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The Biochemical, Pathological and Immunological Effectiveness of Commercial Probiotics in Nile Tilapia, *Oreochromis niloticus*

Mahmoud Tanekhy,[1]* Ryad Khalil,[1] Hanaa Hofi[2] and Emad Hashish[3]
[1]*Fish Diseases Department, Faculty of Veterinary Medicine, Alexandria University, Edfina, Egypt.*
[2]*Animal Health Research Institute, Damanhour Branch, Egypt.*
[3]*Faculty of Veterinary Medicine, Zagazig University, Egypt.*

ABSTRACT

Improved resistance against diseases in Nile tilapia, *Oreochromis niloticus* can be achieved by probiotics. A total of 450 fish (50±5 g) were divided into 5 groups: 1st group received diet with 0.1 ml/kg C.A growth® probiotic, 2nd group received diet with 0.2 ml/kg feed C.A growth® probiotic, 3rd group received diet with 0.15 g/kg feed Tonolest® probiotic, 4th group received diet with 0.3 g/kg feed Tonolest® probiotic, 5th group was fed on probiotic-free diet. Eight weeks later, fish fed C.A growth® probiotic revealed significant increase in phagocytic activity, differential leucocytic count, serum lysozyme and bactericidal activity, total protein and globulin levels than fish fed on Tonolest® probiotic. Moreover, groups receiving C.A growth® probiotic showed significant increase in antibody titre against *Aeromonas hydrophila* than other groups. In addition, Nile tilapia receiving C.A growth® probiotic showed significant decrease in cortisol hormone and bacterial counts in gastrointestinal tract than fish receiving Tonolest® probiotic in all weeks. C.A growth® probiotic evoked no/limited cellular damage and decreased pathological lesions in groups challenged with *A. hydrophila*. On the other hand, Tonolest® probiotic evoked more pathological damage in organs especially with high dose and less effective in decreasing pathological changes in groups challenged with *A. hydrophila*.

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Authors' Contribution
MT and RK designed the study. HH performed field work and collected serum samples. RK analyzed serum samples. MT processed and analyzed the data and wrote the article. EH performed histopathological and fish physiology analyses.

Key words
Tilapia, innate response, histopathology, *Aeromonas hydrophila*, *Oreochromis niloticus*

INTRODUCTION

The intensive rearing of fish species in aquaculture generates a potentially stressful environment to fish, with the possible suppression of the immune system, rendering the fish more susceptible to different diseases (Austin and Austin, 1999). The routine use of antibiotics during fish culture to minimize the risk of disease is not advisable since it may adversely affect the indigenous microflora of juveniles or adult fish and may increase the risk of promoting antibiotic-resistant organisms (Alderman and Hastings, 1998**)**. Thus, the use of probiotics, in the culture of aquatic organisms, is increasing with the demand for more environment-friendly aquaculture practices (Gatesoupe, 1999).

Probiotics are usually members of the healthy intestinal microbiota; therefore, they may provide an alternative way to reduce the antibiotics use, since their addition can assist in returning a disturbed microbiota to its normal beneficial composition. In the past 10 years there has been a growing interest in fish farming to control diseases through alternative methods, such as probiotics (Irianto and Austin, 2002).

Probiotics in aquaculture have been shown to have several modes of action; competitive exclusion of pathogenic bacteria through the production of inhibitory compounds (Servin, 2004), improvement of water quality (Verschuere *et al.*, 2000), enhancement of immune response of host species (Ortuno *et al.*, 2002) and enhancement of nutrition of host species through the production of supplemental digestive enzymes (Tovar-Ramirez *et al.*, 2002). Probiotics have the ability to improve fish health and prevent bacterial diseases in fish. Consequently, the use of probiotics as a new technique to confer protection in the host fish against pathogenic bacteria in the most economic and environment-friendly manner is certainly worth evaluating in aquaculture (Thomas and Chhorn, 2011).

Probiotics are widely used in poultry and swine rearing farms but little has been done to incorporate them into aquaculture. Thus, the current study was aimed to evaluate, immunologically and pathologically the efficiency of some microbial approved probiotics on the culture of Nile tilapia.

MATERIALS AND METHODS

The experiment aimed to evaluate the effects of 2 commercial probiotics (C.A. growth® and Tonolest®) on the immune response and health conditions of Nile tilapia.

* Corresponding author: tanekhyvet2020@yahoo.com
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Fish

Total of 450 apparently healthy Nile tilapia fish (50±5g) were kept in prepared glass aquaria (90×50×35 cm). These aquaria were used for holding the experimental fish throughout the study period (triplicate each treatment), supplied with chlorine free tap water (Innes, 1966).

All fish were apparently free from any pathogenic bacteria and free from parasitic infestation with similar size (averaging 50±5 g in weight). They were transferred in polyethylene bags to the laboratory where the experiments were carried out. Daily food was offered as two equal meals / day (at 9:00 AM and 12:00 PM). Moreover, the fish mortality was recorded daily. Fish were fed once a day at 3% of their body weight on a commercial fish diet containing 25% crude protein.

Probiotics

a) C.A. growth® probiotic which contains lactobacillus, pediococcus, saccharomyces and gluconacetobacter.

b) Tonolest® probiotic is dry yeast used for animal as a growth promoter containing vit B12. The yeast is highly digestible and good for poultry, animals, and fish as appetizer and growth promoters maintaining the health. With ingredients; *Saccharomyces cerevisiae* active live yeast 15X 10^9 cells/gm.

Procedure adopted

Following acclimation to the laboratory conditions, 21 aquaria were divided into seven groups (3 aquaria in each group). The 1st group (control) fed on basal diet without any treatment. The 2nd group was fed on basal diet and 0.1ml/ kg feed C.A. growth® probiotic. The 3rd group was fed on basal diet and 0.2 ml / kg C.A. growth® probiotic. The 4th group was fed on basal diet and 0.15 g/kg feed Tonolest® probiotic. The 5th group was fed on basal diet and 0.3 g/kg feed Tonolest® probiotic. Two thirds of water was changed every alternate day (Eurell *et al.*, 1978).

Two ml blood were collected from different groups via the caudal vessels from 2 fish using disposable syringe at zero day, 2nd, 4th, 6th, 8th weeks during the experimental period. One ml of blood was collected with syringe containing anticoagulant (0.1 ml of 4% sodium citrate solution/1ml blood) for phagocytic assay and differential leucocytic count and an other one ml was kept in refrigerator and then centrifuged to get the serum. The collected serum was used for biochemical determination (Lied *et al.*, 1975).

Differential leucocytic count

Blood film was prepared and the percentage and absolute value of each type of cells were calculated (Lucky, 1977; Schalm, 1986).

Determination of phagocytic activity and phagocytic index

Phagocytic activity was determined according to Kawahara *et al.* (1991).

Phagocytic activity (PA) = percentage of phagocytic cells containing yeast cells.

$$\text{Phagocytic index (PI)} = \frac{\text{Number of yeast cells phagocytized}}{\text{Number of phagocytic cells}}$$

Results were expressed as means±S.E. and differences were evaluated by Student's t-test.

