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Biological antioxidants (Vitamin C and E) in relation to fish immunity

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Abstract
An antioxidant molecule is capable of slowing or preventing the oxidation and mainly used as stress mitigators in fish nutrition and also used to prevent rancidity of lipid in fish feed. Oxidation transfers electrons from a substance to an oxidizing agent. The term "antioxidant" is mainly used for two different groups of substances- industrial chemicals which are added to products to prevent oxidation and natural chemicals which can be found in body tissues and food having beneficial health effects. Vitamin C is an essential vitamin for many fish species which acts as a co-factor in various hydroxylation reactions in living tissue and is involved in collagen formation, growth, reproductive processes, disease resistance and immune response. Vitamin E is a lipid-soluble vitamin that comprises of eight naturally occurring tocopherols. Vitamin E functions as a metabolic antioxidant, preventing the oxidation of lipoproteins and biological membranes. It has been demonstrated to be an essential dietary nutrient for all fish studied.

Keywords: Anti-oxidants, vitamin C, vitamin E, immunostimulants

Introduction
Aquaculture is one of the fastest growing food producing sector in the world. In order, to meet ever increasing demand of world population, there is a need of convinient natural and artificial feed additive in fish feed which enhances the feed utilization efficiency, digestibility of feed and fulfill the nutrient requirement and enhance immune system of fish. There is large number of feed additives available to improve fish immune system. Some of these additives used in feed mill are chemical products especially hormones and antibiotics which may cause unfavorable side effects. Large number of feed additives is available for the enhancement of fish growth. The efficient utilization of antibiotic growth promoters (AGPs) as feed additives in the aquaculture industry has been criticised by government policies and consumers because of possible development of microbial resistance to these products and their potential harmful effects on human health (Baruah *et al.,* 2008) [9]. Recent studies on fish immunity conclude that immunostimulants are crucial for activating the fish immune system. Phytobiotics, probiotics, vitamins etc. have shown some promising results in growth and immunity enhancement in fishes. World Health Organization encourages the use of medicinal herbs and plants to substitute or minimize the use of chemicals through the global trend to go back to the nature. Attempts to use the natural materials such as medicinal plants could be widely accepted as feed additives to enhance efficiency of feed utilization and animal productive performance (Levic *et al*., 2008) [35]. Now a days herbal based feed additives are also used. These plant based feed additives are eco-friendly and cost effective in order to meet the requirement of Indian fish farmers. Herbs have been widely used in veterinary and human medicine. They are natural products that are not only safe for consumers but also widely available throughout Asia. Profitable fish farming depends a lot on cheap and nutritionally balanced feeds that will result in higher growth rate and also enhance immunity. Dietary supplementation of pyridoxine reduces stress and boosts immunity during culture of *Labeo rohita* fingerlings (Akhtar *et al.*, 2010) [3]. Tewary *et al.* (2008) [59] examined the effect of higher levels of dietary vitamin C on growth, nutritional quality and immunomodulation in the Indian major carp, rohu (*Labeo rohita*). Four groups of *L. rohita* were fed experimental diets containing either no vitamin C (control) or supplemented with vitamin C at 500 mg/kg (Exp-1), 1000 mg/kg (Exp-2) or 1500 mg/kg (Exp-3) for 60 days. Growth parameters (NWG, ADG and SGR), serological parameters (TSP, TSA, TSG, and A:G), haematological parameters (TLC, TEC, Hct, MCV,

