Nutritional Approaches to Mitigate Iron Toxicity in Rainbow Trout and Catfish

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ABSTRACT

Catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) are the leading fish food in the United States. Iron (Fe) is an essential element for fish; however, a higher level could exert toxic effects on fish. Vitamin C is a reducing agent that helps in facilitating iron uptake in the gut. Bentonite clay acts as a chelating agent. Our study investigated the effect of dietary supplementation of vitamin C and bentonite in mitigating the iron toxicity in catfish and trout. *Vitamin C study:* Catfish were exposed with/without iron (9.5 mg/L $Fe3^{+}$ in water) and fed 3 diets [control, low vitamin-C (143 ppm), and high vitamin-C (573 ppm)] for 8 weeks. Results showed that fish growth was lower in iron exposed compared to non-exposed groups. Supplementations of vitamin C increased the growth rate, reduced oxidative stress, and mitigate the organs damages under iron-exposed conditions. *Bentonite study:* Six diets [0% bentonite (Con), 2% bentonite (LB), 4% bentonite (HB), 0.25% iron as $FeSO_4$ (Fe), Fe+2% bentonite (LBFe), and Fe+4% bentonite (HBFe) were fed to trout for 8 weeks. Significantly lower fish growth was observed when fed Fe compared to control, however bentonite supplementation improved the growth. Oxidative status was enhanced in LBFe and HBFe groups compared to the Fe group. The liver showed infiltration with inflammatory cells and necrosis in Fe and HB groups whereas the LBFe group appears to be normal. *Bentonite and Vitamin C study*: Seven diets [500 ppm vitamin-C (Con), 0.25% iron as $FeSO_4$ (Fe)+ Vitamin-C(500ppm) (ConFe), 2% bentonite, 0.25% iron as $FeSO_4$ (Fe) (BenFe), Fe+1500 ppm Vitamin-C (MVCFe), Fe+2% bentonite + 1500 ppm Vitamin-C (MVCBenFe), Fe+3000 ppm Vitamin-C (HVCFe), and Fe+2% bentonite + 3000 ppm Vitamin-C (HVCBenFe) were fed to trout for 10 weeks. The result indicated that the supplementation of bentonite, vitamin C,or in combination exposed to a higher dietary iron augmented ($p < 0.05$) their growth performance compared to the ConFe group. The liver and gills showed a series of histological changes in ConFe, accompanied by a significant rise in hepatic and muscle iron. The supplementation of bentonite, vitamin C, or in combination reduced the augmented concentrations of iron accumulation to that of ConFe group. Also, the MDA level was significantly higher in ConFe groups than in the rest of the groups. Similarly, the expression of the antioxidant gene was upregulated with the

supplementation of bentonite, vitamin C, or in combination. Further, the gene expression of Hepcidin (HAMP, transferrin (Tf), and Hemeoxygenase (HO1) were upregulated in the ConFe group compared to the rest groups. Overall, dietary supplementation of vitamin C and bentonite can be an effective approach to mitigate the iron toxicity in the catfish and trout aquaculture industry.

Keywords: Iron toxicity, vitamin C, Bentonite, Catfish, Rainbow trout

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DEDICATION

This dissertation is dedicated to my lovely grandparents, parents, and siblings for their support and encouragement throughout all the years of my studies.

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Abbreviation

Iron……..Fe

Control…… Con

Control iron……ConFe

Low vitamin C…… LVc

High vitamin C…….. HVc

Low bentonite……LB

Low vitamin C iron……LVcFe

High vitamin C iron……..HVcFe

High bentonite……..HB

Low bentonite iron……...LBFe

High bentonite iron……HBFe

Malonaldehyde……MDA

Catalase……CAT

Superoxide dismutase……SOD

Glutathione peroxidase……...GPx

Glutathione reductase……GR

Hemoglobin……...Hb

Hematocrit……...HCt

Hepcidin……HAMP

Transferrin……Trf

Heme oxygenase……..HO1

Dissolved oxygen……DO

Hydrogen peroxide……..H_2O_2

Water……...H_2O

Oxygen……...O_2

Environmental Protection Agency……..EPA

Crude protein……CP

Crude lipid……CL

Reactive oxygen species……...ROS

Lipid peroxidation……..LPO

Red blood cells……RBC

Carbon dioxide……...CO_2

Long-chain polyunsaturated fatty acids……LC-PUFA

Iron-binding protein……IRP

Anti-nutritional factors……ANFs

Divalent metal transporter 1……DMT1

High environmental iron……...HEI

Fish meal……..FM

Poultry by-product meal……..PBM

Fish oil……...FO

Soybean oil……..SO

Soybean meal……..SBM

Weight gain…….....WG

Feed conversion ratio……..FCR

Hepatosomatic indices……..HSI

Hematoxylin and eosin……H&E

Eicosapentaenoic acid……....EPA

Docosahexaenoic acid……....DH

CHAPTER 1

INTRODUCTION

Aquaculture is the fastest-growing food-producing industry and currently provides more than half of all fish eaten directly by humans (FAO, 2018). Aquaculture and capture fisheries supplied about 179 million tons of fish, which accounted for approximately 88% (or over 156 million tonnes) used for direct human consumption, with the remaining 12% (or approximately 22 million tonnes) used for non-food purposes. About 80% (about 18 million tonnes) of the non-food purposes was utilized for fishmeal and fish oil, and the remainder (4 million tonnes) was primarly used as ornamental fish, fish culture (fry, fingerlings, or small adults for ongrowing), bait, pharmaceutical uses, pet food, or raw material for direct feeding in aquaculture and livestock and fur animal raising (FAO, 2020).

Fish are abundant in high-value protein, important micronutrients (like minerals and vitamins), and important polyunsaturated fatty acids (like omega-3 fatty acids) (Torpy et al., 2006; Skåre et al., 2014). Dietary fish consumption supports the growth of an infant's brain, and in maintaining a balanced cardiovascular system (Torpy et al., 2006). Fish consumption decreases the risk of premature death, heart attacks, high blood pressure and strokes (Torpy et al., 2006; Mozaffarian et al., 2011; Tørris et al., 2018). And as such fish are advised for human consumption as a healthy source of food (Piepoli et al., 2016). Consequently, sufficient stocks of fish are crucial in addressing food security for the increasing world population. However, the production of fish from the natural water bodies (i.e., between 93.55 million and 90.91 million metric tons, from 2000 to 2016) has declined over the past decade (FAO, 2018). While capture fishing appears to be decreasing, there is indeed hope as aquaculture fish production continues to increase (e.g., from 35.60 million to 80.03 million metric tons of fish in 2000 and 2016 respectively) (FAO 2002; FAO, 2018). Aquaculture has the highest average percentage growth rate in the food production sector (i.e., 5.8 % from 2001 to 2016) (FAO, 2018; Edwards, et al., 2019). Despite this apparent fact,

however, the production of edible aquatic food from aquaculture is also far behind in terms of volume (i.e., 50 million and 324 million metric tons) relative to terrestrial animal food production (Edwards et al., 2019). Therefore, to ensure that fish production from aquaculture increases in quantity, viable and improved aquaculture development strategies are required.

1.1. Factors affecting Water Quality

Aquaculture production depends on several factors including level of intensity, culture units, and species combination. Being said that water is most important for aquatic life to support which is determined by parameters like dissolved oxygen (DO), pH, NH_3, NO_2^-, NO_3^-, carbons and others. Intensive method involves stocking at higher densities, increased aeration and heavy feeding, algaecide applications etc. have enhanced in the overall production of fish. In these conditions, however, fish may have to survive on less water, food, and spaces at higher densities, which also deteriorates the water quality. Moreover, unconsumed feed and fish metabolic wastes are the causes for raising ammonia levels and it often contributes to iron accumulation in the water.

In aquatic environments, metals are continuously released from natural and anthropogenic sources such as industrial and domestic sewage discharges, mining, agriculture, electronic waste, anthropogenic accidents, navigation traffic, and climate change events such as floods (Sandu et al., 2008; Zhang et al., 2016). In addition, metals are readily dissolved in water and ultimately ingested by aquatic species like fish and invertebrates that cause a broad range of biological effects, from being vital to living organisms to being fatal. Despite the fact that certain metals are important for living organisms at low concentrations, like (i) micronutrients (Cu, Zn, Fe, Mn, Co, Mo, Cr, and Se) and (ii) macronutrients (Ca, Mg, Na, P, and S); at higher doses, they may provoke toxic effects that disrupt the growth, metabolism, or reproduction of organisms with implications for the whole trophic chain, as well as for humans (Stankovic et al., 2014). Among them, iron is one of the most common highly toxic water pollutants (Salimi et al., 2020; Jagaba et al., 2020).

1.2. Iron and its occurrence in the environment

Iron is a necessary element for all species of life. Iron's importance in biogeochemical processes is highlighted by its distinctive purpose of serving as both an electron donor and acceptor and can play a significant role in the biological pathways of several organisms.

Iron can be toxic at higher concentrations because of its ability to donate and accept the electrons. It can catalyze the conversion of hydrogen peroxide into free radicals within the cell. Free radicals can harm a wide range of cellular structures and eventually kill the cell (Crichton et al., 2002). To avoid such harm, living organisms have adapted a biochemical defense mechanism that involves binding iron atoms to proteins. This enables the cells to benefit from iron while also limiting their ability to harm them (Andrews, 1999). Heme is an iron-binding protein containing iron at its centers helping an organism to perform redox reactions and electron transport processes via heme variants. Iron is needed for oxidative phosphorylation, the primary source of energy for cells; without it, cells would die. Iron is also required for the formation of myoglobin, which stores oxygen in muscle cells in higher organisms.

There are two types of iron in nature: soluble ferrous iron (Fe^{2+}) and insoluble ferric particulate iron (Fe^{3+}). In oxygenated water, insoluble ferric (Fe^{3+}) iron predominates over the bivalent (Fe^{2+}) iron, which is toxic to aquatic animals (Davidson, 1993). Water appears reddish color in presence of a higher concentration of iron and causes staining, taste issues, stock losses in aquaculture, and pipe fouling. Iron is classified as a secondary contaminant by the Environmental Protection Agency (EPA) because it poses no health risk to humans. The EPA has established a secondary maximum contaminant level of 0.3 mg Fe/L for total iron in public water systems (EPA, 2013). High iron overload in water sources is a global problem caused by either natural iron source or because of anthropogenic activity. The range of concentration of iron that are present in different countries and United States are mentioned in table (1.2.1).

Table 1.2.1: Summary of iron concentration in different parts of the world

Country	Place	Iron Concentration (mg/l)	References
India	North Eastern Indian rivers	0.002-14.44	Singh et al., 2019
Indonesia	Indramayu	0.10-25.2	Rusydi et al., 2021
Nigeria	Bayelsa State	5.32-9.96	Angaye et al., 2015
Germany	Lusatia, Brandenburg	5.0-10.0	Steffens et al., 1993
China	Marshy Rivers	0.43-4.87	Yan et al., 2010
Brazil	Monjolinho River	11.48-109.36	Ruas et al., 2008
Vietnam	Hanoi area	0.4-10.0	Van dan and Dzung, 2002
United States of America			
Alaska	Fairbanks Experimental Fish Hatchery	5.0-6.0	Fish, 2009
Texas	Ogallala Aquifer	0.001-69.6	
	Gulf Coast Aquifer	0.00-139	
	Carrizo-Wilcox Aquifer	0.0005-20.2	
	Trinity Aquifer	0.001-12.2	Reedy et al., 2011
Louisiana, Arkansas	Lower Mississippi River Alluvial Aquifer (MRAA)	16.00	Borrok et al., 2018
Idaho	Lake Coeur d'Alene	41	Cummings et al., 2000

1.3 . Effect of chemical and biological parameters on iron

Temperature: Water temperature is positively correlated with iron. An increase in water temperature increases bacterial growth along with the biological oxidation of soluble Fe^{2+} into insoluble Fe^{3+}. Also, the studies have shown that watercolor is an indication of an indirect measure of total organic matter. This indicates that iron has a positive correlation with watercolor.

Hardness and pH: The pH is more stable in hard water (250 mg/l) compared to the medium (100 mg/l, and soft water (50 mg/l). The study conducted by Oliver and Fidler (2001) showed that the maximum iron concentration obtained in hard water with a pH of at least 5 was 54 mg/L. This value fell to 36 mg/L in saltwater, 21 mg/L in well water, and 7 mg/L in soft water.

Alkalinity: Alkalinity is negatively correlated with iron concentration. This is because of increase in alkalinity increases the buffering capacity, keeping water pH more stable.

Turbidity: Turbidity is positively correlated with the iron concentration in the water, indicating that turbidity increases with the increase in the concentration of iron.

1.4. Sources of Iron in Aquaculture

In the aquatic environment, Fe is concentrated in two forms i.e., ferric water insoluble (Fe^{3+}) and ferrous water soluble (Fe^{2+}). The latter is more bioavailable, and it is readily absorbed by aquatic species via divalent metal transporters present in the gills and intestines (Gunshin et al., 1997; Bury et al., 2003). Despite the fact that fish can absorb soluble Fe through the gill membrane and intestinal mucosa (Roedar and Roedar, 1968), the diet is the primary source of Fe due to low solubility of Fe concentrations in natural waters (NRC, 2011).

Iron exists in two forms in feeds: heme iron and non-heme iron. Heme iron is primarily found in animal feed ingredients (e.g., fish meal, animal meat, and blood meal). Non-heme iron refers to iron sources in feeds that are not heme or inorganic contaminants from ingredient and/or feed processing. A trace amount of Fe may be present in cereal grains as an iron phytin complex.

Blood meal and poultry offal meal are the good sources of iron, however, the use of blood meal in species like Murray cod, *Maccullochella peelii,* and rainbow trout, *Oncorhynchus mykiss*, express negative growth response, high mortalities when fed at higher inclusion levels (Abery et al., 2002; Bahrevar and Faghani, 2015).

Table 1.4.1: Summary of feed ingredients and it's nutrient composition.

		Ingredients							
Nutrient composition	CP (%)	Soybean meal	Wheat gluten	Soybean protein concentre	Cottonseed meal	Fish meal	Poultry offal meal	Blod meal	Feather meal
		52.6	85	84.4	45.5	70.6	60.2	93.9	85.5

	CL (%)	1.8	1.7	2.5	10.2			2	
Macro-minerals (g/100g)	Ca	0.38	0.1	0.16	0.21	4.48	2.03	0.34	1.3
	P	0.77	0.19	0.57	1.31	2.86	1.01	0.23	0.89
	K	2.46	0.13	0.23	1.64	0.85	0.41	0.38	0.14
	Mg	0.34	0.04	0.08	0.6	0.24	0.07	0.02	0.08
	Na	0.079	0.061	0.276	0.09	1.147	0.27	0.387	0.132
Trace minerals (mg/kg)	Zn	54.6	46	66	44	107	67	24	138
	Mn	38	26	10	23	19	18	6	16
	Fe	38.2	53	88	170	382	5107	2202	536
	Cu	1.79	25	16	117	12	41	12	10

1.5. Effects of higher concentration of iron in fish via water and feed

Iron toxicity varies according to fish species and size. Very small iron particulates are lodged in the gill lamellae of fish, acting as a mechanical filter. This lodged small iron particulate irritate gill tissues, causing gill damage and increasing the incidence of secondary bacterial and fungal infections. Iron promotes the dissociation of oxygen molecules into reactive oxygen species (ROS). ROS can damage lipids, proteins, and DNA macromolecules, affecting the overall physio-biochemical and metabolic performance of fish and, in extreme situations, leading to cell death (Bresgen and Eckl, 2015; Mohanty and Samanta, 2018; Papanikolaou and Panto, 2005). Furthermore, it has been shown that high levels of iron in water can coat the gill surface and cause cellular damage, limiting the gill's functional capacity (Debnath et al., 2012; Sevcikova et al., 2011; Slaninova et al., 2014; Teien et al., 2008). It has been illustrated that iron causes oxidative stress (Sevcikova et al., 2011). Ruas et al. (2008) reported higher levels of lipid peroxidation (LPO) in the erythrocytes of cichlid fish from a river with high iron levels.

Li et al., (2009) reported lipid peroxidation and changes in antioxidant enzyme activity in embryonic and adult medaka *Oryzias latipes* exposed to nano-iron. In medaka embryos, there was dose-dependent inhibition of SOD activity and

increased production of malondialdehyde (MDA). SOD activity in the liver and cerebral cortex of adult medaka was initially reduced after nano-iron exposure, but increased with exposure time. Because there was no evidence of oxidative damage in adult fish, this study concluded that medaka embryos are more sensitive to nano-iron exposure than adults. In addition, Baker et al. (1997) found that feeding an iron-rich diet to the African catfish *Clarias gariepinus* induced LPO in the liver and heart. DNA damage, lipid peroxidation (LPO), and protein oxidation are all negative effects of iron. A higher iron concentration in the human body can cause hemochromatosis, which can lead to end-organ damage (Tembo, 2016).

