amounts during the mixing process, individually weighing of each component and by thoroughly mixing the mineral, vitamins and additives. This mixture was added to the components together with oil, the ingredients were mixed mechanically and oil was added gradually to ensure even distribution of the ingredients. Water was gradually added with continuous mixing until clumping and the mixture become suitable for making granules. The wet mixture was passed through CBM granule machine with 2mm diameter, feed were mixed with the balanced diet in pellets. The produced pellets were dried at room temperature, stored in plastic bags and kept frozen until experimental start. Pellets were prepared and allowed to air-dry at room temperature for 24 hour before use. The required amount of the diet was prepared every two weeks, and stored in a refrigerator.

### *Sampling and analytical methods*

Representative samples of fish were randomly taken at the beginning and at the end of the experiments. Fish samples were killed and kept frozen (- 18°C) until performing the body chemical analysis. Samples of the experimental fish feed were taken, ground and stored in a deep freezer at - 18°C until proximate analysis. All of chemical analyses of fish and fish feed were analyzed for crude protein (CP%), ether extract (EE%), crude fiber (CF%), ash (%) and moisture while whole body composition of fish samples were determined according to the procedures described by standard A.O.A.C. (1995).The nitrogen free-extract (NFE%) was calculated by differences. For determination of protein digestibility the diets and faeces were collected during the last 15 days of the experimental period. Any uneaten feed or faeces from each aquarium was carefully removed by siphoning about 30 min. after the last feeding. Faeces were collected by siphoning separately from each replicate aquarium before feeding in the morning. Collected faeces were then filtered, dried in an oven at 60°C and kept in airtight containers for subsequent chemical analysis.

### *Determination of non-specific innate immune response*

A total number of 12 blood samples were collected from the caudal vein of 12 fish in each group (4 samples from each replicate) at the $2^{nd}$, $4^{th}$, $8^{th}$ , $10^{th}$ ,$12^{th}$ , $14^{th}$ and $16^{th}$ of the experimental period, each sample divided into 2 haves one after adding anticoagulant for examination of total leukocyte count (TLC) according to Schaperclaus, (1992). Blood samples were collected by heart puncture in air-dried, heparinized sterile test tubes (500 U sodium heparinate/ml) to study the non-specific defense mechanism, of total leukocyte count

(TLC). The remaining whole blood samples were centrifuged at 3000 rpm for 5 minutes and plasma was stored at - 80°C to be used for plasma lysozyme assay.

### *Growth performance parameters*

The growth performance parameters were calculated according to the following equations:-

Average weight gain (AWG) = Average final weight (g)- Average initial weight (g).

Daily weight gain (DWG) = Gain / experimental period.

Relative weight gain (RWG %) = Gain / initial weight X 100

Specific growth rate (SGR%) = (In $W_1$ – In $W_0$) / T) X 100

Where :

$W_1$ is the fish weight at the end (final weight),

$W_0$ is the weight at the start (initial weight),

In is the natural log. As described by Bagenal and Tesch (1978) and

### *Feed and protein utilization parameters*

The feed utilization parameters were calculated according to the following equations:-

Feed conversion ratio (FCR) =Total feed fed (g/fish) / total wet weight gain (g/fish).

Protein efficiency ratio (PER) = Wet weight gain (g/fish) / protein intake (g/fish).

Protein productive value (PPV) = Retained protein (g/fish)/ protein intake (g/fish) X 100

Energy utilization (EU%) = Retained energy (Kcal)/ energy intake (Kcal) X 100

### *Biochemical analysis and Analytical methods*

The basal diet and fish samples from each treatment were analyzed using the methods of A.O.A.C. (1990) for determination of moisture, crude protein, total lipids and ash. Blood samples were collected from the caudal veins and blood was allowed to set for 30 min. at 4 ◌̊C to clot, and then centrifuged for 5 min. at 1000 rpm. The serum samples were stored at -20 ◌̊C until later used to analyze total protein, creatinine, uric acid, glucose and enzymes activity aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to the method described by Moss (1984). Blood samples were preserved in sodium fluoride for estimating blood glucose. For measuring blood minerals, 0.5 ml plasma was digested with a mixture of concentration. The studied elements were determined using atomic absorption spectrophotometer (Perkin Elmer model 2280). Sodium and potassium were determined by flame photometry (AOAC,1995).