Determination of serum protein

Serum total protein was determined using commercial kits produced by Pasteur Lab (Domuas *et al.*, 1981). Serum albumin was determined (Reinhold, 1953) using commercially available kits of Chemroy. Serum globulin was determined by subtracting the total serum albumin from total serum protein (Khalil, 2000). Serum albumin/globulin ratio was determined by dividing serum albumin value with serum globulin value (Khalil, 2000).

Determination of cortisol-cortisone shuttle

Radioimmunoassay of cortisone was done to assess the status of the cortisol-cortisone shuttle after extraction and Celite chromatography (Gilles *et al.*, 1997).

Determination of seral bactericidial activity

Serum bactericidal activity to *Aeromonas hydrophila* was determined and the results were recorded as survival index (SI) (Biller-Takahashi *et al.*, 2013). Values were calculated as: SI= CFU at end/CFU at start × 100.

Determination of lysozyme activity in serum

Lysozyme activity in serum was measured with the turbidimetric method (Engstad *et al.*, 1992). The result was expressed as one unit of activity was defined as a reduction in absorbancy of 0.001/min. activity of Lysozyme = (A0- A) / A.

Determination of total bacterial, total enterobacteriaceae and total coliform counts

One gram of mucous was collected from colonies of the all treated groups. All plates were incubated at 28ºC for 24-48 h (APHA, 1992).

Detection of immune response to *A. hydrophila* was evaluated by microagglutination (MA) test (Khalil, 2000). Agglutination titers were expressed as $\log^2$ of the highest serum dilution still giving a clear agglutination

(Khalil, 2000). The negative controls consisted of i) one drop of sterile physiological saline and one drop of tested serum. ii) one drop of sterile physiological saline and one drop of stained antigen. The positive controls were carried out using collected positive antiserum.

Challenge test

At the end of 7th week, ten fish from each group clinically examined and determined to be free from bacterial infection, were injected intraperitoneally with 0.2 ml of culture suspension of pathogenic *A. hydrophila* previously adjusted to 10^4 and specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation. The relative level of protection (RLP) among the challenged fish was determined (Ruangroupan *et al.*, 1986) using the following equation:

$$RLP = 100 - \frac{\text{Percentage of immunized mortality}}{\text{Percentage of control mortality}} \times 100$$

$$\text{Mortality \%} = \frac{\text{No. of death in a specified period}}{\text{Total population during that period}}$$

Histopathological studies

Liver, kidney and gills were harvested from fish of the experimental groups; their histological sections were cut which were stained with hematoxylin and eosin (H&E) (Culling, 1983).

Statistical analysis

The data of hematological and biochemical examinations of exposed fish were statistically analyzed using t-test, Duncan-test after ANOVA and simple correlation to examine the significant effect of the main variables on the studied parameters. After that the results presented in the form of figures according to Harvard graphics (HGW-4) computer program.

RESULTS

Differential leucocytic count

Table I shows the significant ($P<0.05$) effect of different treatments on differential leucocytic count in N. tilapia blood over a period of 8 weeks. At zero days (at the beginning of the experiment) results of differential leucocytic count revealed no significant value in all groups.

The lymphocytic count increased progressively in N. tilapia from the 2nd week till 8th week in the groups treated with C.A. growth® probiotic. Higher dose 0.2 ml/kg feed gave higher count compared with all other experimental groups (Table I). Monocytic count showed no significant value in 2nd, 4th, and 6th week but increased significantly in 8th week in the groups treated with C.A. growth® probiotic (higher dose 0.2 ml/kg feed showed higher value 3.67 ± 0.33^{a} than smaller dose 0.1ml/kg feed 2.00 ± 0.58^{b}) than the groups treated with Tonolest® probiotic (1.67 ± 0.67^{b} in the groups received 0.3g/kg feed and 1.67 ± 0.33^{b} in the groups received 0.15g/kg feed) and the control group showed increased significant value (2.33 ± 0.33^{ab}) than the groups treated with Tonolest® probiotic and the groups received small dose of C.A. growth® probiotic (2.00 ± 0.58^{b}) (Table I).

Meanwhile eosinophils, basophils and thrombocytes counts revealed no significant deviation ($P<0.05$) (Table I). Concerning neutrophils count, the groups treated with Tonolest® probiotic (both doses) and control group showed highly significant value ($P<0.05$) than the groups treated with C.A. growth® probiotic in all weeks. Generally, the groups treated with C.A. growth® probiotic (high dose than small dose) has shown the best results in differential leucocytic count than the groups treated with Tonolest® probiotic (both doses).

Phagocytic activity and phagocytic index

There was progressive increase in phagocytic activity and phagocytic index in the groups treated with C.A. growth® probiotic (high dose than small dose) compared with the groups treated with Tonolest® probiotic (both doses) and the control group from the 2nd week till 8th week (Fig. 1).

Lysozyme and bactericidal activity in serum

They were significantly elevated progressively in the groups treated with C.A. growth® probiotic (high dose than small dose) than the groups treated with Tonolest® probiotic (both doses) and the control one from the 2nd week till 8th week (Fig. 2).

Cortisol level

Cortisol hormone levels were significantly decreased progressively in the groups treated with C.A. growth® probiotic (high dose than small dose. The groups treated with Tonolest® probiotic (both doses) showed significantly increased cortisol level than other treated groups on day 0, 2nd, 4th, 6th and 8th weeks (Fig.3).

Total proteins, albumin, globulin and albumin/globulin ratio

The serum total proteins and globulins were significantly elevated progressively in the groups treated with C.A. growth® probiotic (high dose than small dose) than the groups treated with Tonolest® probiotic (both

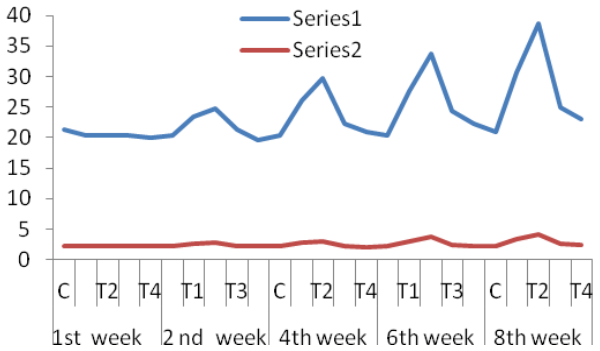

Fig. 1. Phagocytic activity and phagocytic index. C, control; T1, C.A growth 0.1 ml/kg feed; T2, C.A growth 0.2 ml/kg feed; T3, Tonolest 0.15 g/kg feed; T4, Tonolest 0.3 g/kg feed.

For each week: Means within the same column of different letters are significantly different at ($P<0.05$).

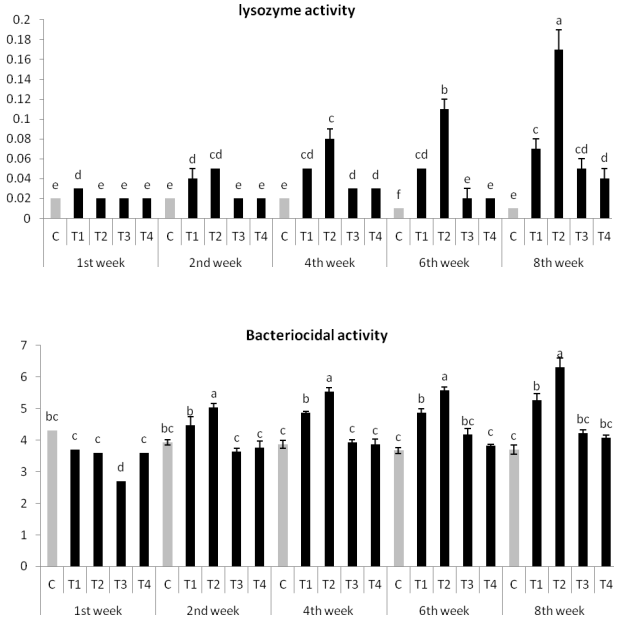

Fig. 2. Serum lysozyme and bactericidal activity.