Table 1.5.1: Form's of iron used and it's negative effect on fish health.

Name of Iron Supplements		Dosage		Side Effects	
		Feed (mg/Kg)	Water (mg/L)		
$FeSO_4.7H_2O$	African Catfish	6354.40		Suppressed growth, increase oxidative stress (higher MDA in the Liver and Heart)	Baker et al., 1997
$FeSO_4.7H_2O$	Grass carp	80-120		Reduce growth, increased MDA, PC content in muscle, reduce GST, GPx, GR, increased cooking loss and shear force in muscle	Zhang et al., 2016
$FeSO_4.7H_2O$	Rainbow trout	1975		Increased iron accumulation in the intestine, liver, stomach	Carriquiriborde et al., 2004
Iron hydroxide	Coho salmon		1.27	Reduce growth	Smith & Sykora 1976
	Rainbow trout		5	Detrimental effect on growth and feed conversions	Steffens et al., 1993
$FeSO_4.7H_2O$	Labeo rohita		8.25-33.01	Increased lipid peroxidation, reduce antioxidant capacity (SOD, Cat), accumulation of iron in liver, tissue injury etc	Singh et al., 2019
$FeCl_3$	Indian Knife fish		0.75	Increased iron accumulation in muscle, liver, gills, increase lipid peroxidation, reduce SOD, CAT, GPx activity	Mohanty & Samanta 2018

1.6. Approaches for the removal of iron from water

The aforementioned studies show that the high iron in water or feed can have a negative impact on fish performance, and, as a result, reduce overall production. As a result, fish farmers, toxicologists, and water quality managers face a significant challenge in reducing the toxic effects of higher iron in water or feed. Several strategies have been applied and the table 1.6.1 summarizes the methods that have been applied for the removal of iron.

Table 1.6.1: Summary of different methods used for iron removal (Adopted from Phadke, 2014).

S.N.	Method	Comments	References
1	Oxidation by aeration	Effective to remove low concentrations of iron. Works best at pH 8	Chaturvedi et al., 2012
2	Oxidation by using chemical oxidants	Useful in removing organically complexed iron. Chemical oxidants Cl, ClO_2, O_3, H_2O_2 and $KMnO_4$. Effective for pH 7 to 9. Shorter reaction time compared to aeration, toxic to aquatic organisms, and phytoplankton's	Chaturvedi et al., 2012 Tekerlekopoulou et al., 2006; Boyd, 1990
3	Iron sequestering	Used to remove soluble iron (Fe^{2+}). Polyphosphates or orthophosphates are effectively used in anaerobic conditions. effective in fast-moving water.	Seelig et al., 2013
4	Biological removal	Used for groundwater having iron bacteria and low pH.	Tekerlekopoulou et al., 2006;
5	Ion exchange	Typically used to remove small amounts of iron, such as at the household treatment level.	Chaturvedi et al., 2012 Sharma, 2001
6	Membrane filtration	Reverse osmosis and nanofiltration are included. operating expenses are high.	Holmden, 2013
7	Electrocoagulation	Removal efficiency is nearly 100%. Solar energy can be used. Time-consuming and puts a lot of strain on the electrodes, causing wear	Chaturvedi et al., 2012

		and tear. Shorter life span of the electrodes	
8	Adsorption-oxidation	Can be used under anoxic and low p^H conditions	Chaturvedi et al., 2012 Sharma, 2001

Aeration-assisted oxidation is used for the removal of lower iron concentration and is ineffective for the removal of organic complexed forms of iron. Additionally, a longer period of time is required to complete this process. The aeration must be controlled in order to keep the amount of dissolved oxygen constant in the water. After aeration, a minimum of 20 minutes of detention time is needed before water filtration with a desirable p^H value (Ityel, 2011). At higher alkalinity, carbonates dominate over the iron precipitate after aeration, whereas the use of oxidants results in hydroxide precipitates (Ghosh et al., 1966; Cleasby, 1975).

The chemical oxidation method is used for the removal of complex forms of irons because of its easy availability. The iron precipitates in the form of iron oxide or oxyhydroxides that are gelatinous in nature and hard to remove. Also, this method requires filtration for the removal of iron precipitates and has a problem like headloss and a high number of backwash cycles. Frequent backwash reduces the efficiency of filter. Also, the application of chemical oxidation like permanganate is toxic to phytoplankton and aquatic life (Boyd, 1990).

The process of combining ferrous iron with other molecules to prevent it from converting to a ferric state is known as iron sequestering/ chelation. As a result, this method can be used to treat groundwater with high ferrous iron concentrations. It's inexpensive to set up and simple to use. Phosphates, polyphosphates, and sodium silicates are the sequencers that are used. These agents, however, embed ferrous iron into colloidal forms, making removal more difficult (Robinson, 1990).

Biological removal process does not use chemical oxidation and has a higher filtration rate. Bacteria like Gallionella, Crenothrrix, Sphaerotilus, and Leptothrix help in the oxidation of iron (Ankrah et al., 2009). Iron load, types of oxidation, p^H, temperature, and co-precipitates formed play a vital role in the biological removal

process. Typically, p^H of 6.5-7.2. low dissolved oxygen and temperature around 50-75°F are required for successful biological iron removal (Summerfelt, 1999).

Ion exchange can be performed on both cations and anions using oppositely charged ions. Hydrophilic structure, high exchange rate, physical and chemical stability are the characteristic of an ideal exchanger (Harland, 1994). Ion exchange has the advantage of being able to operate at different flow rates, and pH, however, it has no effect on water turbidity, total solids, or alkalinity, limiting its application (General Electrical Company, 2012).

Adsorption is a low-cost process that does not require the use of chemicals and produces less effluent in the removal of iron. This is because of the adsorption of iron particles to the media that provides a higher surface area and more surface for adsorption. Also, this method is promising because of its uncomplicatedness, rapidity, reliability, and cost-effectiveness (Vafaeifard et al., 2016; Salimi et al., 2022).

The above-mentioned method's drawbacks necessitate investigations of a nutritional approach to directly mitigate iron-induced toxicity in fish. In this scenario, supplementation of vitamin C and bentonite may be a cost-effective approach because of their easy availability. Vitamin C is an essential micronutrient for most fish species (including rainbow trout, channel catfish; NRC, 1993), and is well known for free radical scavenger, protecting cells from oxidative damage. Similarly, the inclusion of natural zeolites (like Bentonite, clinoptilolite, etc), because of their crystalline nature, thermal stability, catalytic, sieve, and ion-exchange properties, along with their low cost, easy availability of large quantities, global presence made potential candidate for reducing or eliminating the heavy metals in the wastewater treatment process (Akgul et al., 2006; Siljeg et al., 2010).

1.7. Nutritional approach to mitigate iron toxicity

1.7.1 Vitamin C

Vitamin C is a micronutrient that advances the productivity of aquatic animals (Gao et al., 2014; Shahkar et al., 2015) such as ordinary development and the upkeep

of the physiological capacities of most aquatic animals. Fish and crustacean species require the ability to synthesize vitamin C (Lightner et al., 1978), leading to the need for an L-gluconolactone oxidase capable of ascorbic synthesis. Side effects like diminished development, skeletal and cartilaginous deformations, drowsy wound recuperating, expanded mortality, and decreased broodstock richness of females and sibling (Kitamura et al., 1965; Halver et al., 1969; Lovell, 1973; Lim & Lovell, 1978; Li & Lovell, 1985; Chavez, 1990; Frischknecht et al., 1994; Blom & Dabrowski, 1996) are observed from vitamins c deficiency. Vitamin C insufficiency diminishes the retention of intestinal iron in trout (Lipschitz et al., 1971; Hilton et al., 1978).

Vitamin C functions as a coenzyme within the hydroxylation response and is an antioxidant that ensures cells from free radicals (Adham et al., 2000; Sahoo and Mukherjee, 2002; Pierce et al., 2003; Sau et al., 2004; Kiron et al., 2004; Chen et al., 2004). A few researchers have recorded a higher level of vitamin c addition in few freshwater species i.e. catfish channels (Li & Lovell, 1985), rainbow trout (Verlhac & Gabaudan, 1994), bagrid catfish (Anbarasu & Chandran, 2001), Indian main carp (Misra et al., 2007; Nayak et al., 2007), tiger puffer (Eo & Lee 2008) enhances their immune functions. Nonetheless, Kikuchi et al. (1994) and Li et al. (1993) indicated that the elevated levels of vitamin C didn't enhance their unspecific immune responses.

The innate immune system of fish that includes; lysozyme, complements (C3 and C4), immunoglobin (IgM) and acidic phosphate (ACP) is involved as the borderline of protection against a wide spectrum of pathogens, so their activities are essential indicator of innate immunity in fish. In addition, tumor necrotic factor-a (TNF-α) is a pro-inflammatory cytokine involved in systemic inflammation and immune cell regulation generated primarily by activated macrophages, while Interleukin 8 (IL-8) is a chemotactic factor attracting neutrophils, basophils and T-cells during the inflammatory phase (Hong et al., 2013). Tumor necrosis factor-α is a growth factor that activates cell proliferation, apoptosis, and necrosis leading to both physiological and pathological processes (Hong et al., 2013). Both cytokines initiate activation of endothelial and fibroblast cells (Yarilina et al., 2008; Hong et al., 2013).

1.7.2 Bentonite

Bentonites are crystalline, hydrated aluminosilicate salts composed of sodium (Na), potassium (K), calcium (Ca), iron, manganese, nickel whose surface is covered by ionic cations describing their physical and chemical characteristics along with their strength and velocity of adsorption (Pasha et al., 2008; Khanedar et al., 2012; Di Gregorio et al., 2014). The key basic characteristics such as adsorption, high cation exchange efficiency, natural occurrence and low cost make it beneficial for a number of applications including its ability to reduce ammonia and mineral levels (Murray, 2000; Shu-li-et al., 2009). Bentonites are 'generally recognized as food additives (GRAS)' for humans and various domestic animals including poultry, pigs, lambs, dairy cows, and goats (Fenn and Leng, 1989; Ivan et al., 1992; Walz et al., 1998). Dietary inclusion of bentonite in livestock has been documented to improve growth efficiency, feed conversion ratios and animal health, due to efficient nitrogen feed mobilization (Fenn and Leng, 1989; Ivan et al., 1992; Walz et al., 1998). The importance of utilization of bentonite as a feed additive for aquaculture practices is slowly growing in the current context. Obradovic et al. (2006) reported that the introduction of 1.0 percent of bentonite in the diet of rainbow trout (*Onchorhynchus mykiss*) improved growth and feed conversion efficiency. Similarly, Mumpton (1999) reported that adding less than 2.0 % bentonite (as zeolite) to the diet of trout resulted in a substantial weight gain increase over a 64-day feeding period. In addition, Eya et al. (2008) indicated that the inclusion of 5 % bentonite in juvenile rainbow trout diets enhanced the growth performance and whole-body composition. The increase in growth performance was associated with a reduced accumulation of toxic endogenous ammonia. Evidence indicates that tilapia (*Tilapia zillii*) fed with 1.0 percent and 2.0 percent bentonite substantially reduced the production of ammonia and ammonia as opposed to those fed control without bentonite (Yildirim et al., 2009). Similarly, Ergün et al. (2008) also reported that feeding the dietary trout mixed with 2.5% zeolite (bentonite) decreased the fish's discharge of ammonia by 24% relative to the control group.

Bentonites are rich in smectite minerals. Smectites are 2:1 type of crystal lattice aluminosilicates consisting of two-dimensional layers where a central octahedral

alumina sheet is fused into two external layers of silicate (Al-anber, 2010). Inside the layers, isomorphic substitution produces negative charges, counterbalanced by conveniently replaceable alkaline or alkali earth cations. Those are classified as exchangeable cations (Lunsdorf et al., 2000; Hua et al., 2013).

Sodium montmorillonite and calcium montmorillonite (Eya et al., 2008; Carraro et al., 2014) are the dominant minerals in the smectite group of clays. There may be substitutions for both octahedral and tetrahedral sheets in smectites, which produces a charge imbalance in the 2:1 layer. Alumina replaces silica in the tetrahedral layer and aluminum substitute in the octahedral sheet with iron and magnesium (Lira et al., 2014a; Safaei et al., 2014), creates a deficit in negative charges and is offset with the help of exchangeable cations on the edges as well as in between the layers. The dominance of exchangeable cations in the smectite group determines the montmorillonite group such as sodium montmorillonite (exchangeable cation Na) and calcium montmorillonite (exchangeable cation Ca) (Murray, 1991, 2000; Jovic-jovicic et al., 2010).

Claystone minerals (Smectites or montmorillonite) are thought of as a simple and efficient tool for the chemical prevention of a variety of harmful compounds, not only in the environment but also in living organisms, due to their sorption ability and lack of primary toxicity. The additionof clays in feed can bind and immobilize harmful substances in an animal’s gastrointestinal tract, significantly reducing their biological availability and toxicity to the body (Phillips, 1999; Lemke et al., 2001; Phillips et al., 2002; Trckova et al., 2004). Numerous research have supported the decontaminating abilities of clay minerals. They have the capacity to bind aflatoxin (Thieu et al., 2008; Hussain et al., 2017), plant metabolites (Dominy et al., 2004), heavy metals (, 2008; Abbes et al., 2007; Kim et al., 2011).

The liver is an important organ for the detoxification process. Heavy metals can bind the nuclear proteins like metallothioneins, increasing the accumulation of heavy metals ingested either via the gill or the gastrointestinal tract. Therefore, during heavy metal stress liver exhibit higher burdens of heavy metals. Exposure of carp to dietary cadmium (Cd) resulted significantly higher accumulation in the liver (Kim et al., 2009).

However, supplementation of montmorillonite at 0.5% in the diet significantly reduced the Cd load in the liver. A similar finding was seen in tilapia when fed with lead (Pb) with or without supplementation of montmorillonite (Dai et al., 2010). This might be because of the adsorption of Cd, and Pb via ion exchange reactions (Barbier et al., 2000), which reduces Cd, and Pb bioavailability and uptake at the gastrointestinal tract (dai et al., 2010).

Heavy metals are well known for oxidative damage (Dogru et al., 2008; Moreira et al., 2001). Supplementation of montmorillonite in the diet reduces MDA content and enhanced its antioxidant capacity (Kim et al., 2009; Dai et al., 2010). This indicates that montmorillonite is able to prevent oxidative damage caused by heavy metals.

1.7.3 Microbial control in marine fish larvae

Marine aquaculture's juvenile production is hampered by fluctuating larval performance, which has been connected to unfavorable interactions with microorganisms (Vadstein et al., 1993, 2004). Infected by opportunistic microorganisms, newly hatched marine larvae depend on the body's overall immune system. Three strategic plans have been mentioned for controlling the microbes for rearing marine larvae (Vadstein et al., 1993). One of the strategies is to limit the abundance of bacteria focusing on reducing the microbial carrying capacity (CC) of the system by reducing input and increasing the removal of organic matter. The primary growth-limiting substrate for heterotrophic bacteria in rearing water is dissolved organic matter (DOM), which is provided by decaying hatching leftovers, fecal waste, and live feed. When marine larvae first feed, many sorts of particles are frequently introduced to the raising water.

Supplementation of microalgae to the culture water has a beneficial effect on the survival and growth of marine larvae in intensive farming (Reitan et al., 1993; Salvesen et al., 1999; Lazo et al., 2000). According to Naas et al.,(1992), turbidity is responsible for the “green effect”, which enhances foraging conditions by altering prey contrast and larvae dispersion. However, live microalgae are the source of release of organic matter (Baines and Pace, 1991) and senescent and decaying phytoplankton

serves as bacterial substrates (Cole et al., 1984). According to Skjermo and Vadstein (1993) and Salvesen et al. (1999), adding live microalgae to the larval rearing tanks can alter the bacterial population there by providing DOM and bacteria to the water on a daily basis.

Contrary to microalgae, clay makes only a small portion of the DOM or microbial load in fish tanks. As a result, by using clay to condition the rearing water instead of microalgae, the supply of bacterial substrate can be decreased. Additionally, the use of clay may help with the direct adsorption and precipitation of bacteria and organic debris, and germs from the fish tanks. For e.g. Clay has been shown to the improve performance of larvae of Pacific oyster (Crassostrea gigas), Atlantic halibut (Hippoglossus hippoglossus), walleye (Stizostedion vitreum), and other species by increasing ingestion rates (Naas et al., 1995), Bristow and Summerfelt, 1994, Bristow et al., 1996, Rieger and Summerfelt, 1997). (Matson et al., 2006). The first feeding of halibut is increasingly using clay, which is more economical than using either live algae or algal paste (Harboe and Reitan, 2005; Björnsdóttir, 2010).