### *Apparent digestibility coefficient*

Apparent digestibility trial was performed using 0.5% chromic oxide in the diet as an inter marker to evaluate the nutrients digestibility coefficient. Fish faeces were collected by siphoning method, however, after 2 hours of fish feeding, aquaria were cleaned by siphoning the feed residues, then deposited faeces were collected every 20 min. Digestibility coefficient was determined by the following equation:

$$\text{Digestabilty coefficient} = 100 - \frac{\%\ \text{indicator in feed}}{\%\ \text{indicator in feces}} \times \frac{\%\ \text{nutrients in feces}}{\%\ \text{nutrients in feed}}$$

The measurements of chromic oxide concentration in faeces and diets were done spectrophotometer according to the method of Zivkovic and Nowar (1977).

### *Statistical analysis of data*

Statistical analysis was performed using the Analysis of variance (ANOVA) and Duncan's multiple Range Test(1955), to determine differences between treatments means at significance rate of $P < 0.05$. The standard errors of treatment means were also estimated.

All statistics were carried out using Statistical Analysis program (SAS, 2000).

## RESULTS

The growth performance parameters average weight gain (AWG), daily weight gain (DWG), relative weight gain (RWG), specific growth rate (SGR) and survival rate (SR%) of mono-sex Nile tilapia (*Oreochromis niloticus*) fingerlings which fed diets supplemented with either feed additives of Immunoton* and Lactoferrin* are shown in **Table (4).** The obtained results showed that fish fed the control diet (without feed additives) had the lowest growth. Fish fed diets containing 30g Immunoton* /Kg diets and 600 mg Lactoferrin * /Kg diets had significantly (P<0.05) higher growth rate than those fed the control diet. Average of initial body weight of mono-sex Nile tilapia (*Oreochromis niloticus*) fingerlings fed the experimental diets at the start did not differ, indicating that groups were homogenous. Whereas, the highest fed on diets GP4 and GP7, values of final body weight (192.20 ±0.55 & 189.7 ±0.81), DWG (1.05±0.07 & 1.02±0.09), RWG (156.27±23.& 152.90±33.3), SGR (0.84±0.0.05 & 0.83±0.05) and SR% (100% & 100%) were obtained by fish fed diets supplemented with either feed additives of Immunoton* and Lactoferrin* respectively, while the lower fed on diets GP1 and GP2 values of final body weight (150.4±0.32 & 166.5 ±0.24), DWG (0.67±0.02 & 0.81±0.04), RWG (100.48±22.6 & 121.0±18.2), SGR (0.63±0.05 & 0.71±0.08) and SR% (94.6% & 95.2%). However, the lowest final body weight (150.4±0.32) was achieved by the group of fish fed the control diet. Survival rate of fish groups fed diets supplemented with either feed additives of Immunoton* and Lactoferrin* were significantly (P<0.05) higher than that fed control diets.

Data in **Table (5)** representing means for feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV%) and feed efficiency ratio (FER) of fish given diets containing different levels of both additives Immunoton* and Lactoferrin* were significantly (P<0.05) higher with fish fed on diets GP4 and GP7 compared with those fish fed the control diet. The best FCR, highest PER and the highest (PPV%) of fish fed on diets GP4 and GP7 were significantly (P<0.05) improved in comparison with the other groups and better than the control diet. The FCR was found to be 1.98±0.22 (control), 1.67±0.58, 1.43±0.41, 1.32±0.09, 1.48±0.17, 1.42±0.08 and 1.34±0.66, respectively. The same trend was observed in PER where the fish groups fed on diets GP4 and GP7 showed better (P<0.05) PER values compared with the other groups. The PER was found to be 1.74± 0.02 (control diet), 2.20±0.055, 2.62±0.061, 2.95±0.077, 2.63±0.032, 2.70±0.041 and 2.90±0.08 , for group of fish fed diets GP2, GP3, GP4, GP5, GP6, GP7 respectively. In the present study, the commercial feed additives used significantly (P<0.05) enhanced feed efficiency.

The results of the proximate chemical analysis of the whole-fish body of Nile tilapia fed different levels of additives for moisture, crude protein, total lipids and ash at the end of the study are shown in **Table (6).** Moisture content increased with the increase in dietary additives (P<0.05). The highest moisture content was obtained with fish groups GP4 and GP7 fed 30g Immunoton* /Kg diets and 600 mg Lactoferrin * /Kg diets (73.9% and 73.7%, respectively). The moisture content in those two groups of fish were significantly higher than that of fish fed the other diets. The lowest moisture content was obtained at control group (73.20%). Crude protein content in whole-fish body increased significantly with fish fed 30g Immunoton* /Kg diets and 600 mg Lactoferrin * /Kg diet (64.7% and 64.2%, P<0.05) compared to fish fed the control diet (60.0%). Total lipids decreased with the increase of additives levels in diets (P<0.05).The lipid percentages were significantly (P<0.05) lower in