For abbreviations and other statistical details, see legend of Figure 1.

doses) and control from the 2nd week till 8th week. The groups treated with C.A. growth® probiotic (high dose than small dose) revealed also decreased values in serum albumin and albumin/globulin ratio. Tonolest® probiotic (both doses) groups showed decreased values in serum total proteins, and globulin and increased significant values in serum albumin and albumin/globulin ratio than other treated groups throughout experimental week (Table II).

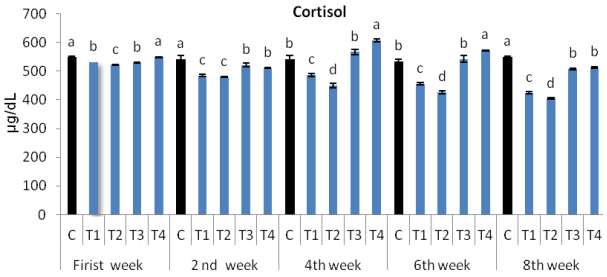

Fig. 3. Cortisol level among different groups in different weeks.

For abbreviations and other statistical details, see legend of Figure 1.

Total bacterial, enterobacteriaceae and coliform counts in gut

Data of total bacterial, total enterobacteriaceae and total coliform counts were transformed to logarithmic scale. At day zero and 2nd week these bacterial counts showed no significant change. At 4th, 6th, and 8th weeks, bacterial counts decreased progressively in the groups treated with C.A. growth® probiotic (high dose than small dose), while the groups treated with Tonolest® probiotic (both doses) showed significantly increased values in all bacterial counts at 4th, 6th, and 8th week compared to other treated groups (Table III).

Antibody titer

The antibody titration differed significantly among different treated groups at different weeks according to the effect of probiotic used. The higher antibody titers were observed in the groups treated with C.A. growth® probiotic (high dose than small dose) than the groups treated with Tonolest® probiotic (both doses) and the control group (Fig. 4).

Mortality after challenge with A. hydrophila *and relative level of protection*

The mortality increased in the groups treated with Tonolest® than the groups treated with C.A. growth® probiotic (both doses). Meanwhile, the relative level of protection showed higher level in C.A. growth® treated groups (70% in high dose and 50% in small dose) than Tonolest® treated groups (20% in both doses). Chi square analysis revealed high significant value ($P<0.01$) in mortality and protection percent between the treated groups (Fig. 5).

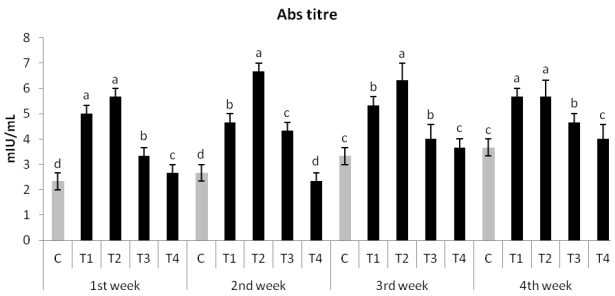

Fig. 4. Antibody titer (log_2) among different groups in different weeks.
For abbreviations and other statistical details, see legend of Figure 1.

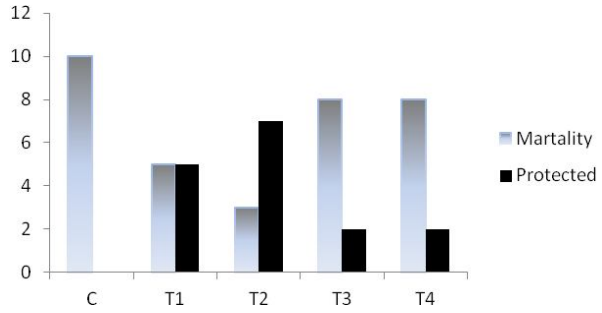

Fig. 5. Mortality and relative level of protection after challenge with pathogenic *A.hydrophila* among different treated groups.
For abbreviations and other statistical details, see legend of Figure 1.

Histopathological examination

Table IV shows histopathological changes in gills, hepatopancreas and posterior kidney of groups fed on diet with probiotic (C.A. growth 0.1 and 0.2 ml/kg and Tonolest 0.15 and 0.3 g/kg feed). The histopathological changes in gills, hepatopancreas and posterior kidney of groups challenged with *A. hydrophila* are shown in Figures 6-8. The gills in control group showed diffused lamellar fusion with basal epithelial necrosis and epithelial lifting of some filaments featuring separation of covering epithelium from capillary beds (Fig. 6A). The group fed on diet with C.A growth exhibited filamentous congestion with multifocal filamentous epithelial lifting (Fig. 6B). Meanwhile, group fed on diet with Tonolest showed multifocal lamellar fusion (Fig. 6C). In hepatopancreas, the control group showed multifocal hepatic lytic necrosis (Fig. 7A), whereas the group fed on diet with C.A. growth showed diffused moderate to advanced hepatic fatty change (Fig. 7B). The group fed on diet with Tonolest showed diffused moderate to

