Enhancement of Innate Immunity in Common Carp *Cyprinus carpio* Using local Probiotic

تحفيز االمناعة الامية في اسماك الكارب الشائع *Cyprinus carpio* باستعمال المعزز الحيوي المحلي

Sundus A. A. Alsaphar **Jamal K. A. Al-Faragi***
Quality Control/ Al-Kindi Co. for Production of Vet Vaccines &drugs
*College of Vet. Med/ Baghdad University
سندس عون علي **جمال خلف عطية الفراجي***
قسم السيطرة النوعية/ شركة الكندي لإنتاج اللقاحات والأدوية البيطرية
* كلية الطب البيطري/ جامعة بغداد

Abstract

The objective of this study was to determine the effect of four levels of local probiotic compared with commercial probiotic on immune status of common carp *Cyprinus carpio* as biological control. One handared and eight speciement of *Cyprinus carpio* average weight of 57.85±0.3g were fed during 42 days at 0.05,0.1,0.5 and 0.75mg local probiotic per Kg diet compared with 2mg commercial probiotic per Kg diet in comparison with the control group without additive. The phagocyctic index of blood macrophages, reduction of nitrobluetetrazolium (NBT) by radical oxegen produce from neutrophils cell ,and alternative complement pathway (ACP) activities were evaluated. A positive correlation was found between the levels of phagocytosis, nitrobluetetrazolium index and alternative complement pathway activities in blood and the level of inclusion of local probiotic compared with commercial probiotic and control group.

Keywords: Phagocytic index, NBT index.Nitrobluetetrazolium, (ACP) Alternative complement pathway and *Cyprinus carpio*

الملخص

هدفت الدراسة تحديد تاثير اربع تراكيز من المعزز الحيوي المحلي مقارنة بالمعزز التجاري في تحسين المناعة الامية لسمك الكارب الاعتيادي *Cyprinus carpio* كسيطرة بيولوجية. مئة وثمانية عينة من اسماك *Cyprinus carpio* بمعدل وزن 0.3±57.85غم غذيت لمدة 42 يوما معزز حيوي محلي بنسبة 0.05و0.1و0.5 و0.75 ملغم/كغم علف قورنت بالمعزز التجاري 2ملغم لكل كيلو غرام علف ومجموعة السيطرة غذيت بدون اضافة. درس مؤشر البلعمة لخلايا البلعمة في الدم ومؤشر اختزال صبغة النايتروبلوتترازوليوم بالاوكسجين الحر المتحرر من الخلايا المتعادلة في الدم وكذلك مستوى فعالية المتمم. وجد إن هناك علاقة ارتباط ايجابية بين مستويات المناعة الامية ومعدل اعطاء المعزز الحيوي المحلي مقارنة بالمعزز التجاري ومجموعة السيطرة.

الكلمات المفتاحية: مؤشر البلعمة, اختزال النايتروبلوتترازوليوم, فعالية المتمم و الكارب الشائع .

Introduction

The immune system of fish has evolved with both non-specific (innate immunity) and acquired immune functions (humoral and cell mediated immunity) to eliminate invading foreign living and non-living agents [1]. Recently studies have focused on understanding how the fish immune system respond against pathogen agent or how the innate can be selected by breeding to produce stock of fish that are resistance to infectious agent [1,2]. Amara *et al*.. (2004) [3] demonstrated the benefits of immunostimulants on the fish immune system .The application of immunostimulants in aquaculture is described as an innovative approach to enhance the non-specific defence mechanism of fish to diseases [4]. This approach has the multiple benefit of being effective against a wide range of bacteria and viruses, suitable for many species of fish and easy to apply as it can be administerated orally in feeds. Immunostimulants are naturally inevitable occurring compounds that modulate the non-specific immune mechanisms by enhancing the host resistance against diseases [5]. Immunostimulants are considered to be safer and more environmental friendly than chemotherapeutics, and their range of efficacy is often wider than that of vaccination [6]. According to Andorson (1992) [7], immunostimulants are often grouped by either their functions, origin sources) and consistance of heterogeneous groups (biological substances, bacterial, algae-derived, animal derived, nutritional factors, herbal/medicinal plants, synthetic products and hormones). Probiotics can modify the immune response of the host by interacting with intestinal epithelial cells and by modulating the secretion of anti-inflammatory cytokines, which could result in a reduction of inflammation. Stafan *et al* (2009) [8] showed that inter leukine1β(IL-1β), IL-8, tumor necrosis factor-α (TNF-α), and tumor growth factor-β (TGF-β) expression was not induced in rainbow trout *Oncorhynchus mykiss* gut cells following