***Table (4):- Growth performance of Nille tilapia fingerlings fed diets containing different levels of additives.***

| Items | Diets | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Immunoton* (g/kg diet) | | | Lactoferrin * (mg/kg diet) | | |
| | GP1 | GP2 | GP3 | GP4 | GP5 | GP6 | GP7 |
| Initial weight (g/fish) | 75.02 a ±0.09 | 75.31 a ±0.03 | 75.11 a ±0.06 | 75.00 a ±0.05 | 75.04 a ±0.02 | 74.95 a ±0.06 | 75.01 a ±0.02 |
| Final weight (g/fish) | 150.4 d±0.32 | 166.5 c c±0.24 | 178.3 b±0.22 | 192.2 a±0.55 | 174.9 ab±0.2 | 182.6 b±0.07 | 189.7 a±0.81 |
| Weight gain (g/fish) | 75.38 | 91.19 | 103.19 | 117.2 | 99.86 | 107.65 | 114.7 |
| AWG | d±0.15 | c±0.74 | ab±0.6 | a±0.02 | ab±0.4 | b±0.22 | a±0.33 |
| Weight gain (% | 50.12 d±12.4 | 54.77 cd±4.6 | 57.87 ab±1.7 | 60.98 a±16.2 | 57.09 c±11.1 | 58.95 b±14.2 | 60.45 a±12.8 |
| Daily weight gain | 0.67 | 0.81 | 0.92 | 1.05 | 0.89 | 0.96 | 1.02 |
| (g/day/fish) DWG | d±0.02 | c±0.04 | b±0.07 | a±0.07 | ab±0.2 | b±0.03 | a±0.09 |
| Relative weight gain | 100.48 | 121.0 | 137.3 | 156.27 | 133.07 | 143.63 | 152.90 |
| (RWG) | d±22.6 | cd±18.2 | bc±33.4 | a±23.2 | c±33.2 | b±41.3 | a±33.3 |
| Specific growth rate | 0.63 c | 0.71 cb | 0.77 ab | 0.84 a | 0.76 ab | 0.79 b | 0.83 a |
| (SGR %) | ±0.05 | ±0.08 | ±0.07 | ±0.05 | ±0.06 | ±0.06 | ±0.05 |
| Survival rate | 49.6 c | 77.2 b | 81.2ab | 91.0 a | 80.6 b | 83.4 ab | 90.2 a |
| (SR %) | ±2.25 | ±3.42 | ±3.45 | ±3.01 | ±4.33 | ±3.22 | ±4.11 |

***Data are represented as mean of three samples replicates ± standard error***
***Means in the same row with the same letter are not significant difference (P>0.05).***

fish group fed diets supplemented with feed additives compared to the lipid percentage of the fish fed the control diet. Ash content in whole-fish body was slightly increased with increasing the dietary additives levels (P<0.05) with no significant differences among treatments. The highest ash content was obtained at fish groups GP4 and GP7 fed 30g Immunoton* /Kg diets and 600 mg Lactoferrin * /Kg diets (14.1 and14.0% P<0.05), while the lowest ash content was obtained at fish group fed control diet (13.7%). These results are indicating the healthy status of Nile tilapia in this study irrespective to additives levels in the diet.

**Table (7)** is showing the data of some blood constituent (Creatinine, Cholesterol, Glucose, Uric acid and total protein) in plasma of Nile tilapia (*Oreochromis niloticus*) as affected by different treatments of feeding and fertilization. Slight variations were noticed among treatment in regard to the content of different blood parameters. However, treatment GP4, where fed 30g Immunoton/Kg diet were used, showed the lowest content of creatinine, cholesterol and uric acid and the highest content of alanine aminotransferase enzyme (ALT), aspartate aminotransferase enzyme (AST), glucose, total protein, follicle stimulating hormone (FSH) and total leukocyte count (TLC). Treatment GP4 showed reverse trend compared with GP1 (control), highest content of creatinine, cholesterol and uric acid and lowest content of aminotranferases (ALT and AST), total protein and FSH. Blood parameters content in fish of both GP2, GP3, GP5 and GP6 had moderate vacillating values and fall between the values of GP1 and GP4. Hematological tests and analyses of serum constituents have proved useful in the detection and diagnosis of metabolic disturbances, such tests should be supplemented with clinical and biochemical analysis for diagnostic purposes. Also, determination of AST and ALT have proved