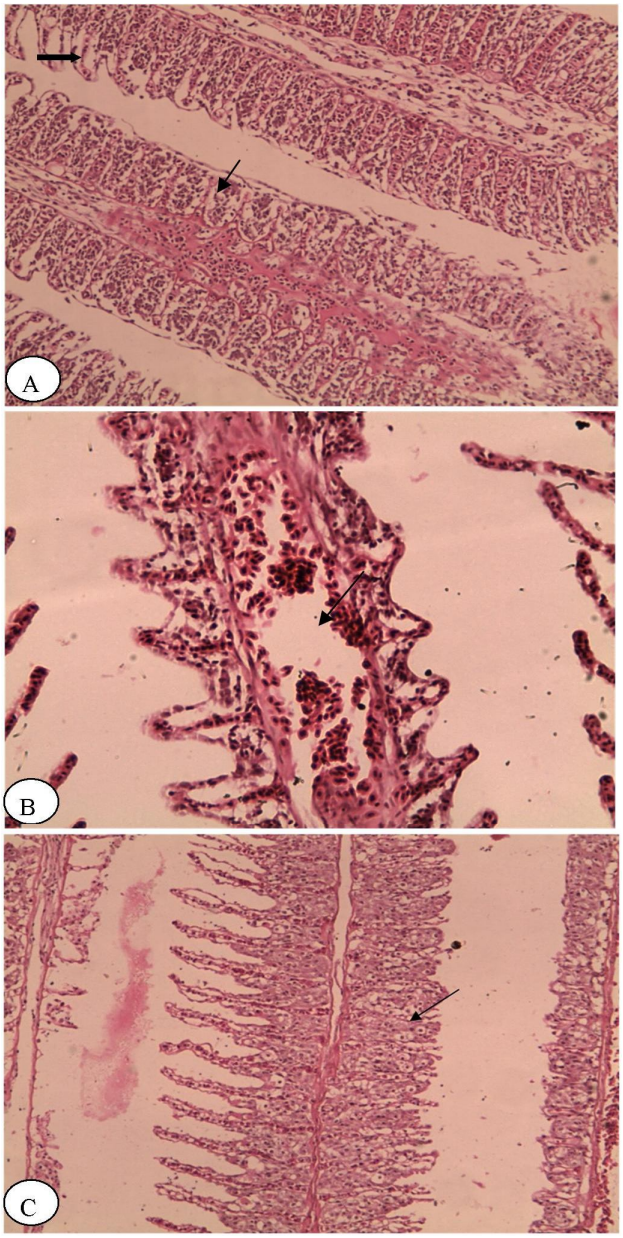

Fig. 6. Histological structure of gills of Nile tilapia challenged with *A. hydrophila*. A, Control group showed diffuse lamellar fusion with basal epithelial necrosis (arrow) and epithelial lifting of some filaments featuring separation of covering epithelium from capillary beds (thick arrow). B, Group fed on diet incorporated with C.A growth showed filamentous congestion with multifocal filamentous epithelial lifting. C, Group fed on diet incorporated with Tonolest showed multifocal lamellar fusion (arrow).
Stained: H &E: Magnification: X400

advanced hepatic fatty change (Fig. 7C). In posterior kidney the control group showed multifocal tubular necrosis, necrosis and depletion of intertubular hemopoietic tissue and tubular epithelial lining contain hyaline droplets (Fig. 8A). Meanwhile, group fed on diet with C.A. growth showed focal hydropic degeneration of tubular epithelium and activation of melanomacrophage center (Fig. 8B). On the other hand, group fed on diet with Tonolest showed hypercellularity of intertubular hemopoietic tissue (Fig. 8C).

DISCUSSION

Fish culture is rising on wide scales to meet the needs for animal proteins, as the production of fish is increasing tremendously all over the world, the need for new strategies for disease control is evoked. The parallel use of biological products namely probiotic either alone or in combination is the recent the goal of the disease bio-control strategy in aquaculture as they improve the fish health and modify the fish associated microbial community (Gibson and Roberfroid, 1995). This study was planned to evoke the differential aspects of using commercial probiotics in N. tilapia and their effects on the immune response of treated fish with pathogenic bacteria, changes in gut microbiota and cortisol hormone level as well as the histopathological studies of treated fish.

The high non-specific immune response developed by increasing the number of lymphocytes and monocytes in the differential leucocytic count and the neutrophils count in groups treated with Tonolest® probiotic (both doses) in addition to the control group showed higher significant value ($P<0.05$) than the groups treated with C.A. growth® probiotic (both doses). These results also are directly proportional to the results of cortisol levels in these groups. This means that leucocyte profiles are particularly useful in the field of conservation physiology because they are altered by stress and can be directly related to stress hormone levels (Harris and Bird, 2000). In general, acute stress induces both neutrophilia and lymphopenia in fish (Pulsford *et al.*, 1994), although sometimes only lymphopenia is reported (Larsson *et al.*, 1980), and these stress-induced changes have been shown repeatedly to be related to elevated glucocorticoids. In the present study the increased value of neutrophils in the groups treated with Tonolest® probiotic (both doses) and control group, means that these groups have decreased ability in controlling stress conditions than the groups treated with high and small dose of C.A. growth® (0.2 and 0.1ml/kg feed, respectively).

The increased value in leucocytic count in C.A. growth® treated groups could be attributed to the fact that

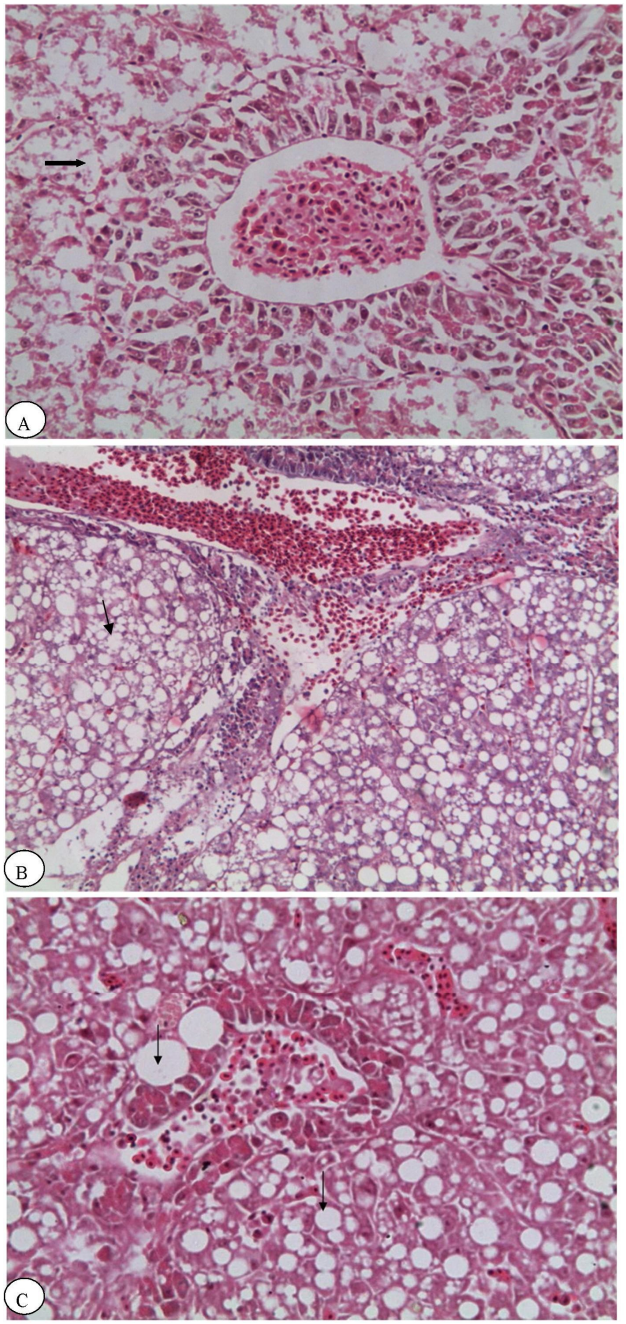

Fig. 7. Histological structure of hepatopancreas of Nile tilapia challenged with *A. hydrophila*. A, Control group showed multifocal hepatic lytic necrosis (thick arrow; X400). B, Group fed on diet incorporated with C.A growth showed diffuse moderate to advanced hepatic fatty change (arrow). C, Group fed on diet incorporated with Tonolest showed diffuse moderate to advanced hepatic fatty change (arrows).
Stained: H &E. Magnification: A, B, C, X250.