and MCH) and different non-specific immunological parameters (PR, PI, respiratory burst activity, and bactericidal activity) were evaluated during the experimental trial. Fish fed with vitamin C supplemented diet showed higher specific growth rate (SGR) up to 1000 mg/kg compared with control fish. Different haematological and serological parameters along with non-specific immune parameters were influenced by vitamin C supplementation. Higher levels of dietary vitamin C significantly ($P \leq 0.05$) enhanced protection against *Aeromonas hydrophila* (AH1) infection compared with controls. Vitamins C (ascorbic acid, AA) and vitamin E (tocopherol) have been extensively studied in fish nutrition (Halver, 2002) [23]. As the world's human population continues to expand beyond 6 billion, it's realiance on farmed fish production as an important source of protein will also increase (Naylor, 2000) [41]. Aquaculture, the fastest growing food production sector in the world is expected to bring most of the increase in fish production. To meet this growing demand of world population, there is a need of appropriate natural and herbal feed additive in aqua feed, which enhances the feed utilization efficiency, digestibility of feed and fulfill the requirement of nutrients and enhance fish immune system. Moreover, herbal plant based feed additives are eco-friendly and cost effective to meet the requirement of Indian aqua farmers. The fish lives in the aquatic environment and prone to various stressors and the attack of opportunistic pathogens such as bacteria, viruses, fungi and parasites. Many farmers faced mortality associated with bacterial and viral diseases in fishes/shellfishes. Hence, there is an urgent need to stimulate the immune system of the cultured aquatic animals. There is large number of feed additives available to improve fish immune system. Some of these additives used in feed mill are chemical products especially hormones and antibiotics which may cause unfavorable side effects. Recent studies on fish immunity conclude that immunostimulants are crucial for activating the fish immune system. Vitamin C and vitamin E function as biological antioxidants to protect cellular macromolecules (DNA, protein and lipids) and other antioxidant molecules from uncontrolled oxidation by free radicals during normal metabolism or under the conditions of stress, pollution, disease or infection Vitamins C and E are often considered together for dietary requirements due to their potential for interaction. Vitamins C (ascorbic acid, AA) and E (tocopherols) have been extensively studied in fish nutrition (Halver, 2002) [23], humans and other animals (Hamilton *et al*., 2000) [24] and considered as are strong antioxidants. Vitamin C plays an important role in growth and immunity of fish (Lin *et al.*, 2005) [33]. The lack of L-gulonolactone oxidase leads to unablity to synthesize ascorbic acid in most teleosts due to which it is necessary to convert L-gulonolactic acid to AA and an exogenous source of vitamin C is must in artificial fish diet (Wilson *et al.*, 1973) [68]. Vitamin E requirement being directly related to dietary HUFA levels since they are fatty acid highly prone to oxidation (Udilova *et al.*, 2003) [63]. Nazir and Chauhan, 2008 [43] concluded that the incorporation of the phytobiotics along with vitamin C and Chitosan in diet of *Cyprinus carpio haematopterus* showed higher growth and survival without having any adverse impact on health of fish as well as on aquatic environment. Antioxidants plays a significant role in defence and oxidative stress in fish semen as well as Antioxidant Supplementation in cryopreservation media, in order to establish perspective for future studies. Antioxidants have the effects on post thaw sperm quality also. (Ladysandoval vargas *et al.*, 2020) [52]. Diet supplement with Nano Se, vitamin C and vitamin E in Experimental trial on Rainbow trout could increase growth performance, antioxidant and immune responses in juvenile Rainbow trout exposed to ammonia stress. (Mohammad Harsij *et al*., 2020) [26].

Effects of Vitamin C

The vitamin C requirement can vary with fish size, age, species and rearing conditions. These requirements of vitamin C supplemented feed have been investigated in various fish species and found that fish requirements for vitamin C ranged between 20 and 50 mg kg-1 feed (NAP, 1993) [40]. Sobhana *et al.* (2002) [53] examined two groups of 3-day-old hatchlings of *Cirrhinus mrigala* were fed with vitamin C supplemented (at 1000 mg vitamin C/kg diet) for a period of 4 months. Fishes were investigated for their disease susceptibility and inflammatory response to a virulent strain of *Aeromonas hydrophila* and concluded both kidney and liver ascorbic acid levels were significantly higher ($P< 0.05$) for the vitamin C supplemented group compared to the non-supplemented group. It was also observed that the vitamin C non-supplemented group had a significantly higher ($P< 0.05$) mortality rate compared to the supplemented group. Tawwab *et al*. (2004) [58] studied effect ascorbic acid (vitamin C) on mercury detoxication, growth performance and physiological aspects of nile tilapia and concluded that vitamin C enhanced weight gain, Specific Growth Rate (SGR) and survival rate, while they decreased significantly with high dose of mercury. Feed intake linearly decreased in fish exposed to high dose of Hg.

Falahatkar *et al.* (2006) [17] evaluated the effects of dietary vitamin C on growth, blood, body chemical composition and alkaline phosphatase (ALP) activity in juvenile great sturgeon (*Huso huso*) and concluded that mortality was low in all treatments The highest and lowest weight gain for fish that were fed different levels of vitamin C were in the 100 mg kg^{-1} and in 1600 mg kg^{-1} treatments, respectively. The best food conversion ratio was observed at the level of 400 mg kg^{-1} and 800 mg kg^{-1}. Shalaby (2009) [54] studied the opposing effect of ascorbic acid (vitamin C) on Ochratoxin toxicity in Nile Tilapia (*Oreochromis niloticus*) and concluded that significant decrease in total erythrocyte count (TEC), haemoglobin content (Hb) and haematocrit value (Hct) in fish exposed to the low or high level ochratoxin while the vitamin C enhanced the blood parameters in fish exposed to both levels of ochratoxin to values close to those of control fish. Alam *et al.* (2009) [5] studied the effects of vitamin C of formulated feed on feed, growth and body composition of *Heteropneustes fossilis* and concluded that there is a significantly positive effect of Vitamin C I diet (Diet C - 1200 mg/ kg of feed) on the growth, feed utilization & body composition. Vitamin C did not affect the survival rate. The effect of Vitamin C on reproductive performance was not highly significant. El-Sayed *et al.* (2010) [16] evaluated effect of including dried citrus pulp in Nile Tilapia diets on growth, digestibility and immune status and observed that dried citrus pulp could replace up to 10% of yellow corn in Nile tilapia fingerling diets without any adverse effects on different parameters or immune status. Moein (2012) [38] examined the influences of vitamin C on Common Carp (*Cyprinus carpio*) and observed that levels of vitamin C significantly influenced the weight of Common Carp during his study period. Weight gain increased with an increase in levels of vitamin C. Pimpimol *et al.* (2012) [48] observed the effects of vitamin C supplementation in feed on the hematological parameters of Mekong Giant Catfish (*Pangasianodon gigas Chevey*) and concluded that