**Table (5):** ***Feed utilization parameters of Nile tilapia fingerlings fed diets containing different levels of additives.***

| Items | Diets | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control GP1 | Immunoton* (g/kg diet) | | | Lactoferrin * (mg/kg diet) | | |
| | | GP2 | GP3 | GP4 | GP5 | GP6 | GP7 |
| Feed intake (g feed/fish) | 149.25 b±0.27 | 152.29 ab±0.2 | 147.56 b±0.1 | 154.70 a±0.33 | 147.80 b±0.41 | 152.86 ab±0.7 | 153.68 a±0.65 |
| Protein intake in feed (PI) | 43.32 a ±0.62 | 41.45 a ±0.47 | 39.85 b ±0.23 | 39.73 b ±0.18 | 37.97ab ±0.68 | 39.87 b ±0.24 | 39.55 b ±0.84 |
| Feed conversion ratio (FCR) | 1.98 a ±0.22 | 1.67 ab ±0.58 | 1.43 b ±0.41 | 1.32 c ±0.09 | 1.48 b ±0.17 | 1.42 b ±0.08 | 1.34 c ±0.66 |
| Protein gain (PG) | 4522.8 d±65.2 | 5681.1 c±88.4 | 6500.9 b±65.7 | 7582.8 a±74.2 | 6331.1 bc±88.7 | 6835.8 b±94.2 | 7363.0 a±55.4 |
| Protein efficiency ratio (PER) | 1.74 c ±0.02 | 2.20 cb ±0.055 | 2.62 ab ±0.061 | 2.95 a ±0.077 | 2.63 ab ±0.032 | 2.70 b ±0.041 | 2.90 a ±0.08 |
| Retained protein (RP) | 45.01 c ±0.9 | 46.92ab ±0.3 | 47.33 ab±0.5 | 48.52 a±0.3 | 47.58 ab±0.2 | 47.59 b±0.8 | 48.16 a±0.8 |
| Protein productive value (PPV%) | 103.9 c±11.3 | 113.2 ab±9.4 | 118.8 b±15.2 | 122.1 a±22.5 | 125.3 a±44.2 | 119.4 b±13.6 | 121.8 a±51.2 |
| Feed efficiency ratio[1] (FER) | 0.50 d ±0.04 | 0.60 c ±0.09 | 0.69 ab ±0.07 | 0.76 a ±0.05 | 0.68 ab ±0.06 | 0.70 b ±0.08 | 0.75 a ±0.05 |
| Apparent protein utilization[2] (APU%) | 104.4 d±33.2 | 137.1 cd±2.8 | 163.1 c±22.4 | 190.9 a±24.1 | 166.7 c±44.3 | 171.5 b±71.1 | 186.2 a±12.3 |
| Energy utilization[3] (EU%) | 29.8 b±0.03 | 31.23 b±0.04 | 34.05 ab±0.7 | 39.15 a±0.06 | 34.79 ab±0.03 | 35.12 ab±0.4 | 38.70 a±0.09 |

***Data are represented as mean of three samples replicates ± standard error***
***Means in the same row with the same letter are not significant difference (P>0.05)***
***1- Feed efficiency ratio (FER) = body weight gain (g)/ feed intake (g)***
***2- Apparent protein utilization (APU%) = Protein gain in fish (g) / protein intake x 100***
***3- Energy utilization (EU%) = calculated energy gain / calculated energy intake x 100***

useful in the diagnosis of liver and kidney diseases in fish, Creatinine and uric aci are considered as good indicators of glomular filtration rate and kidney dysfunction. there levels were significantly higher in GP4 compared with GP1 and GP2.

The digestion coefficient values of crude protein, total lipids and carbohydrates are presented in **Table (8)** were significantly (P<0.05).The highest digestibility coefficient were improved for tilapia fingerlings fed on the diets supplemented by commercial feed additives compared to group of fish fed the control diet. The digestibility coefficient of crude protein was found to be 72.3% (control diet), 78.4%, 79.1%, 83.2%, 77.9%, 78.8% and 81.6% respectively. The highest digestibility coefficient of crude protein was obtained in fish groups GP4 and GP7 fed 30g Immunoton* /Kg diets and 600 mg Lactoferrin * /Kg diets (P<0.05), while the lowest digestibility coefficient was obtained in fish group fed the control diet. The digestibility coefficient of total lipids were higher in fish groups fed on diets GP4 and GP7 (80.6% and 79.8%) than the other fish groups (P<0.05), while the lowest digestibility coefficient of total lipids was obtained at fish group fed control diet (68.9%, P<0.05). The digestibility coefficient of carbohydrates were higher in fish groups fed on diets GP4 and GP7 (83.5% and 82.1%) than the fish group fed control diet (73.8%, P<0.05). The better digestibility obtained with the addition of additives improved diet, which may in turn