administration of the probiotic bacteria *Carnobacterium maltaromaticum*B26 and *Carnobacterium divergens*B33. However, detection of significantly higher IL-1β and TNF-α expression in head kidney cells indicates induction of an ant inflammatory effect [9]. Many probiotic agents stimulate immune response and diseases resistance, and have a positive effect on fish health [10,11].The present study was planned to enhance innate immunity in common carp *Cyprinus carpio* using different levels of local probiotic.

Materials and Methods

Probiotic Microorganism

Micro organism	Count Cfu/ml
Saccharomyces serevisiae	**10^{10} Cfu/ml×1**
Bacillus subtilus	**10^{9} Cfu/ml×1**
Lactic acid bacteria J6	**10^{11} Cfu/ml×1**

Experimental Design

One hundred and eight specimen of *Cyprinus carpio* average weight of 57.85±0.3g were obtained from a carp farm at Al-Musiab, Babyl Iraq. Fish were acclimatized to a laboratory conditions for two weeks prior to the experiment and fed on commercial diet. They were stocked in two aquaria 150 × 80 ×50 cm, then distributed into 12 aquaria filled with chlorine free tap water at rate of 10 fish per aquarium with two replicates for each of the six groups . fish were fed 3% of body weight twice a day for 42 day. Every day aquaria were cleaned and water partially changed. Dissolved oxygen, pH and temperature of aquarian water were measured. At the end of the trial blood was obtained from caudal sinus vien puncture with a 1 ml plastic syringe. No anesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize the stress effects. The first aliquot of blood was transferred to a coated Eppendorf with lithium heparin as anticoagulant and used for measurements. The phagocyctic index of blood macrophages, reduction of nitrobluetetrazolium (NBT) by radical oxygen produce from neutrophils cell. The second aliquot was transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at -20C° for measuring the alternative complement pathway (ACP) activities.

Determination of Immuneresponce

Nitrobluetetrazolium (NBT) activites, phagocyctic activity were determined according to the methods described by [12,13]**.**

Nitroblue tetrazolium (NBT) activity

Reactive oxygen radical production by neutrophil during respiratory burst activity was evaluated by the reduction of nitroblue tetrazolium (NBT) to formazan blood samples were mixed with 0.2% NBT in equal proportion (1:1) and incubated for 30 min at 25C°. 50µl of this mixture was taken out and 1 ml of dimethyl formamide (SRL, India) was added to solubilize the reducted formazan product. Then centrifuged at 2000rpm for 5 min and the supernatant was taken. The reduced extent of NBT was measured at an optical density of 540 nm with dimethyl formamide as the blank by using UBK Spectrophotometer.

Phagocytic Activity

The heparinized blood was immediately used for the phagocytic assay. Briefly, 1×10^{8} cells/ml of *Staphylococcus* sp. in 0.1 ml of phosphate buffer solution (PBS) were added to 0.1 ml of blood samples in a micro plate and incubated for 30 min after thoroughly well mixing. After incubation, the plate content was mixed gently and 0.05 ml of this suspension was smeared on the glass slide. After air drying, the smears were fixed in ethanol, and cells and phagocytised bacteria were counted. Phagocytic Activity was calculated according to the following equations:

Percentage of phagocytosis =

No. of ingesting phagocytes / Total number phagocytes including non-ingesting cells.

Phagocytic index = No. of ingested *Staphylococcus* cells/No. of ingesting phagocytes.