1.7.4 References

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Iron (atomic number 26) is the 4th most abundant atom, comprises about 5% of the earth's crust and exists at a wide range of oxidation states between -2 to + 6 (Aggett, 2012; Kosman, 2013). Fe^{2+} and Fe^{3+} are the most common iron states in aquatic environments. Iron is an important mineral for living organisms, partially because of its ability to undergo consistent redox reactions. It plays a crucial role in maintaining proper health, cellular respiration (metabolism), mitosis (growth and development, and repair and replication of DNA) including gene expression control (Lim et al., 1996; Lasocki et al., 2011). Also, it is one of the most powerful determinants of animals' capacity to tolerate disease (McDowell and Sticco, 2019). Stage mineral control in animals involves many interconnected causes, including systemic associations with liver, spleen, and kidney metabolism in aquatic animals resulting in the absorption of iron through the gill, skin, and gastrointestinal tract. Such paths diverge with the specific elements' homeostasis. The iron requirement for fish species like channel catfish and rainbow trout is 30 mg/kg, 60 mg/kg, respectively (NRC, 1993).

2.2 Iron in animals and Fish

Iron is present in almost all living cells of the body that is available in the form of complex with protein molecules, functions depends on the attachment to the protein. Also, it plays a crucial role in nutrition and metabolism. Iron is found as a heme protein like hemoglobin, myoglobin, and cytochromes or as a nonheme proteins such as transferrin, ferritin, and hemosiderin. In non-heme compound, iron is bound to the sulfur atoms of cysteine residue.

Iron is a part of hemoglobin in red blood cells (RBC) in vertebrates, and myoglobin in muscle cells (Davis and Gatlin, 1996; Aggett, 2012). These globular proteins play an important role in the transportation of vertebrate oxygen through

blood, in the preservation of oxygen in the tissues, and in the transport of carbon dioxide (CO_2) to the lungs for cell metabolism expiration (Harvey, 2008; Poulos, 2014). Also, iron plays a critical role in electron transfer pathways as a co-enzyme for ribonucleotide reductases, cytochromes, peroxidases and catalases due to its capacity to donate electrons (Aggett, 2012; Smith, 2014; Lasocki et al., 2014). Iron also facilitates biosynthesis of long-chain polyunsaturated fatty acid (LC-PUFA) in fish (Senadheera et al., 2012).

In addition, the heme proteins contain two essential enzymes, catalase and peroxidase, for example. To extract harmful oxygen products such as hydrogen peroxide (HOOH) and organic peroxidases (R-OOH), these two enzymes are used. Heme enzymes such as endoperoxide synthase and myeloperoxidase are essential components, respectively, of fatty acid peroxidation and of the immune system.

For all organisms, iron concentrations for biological fluids are strictly controlled to supply iron as required and to prevent toxicity, since the excess iron will lead to reactive oxygen generation (Braun and Killmann, 1999). High levels of free ferrous iron in the blood can combine with peroxides to create highly reactive free radicals which can damage DNA, proteins, lipids and other cellular components and damage gastrointestinal tract cells (Grobois et al., 2005; Smith, 2014). Iron surcharge can lead to mammalian cirrhosis, diabetes, cardiomyopathy, arthritis and testicular failure (Aggett, 2012; McDowell and Sticco, 2019). Such anomalies arise when the iron levels surpass the available transferrin (iron-binding glycoprotein plasma, transferring iron to different tissues via the blood) for binding. At the other hand, the iron deficiency is similarly deleterious and may result in the degradation of tissue iron concentrations. In this case, the body uses all of its stored iron in ferritin (an iron-containing protein that is the primary site of iron storage in the cells) so that it can not make hemoglobin (Harvey, 2008). Latent iron (low serum ferritin without low hemoglobin) deficiency can also occur without anemia (Lieu et al., 2001), while anemia with an iron deficiency is characterized by an insufficient number of healthy red blood cells due to insufficient iron (Grobois et al., 2005; Raza et al., 2014).

Iron deficiency characterized by both an inadequate number of red blood cells and a lack of hemoglobin can lead to severe conditions such as hypochromic microcytic anemia (Grobois et al., 2005). Iron deficiency in humans leads to anemia, pica (commonly in pregnant women and small children) (López et al., 2004), irritability, the condition of restless legs and fatigue (Smith, 2014). Iron deficiency leads to inadequate numbers of red blood cells contributing to anemia in fish such as yellow tail, common carp, red sea bream and brook trout (Kawatsu, 1972; Ikeda et al., 1973; Sakamoto and Yone, 1978a; Sakamoto and Yone, 1978b; Grobois et al., 2005). Iron deficiency induces stunted growth in channel catfish, reduced red blood cell counts, hematocrit and hemoglobin, reduced feed quality, and reduced saturation of plasma iron and transferrin (Gatlin and Wilson,1986). Iron deficiency in rainbow trout (Desjardines 1985) and yellowish white liver disease in carp (Sakamoto and Yone,1978b) also causes slow egg hatching.

2.3 Biochemistry and Physiology

Most of the iron in the body functions in heme protein, complexes that carry oxygen as hemoglobin and myoglobin. Around two-thirds of the body iron is in hemoglobin, with a composition of 68,000 MW comprising four subunits of heme, a protoporphyrin ring of iron in the middle and four polypeptide chains (two Alpha- and Beta-globin chains each). Oxygen binds directly to the iron atom for transport through hemoglobin, stabilized in an oxidation state of Fe^{2+} surrounded by the protoporphyrin ring and histidine residue. Hemoglobin iron binds and releases oxygen quickly, and circulates in the blood. Myoglobin, comprising of a single heme atom and globin, empowers the transfer of oxygen from erythrocytes to mitochondrial cells in cytoplasmic muscle (Hunt, 2005; Das and Abera, 2018). Small portions of heme iron take part in mitochondrial cytochrome involved in electron transport, oxygen utilization along with the production of ATP. Hydrogen peroxidases like catalase (catalytic function) are heme-containing protein that safeguards from the excessive accumulation of hydrogen peroxide by converting them into hydrogen and water. In this process, a small fraction of body iron is involved (Hunt, 2005; Das and Abera, 2018).

Additionally, non-heme proteins (iron-sulfur complex) like aconitase, NADH dehydrogenase, and succinate dehydrogenase enzymes take part in energy metabolism. The sensitivity of aconitase depends on the concentration of iron in the mitochondria and cytosol. In the abundance of iron, the aconitase enzyme forms a complete iron-sulfur cubic structure, involved in carbohydrate metabolism, but it loses its activity at the lower concentration of iron and functions as an iron-binding protein (IRP). The interaction of IRPs and iron response elements (IREs) of the mRNA helps in protein synthesis as well as takes part in the transport of iron, storage, and use depending on the changes in the cellular iron concentration (Hunt, 2005; Das and Abera, 2018)

2.4 Iron Bioavailability and factors affecting bioavailability

A nutrient's bioavailability can be defined as the proportion of that particular nutrient in the food which an organism uses for its normal body functions (Heaney, 2001; Solomons, 2003). The fraction of the consumed nutrient influences its metabolism availability within the body, and hence its bioavailability. The bioavailability of iron depends on the chemical nature of the element, chelating agents' presence, chemical and physical characteristic of feed stuffs, presence of iron in animals, and antagonistic effects along with other nutrients. Hemoglobin repletion assay, slope ratio method, radioisotopes, and injectable forms approaches are used to determine the bioavailability studies. Johnson et al. (1994) and Benito and Miller. (1998) studied the factors that can affect the bioavailability of iron in animals.

1. Species: Iron bioavailability can vary from species to species in few feed stuffs. For e.g., 84% iron is available in corn gluten meal for chicks whereas only 20% is accessible for cats compared to 100% available for ferrous sulfate in both species.

2. Iron status: The presence of iron in individual species is a crucial physiological factor and its absorption increases with the depletion of body iron (Bothwell et al. 1979; cook et al. 1974; Cook. 1990). Also, the concentrations of stored iron in the body directly regulate the absorption of iron across the duodenal lining (Davidsson and Tanumihardjo 2013).

3. Stomach physiology: Acid secretions in duodenum (Cook, 1964) and mucus secretion in digesta (Quarteman, 1987) are significant factors influencing the absorption of iron.

4. Ascorbic acid: The uptake of iron can be enhanced because of chelating properties of ascorbic acid. These vitamins are also responsible for chelating nonheme iron.

5. Phytate: Phytate reduces metal absorption. Anti-nutritional factors (ANFs) including phytate typically found in plant-based diets (Fransis et al., 2001) bind non-heme iron and restrict its digestibility and luminal absorption efficiency (Hallberg and Hulthen 2002). The bioavailability of iron decreases in calves with the formation of insoluble iron-phytate complexes (Bremner and Dalgarno. 1973). Additionally, bioavailability depends on the form of phytate. In rats (Moms and Ellis.1982), the bioavailability (relative to ferrous sulfate) of iron from monoferric wheat bran phytate was found to be 99 %. Other types of phytate (di or tetraferric phytate) demonstrated low bioavailability in the same experiment.

6. Protein: The availability of iron depends on the source of protein. Iron availability is more in animal source compared to plant source (Morris. 1987) because of heme iron content in animal source. Heme iron contains the largest fraction of dietary iron from animal sources and is easily digestible and readily absorbable (Chanda et al., 2015). Non-heme iron is commonly found in metabolism-related enzymes and iron-storage proteins such as transferrin and ferritin (Aggett 2012). Non-heme iron is the largest percentage of dietary iron supplied by plants. It is not easily digestible and, thus, less absorbable than heme iron (Beck et al., 2014; Skolmowska and Głąbska 2019; Van Campen. 1972). The bioavailability of iron is also influenced by the iron source like cysteine, histidine, and lysine (inorganic iron or organic iron) used in fortification cases (Sun et al., 2015; Jarosz et al., 2016). As iron is chelated with amino acids (i.e., in organic form), its quality and state are maintained and covered within the chelated form, and thus easily absorbed by active transport along with the amino acids (Chanda et al., 2015). Iron uptake is reduced because of low protein diets (Coons 1964;

Laryisse et al., 1968). Similarly, depletion of body iron is noted in rats, influencing iron absorption (Thompson and Erdman. 1984).

7. Pectin: Increase in absorption of iron and hemoglobin regeneration efficiency was increased in anaemic rats (Kim and Atallah. 1992, 1993). This might be because of complex formation of free carboxyl groups of pectin with polyvalent cations, including iron.

8. Feed or food processing: Wood et al. (1978) reported increase in iron availability in chicks with heat and pressure of ferrous sulfate, sodium ferric pyrophosphate, ferric orthophosphate, or ferric pyrophosphate. The iron bioavailability gets reduced in human meals because of prolonged warming (Hallberg et al., 1982).

9. Other factors: Cellulose and oxalate can lead to increased ferrous iron bioavailability (Gordon and Chao, 1984). Nevertheless, Reinhold et al. (1986) discovered that corn or wheat fiber reduced retention of iron. Carbohydrate might affect iron metabolism (Amine and Hegsted. 1971). Johnson and Gratzek (1986) noted decrease in absorption of iron and hemoglobin in rats because of carbohydrates, but starch had no impact.

2.5 Interactions with other minerals

Some minerals may also have an effect on dietary iron boavailability. Higher amount of copper exerts an antagonistic effect on iron use (Underwood, 1977). Cobalt has an electrical structure which is identical to iron. Thus, the absorption of iron in the diet is competitively hindered by equimolar quantities of cobalt (Thomson et al., 1971). In the same way, high levels of manganese have been found in feed or water impedes the absorption of iron in ruminants (Hartman et al., 1965). Sugars, especially fructose, can improve the absorption of iron (Pollock et al., 1964). Ascorbic acid plays a dual role in the absorption of iron. This vitamin helps in the formation of a stable iron complex or reduces iron to a ferrous form (Forth and Rummel, 1973). Reducing agents like glutathione, cysteine, succinate, pyruvic and lactic acids also seem to enhance absorption through a similar process (Forth and Rummel, 1973). Dietary components like oxalates, phosphates, orthophosphates, pyrophosphate, and phytate decrease

iron absorption (Forth and Rummel, 1973). This might be because of the tightly binding capacity of phosphate compounds to iron and thus reducing iron absorption. The absorption of iron is also reduced in chicks (Waddell and Sell, 1964; Sell, 1965) and rats (Amine and Hegsted, 1971) becaan use of increase in dietary calcium and/or phosphorus. Further, the absorption of iron was also reduced in domestic animals with the addition of dietary cobalt (Forth et al., 1973), copper (Bradley et al., 1983), and zinc (Settlemire and Matrone, 1967a, b). Hemoglobin in young lamb (Hartman et al., 1955) and chicks (Baker and Halpin, 1991) was reduced because of excess manganese in the diets. Nickel-deficiency in the diet can reduce the absorption of iron (Morris, 1987).

2.6. Mechanism of Iron absorption

2.6.1 Absorption

Since ferric iron (Fe^{3+}) is poorly soluble near neutral pH, aquatic organisms find difficulties in acquiring iron (Molot, 2003; Shaked, 2004; Sandy and Butler, 2009; Xing and Liu, 2011). Consequently, these species uptake iron in complexes or rarely as ferrous iron (Fe^{2+}) before oxidizing it back to ferric iron (Fe^{3+}) (Lammers, 1982; Hudson, 1990; Xing and Liu, 2011). The uptake/absorption of iron in fish occurs through the gill membrane and gastrointestinal tract (Roedar and Roedar, 1968; Bury et al., 2011). The iron-importer, protein divalent metal transporter (DMT1), and iron exporter protein ferroportin (Bury et al., 2011) assist in this process. Briefly, Fe^{2+} reaches the mucosal cells assisted by DMT1 after iron ingestion, where it is temporarily held in the form of ferritin until the cells are physiologically saturated. A mucosal block forms at this stage to prevent further absorption of iron (Granick, 1946).

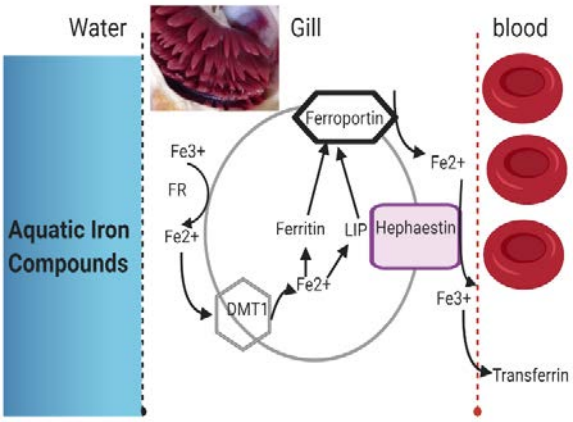

Fig: 2.6.1 Absorption of Iron through gills

Adopted from Bury et al., (2011)

Iron then spreads to the ferroportin-assisted bloodstream where it binds to a specific glycoprotein (transferrin) for transportation to other body cells (Tracey, 2003).

Among fish, absorption of iron occurs predominantly through the gastrointestinal tract (Watnabe et al.,1997; Chanda et al., 2015). Studies have shown increased growth in fish such as channel catfish, rainbow trout, swordtail, carp, and platy fish with iron ($FeSO_4$) added to diet (Roeder and Roeder, 1966; Gatlin and Wilson, 1986a; Sakamoto and Yone, 1978a). In reality, the dietary iron is the key source of iron for fish (NRC, 1993; Chanda et al., 2015).

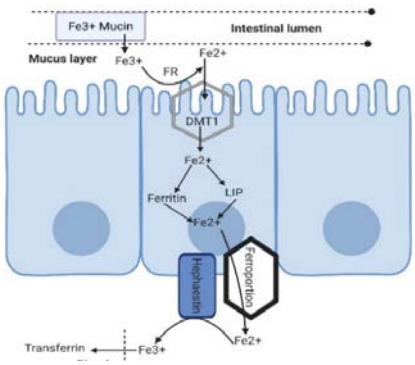

Fig: 2.6.2 Absorption of Iron through gastro-intestinal tract: Adopted from Bury et al., (2011)

The mucosal cells take up heme and nonheme-iron through two various pathways. Complex processes involving three stages are all absorption pathways, including absorption from the intestinal lumen into mucosal cells, transit through the cell, and release from the cells into the body. The complex of the heme enters the intact mucosal cell. It is dissociated from the porphyrin ring by heme oxygenase (Rafin et al., 1974) once within the cell.