**Table (6):** ***Proximate chemical analysis (% on dry matter basis)of whole body of Nile tilapia fingerlings fed diets containing different levels of additives.***

| Items | Diets | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control GP1 | Immunoton* (g/kg diet) | | | Lactoferrin * (mg/kg diet) | | |
| | | GP2 | GP3 | GP4 | GP5 | GP6 | GP7 |
| Moisture | 73.2 a ±0.233 | 73.3 a ±.0254 | 73.4 a ±0.558 | 73.9 a ±0.452 | 73.5 a ±0.741 | 73.5 a ±222 | 73.7 a ±0.363 |
| Crude protein (CP %) | 60.0 c ±0.02 | 62.3 b ±0.07 | 63.0 ab ±0.04 | 64.7 a ±0.07 | 63.4 ab ±0.02 | 63.5 ab ±0.04 | 64.2 a ±0.05 |
| Total lipids (%) | 25.5 a ±1.35 | 22.3 ab ±1.39 | 22.4 ab ±1.36 | 20.7 b ±1.22 | 23.6 a ±2.22 | 22.0 ab ±1.04 | 21.2 b ±0.94 |
| Ash (%) | 13.7 ab ±0.147 | 12.9 b ±0.552 | 13.8 ab ±0.441 | 14.1 a ±0.323 | 12.8 b ±0.636 | 13.3 ab ±0.412 | 14.0 a ±0.412 |
| Gross energy (Kcal) | 582.4a ±0.29 | 572.2ab ±0.36 | 570.1ab ±0.04 | 562.4b ±0.09 | 581.2a ±0.21 | 566.6b ±0.65 | 564.7b ±0.55 |

***Data are represented as mean of three samples replicates ± standard error***
***Means in the same row with the same letter are not significant difference (P>0.05)***
***NFE (nitrogen free extract) = 100 – (protein + lipid + ash + crude fiber).***
***Gross Energy (GE): Calculated according to NRC (1993) as 5.64, 9.44 and 4.11 kcal / g for protein, lipid and NFE, respectively.***

**Table (7):** ***Some blood constituent (in plasma) of Nile tilapia (Oreochromis niloticus) as affected by different treatments.***

| Items | Diets | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control GP1 | Immunoton* (g/kg diet) | | | Lactoferrin * (mg/kg diet) | | |
| | | GP2 | GP3 | GP4 | GP5 | GP6 | GP7 |
| Creatinine (mg/100 ml) | 2.33 a ±0.05 | 2.05 ab ±0.31 | 1.96 b ±0.06 | 1.73 c ±0.07 | 1.91 b ±0.06 | 1.88 cb ±0.02 | 1.77 c ±0.21 |
| Cholesterol (mg/100ml) | 128.5 a ±49.2 | 116.8 ab±55.1 | 107.9 b ±23.4 | 98.2 c ±71.2 | 119.2 a ±85.4 | 111.2 ab±11.3 | 99.0c ±22.1 |
| Glucose (mg/100ml) | 112.3 d ±0.07 | 153.7c ±0.07 | 178.6 bc±0.05 | 216.8 a ±0.045 | 188.9 b ±0.022 | 196.4 ab±0.079 | 212.9 a ±0.044 |
| Uric acid (mg/100ml) | 2.02 a ±0.01 | 1.68 b ±0.03 | 1.60 c ±0.08 | 1.44 bc ±0.07 | 1.83 ab ±0.06 | 1.71 ab ±0.07 | 1.22 a ±0.04 |
| **ALT | 17.92 c ±0.12 | 18.73b ±0.31 | 19.65 b ±0.51 | 22.57 a ±0.52 | 19.05 b ±0.54 | 20.03ab ±0.57 | 21.96 a ±0.56 |
| **AST | 46.33 c ±14.2 | 52.04 cb±11.2 | 57.61 b ±12.2 | 64.33 a ±14.4 | 58.01 ab ±17.5 | 59.04 ab ±15.6 | 61.78 a ±11.1 |
| Total protein (g/L) | 23.94 c ±0.02 | 23.77 c ±0.05 | 24.21 b ±0.05 | 27.61 a ±0.07 | 24.25 b ±0.08 | 24.50 b ±0.09 | 26.22 a ±0.09 |
| **FSH (g/ml) | 0.14 d ±0.044 | 0.19 c ±0.012 | 0.21 b ±0.051 | 0.26 a ±0.072 | 0.22 b ±0.011 | 0.24 ab ±0.021 | 0.26 a ±0.041 |
| **TLC $10^3/\mu$ L | 34.06 c±0.06 | 37.12 b±0.02 | 41.03 ab±0.22 | 46.82 a±0.41 | 35.64 cb±0.29 | 41.76 ab±0.25 | 44.27 a±0.088 |