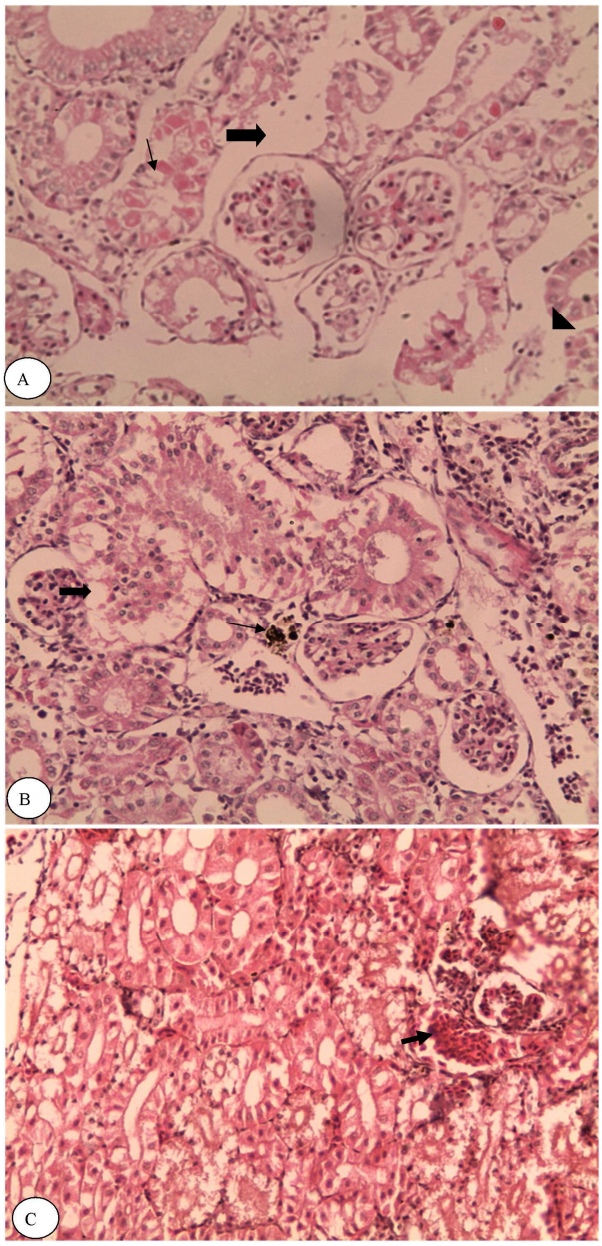

Fig 8. Histological structure of posterior kidney of Nile tilapia challenged with *A. hydrophila.* A, Control group showed multifocal tubular necrosis (arrow head), necrosis and depletion of intertubular hemopoietic tissue (thick arrow) and tubular epithelial lining contain hyaline droplets (thin arrow). B, Group fed on diet incorporated with C.A growth showed focal hydropic degeneration of tubular epithelium and activation of melano-macrophage center (arrow). C, Group fed on diet incorporated with Tonolest showed hypercellularity of intertubular hemopoietic tissue (arrow).
Stained: H &E. Magnification: A, B, C, X250.

the used probiotic increased the level of blood parameters as a result of hemopoeitic stimulation. The effect of using two commercial products containing probiotics (Daimond-V Yeast® and Megalo®) on the hematology of cultured N. tilapia and mixed thoroughly with the prepared basal fish diet during its preparation (Marzouk *et al.*, 2008) gave the same effects. The results of hematogram revealed a significant increase in RBCs count, Hb value, PCV, WBCs and differential leucocytic count in the two groups treated with probiotics. There were better concentrations of % haematocrit, ESR, RBC and WBC observed in *C. gariepinus* fingerling maintained on the diet supplemented with *L. acidophilus* which showed significant differences ($P<0.05$) from the control (Al-Dohail *et al.*, 2009). It supports the hypothesis that fish fed with probiotic-supplemented diets were healthier than the controls probably due to the decreased cortisol levels in the blood plasma (Carnevali *et al.*, 2006; Rollo *et al.*, 2006) in Sea bream *(S. aurata).* Recently the viability of probiotics affected the immune response of N. tilapia fed a commercial preparation including *S. cerevisiae, Bacillus subtilis, Lactobacillus acidophilus* and *Clostridium butyricum* (Alchem Poseidon®; Alchem-Korea Co. Ltd., Wonju, Korea), but the specific importance of yeast viability was not considered (Taoka *et al.*, 2006). A commercial preparation of live *Saccharomyces cerevisiae* and *Lactobacilllus coagulans*, gave better results in Indian carp fry (Gatsoupe, 2007).

Probiotics can effectively trigger the phagocytic cells in host and enhancement of phagocytic activity by LAB group of probiotics such as *Lactobacillus rhamnosus, L. lactis* and *L. acidophilus* has already been observed in several animals (Rutherfurd-Marwicks and Gill, 2004). The groups receiving large dose (0.2ml/kg feed) C.A. growth® showed increased significant value ($P<0.05$) in phagocytic assay than the groups received smaller dose (0.1ml/kg feed). In addition both doses showed increased significant value ($P<0.05$) in phagocytic activity and phagocytic index than the groups treated with Tonolest® probiotic and the control group from the 2nd week till 8th week (Fig. 1) which are directly proportional to the results of differential leucocytic count and results of histological examination in these groups which confirms that C.A. growth® probiotic showed enhancement in the non-specific immune stimulation than fish groups receiving Tonolest® probiotic. Recently, the viability of probiotics affected the immune response of N. tilapia fed a commercial preparation including *S. cerevisiae*, *B. subtilis*, *Lactobacillus acidophilus* and *Clostridium butyricum* (Alchem Poseidon®; Alchem-Korea Co. Ltd., Wonju, Korea) (Taoka *et al.*, 2006), but the specific importance of yeast viability was not

Table I.- Differential leucocytic count among different groups in different weeks.