Alternative complement pathway (ACP) activities.

The natural hemolytic complement activity was assessed using rabbit red blood cells (rRBC) as targets [14]. RBCs were washed and resuspended at 3% (v/v) in phenol red free hanks balanced salt solution (HBSS) containing 10 Mm Mg^{+2} and 10Mm ethelindiamine tetra acetic acid (EDTA). 100µl of serum

was serially diluted in HBSS and was mixed with an equal volume of rRBC in a 96 well titer plate. After incubation for 1 hr at 22°C.The reciprocal of the serum dilution causing 50% lysis of RBC is designed as ACH50 and the results were presented as ACH50 units $ml^{-1}$the values of maximum and minimum haemolysis were obtained by adding 100µl of distilled water or HBSS respectively to 100 µ of rRBC.

Results

The dissolved oxygen content of water throughout the experimental period ranged between 6.4-6.6 mg L^{-1}, pH ranged between 6.5-6.8, water temperature in the aquarium ranged between 24-26C°.

Respiratory burst activity

The respiratory burst activity (NBT reduction) of neutrophils of *C. carpio* of the experimental groups is shown in Fig (1) . Higher respirotary burst activity was found after oral supplementation of local probiotic and commercial probiotic in comparison to the control group during 42 day. Optical density of NBT in all the groups showed a significant difference ($p \leq 0.05$) that were 1.67,1.56,1.55, 1.55 and 1.54 respectively compared with control group (0.682) and no significant difference ($p \leq 0.05$) between treatment groups.

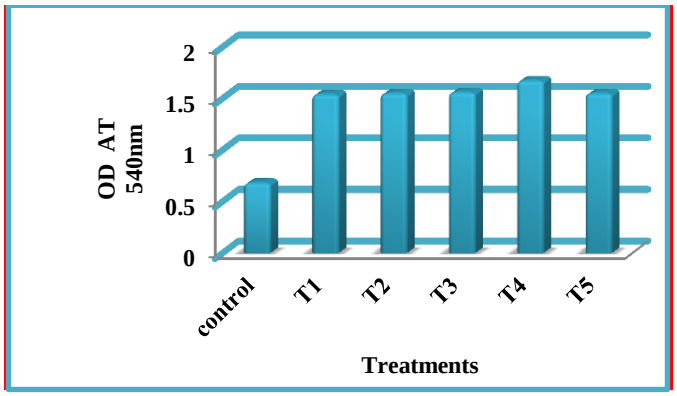

Fig. (1): Respiratory burst activity (NBT reduction) of *C. carpio* neutrophils following 42 days feeding diets with different levels of probiotics. Values are mean ± S.E.

Phagocytic activity

The phagocytic index of blood monocytes and phagocytosis were significantly increased in the fish groups fed on diet supplemented with probiotic in comparison with control group Figures (2 and3).

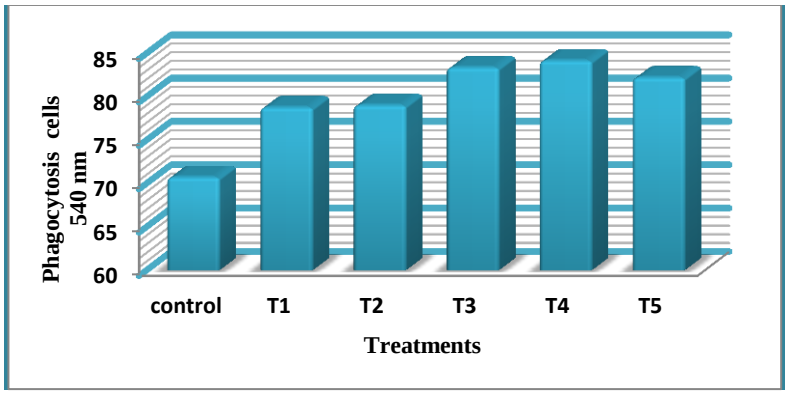

Fig. (2): Phagocytosis assay one hundred and eight of *C. carpio* monocytes following 42 day feeding diets with different levels of probiotics. Values are mean ± S.E.