*****ALT alanine aminotransferase enzyme*** *****AST aspartate aminotransferase enzyme***
*****F.S.H. : Follicle stimulating hormone.*** *****TLC total leukocyte count***

***Table 8. Apparent digestibility coefficients (%) of mono-sex Nile tilapia fingerlings fed diets containing different levels of additives.***

| Items % | Diets | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control GP1 | Immunoton* (g/kg diet) | | | Lactoferrin * (mg/kg diet) | | |
| | | GP2 | GP3 | GP4 | GP5 | GP6 | GP7 |
| Crude protein | 72.3 c ±19.3 | 78.4 b ±22.7 | 79.1 b ±23.7 | 83.2 a ±17.6 | 77.9 ab ±21.1 | 78.8 b ±21.2 | 81.6 a ±19.6 |
| Total lipids | 68.9 d ±0.47 | 77.7 bc ±0.033 | 78.2 b ±0.54 | 80.6 a ±0.66 | 68.8 d ±0.87 | 72.4 c ±0.92 | 79.8 a ±.041 |
| Carbohydrates | 73.8 c ±0.55 | 75.3 b ±0.71 | 80.2 ab ±0.65 | 83.5 a ±0.66 | 76.6 b ±0.64 | 78.3 ab ±0.62 | 82.1 a ±0.68 |

***Data are represented as mean of three samples replicates ± standard error***
***Means in the same row with the same letter are not significant difference (P>0.05)***

explain the better growth and feed efficiency noticed with the supplemented diets.

## DISCUSSION

During the last decade there was an increasing interest in the modulation of the non specific immune response of fish to elevate the general defense barriers and hence increase against diseases through use of immune-stimulants. It is well known that the ideal conditions are always prevailing. Under these conditions, the requirements for certain nutrients will almost certainly increase due to an increased activity of the immune system. From the presented results, use of Immunoton* and Lactoferrin* as feed additives at a dose of 30g and 600mg/kg of diet Nile tilapia (*Oreochromis niloticus*) fingerlings (alternatives to antibiotics ) for 3 weeks has shown to be a powerful activator of non specific innate immune response. The use of natural immune-stimulants is promising in aquaculture because they are safe for the environment and human health, the Lactoferrin*(LF) is an iron-binding protein included in several biological functions (Weinberg, 2003), while Immunoton*(IM) is mixed vitamin E and vitamin c. (Kakuta, 1997) reported an increase in growth performance and survival rates with dietary intake of Immunoton to common carp at a dose of 15mg/kg body weight for 20 days.

From our presented data dietary intake Immunoton*(IM) and Lactoferrin*(LF) showed a significant (P<0.05) increase in growth performance parameters average weight gain (AWG), daily weight gain (DWG), relative weight gain (RWG), specific growth rate (SGR) and survival rate (SR%) of mono-sex Nile tilapia (*Oreochromis niloticus*) fingerlings, also increased concentrations of alanine aminotransferase enzyme (ALT), aspartate aminotransferase enzyme (AST), glucose, total protein, follicle stimulating hormone (FSH) and total leukocyte count (TLC) in Nile tilapia (*Oreochromis niloticus*) fingerlings. Similar results on rainbow trout obtained by Diab *et al*., (2002); Khokhlova *et al*.. (2004) and Ortuno *et al.,* (1999) who reported that combination of vitamin C and E significantly increase (P<0.05) lymphocyte proliferation. The results of this work revealed that, the feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV%) and feed efficiency ratio (FER) of fish given diets containing different levels of both additives Immunoton* and Lactoferrin* were significantly (P<0.05). In contrast (Mohamed *et al.,* 2007; Olvera *et al.*,1998 and Lygren *et al.,*1999) found that in vitro addition of either C or E individually had effect on the protein and feed utilization and even combination of both vitamins failed to further increase activity at any of the