2.6.2 Mucosal iron Uptake

The absorption of iron into the mucosal cells of the intestinal lumen constitutes the first stage in the iron absorption process (Manis and Schachter, 1962). Iron is absorbed by enterocytes. Conard et al., (1994) proposed that mucin, in an accessible form for absorption, plays a role in transmitting iron to the enterocytes. They also indicated that mucin was capable of binding iron at acid pH and retaining the iron at neutral p^H in solution. Much of the Fe^{3+} is transferred to Fe^{2+} before absorption, and consequently oxidized into Fe^{3+} after entering the cell.

2.6.3 Transit through Mucosal Cell

Not much information is available about the mucosal transport of iron from the brush borders (Benito and Miller, 1998). The transport of free form of iron to the brush border may cause membrane damage because of free radical (Halliwell and Gutteridge, 1984). Probably, intracellular protein is involved for the primary storage of iron. Conard et al. (1994) stated that two-iron binding proteins (mobilferrin and integrin) in the intestinal mucosa that differs biochemically and immunologically from iron-binding proteins (Ferritin and transferrin). Mucin helps in binding of iron in the stomach at acidic p^H. This also helps in facilitating for the absorption of iron in the intestine at alkaline $P^{H.}$ Integrin, mucosal protein, present in the duodenal mucosa or microvilli helps in transport of iron into the cell. The iron from the integrin is transferred to the cellular cytosol via carrier mobilferrin. The exchange of mucosal iron to the serum occurs at the basal surface of the cell via integrins. Ferritin can be produced as that of the top of a cell detach intracellular iron in iron overload conditions and avoids cell injury from free iron catalyzed oxidation (Conard et al., 1994).

2.6.4 Release of iron from the intestine to circulation and delivery tissue

Iron is normally bound to transferrin and is released via transferrin cycle to the tissue (Morgan, 1980). The transferrin has two ferric binding sites where ferric ions binds. Endosomes is formed by encapsulating transferrin and transferrin receptors. The endosomes provide the acidic environment for the release of iron from transferrin. The released iron binds with the hemoglobin or other complexes or stored in ferritin.

2.6.5 Distribution

Iron in the blood is transported by transferrin (Tf), a synthesized glycoprotein in the liver, to storage and target cells (Yang et al., 1984; Lane and Richardson, 2014; Kawabata, 2019). Fe^{2+} is oxidized to Fe^{3+} using the enzyme ferric oxidase (haphestin) once absorbed into the bloodstream. The Tf then uses a chelating carbonate anion, one nitrogen, and three oxygens to enclose the Fe^{3+} ions in the middle of its two domains (Aggett, 2012). The bound iron ions (Fe (III)) are then transported, for metabolism and storage, to other cells (Tracey, 2003). Transferrin is an iron-binding

glycoprotein (76-kDa), consisting of a single polypeptide chain folded into two homologous domains-an amino lobe (N-terminal) and a carboxyl lobe (C-terminal) with a small joint region (Yang, 1984; Baker et al.,1987; Kawabata, 2019). The homologous domains are the active iron-binding sites-each binding to a ferric iron ($Fe^{3+}$and a total of two Fe^{3+} per Tf molecule (Yang et al., 1984; Liu et al., 2010a; Aggett, 2012; Kawabata, 2019). Blood saturation for transferrin is an indication of blood iron levels or iron deficiency (Kawabata, 2019). A Tf concentration of less than 20 % suggests iron deficiency; while iron excess is over 50 % (Peyrin-Biroulet et al., 2015; Kawabata, 2019). Transferrin exists in several families of proteins sharing homologous amino acid sequences. Which include serotransferrin (serum Tf), lactotransferrin (in body secretions), ovotransferrin (in birds), and melanotransferrin (Spik et al., 1975; Yang, 1984; Liu et al., 2010a). The commonly studied class in vertebrates is the Tf serum, which is primarily responsible for transporting iron inside the plasma of the blood (Spik et al.,1975; Liu et al., 2010a). In vertebrates, a Tf gene (TF) (Yang, 1984) programs serum Tf expression. The Tf gene is well characterized in channel catfish and is expressed in liver, head, and trunk kidney, stomach, spleen, brain, ovary, back, and intestine (Liu et al., 2010a). Transcription of the Tf gene in vertebrates including fish is up-regulated or down-regulated mainly in response to levels of blood iron and other factors such as pathogenic infections, levels of estrogen hormones, and inflammation (Liu et al., 2010a; Cherayil, 2011; Gkouvatsos et al., 2012). One study showed that the iron deficiency induced an increase in the rate of synthesis of Tf mRNA in the liver (McKnight at al., 1980). This led to a rise in Tf mRNA cellular level, Tf synthesis rate and Tf serum levels (McKnight et al., 1980). It has also been found that transferrin plays several other functions in the immune system against microbial infections, controls enzyme activity and promotes cell proliferation (Liu et al., 2010a; Gao et al., 2012; Martínez et al., 2017; Kawabata, 2019).

2.6.6 Cellular uptake

Cellular iron uptake is promoted by glycoproteins (TfR), a cell membrane transferrin receptor. A TfR is a homodimeric transmembrane glycoprotein that is primarily responsible for mediating the uptake of iron into cells via an endocytosis loop

of iron-laden transferrin and apoTf (iron-free transferrin) exocytosis (Lawrence et al.,1999; Kawabata, 2019). Transmembrane Tf receptor proteins (TfRs) are taken up into the cell by iron loaded Tf at the surface of the target cell via endocytosis in a TfR-Tf complex (Ponka and Lok, 1999; Aisen et al., 2001; Cheng et al., 2004). In the endosome, iron dissociates from the iron charged Tf because of the endosomal acid pH (almost 6.3) and moves into the intracellular chelatable pool where it is used for metabolic needs of cells (West et al., 2000; Shen et al., 2018). The remaining iron is stored in Tf and incorporated into ferritin (Chahine and Pakdaman, 1995; Armah, 2014). The apoTf stays bound to TfR until it reaches the cell surface where, due to higher blood pH, it dissociates into the blood (~ 7.4). The process then repeats itself (Lawrence et al., 1999; West et al., 2000). Two TfR subtypes have been studied extensively: 1) the transferrin receptor 1 (TfR1), and 2) the transferrin receptor 2 (TfR2) (Lawrence et al., 1999; Shen et al. 2018; Kawabata 2019; Quanyuan et al. 2019). The TfR1 is expressed in the entire body in tissues and plays the greatest role in transferrin cellular uptake (West et al., 2000). TfR1 is encoded by the gene TRFC. The transcription of the TRFC gene is upregulated in response to a cellular iron deficiency, which activates the interaction of particular iron-responsive proteins (IRP1 and IRP2) and iron-responsive elements (IRE) in the TfR1 mRNA transcript five prime untranslated region (5' UTR). This promotes transcription of TRFC, and the opposite happens with repletion of cellular iron (Hunt 2010; Hentze et al., 2010; Armah, 2014; Shen et al., 2018; Kawabata, 2019). Other causes, such as oxidative stress, inflammation, and hypoxia, can also influence TfR1 gene transcription regulation (Quanyuan et al., 2019; Kawabata, 2019). In comparison, because of the particular localized functions that it performs, TfR2 is selectively expressed in different tissues. For example, TfR2 is expressed primarily in the liver where it promotes the hepatocyte sensing of the level of transferrin iron and thus the expression of hepcidin (iron regulating hormone) (Lasocki et al., 2014; Shen et al., 2018). TfR2 also expresses itself highly in erythroid cells where it regulates erythropoiesis (Kawabata, 2019). Like TfR1, however, TfR2 also mediates intracellular iron transportation, but is non-iron-dependent (Shen et al., 2018; Kawabata, 2019). TfR2 is encoded by the gene TfR2 (TFR2). The expression TfR2 is upregulated to induce hepcidin production at elevated

serum Tf levels, which in turn inhibits DMT1 (iron importer in duodenal lining) iron absorption and ferroportin (intracellular iron exporter) iron release from the body stores (Nemeth 2004; Gkouvatsos et al., 2012; Ganz 2013; Lasocki et al., 2014). The TfR also plays several other immunity functions in vertebrates against microbial infections (Quanyuan et al., 2019), cancer (Shen et al., 2018), and ferroptosis (iron-dependent programmed cell death) (West et al., 2000; Cao and Dixon, 2016).

2.6.7 Iron Storage

In the red blood cell, hemoglobin nearly two-thirds of the physiologically active iron within the body is contained (Harvey, 2008; Lieu et al., 2001). Hemoglobin is a metalloprotein that is mainly responsible for oxygen delivery in animals within the red blood cells. Hemoglobin accounts for around 97 % of the dry mass of red blood cells (Lieu et al., 2001; Smith, 2014). Hemoglobin consists of four polypeptide chains, each carrying a heme group with one Fe^{2+} atom (Aggett, 2012; Poulos, 2014) and is commonly assessed in iron bioavailability studies (Wienk et al.,1999). The remainder of the iron is contained in a glycoprotein known as ferritin (450 kDa molecular weight) located in the liver and reticulo-endothelial system (Aggett, 2012; Ganz 2013). Ferritin controls the amount of intracellular iron in the cytoplasm of cells by storing and releasing the excess iron in a regulated manner in cases of deficiency (Harrison et al., 1990; Liu et al., 2010b, Harvey, 2012). Ferritin is a globular protein that stores iron in an oxidation state (Fe^{3+}) incorporated in the mineral ferrihydrite, bonded within its inner wall (Theil, 1987; McDowell, 1992). In vertebrates, ferritin molecules consist of subunits of the heavy (H), middle (M) and medium (L) chain encoded by their respective genes. The gene Ferritin H is well characterized in catfish channels, and its expression is primarily upregulated by increased levels of cellular iron and other factors such as pathogenic infections (Liu et al., 2010b). Ferritin H also plays a role in microbial infection tolerance and has ferroxidase activity (Liu et al., 2010b; Lei et al., 2012). A small amount of iron is also contained in muscle myoglobin, haemosiderin in the liver and some in enzyme systems (Aggett, 2012; Smith, 2014).

2.7 Iron Toxicity

Toxicity is the toxic agent 's inherent capability and potential to cause harmful consequences to living organisms (Borges et al., 2018). Toxicity, in general, is proportional to the toxic agent concentration at the site of action (Kluver et al., 2015). The toxicity of the substance depends upon the organism type, dose of substance, and routes of administration (Manahan, 1991).

Streams containing coal mine effluents might contain higher load (concentration) of ferrous iron (Gale et al., 1976). The ecosystem of streams may get affected by reduction in the populations of phytoplankton and zooplankton because of oxidation of ferrous to ferric iron that depends upon the temperature.

Several studies have indicated iron toxic effects on livestock (NAS, 1980). The toxic effects are seen with iron deficiency and excess supplementation in the diet. Acute toxicosis symptoms like anorexia, oliguria, diarrhea, hypothermia, diphasic shock, metabolic acidosis, and ultimately death with the supplementation of iron at a higher level via oral administration (Boyd and Shanas, 1963). Several reports have suggested iron toxic effects on livestock (NAS, 1980). The harmful effects are associated with the iron deficiency and unnecessary supplementation in the diet. Acute toxicosis symptoms include anorexia, oliguria, vomiting, hypothermia, diphasic shock, metabolic acidosis, and eventually death with the supplementation of iron at a higher level by oral administration (Boyd and Shanas, 1963). Increased serum iron levels triggered the iron toxicosis. Histology of the gastrointestinal tract, liver, kidneys, lungs, liver, spleen, adrenals and thymus showed cellular deformities. Supplementation of ferrous sulfate to dogs at amounts of 150 to 600 mg of iron per kg body weight, various symptoms varying from vomiting, and diarrhea to inflammation of the gastrointestinal tract resulted (Reissman and Coleman, 1955; D’Acry and howard, 1962). Lungo and Bjomson (1954) observed hepatic congestion in the rabbit within 24-48 hours of supplementation of ferrous sulfate (750 mg/kg BWAll rabbits were killed after a few hours of receiving 2g/kg of ferrous sulfate.

Over-burden of iron leads to disorder in iron metabolisms like hemosiderosis, hemochromatosis, and hemolytic jaundice. This results in the accumulation of large numbers of hemosiderin granules in the parenchymatous tissue of the liver. In this condition, nonheme soluble form iron (ferritin) is stored in lysosomes and degraded into insoluble hemosiderin (Bradford et al., 1969; Crichton, 1971; Trump et al., 1973). Hemolytic jaundice can also lead to increased lysis of the erythrocytes in subjects. In animals, low dietary iron levels cause symptoms of chronic iron toxicosis. This results in lower feed consumption, lower growth rates, and reduced feed conversion efficiency for most organisms (Standish and Ammerman, 1971). Rainbow trout fed a diet containing 5964 mg/Kg iron showed a high level of fecal iron efflux (Desjardins, 1985). The concentration of iron, copper, and zinc got increased in these fish with an increased level of iron in their diets. The concentration of iron in the liver, kidney, and spleen got increased by two folds and were 536 and 493 at a dose of 5964 mg iron/kg (Desjardins, 1985).

Iron can interact with harmful elements such as cadmium, lead and aluminum (Goyer, 1997). The deficiency of iron increases those elements' absorption. In the skeletal system, cadmium and aluminum associate with calcium for generating osteodystrophies. Lead reacts with calcium in the nervous system and this contributes to cognitive developmental disability. Calcium deficiency and including low dietary intake of magnesium may lead to degenerative nervous disease in fish induced by aluminum.

In rainbow trout, diets fed with iron/kg greater than 1380 mg (Desjardins, 1985), iron toxicity signs develop. Low production, inadequate use of feed, rejection of feed, increased mortality, diarrhea and histopathological damage to liver cells are the main consequences of iron toxicity. The maximum tolerable limit will depend on the dietary iron 's biological availableness. Supplementation of iron higher than optimal requirement have retarded growth in several species, like grass carp (73.5 mg/kg vs 120 mg/kg), yellow catfish (55.73 mg/kg vs 118.25 mg/kg) and stinging fish (86.6 mg/kg vs 146.9 mg/kg) (Zhang et al., 2016; Zafar and Mukhtar 2020; and Luo et al., 2017). Similarly, the studies conducted by Zhang et al., (2016), Zafar and Mukhtar

(2020), and Luo et al., (2017) in grass carp (73.5 mg/kg vs 120 mg/kg), Stinging catfish (86.6 mg/kg vs 146.9 mg/kg), yellow catfish (55.73 mg/kg vs 118.25 mg/kg) showed decreased in SOD, CAT, GR activity when fed iron-containing diets at a higher level compared to their optimum level of inclusion in their diets.

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CHAPTER 3

POTENTIAL AMELIORATION OF WATERBORNE IRON TOXICITY IN CHANNEL CATFISH (*Ictalurus punctatus*) THROUGH DIETARY SUPPLEMENTATION OF VITAMIN C

3.1 Introduction

Iron is an essential element which plays a vital role in several physiological functions of most organisms, including fish (Aisen et al., 2001). However, high environmental iron (HEI) can cause several toxic effects that negatively impact fish production. Globally, water sources with iron overload are an issue either due to natural iron sources or due to increasing anthropogenic activities. Some examples of HEI include up to 14.4 mg/L (in North Eastern Indian rivers; Singh et al., 2019), 5.32-9.96 mg/L (Bayelsa state, Nigeria; Angaye et al., 2015), 5-10 mg/L (Lusatia, Brandenburg, Germany; Steffens et al., 1993), and 5-6 mg/L (Fairbanks Experiment Fish Hatchery, Alaska, USA; Fish, 2009)

Various toxic effects from HEI can occur, but primarily is due to cellular damage when a certain threshold of iron is exceeded in the body as well as due to the iron's catalytic property in triggering the Fenton reaction (Fenton, 1894), which results in the production of free radical species (ROS) (Baker at al., 1997; Li et al., 2009). Accumulations of ROS can indiscriminately damage lipids, proteins and DNA macromolecules; thereby negatively affecting the overall physio-biochemical and metabolic performance of fish, and in extreme cases may lead to cell death (Bresgen and Eckl, 2015; Mohanty and Samanta, 2018; Papanikolaou and Panto, 2018). In addition, it has been documented that elevated levels of iron in water can cover the gill surface and induce cellular damage, which will ultimately limit the gill's functional capacity (Debnath et al., 2012; Sevcikova et al., 2011; Slaninova et al., 2014; Teien et al., 2008).