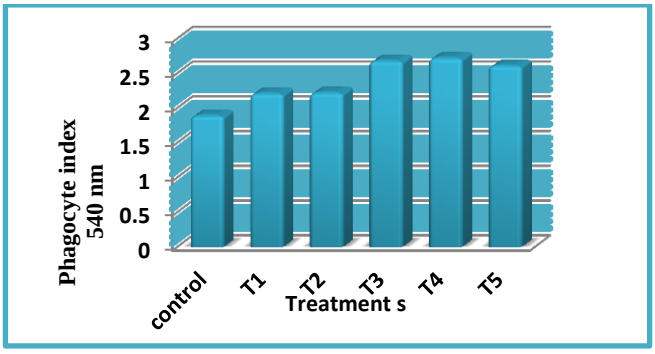

Fig. (3): Phagocyte index of *Cyprinus carpio* fed diets with different levels of probiotic for 42 day.

The results indicated that the percentge of phagocytosis and phagocytic index in *C. carpio* groupT4 using local probiotic was the best (84.2% and 2.72) respectively followed by *C. carpio* T3,T5,T2 and T1 in which the values were 83.4% ,82.2%,79.05%,78.8% and 2.68,2.61,2.22, 2.21 respectively in comparison to *C. carpio* kept on a basal diet which referred to 68.6 and 1.89 respectively.

Complement activity (serum natural haemolytic)

Data on complement activity presented in figure (4) for treatments T1,T2,T3,T4and T5 increased significantly ($P \leq 0.05$) were 35, 40, 80, 90 and 75ACH50 Unit/ml respectively compared with control (20ACH50Unit/ml) post treatment with probiotic during 42 day. T4 was showed the best value followed by T3 and T5.

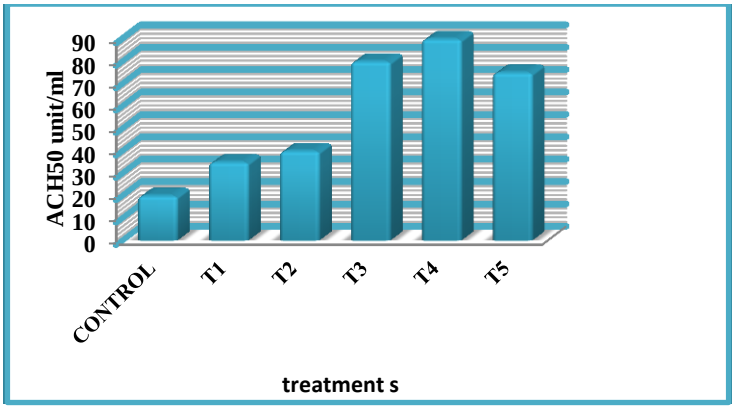

Fig. (4): Alternation complement activity in *C. carpio* during 42 day feeding on diets with different levels of probiotics. Values are mean ± S.E. ACH50 means 50% hemolysis)

Discussion

Using of commercial probiotics in fish diet is relatively ineffective as most commercial preparations are based on strains isolated from non-fish sources that are unable to survive or remain viable at high cell density in the intestinal environment of fish during the active growth phase of the fish [15]. Hence, there is elegant logic in isolating putative probiotics from the host in which the probiotic is intended for use. Such strains should perform better because they have already adhered to the gut wall of the fish and, thus, are well-adapted to compare with pathogens for nutrients [16]. Presumably, strains that develop dominant colonies in the fish intestine are good candidates for preventing the adhesion of pathogens on the gut wall [17]. Phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defenses also Phagocytic cells generate reactive metabolites such as superoxide anion, hydrogen peroxide and hypochlorous acid in response to membrane stimulation and these antimicrobial substances, however, may compromise host responses by inducing oxidative damage [18]. Probiotics can effectively trigger the pahgocytic cells in host and enhancement of phagocytic activity and this observation may consistence with our results especially the third, fourth and fifth treatment that showing aggregation of multinuclear cells MNCS