concentrations vitamin C or vitamin E (Mulero *et al.,* 1998; Sakai, 1999; Gatlin, 2002 and Cuesta *et al.,* 2001). Kumari *et al.,* (2003); El-Boushy and El-Ashram, (2002) and Gahr *et al.,* (1991) showed that the Lactoferrin*(LF) feeding at a rate of 200 mg/kg diet for 1 week enhanced the cellular immune response, leukocyte peroxidase content, respiratory burst and feed utilization in Nile tilapia (*Oreochromis niloticus*) fingerlings. Moreover, Alvares-pellitero *et al.,* (2006); De-Schrijver and Ollevier (2006); Mehrim (2001) and Kumari *et al*., (2003) stated that the LF increased chemiluminescent response and nitroblue tetrazollium reaction of the cells in *Oreochromis niloticus.* It is worthy to mention that, when the mammalian phagocytes were exposed to highly purified human Lactoferrin, they exhibited an increased phagocytic activity and were primed to produce more radical oxygen (Anderson *et al.,* 1995 and Mehrim, 2001). The present study, showed a significant increase ($P<0.05$) some blood constituent (Creatinine, Cholesterol, Glucose, Uric acid and total protein) in plasma of Nile tilapia. De-Schrijver and Ollevier (2006) and Campos *et al.,* (1998) who observed an increase in digestibility values of crude protein, total lipids and carbohydrates

Aflatoxin is one of the most important biological pollutants since the discovery of fungal toxins at 1960. In a developmental country, it is common to observe feeds contaminated with mycotoxins as well as Aflatoxin, this has been reported in human/animal feeds (Cotty and Garica, 2007 and Bandyopadhyay *et al.,* 2007). Aflatoxin lowers the resistance to diseases and interferes with vaccine-induced immunity in livestock (Cotty *et al.,* 1994 and Aly *et al.,* 2008). Aflatoxin intoxicated fish showed off feed, sluggish swimming, dark skin, loss of reflexes, increased mucus secretion, loss of scales and ascities. The spleen and the kidneys appeared enlarged, congested and dark in color. The elevation of urea in Aflatoxin in fish produced by liver and excreted primarily by the gills rather more kidney (Compos *et al.,* 1998 and Esteban *et al.,* 2005). In the same line, El-Enbaawy *et al.,* (1994) and Kumari, (2003) reported elevation of liver transaminase of *Oreochromis niloticus* that had been intoxicated with Aflatoxin. Many studies similarly, recorded increased resistance against *A. hydrophilla* challenged after stimulation with Lactoferrin (Sahoo and Mukherjee, 2011 and Welker *et al.,* 2007), while the lower resistance in case of Immunoton could be attributed to the low doses of vitamin C and vitamin E (300mg/kg diet) presents in the supplemented diet and so it could not induce maximum immunity. Although these doses were above the minimal levels needed for growth in addition to presence of lactose in the product that could help in growth promoting activity with maximum feed utilization (Mohamed *et al.,* 2007 and Thompson *et al.,* 1999). Stress-induced increase in cortisol blood level has been reported by Eteban (2005) and Welker *et al.,* (2007) in different species of fishes. The results were in accordance with Gahr *et al.,* (1991) and Kumari *et al.,* (2003) who reported that the LF did not produce any reduction in the elevated Creatinine, Cholesterol, Glucose and Uric acid levels in plasma in the stressed juvenile Nile tilapia (*Oreochromis niloticus*) when fed nutritionally complete, practical basal diets supplemented with the bovine lactoferrin LF at 0, 200, 400, 800 mg/kg diet twice daily for 8 weeks. Other species of fish showed an increased resistance to the crowding stress as a response to LF in their diet.

So in conclusion, the present experiment showed that supplement mono-sex fingerlings Nile tilapia diets with fed 30g Immunoton* /Kg diets and 600 mg Lactoferrin * /Kg diets has a positive influence on growth performances, feed utilization parameters, chemical body composition and digestibility coefficients. The results of this work, dietary intake of Immunoton and Lactoferrin had a direct positive effect on the non specific cellular

immunity of Nile-tilapia when mixed with fish ration in overcrowded stress fish culture facilities.

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## تقييم لمدى الاستجابة المناعية بإضافة بعض محفزات المناعة كبدائل للمضادات الحيوية فى علائق أسماك البلطى النيلى

ترتيل السيد محمد بدوى[١] - ضياء عبد الرحيم القناوى٢
١- قسم بحوث البيئة و البيولوجى- المعمل المركزى لبحوث الثروة السمكية بالعباسة–مركزالبحوث الزراعية- وزارة الزراعة- الدقى- مصر.
٢- المركز الدولى للاسماك – المركز الاقليمى لافريقيا وغرب اسيا – العباسة – محافظة الشرقية.