Group	Thrombocytes	Neutrophils	Eosinophils	Basophils	Lymphocytes
1st week					
Control	3.33±0.33[a]	21.00±1.15[a]	8.33±0.33[a]	5.00±0.58[a]	60.67±0.88[a]
C.A growth 0.1ml/kg feed	3.33±0.58[a]	19.33±1.76[a]	7.00±0.00[a]	5.33±0.33[a]	60.33±1.45[a]
C.A growth 0.2ml/kg feed	3.00±0.33[a]	24.67±1.20[a]	7.00±1.00[a]	4.67±1.20[a]	60.00±0.58[a]
Tonolest 0.15g/kg feed	3.33±0.88[a]	25.33±1.45[a]	7.33±0.33[a]	4.00±0.00[a]	60.33±1.86[a]
Tonolest 0.3 g/kg feed	3.67±1.20[a]	23.67±2.91[a]	7.67±0.67[a]	6.00±1.53[a]	56.33±0.67[a]
2nd week					
Control	2.67±0.33 [a]	20.33±1.76 [a]	8.00±0.58 [a]	6.00±0.58 [a]	59.33±1.20[cd]
C.A growth 0.1ml/kg feed	3.00±0.58 [a]	19.67±1.67[ab]	8.00±0.58 [a]	5.33±0.33[a]	64.00±0.58[ab]
C.A growth 0.2ml/kg feed	3.33±0.67 [a]	11.67±0.88 [b]	8.33±0.33 [a]	5.00±0.33[a]	66.67±0.88 [a]
Tonolest 0.15g/kg feed	3.67±0.33 [a]	16.33±2.33[ab]	8.00±0.58 [a]	6.20±0.58[a]	61.00±2.08[bc]
Tonolest 0.3 g/kg feed	4.00±0.00 [a]	21.67±1.45 [a]	8.33±0.67 [a]	6.23 ±0.33 [a]	56.00±2.08 [d]
4th week					
Control	2.33±0.33[a]	25.33±0.33[a]	6.67±0.67[a]	6.00±0.58[a]	59.00±0.58[c]
C.A growth 0.1ml/kg feed	3.00±0.58[a]	15.67±0.67[b]	6.67±0.33[a]	6.33±0.88[a]	67.00±0.58[a]
C.A growth 0.2ml/kg feed	3.67±0.67[a]	13.67±1.33[b]	7.67±0.33[a]	5.67±0.33[a]	67.67±0.33[a]
Tonolest 0.15g/kg feed	3.33±0.33[a]	22.00±1.15[a]	7.33±0.33[a]	5.67±0.33[a]	59.00±0.58[b]
Tonolest 0.3 g/kg feed	3.33±0.33[a]	23.33±0.88[a]	6.67±0.33[a]	6.33±0.33[a]	58.00±0.58[b]
6th week					
Control	3.00±0.58[a]	23.33±0.67[a]	8.33±0.33[a]	6.33±0.33[a]	56.67±0.88[c]
C.A growth 0.1ml/kg feed	3.33±0.33[a]	14.67±0.33[c]	7.00±1.00[a]	6.67±0.88[a]	67.33±0.33[a]
C.A growth 0.2ml/kg feed	3.67±0.33[a]	12.00±0.58[d]	7.67±0.33[a]	6.67±0.33[a]	68.33±0.33[a]
Tonolest 0.15 g/kg feed	4.33±0.67[a]	20.00±1.00[ab]	8.33±0.67[a]	7.33±0.33[a]	58.33±0.88[bc]
Tonolest 0.3 g/kg feed	4.33±0.33[a]	18.33±0.88[b]	7.67±0.33[a]	7.00±1.00[a]	60.33±0.88[b]
8th week					
Control	3.33±0.33[a]	22.00±2.08[a]	7.00±0.58[a]	6.33±0.33[a]	59.00±0.58[c]
C.A growth 0.1ml/kg feed	3.67±0.67[a]	11.33±2.19[b]	7.67±0.33[a]	7.00±0.58[a]	68.33±0.33[b]
C.A growth 0.2ml/kg feed	4.00±0.00[a]	7.00±1.73[c]	8.00±0.58[a]	6.33±0.88[a]	71.00±1.15[a]
Tonolest 0.15 g/kg feed	4.00±0.58[a]	18.67±0.88[a]	7.33±0.33[a]	7.67±0.33[a]	60.67±0.33[c]
Tonolest 0.3 g/kg feed	4.00±0.58[a]	22.00±1.00[a]	7.33±0.33[a]	6.00±0.58[a]	59.00±0.58[c]

For each week: Means within the same column of different letters are significantly different at ($P<0.05$).

considered. These results confirm the study done using Megalo® where the percent of phagocytosis and phagocytic index in N. tilapia group receiving *S. cerevisae* and *B. sutilis* was the best 83.1% and 2.63 respectively followed by N. tilapia in group receiving Diamond® composed of *S. cerevisiae* yeast in which the values were 81.7% and 2.27 respectively in comparison to N. tilapia kept on a basal diet with values of 73.9% and 1.9%, respectively (Marzouk *et al.*, 2008).

Baker's yeast stimulates the immune response by increasing the phagocytic activity and respiratory burst activity (Tewary and Patra, 2011). In our study the combination between bacteria and yeast in C.A. growth® probiotic has better results in differential leucocytic count and phagocytic assay than using the yeast type only in Tonolest® probiotic.

The groups receiving higher dose (0.2ml/kg feed C.A. growth®) of probiotic showed increased significant value ($P<0.05$) in serum lysozyme and bactericidal activity than the groups receiving smaller dose (0.1ml/kg feed), but also both doses showed increased significant value ($P<0.05$) serum lysozyme and bactericidal activity than the treated groups with Tonolest® probiotic and the control group from the 2nd week till 8th week as indicated in Figure 2. A previous study stated that combination *in vivo* between *B. subtilis, Lactobacillus acidophilus, Clostridium butyricum, Saccharomyces cerevisiae* (Commercial probiotics preparation) viable or inactivated, enhanced the serum lysozyme and bactericidal activity (mucus and serum) (Taoka *et al.*, 2006a). The increase in serum bactericidal activity of N. tilapia against pathogenic bacteria in comparison to the

Table II.- Serum total protein, albumin, globulin and albumin/globulin ratio among different groups in different weeks.

Groups	A/G ratio	Globulin	Albumin	Total protein
1st week				
Control	1.06±0.24^{a}	2.67±0.33^{a}	2.67±0.33^{a}	5.33±0.33^{a}
C.A growth 0.1ml/kg feed	1.06±0.24^{a}	2.33±0.33^{a}	2.33±0.33^{a}	4.67±0.33^{a}
C.A growth 0.2ml/kg feed	0.56±0.11^{a}	3.00±0.00^{a}	1.67±0.33^{a}	4.67±0.33^{a}
Tonolest 0.15g/kg feed	2.17±0.83^{a}	2.00±1.00^{a}	2.67±0.33^{a}	4.67±0.67^{a}
Tonolest 0.3 g/kg feed	1.06±0.24^{a}	2.67±0.33^{a}	2.67±0.33^{a}	5.33±0.33^{a}
2nd week				
Control	0.72±0.39^{a}	2.67±0.33^{bc}	1.67±0.67^{a}	4.33±0.33^{b}
C.A growth 0.1ml/kg feed	0.81±0.10^{a}	3.33±0.33^{ab}	2.67±0.33^{a}	6.00±0.58^{ab}
C.A growth 0.2ml/kg feed	0.81±0.10^{a}	3.67±0.33^{a}	3.00±0.58^{a}	6.67±0.88^{a}
Tonolest 0.15g/kg feed	2.00±0.50^{a}	1.67±0.33^{c}	3.00±0.00^{a}	4.67±0.33^{ab}
Tonolest 0.3 g/kg feed	2.39±1.33^{a}	2.00±0.58^{c}	3.33±0.88^{a}	5.33±0.33^{b}
4th week				
Control	1.06±0.24^{b}	2.33±0.33^{d}	2.33±0.33^{a}	4.67±0.33^{d}
C.A growth 0.1ml/kg feed	0.64±0.11^{bc}	3.67±0.17^{ab}	2.33±0.33^{a}	6.00±0.29^{b}
C.A growth 0.2ml/kg feed	0.53±0.13^{bc}	4.63±0.43^{a}	2.33±0.33^{a}	6.97±0.12^{a}
Tonolest 0.15g/kg feed	1.67±0.17^{a}	1.83±0.17^{c}	3.00±0.00^{a}	4.83±0.17^{cd}
Tonolest 0.3 g/kg feed	0.88±0.31^{bc}	3.00±0.50^{b}	2.33±0.33^{a}	5.33±0.17^{c}
6th week				
Control	1.28±0.15^{b}	1.83±0.17^{c}	2.33±0.33^{b}	4.50±0.29^{d}
C.A growth 0.1ml/kg feed	0.39±0.07^{c}	4.90±0.40^{b}	1.87±0.23^{bc}	6.77±0.26^{b}
C.A growth 0.2ml/kg feed	0.27±0.02^{c}	5.83±0.22^{a}	1.60±0.10^{c}	7.43±0.27^{a}
Tonolest 0.15 g/kg feed	0.98±0.19^{b}	2.43±0.12^{c}	2.33±0.33^{b}	4.77±0.22^{cd}
Tonolest 0.3 g/kg feed	2.27±0.26^{a}	1.67±0.23^{c}	3.67±0.09^{a}	5.33±0.32^{c}
8th week				
Control	2.84±1.24^{a}	1.40±0.40^{d}	3.00±0.10^{a}	4.40±0.30^{d}
C.A growth 0.1ml/kg feed	0.40±0.04^{b}	5.37±0.24^{b}	2.13±0.12^{b}	7.50±0.15^{b}
C.A growth 0.2ml/kg feed	0.30±0.01^{b}	6.13±0.09^{a}	1.83±0.07^{c}	7.97±0.12^{a}
Tonolest 0.15 g/kg feed	1.05±0.03^{b}	2.67±0.07^{c}	2.80±0.06^{a}	5.47±0.09^{c}
Tonolest 0.3 g/kg feed	1.37±0.23^{b}	2.17±0.24^{c}	2.87±0.12^{a}	5.03±0.12^{c}

