# Effects of Humic Acid as Feed Additive in Improvement of Nonspecific Immune Response and Disease Resistance in Common Carp (*Cyprinus carpio*)

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**Abstract**

Humic acid is a natural organic substance found in natural water systems and thus is compatible with all aquatic life forms. Common carp (*Cyprinus carpio*) was fed on diets supplemented by four of humic acid levels (0.0, 0.4, 0.8, and 1% of the dry weight diet, gps. (1-4)) for 45 days. At the end of the experiment, the body weight gain, specific growth rate and lysozyme activity were evaluated. A subgroup (10 fish each) of gps. (1-4) were challenged with nitric oxide (1.75 mg/L) for five successive days, and other subgroups (10 fish each) were IP challenged with virulent *Aeromonas hydrophila*. Serum was collected from challenged fish with *A. hydrophila* twice with 15 days interval for detection antibody immune level, using agglutination test. The body weight gain, specific growth rate and lysozyme activity were significantly higher in gps. (2-4) than gp. (1). The mortality rates due to nitric oxide challenge were 46.6, 33.3, 20 and 13.3% for gps (1-4) respectively. The mortality rates were 46.6, 26.6, 13.3 and 13.3% due to *A. hydrophila* challenge for gps (1-4) respectively. Titers of antibody were 0.0, 1/160, 1/320 and 1/320 in gps (1-4) respectively at first collection and 0.0, 1/80, 1/80 and 1/160 at the second blood-collection. The addition of humic acid to common carp diet induced effective protection against the virulence of *A. hydrophila* infection and increased the non-specific immune response.

## Introduction

Recently the use of natural immunostimulants for activation of the non-specific immune response against different stressors, as prophylactic measures, is an attractive and promising area to support development and sustain aquaculture production. It is well acknowledged that the non-specific immune defense, in fish, plays an important role in Preventive Medicines (Sahu *et al.,* 2007).

Humic acid is one of the major components of humic substances which are dark brown and major constituents of soil organic matter Humic acid contributes to soil chemical and physical quality and acts as precursor of some fossil fuels. They can be found in peat, coal, many upland streams and ocean water. It arises by the microbial degradation of biomolecules (lipids, proteins, carbohydrates and lignin) dispersed in the environment after the death of living cells (Marinsky *et al.,* 1995). Humic substances from soil and water are major controlling materials for metal speciation, pollutant binding and nutrient availability. An understanding of metal ion binding and competition remains difficult because of the complexity of humic substances which are proposed to be irregular polymers with a number of chemically un-identical

**Table (1): Effect of humic acid fed to common carp for 45 days, on body weight, specific growth rate (SGR) condition factor (CF) and survival rate.**

| Subgroups | Fish No. | Humic acid% | Body Weight (g) | SGR | length (cm) | CF | Survival rate % |
|---|---|---|---|---|---|---|---|
| 1 | 75 | 0.0 | 133.0 $^{C}$ ±0.38 | 1.89$^{C}$ ±0.15 | 20.4 $^{B}$ ±0.07 | 1.56 $^{C}$ ±0.06 | 68.89 |
| 2 | 75 | 0.4 | 171.0 $^{B}$ ±0.64 | 2.03 $^{B}$ ±0.18 | 20.8 $^{A}$ ±0.11 | 1.90 $^{B}$ ±0.08 | 80 |
| 3 | 75 | 0.8 | 192.3 $^{A}$ ±0.31 | 2.12$^{A}$±0.2 | 20.6 $^{B}$ ±0.09 | 2.19 $^{A}$ ±0.02 | 88.89 |
| 4 | 75 | 1.0 | 201.5 $^{A}$ ± 0.3 | 2.16 $^{A}$ ±0.22 | 20.6 $^{B}$ ±0.06 | 2.3 $^{A}$ ± 0.04 | 91.12 |

Means having the same superscript letters in the same column are not significantly different at P <0.05.

acidic functional groups having a variable electric potential (Saar and Weber, 1982).

Bacterial fish pathogens constitute one of the major limiting factors which impair the fish health and cause great economic losses. *Aeromonas hydrophila,* the causative agent of motile *Aeromonas* septicemia, has a worldwide distribution, infecting fishes, birds and mammals and causing heavy mortalities (Stoskoph, 1993). This problem is exacerbated by the lack of chemotherapeutics approved for use in food fish, besides lack of efficient vaccine. Prophylactic measures and immunostimulants are needed to confront the increase in disease risk, including transport, handling and a change in environmental conditions (Raa, 1996).