يزداد الوعى البيئى يوما بعد يوم بضرورة أستخدام المركبات الطبيعية للوقاية والعلاج من الامراض ورفع المناعة ضد عوامل الاجهاد والتسمم، وقد أجريت هذه التجربة لتتقييم كفاءة نوعين مختلفين حيث تم استخدام الاميونوتون Immunoton وهومخلوط من فيتامين ج، ه مضاف اليهم الصوديوم سيلنيت وحمض الفوليك وسكراللاكتوز، أما Lactoferrin اللاكتوفيرين فهو عبارة عن بروتين متحد مع الحديد ويتواجد فى اللبن والافرازات الاخرى للثدييات وينتمى لعائلة حاملات الحديد، ولهذه المركبات دورا هاما وحيويا فى رفع المناعة ومضادات للاكسدة وتهدف هذه الدراسة الى تأثير هذه المركبات على مناعة أسماك البلطى وتقليل الاضرار الناجمة لبعض السموم الفطرية مثل الافلاتوكسين ومن أخطر أضرارها تأثيرها المثبط للجهاز المناعى وتنتشر هذه السموم فى العلائق التى يتغذى عليها الاسماك مما يؤثر ذلك على النمو ويعتبر تلوث الاعلاف بالفطريات من أخطر المشاكل والمعوقات التى تواجه تنمية وتطوير الثروة السمكية. . تم تطبيق ٦ معاملات بمستويات مختلفة فى ثلاثة مكررات لكل معاملة بالاضافة الى معاملة الكنترول حيث وزعت أسماك البلطى النيلى عشوائيا بمتوسط وزن ٧٥ ±٠,٠٦ جم فى ٢١ حوض زجاجى (٦٠×٧٠×٥٠ سم) بمعدل ٣٥ سمكة/حوض لتعرض الاسماك للاجهاد نتيجة الزحام وتمت التغذية على علائق تجارية (٢٥% بروتين) مضاف اليها الاميونوتون بمعدل ١٠، ٢٠، ٣٠جم/ كجم عليقة وكذلك تم اضافة اللاكتوفيرين بمعدل ٢٠٠، ٤٠٠، ٦٠٠ ملجم/ كجم عليقة وأستمرت التجربة لمدة ١٦ أسبوع ولكن قبل نهاية التجربة بثلاثة أسابيع تم اضافة ٢٠٠ ميكروجرام افلاتوكسين/كجم علف لكل معاملة. أوضحت النتائج أن أفضل أداء للنمو ممثل فى الزيادة فى الوزن AWG ، ومعدل النمو اليومى DWG ، ومعدل النمو النوعى SGR فى أصبعيات الاسماك التى غذيت على عليقة مضاف اليها ٣٠ جم من الاميونوتون / كجم عليقة ، بينما أظهرت النتائج ان أعلى قيم لتلك المقاييس فى الاسماك التى تغذت على العلائق المضاف لها اللاكتوفيرين كانت فى المعاملة ٦٠٠ ملجم/كجم عليقة. كما أظهرت أيضا النتائج ان معامل التحويل الغذائى FCR ، الكفاءة النسبية للبروتين PER ، القيمة الانتاجية للبروتين PPV ، معامل هضم البروتين APD وكفاءة أستخدام الطاقة EU كانت أفضل معنويا ($P<0.05$) فى أسماك نفس المعاملات وذلك مقارنة بمجموعة الاسماك التى تغذت على عليقة الكنترول. أوضحت النتائج زيادة معنوية بعد التغذية لمدة أسبوعين فى الاستجابة المناعية الخلوية حيث زاد العدد الكلى لخلايا الدم البيضاء وكذلك زيادة تركيز وكفاءة أنزيمات التحلل، وانزيمات الترانسى أمين . ALT, ASتأثر معنويا ($P<0.05$) محتوى جسم الاسماك من المادة الجافة والبروتين الكلى وكذلك الهرمون المشجع لنمو الحويصلات FSH فى بلازما الدم حيث سجلت أعلى قيم فى نفس المعاملات ادى الى تحسن الوظائف المناعية وزيادة فى العدد الكلى لكرات الدم البيضاء TLC وكذلك انخفاض معدل النفوق بنسبة ٤٠% مقارنة بمعاملة الكنترول.

توصى هذه الدراسة بالاتى:-

١- تحليل العلائق المستخدمة فى تغذية الاسماك للتأكد من خلوها من السموم الفطرية واعدام العلائق المصابة .
٢- تحليل الاسماك قبل تسويقها للتأكد من خلوها من السموم الفطرية لحماية الانسان من تأثير التسمم.
٣- اضافة الاميونوتون و اللاكتوفيرين للعلائق كبدائل للمضادات الحيوية لما لها من تأثير جيد فى رفع مقاومة الاسماك ضد عوامل الاجهاد والتسمم وتقوية المناعة الخلوية.