For each week: Means within the same column of different letters are significant different at ($P<0.05$).

control especially after 2 months may attribute either to the antimicrobial substances that are produced by *L. acidophilus* (Smoragiewicz *et al.*, 1993) or to the increased natural complement, serum peroxidase and phagocytic activities (Salinas *et al.*, 2008). These findings support that the phagocytic activity of the peritoneal macrophages was significantly higher in mice fed either *L. acidophilus* or *L. paracasei* compared with control mice (Paturi *et al.*, 2008). Moreover, the serum bactericidal activities against *A. hydrophila, P. fluorescens* and *Strept. iniae* were lowest in the control group and highest in the group that received mixture of both bacteria *(B. subtilus, L. acidophilus)* after one and two months of the experiment. The viable bacterial counts of *A. hydrophila, P. fluorescens* and *Strept. iniae* were lower in two months than that in one month of the experiment and also in all probiotics treated groups in comparison with untreated control group or bacterial control. In addition to that, the viable bacterial counts in the group that received a mixture of the two bacteria *(B. subtilus, L. acidophilus)* were lower than the group receiving either *L. acidophilus* or *B. subtilus* (Nouh *et al.*, 2009). The serum bactericidal activity was significantly higher in the groups receiving a mixture of probiotics compared to those supplemented with single probiotic species or the control groups (Salinas *et al.*, 2008).

Changes in the physiological state often reflect alteration of hematologic and blood biochemical values. Clinical chemical analysis is a fundamental tool to diagnose and predict the outcome of diseases and to monitor the effects of therapeutic, nutritional and environmental management in medicine. Blood

Table III.- Logarithmic transformation of total bacterial count, total enterobacteriaceae count and total coliform count among different groups in different weeks.

Groups	Total coli form count	Total enterobacteriaceae count	Total bacterial count
1st week			
Control	3.11±0.00[a]	3.41±0.00[a]	3.48±0.00[a]
C.A growth 0.1ml/kg feed	2.85±0.00[a]	3.26±0.00[a]	3.36±0.00[a]
C.A growth 0.2ml/kg feed	2.78±0.00[a]	3.23±0.00[a]	3.30±0.00[a]
Tonolest 0.15g/kg feed	2.78±0.00[a]	3.45±0.00[a]	3.36±0.00[a]
Tonolest 0.3 g/kg feed	2.95±0.00[a]	3.49±0.00[a]	3.34±0.00[a]
2nd week			
Control	3.11±0.00[a]	3.41±0.00[a]	3.51±0.00[a]
C.A growth 0.1ml/kg feed	2.85±0.00[a]	3.26±0.00[a]	3.43±0.00[a]
C.A growth 0.2ml/kg feed	2.78±0.00[a]	3.23±0.00[a]	3.36±0.00[a]
Tonolest 0.15g/kg feed	2.78±0.00[a]	3.45±0.00[a]	3.53±0.00[a]
Tonolest 0.3 g/kg feed	2.95±0.00[a]	3.49±0.00[a]	3.58±0.00[a]
4th week			
Control	2.58±0.10[bc]	3.44±0.01[ab]	3.50±0.02[b]
C.A growth 0.1ml/kg feed	2.63±0.03[b]	3.18±0.02[c]	3.36±0.01[c]
C.A growth 0.2ml/kg feed	2.48±0.00[c]	3.19±0.02[c]	3.27±0.02[d]
Tonolest 0.15g/kg feed	2.67±0.11[bc]	3.42±0.02[b]	3.50±0.02[b]
Tonolest 0.3 g/kg feed	2.90±0.03[a]	3.50±0.00[a]	3.55±0.00[a]
6th week			
Control	2.26±0.14[b]	3.41±0.03[a]	3.45±0.03[a]
C.A growth 0.1ml/kg feed	2.48±0.09[ab]	3.18±0.04[b]	3.38±0.01[b]
C.A growth 0.2ml/kg feed	2.46±0.09[ab]	3.17±0.01[b]	3.25±0.02[c]
Tonolest 0.15 g/kg feed	2.55±0.25[ab]	3.40±0.03[a]	3.46±0.02[a]
Tonolest 0.3 g/kg feed	2.91±0.11[a]	3.43±0.02[a]	3.48±0.02[a]
8th week			
Control	2.36±0.06[c]	3.44±0.01[a]	3.47±0.02[a]
C.A growth 0.1ml/kg feed	2.52±0.04[b]	2.92±0.16[c]	3.24±0.01[b]
C.A growth 0.2ml/kg feed	2.36±0.06[c]	3.01±0.01[b]	3.10±0.01[c]
Tonolest 0.15 g/kg feed	2.72±0.07[ab]	3.36±0.02[a]	3.45±0.01[a]
Tonolest 0.3 g/kg feed	2.85±0.15[a]	3.38±0.01[a]	3.44±0.01[a]

For each week: Means within the same column of different letters are significant different at ($P<0.05$).

biochemical values are not commonly used as a diagnostic tool in fish medicine, partly because of the lack of reference intervals for various fish species, and also because changes in blood analysis associated with specific diseases and metabolic disorders are not well characterized with sufficient background data. Thus, clinical biochemical analysis could be developed to detect metabolic disorders and sublethal diseases that affect the production efficiency (Shalaby *et al.*, 2006).

The groups treated with C.A. growth® probiotic (both doses) showed significantly high value ($P<0.05$) of serum total protein and globulin and decreased A/G ratio and plasma cortisol level than the groups treated with Tonolest® probiotic (both doses) which showed decreased significant value ($P>0.05$) in serum protein and globulin levels and increased plasma cortisol level from the 2nd week to 8th week (Table II, Fig.3). An improvement of fish health when fed *L. acidophilus* supplement diets (Eid and Mohamed, 2008) and a significant increase in total protein and decrease A/G ratio which could be attributed to the immuno-modulatory effect of *S. crevisiae and B. subtilis* than the group treated with dead *Saccharomyces cerevisiae* on the liver cells which activate the anabolic capacity of the hepatocytes to produce blood proteins particularly globulin (Marzouk *et al.*, 2008).