and neutrophile cells. Pieters *et al* (2008) [19] showed that probiotics are often used in aquaculture practices and supplementation of these probiotics either in viable or inactivated form is found to stimulate phagocytic activity in several fish species such as Rainbow trout's. In tilapia *Oreochromisniloticus* a 2 weeks feeding of L. *rhamnosus* significantly stimulated the phagocytic activity while several *in vitro* and in vivo studies showed significant increase in respiratory burst activity by various probiotics in many aquatic animals including fish [20]. Probiotics like *Bacillus subtilis* and certain members of LAB group can stimulate respiratory burst activity in fish [21]. Also probiotics can enhance natural complement activity of fish [22] and dietary as well as water treatment of many probiotics are often reported to stimulate the piscine complement components [11]. All three pathways (alternative, lectin and classical) converge in the lytic pathway, leading to opsonization or direct destruction of the microorganism. The complement system is composed of numerous proteins, and all pathways generate factor C3, which has been described and isolated from teleost species [23]. Complement is a non-specific component of the immune system, which can attract and activate phagocytes (chemotaxis), function as opsonin and thereby increase phagocytosis of complement coated particles causing target cell lyses [11]. Thus, enhanced complement activity in the treatment 4,5 and 3 probiotic supplemented groups may have contributed to an enhanced inflammatory response and this could be one of the mechanisms responsible for the increased disease resistance observed in probiotic supplemented groups. Furthermore [24] showed that fishes fed diet containing *B. subtilis* showed a significant increase in respiratory burst activity (NBT reduction), compared to the control. This confirmed that nonspecific immunity im-proved in fishes fed with feed containing *B. subtilis* this indicated that *B. subtilis* enhances the immunity of major carp *Labeo rohita* to overcome the stress caused by *A. hydrophila*. [25] previously demonstrated that administration of *B. subtilis* in feed could reduce mortality of *Labeo. rohita*. Abdel-Tawwab (2008) [26] suggested that the yeast supplementation could increase the nonspecific immune system of Nile tilapia resulting in resistance to *A. hydrophila*. Also, Taoka *etl*. (2006) [20] investigated the effect of live and dead probiotic cells on the non – specific immune system of Nile tilapia such as lysozyme activity, migration of neutrophils and plasma bactericidal activity resulting in improve resistance to *Edwarsiella tarda* infection. Also, Abdel-Tawwab, *etl.*(2008) [26] proved that the cumulative mortality of Nile tilapia, ten days after I/P injection of *A. hydrophila* decreased significantly with the increased dose of yeast. The previous observations agreed with our result that an revealed supplementation. In the present study can be concluded that local probiotic can be used in aquaculture as an immunostimulator. It is also suggested that some more experiments may be conducted using some other species of fishes in order to establish the role of local probiotic as an immunostimulator.

References

1. Swain, P., Dash, S., Sahoo, P.K., Routray, P., Sahoo, S.K., Gupta, S.D., Meher, P.K. and Sarangi, N. (2007). Non-specific immune parameters of broad Indian major carp *Labeo rohita* and their seasonal variations. Fish and Shellfish Immunol. 22. 38-43.
2. Magnadóttir, B. (2005). Innate immunity of fish (overview). Fish and Shellfish Immunol. 20: 137-151.
3. Amara, E.C., Kirona, V., Satoha, S. and Watanabea, T. (2004). Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss*) associated with dietary intake of carotenoids from natural products. Fish and Shellfish Immunol. 16: 527-537.
4. Robertseen, B., Engstad, R.E. and Jorgensen, J.B. (1994). B-glucan as immunostimulant in fish. In: Modulators of fish immune responses 1 (Stolen, J. S. and Fletcher, T. C. eds). S O S Publications, Fair Haven., N. J. 83- 99.
5. Bricknell, I. and Dalmo, R. A. (2005). The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immunol. 19: 457- 472.
6. Sakai, M. (1999). Current research status of fish Immunostimulants. Aquaculture. 172:63- 92.
7. Anderson, D. P. (1992). Immunostimulants, adjuvant and vaccines carriers in fish: Applications to aquaculture, Ann. Rev. Fish Dis. 2:281-307.
8. Stafan, D.,Yordan, S., Rumyana, M. and Georgi, B. (2009). Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. Inte. Aqua. Res. 1:1-29.
9. Kim, D.H. and Austin, B. (2006). Cytokine expression in leucocytes and gut cells of rainbow trout, (*Oncorhynchus mykiss*), induced by probiotics. Vet.Immunol. Immunopath. 114: 297-304.