sufficient vitamin C supplementation in fish feeds is necessary under intensive culture system for better survival and growth. A 250 mg kg^{-1} vitamin C in fish diet showed significantly better growth and feed conversion ratio in Mekong giant catfish. Mehrpak *et al.* (2015) [37] evaluated protective effects of vitamin C and chitosan against cadmium-induced oxidative stress in the liver of Common Carp (*Cyprinus carpio*).Study demonstrated that vitamin C and vitamin C combined with chitosan protect the fish against the toxic effects of $CdCl_2$ on the examined biochemical parameters in liver tissues. Sharifinasab *et al.* (2015) [55] studied Vitamin C and Chitosan alleviate toxic effects of paraquat on some biochemical parameters in hepatocytes of Common Carp and reported administration of vitamin C is effective in reducing liver toxicity of paraquat. However, administrating combination of both vitamin C and chitosan is more effective and have a synergic effect. They could be used as hepato-protective agent against paraquat-induced hepatotoxicity in fish. Shanmugam *et al.* (2015) [56] examined protective effect of vitamin C in sodium fluoride (NaF) induced toxicity in fishes and concluded that vitamin C effectively reduced the elevation in lipid metabolic profiles and strongly indicated the protective role of vitamin C against toxic effects of NaF in fishes. Asaikkutti *et al.* (2016) [7] evaluated effect of different levels of dietary vitamin C on freshwater prawn, *Macrobrachium malcolmsonii* and proposed that 100 mg/kg of vitamin C could be supplemented for antioxidant defense system and production of *M. malcolmsonii*.Lack of gulonolactone oxidase responsible for the synthesis of vitamin C in liver and kidney of many fishes (Dabrowski, 1990 [13]; Fracalossi *et al*., 2001) [19] demands dietary inclusion to meet the nutritional requirement for optimum growth performance of fish (Ai *et al*., 2004 [1]; Dabrowski, 1990) [13]. During processing and storage loss of supplemented ascorbic acid in fish diets has been reported (Anderson and Sunderland, 2002 [6]; Waagbo *et al*., 1991) [67]. More stable ascorbic acid derivatives with vitamin C activity, like phosphate derivates, are now widely used in fish feeds. Cultured fish require vitamin C, because they are unable to synthesize it [National Academic Press (NAP), 1993 [40]; Papp *et al*., 1995 [47]; Dabrowski, 2001] [14] but controversy surrounds the ability of primitive teleost fish to synthesize ascorbate (Moreau *et al*., 1999a [39]; Hung and Deng, 2002) [29] for presence of L-gulono-1, 4-lactone oxidase, a key enzyme for conversion of glucose to ascorbic acid. Vitamin C has been demonstrated to play an important role in the functioning of the immune system when supplied at dietary levels higher than standard doses in fish groups (Blazer, 1992) [10]. Specific effects of vitamin C on a variety of non-specific resistance mechanisms and the specific immune response have been reported in fish (Hardie *et al*., 1991 [25]; Verlhac and Gabaudan, 1994 [62]; Ortuno *et al*., 1999) [44]. Vitamin C is not stable and usually degraded during feed processing and storage because of the exposure to light, moisture, high temperature and oxygen. Vitamin C deficiency results in various abnormal signs in fish including slow growth rate (Gouillou-Coustans *et al*., 1998) [22], scoliosis and lordosis in rainbow trout (Hilton *et al*., 1978) [27], impaired wound healing (Wahli *et al*., 2003) [66], increased susceptibility to bacterial diseases (Ai *et al*., 2006) [2], lower survival rates (Ai *et al*., 2006 [2]; Wang *et al*., 2003) [66]. The vitamin C requirement can vary with fish size, age, species and rearing conditions. These requirements of vitamin C supplemented feed have been investigated in various fish species and found that fish requirements for vitamin C ranged between 20 and 50 mg kg-1 feed (NAP, 1993) [40].