No marked histopathological alterations was observed after administration of 0.2 ml/kg C.A. growth®. Additionally, better growth performance and nutrient efficiency could possibly be related to lower stressor levels in fish fed probiotic diet. Decreased cortisol levels

Table IV.- Histopathological changes in examined organs (gills, hepatopancreas and posterior kidney) of groups feed on diet incorporated with proboitic (C.A growth 0.1 and 0.2 ml/kg and Tonolest 0.15 and 0.3 g/kg feed).

Histopathological changes	Diet + Tonolest 0.3 g/ kg feed	Diet + Tonolest 0.15 g/ kg feed	Diet + C. A growth 0.2 ml / kg feed	Diet + C.A growth 0.1 ml / kg
Gills				
Epithelial lifting	++	+	+	-
Congestion	+	-	-	-
Lamellar fusion	++	+	+	-
Hepatopancreas				
Necrosis	+	-	-	-
Fatty change	++	++	++	+
Posterior kidney				
Tubular necrosis	+	-	-	-
Hemopoietic tissue depletion	+	+	+	-

-, absent; +, few; ++, moderate to severe

when fish was fed a diet supplemented with *L. delbrueckii* (Carnevali *et al.*, 2006). Confirming that total serum protein, cholesterol and total immunoglobulin concentrations were also significantly better in fish maintained on the diet supplemented with the probiotic, *L. acidophilus,* than in fish fed the control diet (Al-Dohail *et al.*, 2009).

The dietary administration of probiotic strain caused a decrease in the bacterial densities in the gut of the fishes (Bagut *et al.*, 2000). The concentration of probiont in feed in the present study was significant in regard to reduction of gut bacterial counts, emphasizing that a probiotic dose of 10^6 to 10^7 cfu g^{-1} of feed administered continuously is sufficient to obtain a healthy balance between probiotic micro-organisms and other bacteria in the gut (Guillot, 2003). Figure 2 shows that the groups receiving higher dose (0.2ml/kg feed) of C.A. growth® probiotic showed significantly decreased bacterial counts than the groups receiving smaller dose (0.1ml/kg feed), but also both doses showed significantly decreased value ($P>0.05$) in bacterial counts than the treated groups with Tonolest® probiotic and the control group in all weeks which could be attributed to the bactericidal or the bacteriostatic substances produced by *Lactobacillus* and *Saccharomyces* that inhibit the growth of other bacteria (Pybus *et al.*, 1994) and the ability of these probiotic bacteria and yeast to bind the intestinal mucosal cell receptors for some members of enterobacteriaceae and other bacteria. The *L. delbrueckii subsp. Delbrueckii* strain showed good capability to colonize the gut of Sea bass larvae and showed the ability to inhibit colonization and proliferation of Enterobacteriaceae family that includes some opportunistic pathogenic strains that are often involved in larval and adult mortality therefore modifying the gut microbiota, and exerting positive effects on the survival of the treated Sea bass (Silvi *et al.*, 2008). *Lactobacillus* species possess several important properties and therefore can be used as effective probiotic organisms (Ouwehand *et al.*, 1999). These features are: efficient adherence to intestinal epithelial cells to reduce or prevent colonization of pathogens, competitive growth, non pathogenic, and production of metabolites to inhibit or kill pathogen (Reid and Burton, 2002).

The effect of feeding *Lactobacillus*–based probiotics on the gut microflora, and they concluded that, this treatment has inhibitory effect on gram negative bacteria present in gut microflora of *Macrobrachium rosenbergii* post larvae (Himabindu *et al.*, 2004). In the present study the combinations between bacteria and yeast in (C.A. growth®) probiotic give better results than that obtained by using yeast only in Tonolest® probiotic.

Fish groups treated with 0.1 and 0.2ml/kg feed (C.A. growth®) showed high level of protection (50% and 70% respectively) and survival than the groups treated with (Tonolest®) probiotic (20% relative level of protection in both doses) as shown in Figure 5. The protection may be due to the immunostimulatory effect of the living *Saccharomyces* and *Lactobacillus, Pediococus, Glucanobacter* sp. present in C.A. growth® probiotic and also their inhibitory effect to *A. hydrophila*. These results also directly proportional with the results of antibody titer (log_2) in the groups treated with 0.1 and 0.2ml/kg feed (C.A. growth®) ($5.6667\pm.3333^a$ and $5.6667\pm.6667^a$, respectively) and also supports the histopathological examination of groups challenged experimentally with *A. hydrophila* and fed on diet incorporated with C.A. growth® where minimal changes in the examined organs (hepatopancreas, kidney and gills) than control group.

The living yeast cells and bacteria cells are more potent than dead yeast cell in protecting fish against pathogens (Marzouk *et al.*, 2008; Al-Dohai *et al.*, 2009; Nouh *et al.*, 2009; Ajitha *et al*., 2004).

The immunomodulatory effect of C.A. growth® probiotic against pathogenic *A. hydrophila* is possibly due to production of inhibitory compounds from *Lactobacillus* sp such as bacteriocins that inhibit the growth of other microorganisms (Vanderbergh, 1993). Moreover, the yeast cell walls can provide very important non-nutritive compounds that may benefit fish health, including mannoprotein, glucans, chitin, polyamines, and nucleic acids (Rumsey *et al.*, 1992). The beneficial influence of glucans has been demonstrated with various fish species. Enhanced immunological responses including respiratory burst also have been reported for dietary chitin (Esteban *et al.*, 2001). Several factors affect the efficacy of probiotics on disease prevention in fish, especially the type of probiont and dietary dose concentration (dietary concentration + feeding duration). In tilapia, short-term (2 weeks) and long-term (2 months or greater) feeding have all proven to be effective in enhancing disease resistance in tilapia

The histopathological findings of the groups fed on basal diet incorporated with C.A growth® probiotic revealed no marked difference in the microscopic picture in all weeks of the experiment. Similarly, minimal pathological alterations in different supplemented groups with *L. acidophilus* and in groups supplemented with *B. subtilis* and *L. acidophilus* with no remarkable difference between groups or duration of experiments (Nouh *et al.*, 2009). The histopathological findings due to using two commercial products containing probiotics (Daimond-V Yeast® and Megalo®) showed a great activation of melano-macrophage centers and Kupffer cells in the splenic and liver tissues of probiotic-treated fish respectively (Marzouk *et al.*, 2008).

The N. tilapia, of the groups fed on basal diet with Tonolest® probiotic especially at high dose revealed necrotic and degenerative changes in gills, hepatopancreas and posterior kidney. The degree of histopathological changes in organs of N. tilapia fed on basal diet with Tonolest® more than C.A growth® probiotic. These confirm the results of the previous explained parameters which revealed good results obtained with C.A growth® probiotic than that obtained with Tonolest® probiotic.

Due to its apparent effectiveness in improving health and growth in tilapia, besides, enhancing the innate immune response, research and interest in probiotics is likely to continue, which will hopefully fill existing research gaps.

Statement of conflict of interest

Authors have declared no conflict of interest.

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