10. Brunt, B and Austin, B. (2005). Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss*. Fish Dis. 28: 693-701.

11. Wang,Y.B., Tian, Z.O., Yao, J.T. and Li, W. (2008). Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. Aquaculture. 277: 203-207.

12. Siwicki, A.K. and Anderson, D.P. (1993).Immunostimulation in fish: measuring the effects of stimulants by serological and immunological methods. Abstract Symposium on Fish Immunol., Lysekil, Sweden.

13. Siwicki, A.K., Anderson, D.P. and Rumsey, G.L. (1994). Dietary intake of immunostimulants by rainbow trout effects non-specific immunity and protection against furuncluosis. Vet Immunol.Immunopath. 41: 125-139

14. Sunyer, J.O. and Tort, L. (1995). Natural hemolitic and bactericidal activities of seabream *Sparus aurata* serum are affected by the alternative complement pathway. Vet. Immunol. Immunopath. 45:333-345.

15. Moriarty, DJ.W. (1996) . Microbial biotechnology : a key ingredient for sustainable aquaculture .Info. Fish Int. 4/96:29-33.

16. Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete ,W. (2000). Probiotic bacteria as biological control agents in aquaculture. Micro. and Mole. Biol. Rev. 64: 655-671.

17. Bengmark, S. (1998). Ecological control of the gastrointestinal tract. The role of probiotic flora. Gut. 42: 2-7.

18. Nayak, S.K. (2010). Probiotic and immunity : A fish perspective. Fish and shellfish immunol. 29:2-14.

19. Pieters, N., Brunt, J., Austin, B., and Lyndon, A.R. (2008). Efficacy of in-feed probiotics against *Aeromonas* bectiarum and *Ichthyophthirius multifiliis* skin infections in rainbow trout (*Oncorhynchus mykiss*,). Appl Microbiol.105:723-32.

20. Taoka, Y., Maeda, H., Jo, JY., Kim, S.M., Park, S., Yoshikawa, T. and Sakata, T. (2006).Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. Fish.Sci. 72: 755–766.

21. Nikoskelainen, S., Ouwehand, AC., Bylund, G., Salminen, S. and Lilius, E.M. (2003). Immune enhancement in rainbow trout *(Oncorhynchus mykiss)* by potential probiotic bacteria *(Lactobacillus rhamnosus).* Fish and Shellfish Immunol. 15: 443-452.

22. Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayashi, T. and Sugita, H. (2007). Immunemodulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss*upon probiotic feeding. Dev. Comp. Immuno. 31:372-82.

23. Nakao, M. and Yano, T. (1998). Structural and functional identification of complement components of the bony fish, carp (*Cyprinus carpio*). Immunol. Rev. 166:27- 38.90.

24. Rajesh, K., Subhas, C., Mukherjee, R.R. and Nayak, S.K. (2008). Enhauced innate immune parameters in *labeo rohita* following oral administration of *Bacillu ssubtilis*. Fish and shellfish immunol. 24:168-172.

25. Kumar, R., Mukherjee, S.C., Prasad, K.P. and Pal, A.K. (2006). Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita*. Aqua Res. 37(12):1215-1221.

26. Abdel-Tawwab, M., Abdel-Rahman, A.M. and Ismael N.E. **(**2008**)**. Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for Fry Nile tilapia, *Oreochromis niloticus* challenged in situ with *Aeromonas hydrophila*. Aquaculture. 280: 185-189.