Studies listed above clearly indicate that HEI can negatively affect fish performance and ultimately reduce the overall production. Consequently, mitigating

the toxic effects of HEI is a major challenge to fish farmers, toxicologists and water quality managers. Several strategies have been suggested to manage iron level in the fish culture system including application of permanganate; however, it had been reported to pose toxicity threat to phytoplankton and other aquatic life (Boyd, 1990). Other strategies such as intense agitation, spraying through water towers, filtration and microbubble generators are often used in areas with high iron loads, but this is generally viewed as a partial solution because only a fraction of iron is removed. These drawbacks necessitate investigations on a therapeutic approach to directly mitigate iron induced toxicity in fish. In this scenario, dietary supplementations with vitamin C may be a cost-effective approach. Vitamin C is a commercially available essential micronutrient for most fish species (including channel catfish; NRC, 1993), and is also known to be a free radical scavenger which protects cells from oxidative injury. In a number of fish species, anti-oxidant protective effect of vitamin C has been shown against waterborne metals (e.g. lead) toxicity (Nourian et al., 2019; Shahsavani et al., 2017). It is also documented that vitamin C has a chelation capacity for some metals (e.g. arsenic and lead; Flora et al., 2012; Kalia and Flora, 2005; Patrick, 2006), and thus can limit metals bioaccumulation in the tissues. In addition, existing reports suggests that vitamin C also promotes iron absorption from the intestine via reducing Fe^{3+} to a more soluble Fe^{2+} form state (Chen et al., 2015; Lim et al., 2000).

However, most teleosts are not capable of synthesizing vitamin C (due to the lack of L-gulonolactone oxidase; Fracalossi et al., 2001), therefore, an exogenous supplementation of vitamin C is requisite in fish diets. Dietary requirement of vitamin C is dependent on the fish species and size, forms of vitamin C and rearing conditions (Chen et al., 2015; Lin and Shiau, 2004; Zhou et al., 2012). A number of reports on freshwater teleosts recommend 10 - 60 mg vitamin C/kg feed as an effective dose for optimal growth and normal metabolic function (For review, see Dawood and Koshio, 2018). For channel catfish, the minimum dietary requirement of vitamin C to support normal growth was estimated to be 50 - 60 mg/kg (Andrews and Murai, 1975; Lim and Lovell, 1978). Furthermore, El Naggar and Lovell (1991) and Robinson (1992) proposed approx. 45 mg/kg feed as the most appropriate dose when supplementing vitamin C in its stable form (L-ascorbyl-2-polyphosphate). Higher vitamin C

supplements are often used to enhance the resistance of aquatic animals against pathogens (Anbarasu and Chandran 2001; Liang et al., 2017; Lim et al., 2000) as well as mitigate environmental stressors including trace elements (lead, copper, zinc, cadmium; Fox et al., 1980; Jiraungkoorskul and Sahaphong, 2007; Lim et al., 2000; Maage et al., 1990; Shahsavani et al., 2017), elevated ammonia (Mazik et al., 1987; Wang et al., 2005), salinity stress (Merchie et al., 1996), hypoxia (Mazik et al., 1987) and thermal shock (Falcon et al. 2007). Moreover, supplementation of vitamin C (150 mg/kg diet) increased the resistance of channel catfish against *E. tarda* at 23ºc, and morality was reduced significantly compared to the vitamin c deficient diet as well as with the supplementation of vitamin c at 60 mg/kg of diet (Durve and Lovell, 1982). Similarly, the supplementation of vitamin C at megalevels (1000 mg/kg diet) increased the resistance to infection against *E. ictalurid* in channel catfish (Liu et al., 1989). The supplementation of vitamin C at 500mg/kg diet can be used as an immunostimulant in Asian catfish (Kumari and Sahoo, 2005). According to Halver et al (1975), the supplementation of vitamin C at 100 mg/kg diet is sufficient for normal growth for rainbow trout (*Salmo gairdneri*); however, at 1000 mg/kg diet provided maximum rate of wound-healing, indicating that the requirement varies with the metabolic functions of the fish.

In an earlier research on channel catfish, Lim et al. (2000) evaluated the potential effect of vitamin C supplementation against the toxicity mediated by the elevated level of dietary iron (30-300 mg/L). However, in the culture systems and in field situations, fish are challenged with pollutants mainly via waterborne route; as such the direct application of experimental results considering dietary exposure of pollutants remained questionable for most of the aquaculture operations.

Channel catfish is the leading and the most successful aquaculture industry within the United States (US). However, water quality degradation, particularly elevated iron, is threating current as well as future catfish production. In most parts of US, the main source of water for catfish culture is deep underground water resources, which oftentimes have high iron concentrations depending on the aquifer, with Central Arkansas (Maumellae and Winona) being up to 8.4mg/L (Green, 1994). With this

background, this current study aimed at evaluating whether waterborne iron mediated toxicity in channel catfish can be mitigated through dietary supplementation of vitamin C. Two doses of stable form of vitamin C (143 and 573 mg/kg diet) were tested when fish were exposed chronically (up to 8 week) to a sub-lethal 25% 96 h LC_{50} iron dose. To achieve our goal, we investigated: (i) growth performance and feed efficiency by quantifying weight gain (%) and feed conversion ratio (FCR), respectively, (ii) pro-oxidant status by examining oxidative stress and activity of hepatic antioxidant enzymes; (iii) hemoglobin (Hb) and hematocrit (HCt) as indictors of stress; (iv) histopathological examination of organs to obtain insight into the efficacy of any potential protective effect of vitamin C; (v) metals and electrolyte bioaccumulation dynamics; and (vi) fatty acid profile in muscle to assess nutritive quality.

3.2 Materials and Methods

3.2.1 Experiment diets

Three isonitrogenous (35% crude protein 'CP') and isolipidic (9%) experimental diets were formulated with the inclusion of 0, 143 and 573 mg vitamin C per kg diet. Vitamin C was supplemented in the form of Stay-C (35% equivalent, L-ascorbyl-2-poly phosphate (APP) Skretting USA, Tooele, UT, USA). Compositions of the experimental diets are illustrated in Table 1, and with the exception of vitamin C, were formulated to meet the dietary requirements of channel catfish (NRC, 1993). The level of 143 mg/kg diet was chosen based on this being around the level needed for optimal growth and disease resistance (Durve and Lovell, 1982; Li and Lovell, 1985) while the 573 mg/kg would be considered a megadose (Liu et al., 1989). The main dietary protein sources were fish meal (FM), poultry by-product meal (PBM), and soy protein concentrate. The lipid source was fish oil (FO) and soybean oil (SO). Briefly, all experimental diets were cold pelleted using a California pellet mill equipped with a 2.4 mm die. All ingredients were ground with an air swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN, USA) to a particle size of < 200 µm. Diets were cold pelleted at the University of Idaho's Hagerman Fish Culture Experiment Station (HFCES) using a laboratory-scale California pellet mill fitted with a 2.4-mm die. Feeds

were dried in a forced-air dryer at 40°C to less than 10% moisture, then stored at ambient temperature (20-22°C) until being fed.

3.2.2 Fish and Experimental systems

Channel catfish juveniles (22- 25 g) were collected from the fish ponds located within the University of Arkansas Pine Bluff's (UAPB) Aquaculture Research Station. Fish were maintained for at least a month in a 2000 L flow through system. Fish were fed ad libitum with a commercial feed (Rangen, containing 32% CP and 5% crude lipid) once a day. Water quality parameters were temperature 24 ± 2 °C, pH 7.8 ± 0.2, dissolved oxygen 7.8 ± 0.3, total alkalinity 65 ± 8 mg $CaCO_3$/L, hardness 70 ± 15 mg $CaCO_3$/L, total ammonia 0.65–1.60 mg/L, and iron remained below 0.4 mg/L. This study was approved and conducted as per the guidelines of Institutional Animal Care and Use Committee.

3.2.3 Experimental group and set-up

Following acclimation, 480 fish from the holding tank were equally distributed into twenty-four 300 L experimental tanks (n=20 per tank). To ensure water quality, each tank was equipped with an individual recirculating system including a bio-filter with quilt batting. Water circulation also prevented the settling of iron (in iron-exposed tanks) at the tanks bottom. Continuous aeration was provided using an air-stone. Fish were fed ad libitum once a day and acclimated in these experimental tanks for 2 weeks prior to the experimentation.

The set-up consisted of six experimental groups with fish being fed: (i) vitamin C deficient diet and reared in normal freshwater (served as control); (ii) vitamin C supplemented with 143 mg/kg diet and reared in normal freshwater (LVc); (iii) vitamin C supplemented with 573 mg/kg diet and reared in normal freshwater (HVc); (iv) vitamin C deficient diet and exposed to 9.5 mg/L iron (Con+Fe); (v) vitamin C supplemented with 143 mg/kg diet and exposed to 9.5 mg/L iron (LVc+Fe); and (vi) vitamin C supplemented with 573 mg/kg diet and exposed to 9.5 mg/L iron (HVc+Fe). Each of the six groups was randomly assigned in 4 tanks. The exposure level of iron used in the present study represents 25% of 96 h LC_{50} value for channel catfish (Sinha

et al., unpublished data). The experiment was continued for a period of 8 weeks, and fish were fed with their respective diets twice a day (07.30–08.00 and 15.30–16.00 h) to apparent satiation. The amount of feed consumed by fish in each tank was recorded daily by calculating the differences in the feed weights prior to the feeding and after the feeding.

Iron exposure tanks were pre-dosed with a $FeCl_3.6H_2O$ (ACROS Organics, USA) stock solution to bring the concentration to the nominal level. A constant concentration of 9.6 ± 0.20 mg/L iron was maintained throughout the experiment by adding calculated amounts of the $FeCl_3.6H_2O$ stock solution. Iron concentrations were measured (using the FerroZine method, Hach Method 8147 and by flame atomic absorption spectrophotometry, iCE 3000 series, Thermo Scientific, USA) every 24 h after the onset of exposure. All feces and other waste residue were removed daily by suction, and every 3 days, 60–80% of the water was replaced with fresh water containing the respective amount of iron. Water pH was monitored throughout the experimental period using a handheld pH electrode (HACH, Colorado, USA), and was maintained within the range of the control group (7.8 ± 0.2) using diluted HCl and/or KOH.

The percentage (%) distribution of various Fe species in the exposure water.

% of total concentration	Species
0.014	$FeOH^{2+}$
96.487	$Fe(OH)_2^{+}$
2.036	$Fe(OH)_3$ (aq)
1.464	$Fe(OH)_4^{-}$

3.3 Sampling procedure

Fish from each tank were counted and bulk weighed at the beginning and the end of the experiment. Fish were fasted 24 h before the sampling. Growth performance and feed/nutrient utilization of catfish juveniles were evaluated following standard formula

Weight gain (WG %): [(Final body weight in g-Initial body weight in g) / Initial body weight in g] ×100.

Feed conversion ratio (FCR): [Quantity of feed consumed in g ÷ (Final body weight in g-Initial fish weight in g)]

Hepatosomatic indices (HSI): (Weight of liver in g ÷ weight of fish in g) × 100.

Eight fish for each experimental group were randomly sampled from each of the four replicated (n=2) tanks, anesthetized with an overdose (1 g/ L) of neutralized MS222 (ethyl 3 aminobenzoate methanesulfonic acid, Western Chemical Inc., USA) and weighed. Subsequently, a blood sample was collected from the caudal blood vessel (into a heparinized tube) using a heparinized syringe with needle. Following hematological assay (section 2.5.1), the remaining blood samples were centrifuged; plasma was frozen in liquid-nitrogen and stored at −80 °C for latter analysis. The remaining fish were dissected on ice to remove the livers and muscle. After recording the liver weight (for HSI determination, refer above), extracted tissues were frozen in liquid-nitrogen and stored at −80 °C for further bio-chemical assay while the gills and livers from 4 fish (n=1 per replicate tank) were used for histological analysis (section 2.5.3).

3.4 Analytical techniques

3.4.1 Haematological assay

Hematocrit (HCt) analysis in blood samples were collected in micro-capillary hematocrit tubes and the length of red blood cell and total blood (RBC and plasma) was recorded following centrifugation. HCt value (%) is expressed as a percentage fraction of red blood cells in total blood volume (%). Hemoglobin (Hb) concentration was measured using the standard cyanmethemoglobin method (Baker and Silverton, 1976).

3.4.2 Oxidative stress and antioxidant enzymes activity in liver

Malondialdehyde (MDA) content was determined as per methodology described by Hodges et al. (1999) following homogenization in an 80 % ethanol buffer.

The absorbance was read at 440, 532 and 600 nm using a microplate reader (BioTek Instruments, Vermont, USA). The activity of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were assayed using a calorimetric/ flurometric assay kit (Cayman Chemical, MI, USA) as per the manufacturing instructions; absorbance was read at 540, 450 and 340 nm, respectively.

3.4.3 Histological procedure and analysis

For histological analysis of the liver and gills, these were placed in 10% (v/v) phosphate-buffered formalin and Bouin's solution, respectively. After 18 hr, tissues were transferred to 70% (v/v) ethanol for several hours before being processed at increasing concentrations of ethanol. The samples were cleared in xylene and then embedded in paraffin wax. Rotary microtomes (HM 340E, Thermo Scientific) were used to prepare 5 µm sections, and stained with hematoxylin and eosin (H&E). Pictures were then taken with a light microscope (Leica DM 2500 LED).

3.4.4 Fatty acid profile in muscle

Muscle tissue fatty acid composition was analyzed using modified AOAC method 991.39 (AOAC, 1995). In brief, muscle tissue was dried at 50^{o} C (OA-SYS heating system, Organomation Associates, Inc., Berlin, MA, USA) for 5-6 h under a N_2 current. Then, for sample saponification, 2 mL of 0.5 N NaOH was added and incubated at 70^{o} C for 60 min. After cooling the sample, the free fatty acids were methylated in methanol by adding 2 mL 14% boron trifluoride and incubated at 70 ^{o}C for 60 min. After allowing the samples to cool, 2 mL of hexane was added, inverted repeatedly for 60 sec and 1 mL of saturated NaCl was added. The samples were repeatedly inverted for 60 sec, and then centrifuged for 5 min at 2000 xg. For gas chromatography/mass spectroscopy (GC/MS) analysis, an aliquot (100 µL) of the clarified hexane extract was diluted in hexane (1:10) and put into autosampler vials. The injection mode with a helium flow rate and the column temperature followed the protocol stated by Overturf et al. (2013).

3.4.5 Determination of metal-and electrolyte burden in liver

The liver samples were dried in an oven at 60˚C for 48 h. Samples (~100 mg) were then mixed with 3 mL trace-metal-grade HNO_3 (69–70%) and 2 mL 30% H_2O_2 in a microwave digester (SP-D, CEM corporation) at 300 °C and 250 psi for 5 min. A 5-min ramp was used to reach digestion conditions. One mL of internal standard (10 µg/ mL, Bi, Li-6, Sc, Tb, and Y; Accustandard, Inc.) was added to each digested solution, and were then diluted to 100 mL with E-pure water. Metals concentration (Fe, Cu, Zn) and major ion levels (Na, K, Ca, Mg) in the digested tissues were determined with an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500cx). Calibration was performed using a multi-element standard; all standard solutions and samples included an equal quantity of internal standard. Calibration curves showed strong linearity ($R^2 \approx 0.99$) for all elements studied.

3.5 Statistical Analysis

All data are presented as mean ± standard error. The resulting data were tested for normality by the Shapiro Wilk test and variance homogeneity by the Levene test. The data for the iron content were log transformed for the normalization. For comparison among experimental groups, a one-way analysis of variance (ANOVA) was performed, followed by Duncan multiple range tests. Main effects of iron exposure and vitamin C supplementation and their interactions were analyzed by two-way ANOVA.

A probability level (*P* value) < 0.05 was considered significant. All analyses were conducted using SPSS software ver. 20.0.

3.6 Results

3.6.1 Growth performance

After eight weeks, the weight gain (%) was significantly augmented in LVc and HVc groups in comparison to control and other groups (Fig. 3.1A). However, a significant inhibition in weight gain (%) was recorded in Con+Fe, and was also lowest among all groups. The iron exposed fish fed the diet supplemented with vitamin C at

either dose (LVC+Fe and HVc+Fe groups) displayed a significant ($P < 0.05$) increment in weight gain (%) relative to the Cont+Fe. FCR was not affected ($P > 0.05$) by the dietary level of vitamin C or iron exposure or the combination of vitamin C and iron exposure (Fig. 3.1B). Values of HSI in HVc as well as Con+Fe were significantly lower when compared to HVc+Fe (Fig. 3.1C). There was a significant interaction between iron and vitamin C for in WG% and HSI (Table 3.1.2).