Effects of Vitamin E

Tocheri *et al.* (2002) [60] examined the interaction of the dietary antioxidant micronutrient, vitamin E, with antioxidant defence systems and observed decreased dietary vitamin E led to decreased levels of tissue and higher activities of the liver antioxidant enzymes and higher levels of lipid peroxides. Galaz *et al.* (2010) [20] aimed to investigate the essentiality and requirements of vitamin E in diets and a challenge test against *Vibrio angullarum* and suggested that parrot fish require exogenous vitamin E which could be around 38 mg α-TA/kg diet for normal growth and physiology. Over 500 mg/kg dietary α-TA concentration could be required to enhance the nonspecific immune responses and improve the resistance of juvenile parrot fish against *V. anguillarum*. Kelestemur *et al.* (2012) [30] observed the effects of propolis and vitamin E supplementation in diets of juvenile rainbow trout subjected to different flow and concluded that dietary supplementations of rainbow trout with antioxidants alleviated the stress-induced oxidative damages. Two antioxidants (vitamin E and propolis) significantly decreased the negative effects. Udo *et al.* (2013) [64] examined the dietary needs for tocopherols in diets used in aquaculture and concluded that Common carp (*Cyprinus carpio)* was seen to have the highest requirement (200-300 mg kg^{-1}) for tocopherols. Better good performance, survival, better immune response and protection against oxidative damage to fish were positive effects of tocopherols in growers while high fecundity is associated with tocopherols in broodfish. Nasution *et al.* (2014) [42] evaluated the best dose of Vit E added to fish pellets on egg quality of kelabau fish (*Osteochilus kelabau*) and applied doses of Vit E were 0, 100, 150, 200 mg/kg pellets. The results show that the 200 mg Vit E /kg pellets is the best to reach gonad maturity was with three months cultivation period. Laten time was 13.5 hours.Vitamin E is a lipid-soluble vitamin that comprises of eight naturally occurring tocopherols. Among them, D-α-tocopherol has the highest biopotency. Vitamin E functions as a metabolic antioxidant, preventing the oxidation of lipoproteins and biological membranes. It has been demonstrated to be an essential dietary nutrient for all fish studied. Many studies reported its optimum requirement in diets for many fish species. Vitamin E enhances non-specific immune responses in fish and maintain flesh quality, normal resistance of red blood corpuscles (RBC) to haemolysis and capillary permeability but its precise mechanism yet not been demonstrated (Halver, 2002) [23]. A number of studies reported the improved immune responses, disease resistance, reproductive performance, growth performance, meat quality and nutrient digestibility, in most of the fish species as well as in terrestrial animals through feeding of high levels of dietary vitamin E than required amount (Lohakare *et al*., 2006 [36]; Lee *et al*., 2003 [34]; Panda *et al*., 2006 [46]; Samanta *et al*., 2006) [51]. The deficiency signs have been described for Atlantic Salmon (Poston *et al*., 1976) [49], Common Carp (Roem *et al*., 1990) [50], Rainbow Trout (Cowey *et al*., 1983) [12], Yellowtail (Toyoda, 1985) [61] and Korean Rockfish (Bai and Lee, 1998) [8]. In the non-specific defense system of fish, fish phagocytes are considered as one of the most vital components of defense and play a crucial role in both regulation and initiation of immunity similar to other vertebrates (Clem *et al*., 1985) [11]. It has been known that nutrients, such as proteins, vitamins, lipids and minerals could

affect phagocyte function (Landolt, 1989 [32]; Fletcher *et al*., 1988) [18]. Vitamin C and E activates the phagocyte population and immunostimulants (Eo and Lee, 2008) [15]. They extend the duration of specific immune response and at the same time improve the non-specific defense mechanisms (Blazer, 1992) [10]. Ortuno *et al*. (2001) [45] evaluated the effect of the oral administration of high ovulated eggs were 4,807. The ovisomati index was 0.82% and the average diameter of egg after treatments was 0.14 mm. Fertilization rate was 0.61%, with hatching rate was 16.68%. dosages of vitamin C and E on the innate immune system of the seabream and concluded that *in vivo* vitamin C and E exert a synergistic effect enhancing the respiratory burst activity of seabream phagocyte. Gammanpila *et al.* (2007) [21] conducted a experiment to evaluate the effects of dietry vitamin C, E and Zinc supplementation on growth, survival and reproduction of *Oreochromis niloticus* and concluded that supplementation of Vitamin C, E and Zn would improve the number of spawns, seed production, fecundity, hatching rate, sperm motility and viability of *O. niloticus.* Khara *et al.* (2016) [31] examined the effects of different levels of ascorbic acid and α- tocopherol and their combinations on Caspian Brown Trout, *Salmo trutta caspius* juveniles and concluded that a combination of 30 mg.kg diet^{-1} Vit E + 300 mg.kg^{-1} Vit C or 40 mg.kg^{-1} Vit E + 300 mg.kg^{-1} Vit C could be a good option for obtaining appropriate growth and survival in Caspian brown trout juveniles.

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