The potential of humic substances in the treatment of fish diseases has scarcely been investigated, although previous experiments demonstrated a protective effect of humic acid against atypical *Aeromonas salmonicida* infection in carp (ulcer disease) (Kodama *et al.,* 2007). Also, humic acid treatment of fish was effective against parasites through the improvement of the physiological condition of the fish (Meinelt *et al.,* 2008). Humic acid has an effect by oral administration on protection of ayu fish from cold water disease (Nakagwa *et al.,* 2009). The chemical acriflavine is used as a long-term bath to treat external bacterial and protozoan diseases of fish. The treatment with humic acid in protocol reduces the toxicity of acriflavine (Meinelt *et al.,* 2002).

Therefore, the goal of this study was to determine the effects of the orally administered humic acid to *C. carpio* on the non-specific immune response under experimental conditions.

## Material and methods

### Fish

Apparently healthy *Cyprinus carpio* of 15± 2g B. wt. and 10 ±1.2 cm long were randomly collected from a local farm of Central Laboratory for Aquaculture Research (CLAR), Abbassa, Abou–Hammad, Sharkia, Egypt. The fish were maintained in dechlorinated and continuous aeration tap water They were exposed to the natural photo-period and acclimatized to laboratory conditions for 15 days before the start of the experiment. Fish were fed twice daily on standard commercially prepared pellets at satiation.

### Aquaria

Twelve aquaria (77×37×48cm) were prepared using dechlorinated tap water; continuous aeration; maintained temperature at 22$^{o}$C throughout the experiment. About half of the water was changed and fecal matters were siphoned out once daily during the experiment.

### Medicated Rations

A commercial ration was grained and divided into four divisions/groups. Gp. (1) was free from humic acid (control). Gps (2-4) of the ration contained humic acid, at a concentrations of 0.4, 0.8, and 1% of ration weight respectively. The calculated dose was mixed with a small amount of vegetable oil, to avoid rapid dissolution in water, then adsorbed into ration, re-pelleted and dried in an incubator at 25ºC. The humic acid was procured from a Turkish company by an Egyptian sale agent (IBN ELWALED CO. Tanta, Egypt).

### Experimental Design

Four groups of *C. carpio* (75 in each group in three replicates) were prepared. The fish were fed to satiation twice daily

during the experiment for 45 days. At the end of experiment, the following parameters were measured:-

**Growth Parameters**

The fish were weighed and length was measured weekly throughout the experimental period (45 days). The specific growth rate (SGR) and condition factor (CF) were calculated as described by Laird and Needham (1988).

SGR= [(ln of final mean body – In of initial mean body weight (g)) ÷ Time interval (days)] ×100

CF= [weight (g) ÷ (length (cm)) $^{3}$] × 100

**Nitric Oxide Challenge**

At the end of the feeding experiment, 30 fish from each replicate were randomly collected and challenged by exposure to nitric oxide (Baker Chemical Co.) 1.75mg/l for five successive days (Beer *et al.,* 2003).

**Lysozomal activity**

Serum lysozyme was determined by turbidometric assay according to Sankaran and Gurnani (1972).

**Challenge with Virulent *Aeromonas hydrophila***

At the end of the feeding experiment, 30 fish from each replicate were randomly collected and challenged with virulent strain of *A. hydrophila,* which was previously isolated from naturally diseased carp and identified according to standard bacteriological tests as described by Chu and Lu (2005). Eachfish was IP inoculated by 0.5 ml virulent *A. hydrophila* 24 hs culture suspension of containing $10^{8}$ CFU/ ml. The fish were observed for 15 days for mortalities. The dead fish were removed once a day and subjected to bacterial re-isolation to verify death.

**Agglutination Test (titer)**

After 10 days of challenge fish with *A. hydrophila*, blood was collected two times with 15 days interval without anticoagulant for serum separation to measure the stability of specific antiboy production. The serum was serially two fold, diluted and titrated against suspension of *A. hydrophila* strain $6\times10^{8}$ CFU/ml then mixed well. The mixture was incubated at 37$^{o}$C for 1hr. and the results were recorded. The technique was done according to McGraw (1957).

**Statistical Analysis**

The statistical analysis was performed by the one way ANOVA analysis of variance according to (Kachigan, 1991). The multiple tests were carried out to determine the differences among treatment means at significance level of $P< 0.05$. The standard error was determined.

## Results

**Growth Parameters**

The body weight-gain, specific growth rate (SGR), condition factor (CF) and survival percent in in gps. (2-4) were significantly higher than the control (Table 1). There was a significant increase in the body weight-gain and condition factor with gps. (3&4) than gp. (2).