3.6.2 Hemoglobin and hematocrit

The treatment with LVc+Fe had the highest level of hemoglobin (Hb), which was significantly higher than other groups (Fig. 3.2A). Moreover, in comparison to control, Hb level notably ($P < 0.05$) increased in iron exposed fish (Con+Fe). Similar to Hb, HCt (%) was also significantly ($P < 0.05$) highest in LVc+Fe among all groups (Fig. 2B). In addition, relative to control, there was a significant increment in HVc, Cont+Fe and HVc+Fe groups. Significant interaction was noted between iron exposure and vitamin C supplementation for in Hb and HCt (Table 3.1.2).

3.6.3 Histological analysis

Gill structure appeared normal in control, LVc and HVc fish groups with well-defined filaments and primary and secondary lamellae (Figs. 3.3A-C). However, there was a deposition of interlamellar cell mass in the Cont+Fe group (Fig. 3D). There were occasional distortions of primary lamellae in the LVc+Fe group (Fig. 3.3E). Unlike the gills of fish in the control, LVc and HVc groups, there was mucus around the secondary lamellae in all iron exposed groups; however, no structural changes in the architecture of the gills (compared to control) were noted in the HVc+Fe group (Fig. 3.3F).

Similarly, no changes were observed in the sinusoid organization or cellular shape in the livers from the iron unexposed groups, including LVc and HVc (Figs. 3.4A-C). However, occasionally there was enlargement of the bile duct in the iron exposed (Cont+Fe) group, but no evidence of inflammation or necrosis that would indicate injury (Fig. 3.4D). Furthermore, relative to control, no major changes were noticed for LVc+Fe (Fig. 3.4E) and HVc+Fe (Fig. 3.4F) group.

3.6.4 Oxidative stress and antioxidant enzyme activities in liver

Exposure with iron significantly elevated MDA content in the liver compared to the control and other experimental groups (Fig. 3.5A). SOD activity was depressed significantly in the Con+Fe group, and was lowest among all experimental groups (Fig. 3.5B). However, SOD activity remained unchanged in the remaining groups. Likewise, the Con+Fe group showed a significant reduction in CAT activity compared to the other groups. HVc+Fe treatment induced a distinct increment in CAT activity, evident by a significant higher value compared to the control (Fig. 3.5C). GR activity in HVc+Fe treatment group displayed a significant increment relative to the control (Fig. 3.5D). However, no effect of vitamin C supplementation (LVc and HVc) as well as iron exposure (con+Fe) or LVc+Fe were observed for GR activity, as the values remained statistically insignificant compared to the control. Significant interaction in CAT activity was found between iron and vitamin C (Table 3.1.2).

3.6.5 Muscle Fatty acid profile

The composition of muscle fatty acids is illustrated in Table 3.1.3. Regardless of vitamin C supplementation, iron exposure or combination of both, no major remarkable effect was observed for the fatty acids analyzed.

3.6.6 Metal and electrolyte content in the liver

Patterns of iron accumulation in the liver under experimental conditions are shown in Fig. 3.6. A distinct effect of iron exposure was also noted, with the iron content increased significantly in the Con+Fe group in comparison to control and other experimental groups. The major electrolytes, Na, K, Ca and Mg content in the liver remained unaltered under the testing conditions (Table 3.1.4). Likewise, no effect was documented for the Cu content (Table 3.1.4). However, in case of Zn content, a significant difference was noted between HVc and Con+Fe group, with the latter group having a significantly higher ($P < 0.05$) Zn load than the former. Significant interaction was recorded between iron and vitamin C for the pattern of iron accumulation (Table 3.1.2).

3.7 Discussion

The results of the present study show that weight gain was considerably improved by the dietary supplementation of vitamin C at either dose (LVc and HVc). Likewise, several studies have concluded that dietary vitamin C can improve growth and feed utilization in a number of fish species (Affonso et al. 2007; Misra et al. 2007; Tandel et al. 2019; Tewary and Patra, 2008). For example, Affonso et al. (2007) reported a high dose of ascorbic acid (800 mg/kg) in the diet improved growth efficiency and survival rate of matrinxa (*Brycon amazonicus*). A study conducted by Tandel et al. (2019) indicated that a dose of 1000 mg/kg of vitamin C had a beneficial effect on the *Labeo rohita* growth efficiency. Likewise, incorporation of vitamin C in diet improved the growth of channel catfish (Duncan and Lovell,1994) and Indian major carp (*Labeo rohita*) (Tewary and Patra, 2008; Misra et al., 2007).

In the present study, the chronic exposure to waterborne iron resulted in a significantly reduced growth rate in catfish. This indicates that the tested dose of elevated iron (9.5 mg/L) exerts a toxic effect in catfish. Reduction in growth performance in response to iron overload (3 mg/L) had also been reported in coho salmon *(Oncorhynchus kisutch*; Smith and Sykora, 1976). Likewise, chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to the iron polluted water (5 mg/L) resulted in the detrimental effect on growth and feed conversion (Steffens et al., 1993). Iron mediated toxicity is believed to be associated with its capacity to induce lipid peroxidation of biological membranes. As such, an elevated accumulation of iron in the hepatic tissue among iron exposed (Con+Fe) fish might explain the overproduction of MDA, which is the end product of lipid peroxidation and an indicator of oxidative injury. This signifies that iron exposure caused oxidative damage, and lipid peroxidation can ultimately suppress the growth and physiological capacity of fish (Lushchak, 2011). This likely contributed to the reduced growth rate in the Con+Fe group. However, the level of iron/duration of exposure was not excessive to cause liver histopathological damage, such as observing necrosis or lysed cells. On the other hand, there were instances of bile duct enlargement which may indicate a response to maintain lipid digestion/utilization.

Within the cell, there is equilibrium between pro-oxidant production and antioxidant defenses. Oxidative injury instigates when antioxidant defenses systems are compromised and can no longer quench the over production of ROS. SOD and CAT acts as the first line of antioxidant defense; wherein SOD catalyzes the conversion of oxygen radical to H_2O_2 and H_2O while CAT activity breakdown H_2O_2 into H_2O and O_2 (Lushchak, 2011). A reduction in the activities of both of these antioxidant enzymes under iron exposure (Con+Fe group), likely compromised the ability of catfish to prevent lipid peroxidation, eventually resulted in excess accumulation of MDA. Likewise, exposure with elevated iron (8.25 – 33.01 mg/L) also reduced SOD and CAT activity in the liver of *Labeo rohita* (Singh et al., 2019), further reinforcing the notion that iron overload can inhibit the cellular antioxidant level and induce the lipid peroxidation in fish.

There have been several reports that dietary iron toxicity can be ameliorated by dietary vitamin C in Atlantic salmon (Andersen et al., 1998; Maage et al., 1990) as well as channel catfish (Lim et al. 2000). To the best of our knowledge, however, this is the first study to evaluate dietary vitamin C on waterborne HEI. In this study, a significant interaction between iron exposure and dietary vitamin C was seen for weight gain and HSI, signifying that growth performance of channel catfish under iron overload can be mitigated by supplementation of vitamin C. This was also complimented by the finding that LVc+Fe and HVc+Fe exhibited significantly greater weight gain in comparison to the Con+Fe. This scenario emphasizes that dietary supplementation of vitamin C provides some protection against iron toxicity, possibly by chelating Fe^{3+} in the liver as proposed by Lynch and Stoltzfus (2003) in mammalian models. Indeed, this was evident in the present study wherein the hepatic iron burden was remarkably lowered in the iron exposed fish fed with vitamin C (LVc+Fe and HVc+Fe group) relative to iron exposed fish fed with vitamin C deficient diet (Con+Fe). Also, there was a significant interaction between iron exposure and dietary vitamin C for iron accumulation in the liver. Vitamin C also mediated protection against iron toxicity was also seen for oxidative stress/anti-oxidant response. Vitamin C, at both doses, lessened the hepatic MDA content of iron exposed fish to those of control group, and was also evident with a concomitant rise in SOD and CAT activities above

the iron exposed fish. These findings suggest that any excess MDA that might have been generated by iron exposure and was efficiently eliminated by vitamin C mediated increments in SOD and CAT anti-oxidant defense systems. In this context, it is possible that the improved growth observed for LVc+Fe and HVc+Fe relative to Con+Fe may have been a result from the protective effect of vitamin C against iron mediated lipid peroxidation. Besides SOD and CAT, components of glutathione (GSH) based antioxidant defense system (e.g. glutathione peroxidase 'GPX', Glutathione reductase 'GR' and Glutathione-s-transferase 'GST') also play an important in various fish species in eliminating ROS (Lushchak, 2011). In the redox cycle, GR reduces oxidized glutathione to GSH, which is involved in the decomposition of ROS. No change in the GR activity among Con+Fe and LVc+Fe as well as HVc+Fe group suggest that vitamin C may not be a potential trigger for GSH dependent anti-oxidant pathways.

In contrast to the liver, the gills suffered severe structural modifications in response to iron exposure, marked by the formation and deposition of interlamellar cell mass at the interlamellar space. This morphological modification reduced the contact surface area of gills to the point of the secondary lamellae having only the ends exposed to the water. While this can possibly be a response to protect these delicate structures against iron, this can also compromise oxygen (and ion) exchange processes, and eventually cause hypoxia-like conditions. It has been documented that hypoxic conditions incite the formation and release of more red blood cells (from hemopoietic tissue) into the blood stream, as an adaptive response to transport sufficient oxygen (Mazon et al., 2002; Pilgaard et al., 1994). This scenario perhaps explains, in part, an elevated HCt (%) and Hb content in Con+Fe group relative to the control. From the present study it was also apparent that dietary vitamin C supplementation (typically at lower dose) in presence of iron exposure can further raise the HCt (%) and Hb content. This is probably due to the direct effect of vitamin C on erythropoiesis as suggested by Cox (1969) and Lim et al. (2000). Furthermore, iron mediated anomalies in gills structures and function was also supported by the works of Li et al. (2009) and Singh et al. (2019), respectively, in medaka and *Labeo rohita*. However, as oppose to liver, in gills the low dose of vitamin C was not sufficient

to restore iron mediated morphological alterations, as manifested by distorted primary lamellae in LVc+Fe. This might relate to the large amounts of iron likely attached (and accumulated) on the gill (as compared to liver), owing to the fact that gill is the first site to make direct contact with the waterborne contaminants. As for iron, Cu and Zn are also essential elements for the optimal health and growth of fish. Iron exposure and/or supplementation of vitamin C does not appear to interfere with the uptake, accumulation or clearance dynamics of Cu and Zn as well minerals including sodium, potassium, calcium and magnesium in the hepatic tissue. Likewise, in rainbow trout, vitamin C supplementation had no influence on the Cu and Zn content in the liver (Lanno et al., 1985).

It has been reported that vitamin C and iron exposure can influence the fatty acid composition of fish, which directly influences their nutritional value. For example, elevated iron (1450 mg/kg) significantly reduced the EPA and DHA content in the salmon muscle (Sutton et al., 2006). Meanwhile, previous research in red sea bream (*Pagrus major*), and juvenile abalone (*Haliotis discus hannai* Info) reported that the percentages of EPA, DHA and total n-3 fatty acids increased significantly with the rise in dietary vitamin C levels and the percentages of 14:0 and 18:0 were reduced (Gao et al., 2013; Li et al., 2020), whereas in Japanese flounder (*Paralichthys olivaceus*) and thornfish (*Terapon jarbua*) increasing the levels of dietary vitamin C supplementation lowered EPA and DHA (Chien and Hwang, 2001; Gao et al., 2014). Knowing this relationship is important because omega-3 (n-3) fatty acids, especially EPA and DHA, can fight against several degenerative diseases, particularly cardiovascular disease, which underscores the importance of consuming some fish products. Nevertheless, in the present study on catfish, no major changes were noted in fatty acid composition either in response to dietary increment of vitamin C and/or exposure to iron. Such inconsistency in the response might be attributed to the species-specific differences on lipid metabolism and/or protection from unsaturated fatty acids that are more prone to lipid oxidation, but the mechanisms influencing these responses are not well understood.

3.8 Conclusion

In conclusion, exposure to elevated waterborne iron elicited a negative effect on growth that was likely due to hepatic oxidative stress as well as substantial histopathological alterations that would have reduced their surface area and thus function. The latter was supported by changes in the hematological parameters that signify respiratory dysfunction. It was clear, however, that dietary supplementations of vitamin C at either dose (143 or 573 mg/kg diet) were effective at suppressing the negative effects of iron exposure on growth as well as acting as a growth promoter under normal iron levels. It appears likely that vitamin C mitigated iron toxicity by eliminating an overload of iron accumulation in the liver as well as having a potent protective effect against cellular lipid peroxidation, via augmenting antioxidant capacity of ROS scavenging SOD and CAT enzyme activities. This was complimented by less adverse effects to the liver, but especially the gills. In brief, supplementation of vitamin C appears to be a viable therapeutic approach mitigating the toxic effects of waterborne iron in channel catfish. Although both doses of vitamin C were equally effective, the lower tested dose (143 mg/kg diet) is practically recommended from an economical point of view.

3.9 References

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Figures

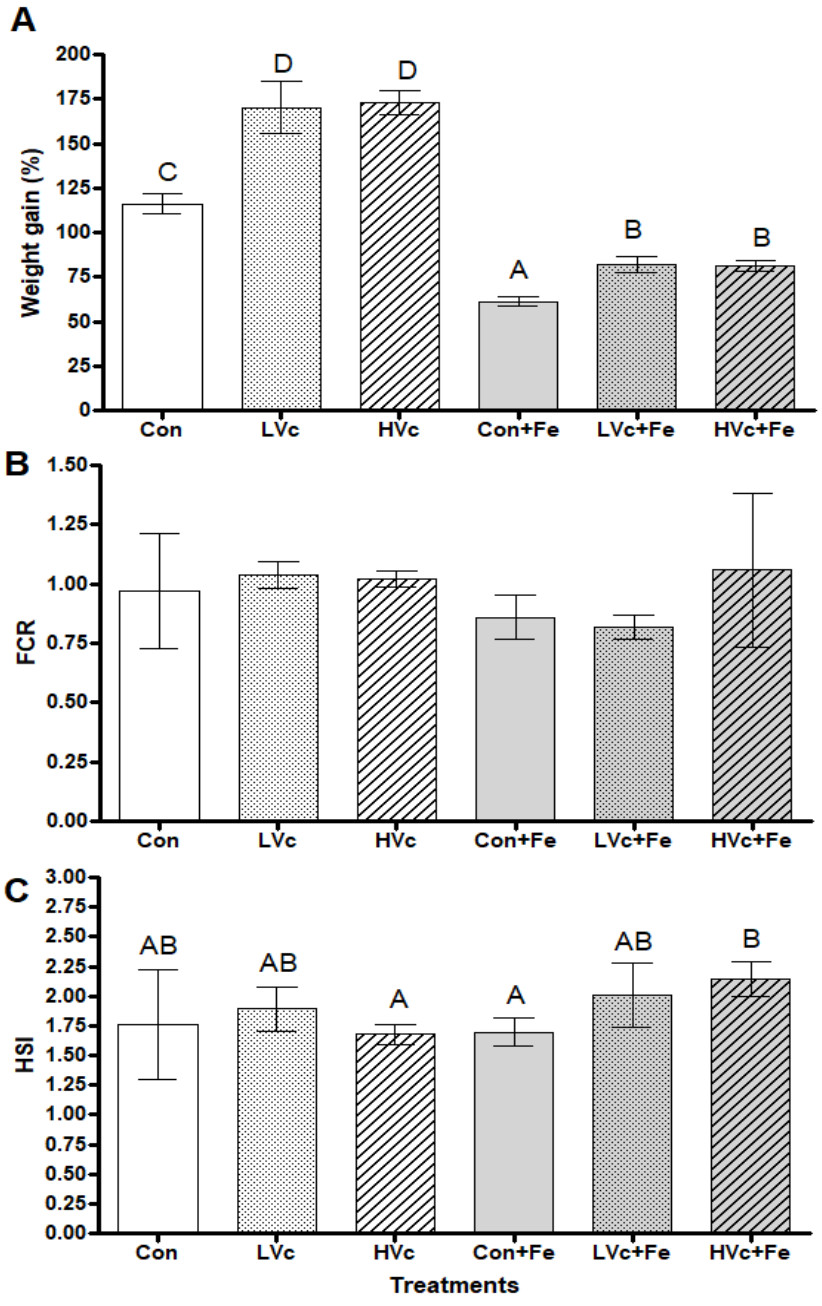

Figure 3.1. Effect of iron exposure, vitamin C supplementations (143 mg/kg 'LVc' and 573 mg/kg diet 'HVc') and their combination on (A) weight gain (%), (B) feed conversion ratio (FCR) and (C) hepatosomatic indices (HSI) of channel catfish after 8 weeks. Values are presented as mean ± S.E. Capital letters (A, B) denote significant differences between experimental groups (Number of replicates (N)=4).

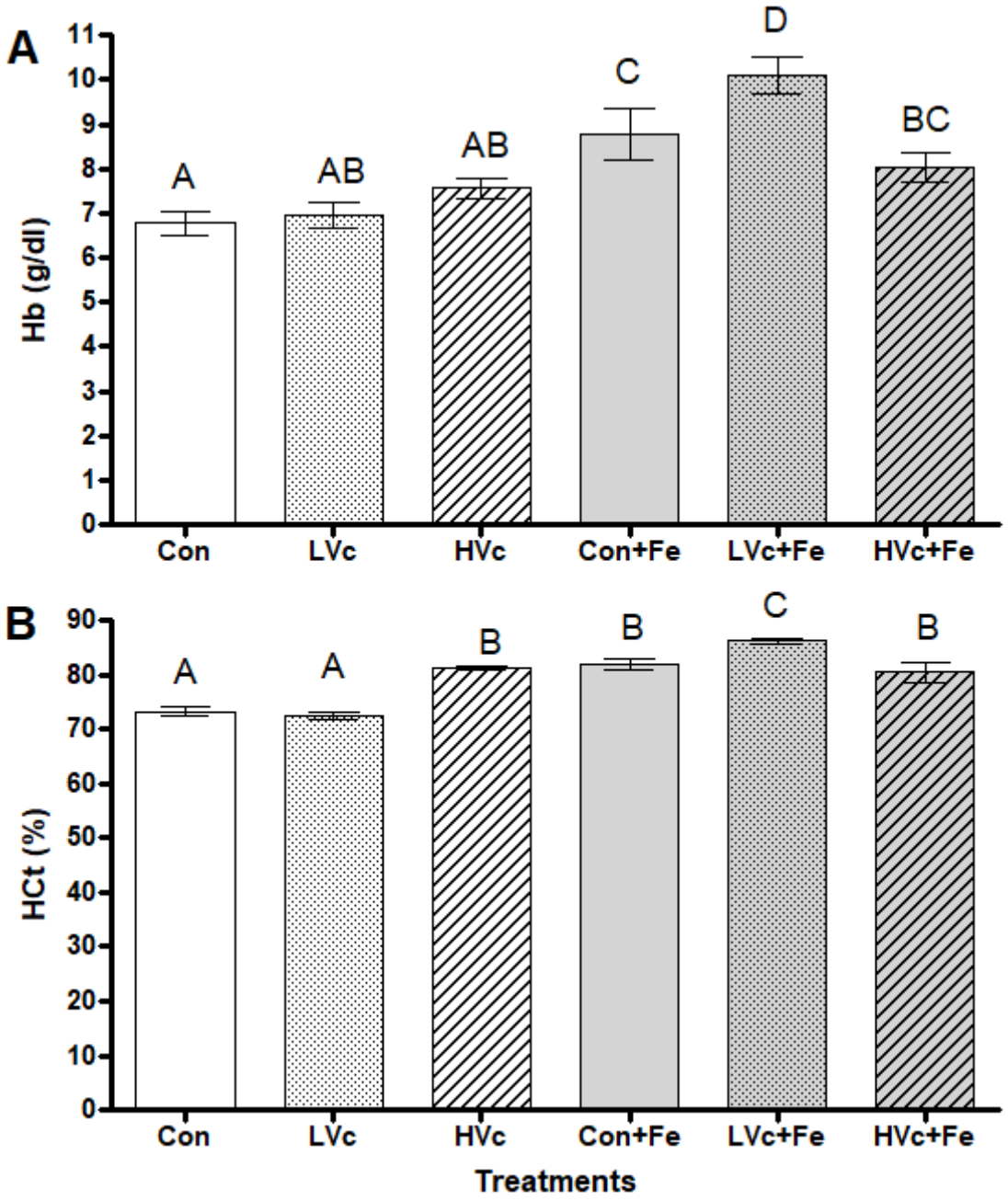

Figure 3.2. Effect of iron exposure, vitamin C supplementations (143 mg/kg 'LVc' and 573 mg/kg diet 'HVc') and their combination on (A) hemoglobin (Hb) content and (B) hematocrit (HCt %) of channel catfish after 8 weeks. Values are presented as mean ± S.E. Capital letters (A, B, C, D) denote significant differences between experimental groups (Number of replicates (N)=4).

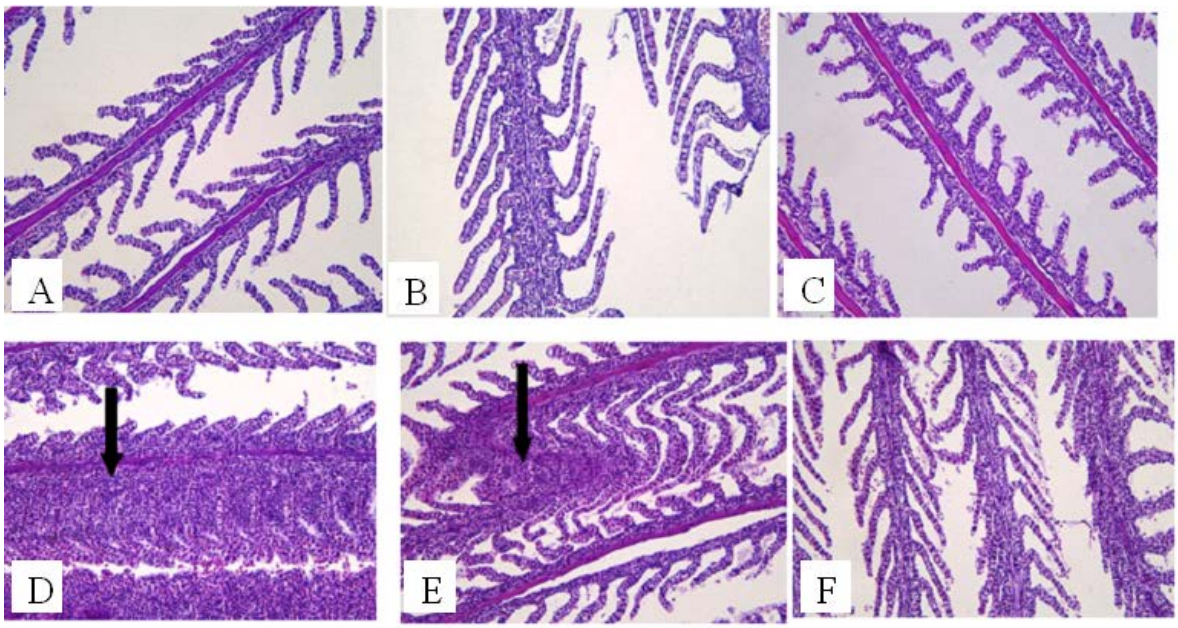

Figure 3.3. Histological sections of the gills from channel catfish under experimental conditions of iron exposure and vitamin C supplementations (143 mg/kg 'LVc' and 573 mg/kg diet 'HVc'). Normal structure of gills observed in (A) control, (B) LVc, and (C) HVc. Deposition of interlamellar cell mass in (D) Con+Fe and occasional disruptions of the primary gill filaments in (E) LVc+Fe groups were observed (arrows). Some mucus production was observed in the (F) HVc+Fe groups, but otherwise had no abnormalities. H & E staining and magnification 100 X.

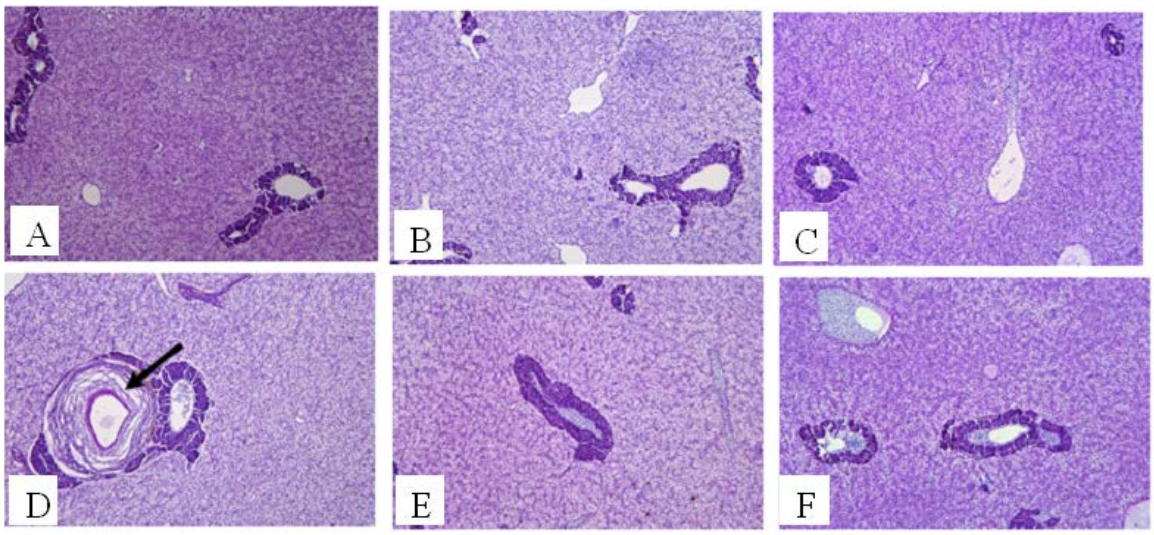

Figure 3.4. Hepatic histological assay of channel catfish under experimental conditions of iron exposure and vitamin C supplementations (143 mg/kg 'LVc' and 573 mg/kg diet 'HVc'). Normal structure of hepatic lobules found in (A) control, (B) LVc, (C) HVc, (E) LVc+Fe and (F) LVc+Fe groups, and enlargement of bile duct in (D) Con+Fe group (arrow). H & E staining and magnification 100X.

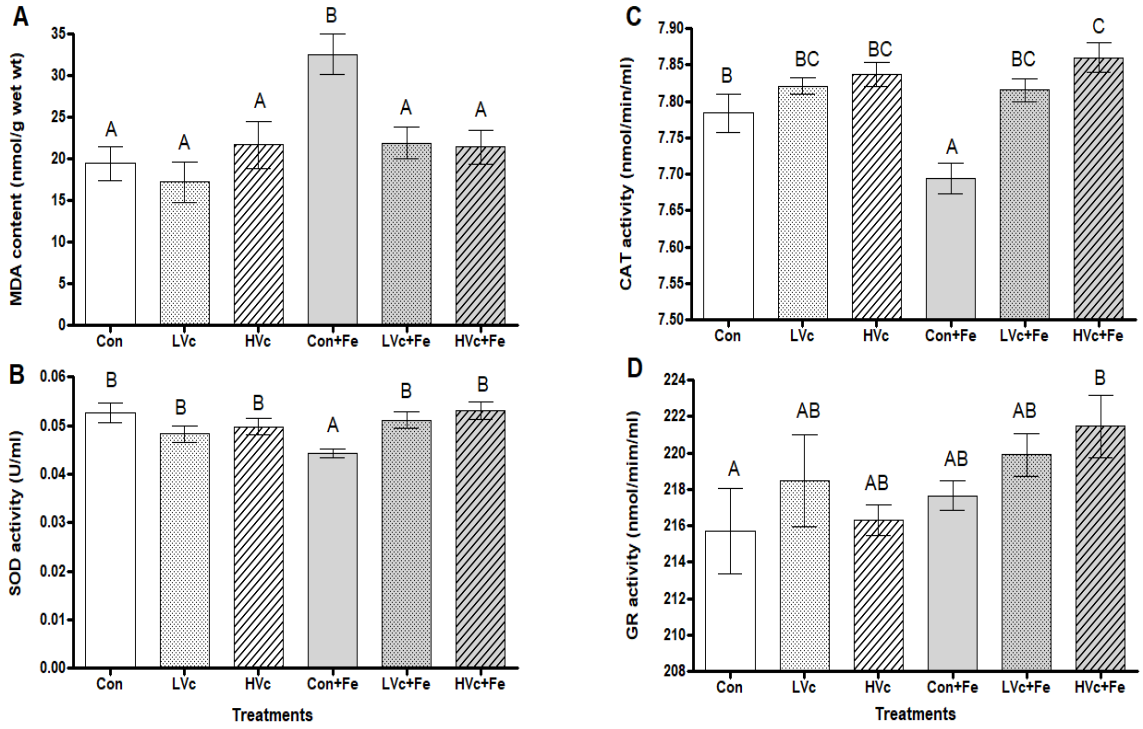

Figure 3.5. Effect of iron exposure, vitamin C supplementations (143 mg/kg 'LVc' and 573 mg/kg diet 'HVc') and their combination on (A) malondialdehyde (MDA) content, (B) superoxide dismutase (SOD) activity, (C) catalase (CAT) activity and (D) glutathione reductase (GR) activity in liver of channel catfish during 8-week trial. Values are presented as mean ± S.E. Capital letters (A, B, C) denote significant difference between experimental groups.

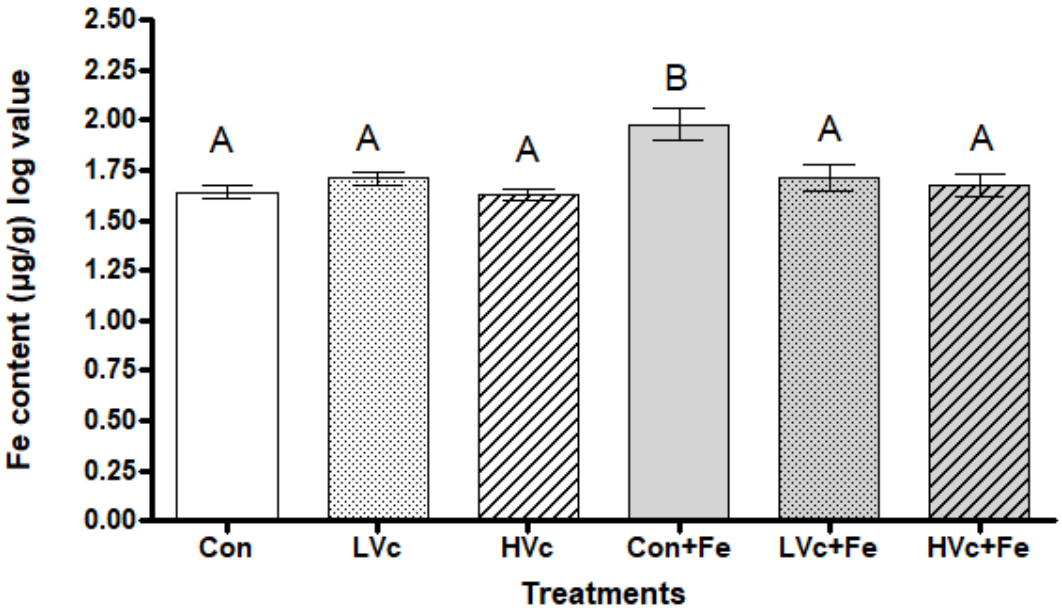

Figure 3.6. Iron accumulation in the hepatic tissue of channel catfish following iron exposure, vitamin C supplementation (143 mg/kg 'LVc' and 573 mg/kg diet 'HVc') and their combination. Values are mean ± S.E. Capital letters (A, B) denote significant difference between experimental groups.

Tables

Table 3.1.1

Formulation of the experimental diets (%), proximate composition (% dry weight) and fatty acid composition (%).

Ingredients		Control	LVc	HVc
Fish meal[a]		10.00	10.00	10.00
Poultry by-product meal[a]		10.00	10.00	10.00
Soy protein concentrate[b]		25.00	25.00	25.00
Corn meal[a]		18.10	18.10	18.10
Wheat flour[a]		27.00	26.88	26.45
Fish oil[a]		3.00	3.00	3.00
Soybean oil[c]		2.14	2.14	2.14
Dicalcium phosphate[a]		1	1	1
Choline chloride (60%)[a]		0.3	0.3	0.3
Vitamin premix, ARS 702[a]		0.1	0.1	0.1
Trace Mineral mixture, Trouw nutrition[d]		0.2	0.2	0.2
Vitamin C, (Stay C-35)[e]		0	0.143	0.573
α-cellulose[a]		2.81	2.7914	2.7355
Methionine[f]		0.35	0.35	0.35
Proximate composition (%)				
Moisture		6.0	6.8	7.0
Crude protein		35.2	34.9	35.1
Crude lipid		8.6	8.7	8.5
Ash		6.9	7.1	7.2
Fatty acid composition (% of total fatty acids)				
C14:0	Myristic acid	1.694	1.752	1.945
C16:0	Palmitic acid	15.56	15.72	15.06
C16:1	Palmitoleic acid	3.055	3.162	3.0191
C18:0	Stearic acid	2.996	2.938	3.062
C18:1n-9	Oleic acid	3.05	3.16	3.01

C18:2n-6	linoleic acid	22.71	23.18	25.65
C18:3n-3	α-Linolenic acid	2.381	2.254	2.661
C20:1n-9	11-Eicosenoic acid or Gondoic acid	5.483	5.151	5.566
C20:3n-3	Eicosatrienoic acid	0.00	0.00	0.00
C20:5n-3	Eicosapentaenoic acid	2.682	2.645	2.726
C22:6n-3	Docosahexaenoic acid	2.506	2.443	2.607
ΣSFA		20.252	20.418	20.071
ΣMUFA		49.463	49.054	46.273
ΣPUFA		30.283	30.527	33.654
Σn-3 PUFA		4.88	4.69	5.26
Σn-6 PUFA		22.71	23.18	25.65
EPA/DHA		1.07	1.08	1.04
DHA/EPA		0.93	0.92	0.95

SFA=saturated fatty acid, MUFA=Mono-unsaturated fatty acid, PUFA=Poly-unsaturated fatty acid.