**Nitric Oxide Challenge**

Table (2) shows effective protection of humic acidagainst nitric oxide challenge. The mortality rate was 46.6% in gp. (1) due to nitric oxide challenge for 5 successive days. In

contrast, the mortality rates were 33.3, 20 and 13.3% in gps. (2-4) respectively.

**Serum lysozome activity**

The current investigation showed that the serum lysozyme was significantly increased in gps. (3&4) at the end of the second week of feeding. The decreased at the end of the 4th week and increased again at the end of the 6th week of the feeding experiment without any significant changes (Table 3). Gp. (2) presented decreasing lysozyme activity throughout the feeding experiment.

**Challenge with Virulent *Aeromonas hydrophila***

(Table 4) shows that gp. (1) challenged with *A. hydrophila*, suffered 46.6% mortality. Gps (2-4) exhibited 26.6, 13.3 and 13.3% mortalities respectively. Fish mortality and development of skin lesions, such as erosions and hemorrhages on the skin, gill cover and mouth were suppressed in fish fed on supplemented humic acid ration. *A. hydrophila* was re-isolated from the gills and the eroded skin of the recently dead fish.

**Agglutination Test (titer)**

The used *A.hydrophila* strain $6\times10^8$ CFU/ml was agglutinated blood at 4HA and so, by the collected serum which was serially two fold diluted and titrated against suspension of 4HA used bacteria then mixed well. The obtained cut point titers were 1/160, 1/320 and 1/320 in gps (2-4) at the first blood collection and 1/80, 1/80 and 1/160 at the second blood collection. The serum gp. (1) unable to inhibit agglutinate blood cells (Table, 5 & 6).

## Discussion

Prolonged stress and inadequate adaptation of fish to aquaculture could compromize the immune defense of fish which predisposes to infection and decrease growth rate (Sahu *et al*., 2007).

Supplemented ration with humic acid (0.8 and 1%) increased the body weight-gain and specific growth rate of common carp. The treated fish expressed better condition factor than control which is probably due to improved of physiological conditions of the common carp fed on the humic acid supplemented diet. (Van Rensburg *et al*., 2001 and Schepetkin *et al*., 2003) through improving hematological parameters together with serum and tissue enzymes. Moreover, humic acid eliminates metal bioaccumulation levels from fish tissues.

Generally, most fish bacterial diseases are caused by opportunistic bacteria, which mean, most bacterial infections result because of changes in the bacteria / fish relationship. This is a very delicate relationship, but generally when an opportunistic bacterium tries to establish an infection the fish host is able to resist it by a variety of defense mechanisms. However, if the numbers or virulence of the pathogen increases or the host resistance is reduced the pathogen may be able to colonize tissue and establish infection. The water quality is important in this aspect. Some signs, such as flashing, may be caused by high nitrite levels. Adding any chemical, as therapy, in these circumstances will exacerbate the malady (Saar and Weber, 1982).

The innate immune responses of fishes play a central role in host defense against infectious diseases (Hanington *et al*., 2009). Humic acid additive to diet, at high level, increased serum lysozyme in the

second week of feeding, compared with the control. Innate immune response, mediated by lysozyme was reported in several fish species (El-Ashram and El-Boshy 2008; El-Boshy *et al*., 2008 and El-Boshy *et al*., 2010). Improved the immune system by a thin protective coating on the animals' intestinal mucosa.

The supplemented diet with humic acid for 45 days proved to be effectiveagainst experimental *A. hydrophila* infection in carp which is in agreement with Kodama and Denso (2007) and Kodama *et al.* (2008) who recorded that humic acid was effective against trypanosome brucie in mice. Humic acid may improve the physiological and immunological condition (Kodama *et al.,* 2008). Humic acid enhanced the production of reactive oxygen species and nitric oxide in mouse peritoneal macrophages that clarifies the role of humic acid in protection of carp after nitric oxide challenge. The empirical evidence is currently accumulating that humic substances are taken up (Beer *et al*., 2003). Once internalized, these substances can induce biotransformation enzymes and stress defense proteins, such as chaperons or heat shock protein in fish and invertebrates (Menzel *et al*., 2005).

Measuring stability of produced specific antibodies against *A. hydrophila* challenge, showed cut point at 1/160, 1/320 and 1/320 in gps. (2-4) respectively at first blood-collection and 1/80, 1/80 and 1/160 at the second blood-collection with the highest in gp. (4). It is therefore reasonable to suppose that humic substances are absorbed via the intestinal tract of the carp to affect the host physiological conditions, such as innate immune responses, thereby conferring protection against *A. hydrophila* infection. Similar findings were described by (Van Rensburg *et al*., 2001).

It could be concluded that the oral administration of humic acid, during the rearing period, is beneficial. As the specific and innate immune responses were enhanced, to resist diseases. Furthermore, it increased the growth rate. The best level of humic acid in the fish diet was 0.8%.

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**Table (1): Effect of humic acid fed to common carp for 45 days, on body weight, specific growth rate (SGR) condition factor (CF) and survival rate.**

| Subgroups | Fish No. | Humic acid% | Body Weight (g) | SGR | length (cm) | CF | Survival rate % |
|---|---|---|---|---|---|---|---|
| 1 | 75 | 0.0 | $133.0^{C} \pm 0.38$ | $1.89^{C} \pm 0.15$ | $20.4^{B} \pm 0.07$ | $1.56^{C} \pm 0.06$ | 68.89 |
| 2 | 75 | 0.4 | $171.0^{B} \pm 0.64$ | $2.03^{B} \pm 0.18$ | $20.8^{A} \pm 0.11$ | $1.90^{B} \pm 0.08$ | 80 |
| 3 | 75 | 0.8 | $192.3^{A} \pm 0.31$ | $2.12^{A} \pm 0.2$ | $20.6^{B} \pm 0.09$ | $2.19^{A} \pm 0.02$ | 88.89 |
| 4 | 75 | 1.0 | $201.5^{A} \pm 0.3$ | $2.16^{A} \pm 0.22$ | $20.6^{B} \pm 0.06$ | $2.3^{A} \pm 0.04$ | 91.12 |

Means having the same superscript letters in the same column are not significantly different at $P < 0.05$.

**Table (2): Protective effect of humic acid fed to common carp for 45 days, against nitric oxide challenge for five successive days, (gps. 1-4).**

| Subgroup | Fish No. | Humic acid% | Nitric oxide | Mortality no., and rate |
|---|---|---|---|---|
| 1 | 30 | 0.0 | 1.75 mg/l | 21 (46.6) |
| 2 | 30 | 0.4 | | 15 (33.3) |
| 3 | 30 | 0.8 | | 9 (20) |
| 4 | 30 | 1.0 | | 6 (13.3) |

**Table (3): lysozyme activity in humic acid fed in common carp for 45 days (gps 1-4).**

| Subgroup | 15th day | 30th day | 45th day |
|---|---|---|---|
| 1 | $0.256\pm0.0^{B}$ | $0.267\pm0.001^{A}$ | $0.261\pm0.0^{A}$ |
| 2 | $0.155\pm0.003^{C}$ | $0.151\pm0.003^{C}$ | $0.149\pm0.003^{C}$ |
| 3 | $0.264\pm0.00^{A}$ | $0.255\pm0.001^{B}$ | $0.26\pm0.001^{A}$ |
| 4 | $0.261\pm0.0^{A}$ | $0.258\pm0.0^{B}$ | $0.266\pm0.0^{A}$ |

Means having the same superscript letters in the same column are not significantly different at $P < 0.05$.

**Table (4): Mortality rate of common carp fed on diet supplemented with humic acid for 45 days and I/P challenged with $0.5\times10^{8}$ ml virulent *A. hydrophila* (gps. 1-4).**

| Subgroups | Fish No. | Humic Acid % | Mortality No. and Rate |
|---|---|---|---|
| 1 | 30 | 0.0 | 21 (46.6) |
| 2 | 30 | 0.4 | 12 (26.6) |
| 3 | 30 | 0.8 | 6 (13.3) |
| 4 | 30 | 1.0 | 6 (13.3) |

**Table (5): First Agglutination Test among blood collection titers against *A. hydrophila* (gps. 1-4).**

| Well No. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Serum | | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 |
| Agglutinin | 1 | -- | - | - | - | - | - | - |
| | 2 | +++ | ++ | ++ | + | + | - | - |
| | 3 | +++ | ++ | ++ | + | + | + | - |
| | 4 | +++ | +++ | ++ | ++ | + | + | - |

+ = agglutination positive; - = non agglutination;

**Table (6): Second blood collection titers against *A. hydrophila* (gps. 1-4).**

| Well No. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Serum dilution | | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 |
| Agglutinin | 1 | - | - | - | - | - | - | - |
| | 2 | ++ | + | + | + | - | - | - |
| | 3 | ++ | ++ | + | + | - | - | - |
| | 4 | ++ | ++ | + | + | + | - | - |

+: agglutination positive; -: no agglutination;