[a]Rangen Inc., Buhl, ID, USA

[b]Profine VF, The Solae Company, St. Louis, MO, USA.

[c]Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

[d]US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as $ZnSO_4 \cdot 7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4 \cdot 5H_2O$), 1.54; I (as KIO_3), 10.

[e]Skretting USA, Tooele, UT, USA.

[f]Sigma Aldrich, St. Louis MQ, USA.

Table 3.1.2

Effects of iron exposure, vitamin C supplementations and their interaction on growth, hematology, oxidative index and antioxidant enzymes, metals/electrolyte parameters and fatty acids in the channel catfish after 8 weeks of the experimentation

Treatment	Iron		Vit. C		Iron × Vit. C	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*
Production indices						
WG%	183.817	0	19.237	0	4.111	**0.044**
FCR	1.354	0.267	1.201	0.335	0.653	0.538
HSI	4.423	0.044	2.864	0.073	3.641	**0.038**
Hematological parameters						
Hb	72.39	0	5.131	0.012	12.088	**0.00**
HCt	106.8	0	6.999	0.003	25.409	**0.00**
Oxidative and anti-oxidant status (Liver)						
MDA	1.31	0.011	1.241	0.424	0.534	0.160
CAT	2.421	0.131	17.354	0	4.659	**0.018**
SOD	13.3	0.001	10.232	0	2.078	0.143
GR	0.301	0.588	0.433	0.653	0.949	0.399
Metals and electrolyte (Liver)						
Fe	12.04	0.002	4.893	0.014	4.119	**0.026**
Cu	0.002	0.968	0.191	0.827	2.171	0.132
Zn	4.149	0.051	2.286	0.119	0.984	0.386
Na	1.876	0.181	2.53	0.097	0.747	0.482
K	0.017	0.897	2.525	0.097	0.24	0.788
Ca	1.118	0.299	1.245	0.302	0.746	0.483
Mg	5.464	0.026	6.046	0.006	0.216	0.807
Fatty acids (Muscle)						
ΣSFA	0.614	0.440	0.255	0.777	1.387	0.267
ΣMUFA	4.344	0.047	0.316	0.732	0.533	0.593
ΣPUFA	8.653	0.007	0.579	0.567	0.199	0.821
EPA	1.288	0.266	0.928	0.408	0.170	0.844
DHA	0.686	0.415	0.242	0.786	0.434	0.652
EPA+DHA	0.728	0.401	0.247	0.783	0.390	0.681
Σn-6 PUFA	15.59	0.001	1.924	0.166	2.080	0.144
Σn-3 PUFA	1.968	0.172	0.290	0.751	0.244	0.785
EPA/DHA ratio	0.003	0.959	1.101	0.347	0.224	0.801
DHA/EPA ratio	0.128	0.723	0.598	0.557	0.868	0.431
n-3/n-6 ratio	0.111	0.742	0.509	0.607	0.730	0.491

Table 3.1.3

Effects of iron exposure, vitamin C supplementations and their interaction on the muscle fatty acid composition (%) after 8 weeks.

Fatty acids		Treatments group					
Type		Con	LVc	HVc	Con+Fe	LVc+Fe	HVc+Fe
Saturates							
C14:0	Myristic acid	1.26 ± 0.07	1.00 ± 0.03	1.07 ± 0.08	1.08 ± 0.12	1.18 ± 0.12	1.08 ± 0.06
C16:0	Palmitic acid	14.17± 0.55	13.39 ± 0.31	14.98± 0.40	13.99 ± 0.74	14.68 ± 0.52	14.43 ± 0.25
C18:0	Stearic acid	4.15 ± 0.07	4.49 ± 0.07	4.53 ± 0.32	5.04 ± 0.23	4.64 ± 0.36	4.29 ± 0.87
ΣSFA		19.58± 0.62	18.89 ± 0.32	20.59 ± 0.51	20.12 ± 0.85	20.51± 0.73	19.81 ± 0.95
Monoenes							
C16:1n-7	Palmitoleic acid	2.86 ± 0.20	2.44 ± 0.26	2.37 ± 0.39	2.49 ± 0.77	2.41 ± 0.62	2.39 ± 0.34
C18:1n-9	Oleic acid	53.32 ± 1.96	54.53 ± 2.38	54.70 ± 1.02	52.58 ± 2.65	51.87 ±1.24	51.90 ± 3.64
C20:1n-9	11-Eicosenoic acid or Gondoic acid	2.46 ± 0.26	1.98 ± 0.27	1.68 ± 0.65	2.61 ± 0.46	2.53 ± 0.38	2.99 ± 0.89
C22:1n-9	Erucic acid	0.71 ± 0.11	0.53 ± 0.04	0.40 ± 0.05	0.64 ± 0.15	0.50 ± 0.11	0.54 ± 0.05
ΣMUFA		59.37 ± 1.36	61.49 ± 0.62	59.09 ± 1.36	57.33 ± 1.72	57.32 ± 1.68	57.84 ± 1.65
Polyunsaturate							
C18:2n-6	linoleic acid	10.92 ± 0.06	10.06 ± 0.23	9.32 ± 0.54	10.70 ± 0.50	10.69 ± 0.70	10.55 ± 0.34

C18:3n-3	α-Linolenic acid	1.13 ± 0.12	0.90 ± 0.04	0.76 ± 0.07	1.10 ± 0.19	1.19 ± 0.17	1.16 ± 0.12
C20:2n-6	Eicosadienoic acid	0.35 ± 0.03	0.33 ± 0.02	0.28 ± 0.05	0.40 ± 0.09	0.37 ± 0.05	0.39 ± 0.08
C20:3n-6	Dihomo-γ-linolenic acid	0.41 ± 0.11	0.54 ± 0.07	0.55 ± 0.09	0.64 ± 0.18	0.71 ± 0.21	0.85 ± 0.36
C20:4n-6	Arachidonic acid	1.09 ± 0.19	1.01 ± 0.05	1.23 ± 0.16	1.36 ± 0.23	1.28 ± 0.27	1.48 ± 0.20
C20:5n-3	Eicosapentaenoic acid (EPA)	0.92 ± 0.03	0.81 ± 0.02	0.88 ± 0.05	0.94 ± 0.05	0.90 ± 0.08	0.93 ± 0.06
C22:5n-3	Docosapentaenoic acid	0.90 ± 0.05	0.83 ± 0.02	0.88 ± 0.10	0.96 ± 0.09	0.85 ± 0.17	0.92 ± 0.08
C22:6n-3	Docosahexaenoic acid (DHA)	5.30 ± 0.75	5.09 ± 0.31	6.38 ± 0.88	6.3 ± 1.10	6.16 ± 1.13	6.03 ± 0.67
ΣPUFA		21.04 ± 0.89	19.60 ± 0.86	20.31 ± 0.88	22.13 ± 1.80	22.16 ± 1.35	22.04 ± 1.68
EPA + DHA		6.22 ± 0.79	5.91 ± 0.32	7.26 ± 0.94	7.24 ± 1.16	7.06 ± 1.21	6.97 ± 0.73
EPA/DHA		0.18 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	0.17 ± 0.03	0.15 ± 0.01	0.16 ± 0.01
DHA/EPA		5.65 ± 0.63	6.23 ± 0.32	7.08 ± 0.62	6.48 ± 0.85	6.66 ± 0.69	6.37 ± 0.35
Σn-3 PUFA		12.62 ± 0.41	11.89 ± 0.58	11.99 ± 0.52	13.03 ± 0.58	13.04 ± 0.66	13.05 ± 0.74
Σn-6 PUFA		0.66 ± 0.06	0.71 ± 0.08	0.73 ± 0.08	0.77 ± 0.10	0.73 ± 0.09	0.67 ± 0.11
n-3/n-6 ratio		0.66 ± 0.06	0.71 ± 0.08	0.73 ± 0.08	0.77 ± 0.10	0.73 ± 0.09	0.67 ± 0.11

Data expressed as Mean ± SE, n = 8 per treatment; values along row with different superscript differ significantly ($P < 0.05$). SFA=saturated fatty acid, MUFA=Mono-unsaturated fatty acid, PUFA=Poly-unsaturated fatty acid, EPA= Eicosapentaenoic acid, DHA= Docosahexaenoic acid.

Table 3.1.4

Electrolyte and metal load in the hepatic tissue (dry wt.) of channel catfish following vitamin C supplementation (143 mg/kg and 573 mg/kg diet), iron exposure and their combination.

Treatment	Na (mg/g)	K (mg/g)	Ca (µg/g)	Mg (mg/g)	Cu (µg/g)	Zn (µg/g)
Control	1.39 ± 0.62	34.89±2.13	4.78±0.68	0.26±0.01	1.57±0.03	3.06 ± 0.06^{AB}
LVc	1.98±0.20	32.93±4.90	4.50±0.60	0.29±0.03	1.99±0.27	3.47 ± 0.57^{AB}
HVc	1.15±0.23	26.76±3.17	3.95±0.25	0.21±0.02	1.45±0.16	2.58 ± 0.28^{A}
Con+Fe	1.34±0.38	34.78±6.52	4.39±0.67	0.32±0.01	1.41±0.07	3.04 ± 0.07^{B}
LVc+Fe	1.18±0.41	37.12±3.82	4.52±0.84	0.32±0.02	1.59±0.22	3.32 ± 0.34^{AB}
HVc+Fe	2.04±0.43	37.05±4.91	4.20±1.16	0.25±0.01	1.46±0.06	2.85 ± 0.09^{AB}

Values are mean ± S.E. Superscripts (A, B) denote significant difference between experimental groups.

CHAPTER 4

BENTONITE MITIGATES THE FEED BORNE IRON TOXICITY IN RAINBOW TROUT, *Oncorhynchus mykiss*

4.1 Introduction

Water quality plays an essential role in the wellbeing of fish, but unfortunately some water sources are naturally high in iron (Fe) (Ohimain et al., 2013), For example, in northern Idaho where many rainbow trout farms are located, the valley-fill aquifers dissolved solid concentration ranges from 250-500 mg/l and concentration of Fe in interstitial water is 41mg/l (Cummings et al., 2000). Fe is an essential mineral for all animals, including fish, and forms an important functional component with proteins and aids in different metabolic processes, sucha as DNA synthesis, oxygen transfer, and energy production (Bury and Grosell, 2003; Ganz and Nemeth, 2015; NRC, 2011; Luo et al., 2017; Tarifeno -Saldivia et al., 2018). However, excess Fe accumulation in the tissue can cause toxic effects (Aisen et al. 2001; Lebster, 2014), which mainly includes playing a catalytic role in Fenton reactions (Fenton, 1894), and promotes free radical species (ROS) generation (Li et al., 2009). ROS accumulations can trigger lipids, proteins, and DNA damage, thus negatively affect the overall physio-biochemical and metabolic performance of fish, and/or cause cell death in extreme cases. (Bagnyukova et al., 2006; Bresgen and Eckl, 2015; Li et al. 2009).

In the aquatic environment, Fe is concentrated in two forms i.e., ferric water insoluble (Fe^{3+}) and ferrous water soluble (Fe^{2+}). The latter is more bioavailable, and it is readily absorbed by aquatic species via divalent metal transporters present in the gills and intestines (Gunshin et al. 1997; Bury et al. 2003). Ferrous (Fe^{2+}) iron has been found to be detrimental to aquatic organisms because it binds to the surface of the gill and is oxidized into insoluble iron (Fe^{3+}) covering the surface of the gill and causing damage to the cells followed by respiratory dysfunction (Debnath et al., 2012; Sevcikova et al., 2011; Slaninova et al., 2014). The above negative effects result in reduced surface area for gas exchange as well as increased diffusion distance,

ultimately limiting functional capacity of the gills and reducing the rate of oxygen diffusion into the blood. (Tuurala and Soivio,1982; Yasser and Naser, 1983). Despite the fact that fish can absorb soluble Fe through the gill membrane and intestinal mucosa (Roedar and Roedar, 1968), the diet is the primary source of Fe due to low solubility of Fe concentrations in natural waters (NRC, 2011). The toxic effect of iron in rainbow trout is seen at the higher rate (1380 mg/kg) of inclusion of iron in their diets (Desjardins, 1985).

In Fe overload conditions, the involvement of free or loosely chelated Fe in the production of ROS and tissue damage is still a prevalent feature. As a result, effective scavenging of excess Fe may be a viable way to limit and mitigate free radical-mediated tissue damage. Fe chelation is gaining popularity as a treatment to not only remove Fe from the body, but also scavenge and bind free Fe to avoid the production of reactive ROS (Hatcher et al., 2009; Hershko, 2010). The dietary supplementation of chelators that are cheaper and readily available might be an alternatives source for mitigating the feed borne Fe toxicity. One compound that is known for their superior binding (adsorption) properties and high cation exchange capacity are bentonites.

Bentonites are crystalline, hydrated aluminosilicate salts composed of sodium (Na), potassium (K), calcium (Ca), with traces of Fe, manganese (Mn), and nickel (Ni), and having higher negative charges that are balanced by cations like Na, K, and magnesium (Mg) present in the cavities (Lira et al., 2014; Safaei et al., 2014). The neutral pH or slightly alkaline pH makes bentonite as an inert material and thus, does not react with food ingredients (Khanedar et al., 2012; Di Gregorio et al., 2014). Other desirable characteristics of bentonites are their natural occurrence, low cost and 'generally recognized as food additives (GRAS)' for humans and various domestic animals including poultry, pigs, lambs, dairy cows, and goats (Fenn and Leng, 1990; Ivan et al., 1992; Murray, 2000; Shu-li-et al., 2009). Obradovic et al. (2006) reported that dietary bentonite of 1.0 % improved growth and feed conversion efficiency in rainbow trout (*Onchorhynchus mykiss*). Similarly, Mumpton (1999) reported that adding less than 2.0 % bentonite (as zeolite) to the diet of trout resulted in a substantial weight gain increase over a 64-day feeding period. In addition, Eya et al. (2008)

indicated that the inclusion of 5% bentonite in juvenile rainbow trout diets enhanced their growth performance and whole-body composition. Tilapia (Tilapia zillii) fed with 1.0-2.0% zeolite had improved feeding efficiency and was attributed, at least partly, to reducing the amount of ammonia excreted (Yildirim et al., 2009). Similarly, Ergün et al. (2008) also reported that feeding trout with dietary zeolite at 2.5% decreased ammonia discharge by 24% relative to the control group.

The current study was aimed to investigate the effects of dietary bentonite supplementation and its capacity to mitigate Fe toxicity in rainbow trout by measuring: (i) weight gain (%), and feed conversion ratio, ii) Fe and mineral load in the liver, plasma, and digesta, (iii) hepatic antioxidant enzyme activity, (iv) histopathological examinations of liver; and (v) muscle fatty acid profile.

4.2 Materials and Methods

4.2.1 Fish, diet preparation and experimental set-up

Rainbow trout (average weight: 1.5 ± 0.01g) were obtained from a clear springs Foods located at Buhl, Idaho, and transported to the cold-water laboratory of the Aquaculture Research Institute, University of Idaho, Moscow, Idaho. Upon arrival, fish were stocked in two 360-L fiberglass tanks. During acclimation period, the fish were fed skretting oncor trout diet, twice daily until they attained a size of 2-3 g.

Six experimental diets were formulated to include: 1) a positive control 0% iron (Con); 2) 2% bentonite (LB); 3) 4% bentonite (HB); 4) negative control (0.25% Fe); 5) 0.25% $FeSO_4$+2% bentonite (LBFe); and 6)0.25% $FeSO_4$ + 4% bentonite (HBFe). All the diets were isonitrogenous (47% crude protein) and isolipidic (18% crude lipid) as depicted in Table 1. The feed ingredients were thoroughly mixed using an industrial mixer (Hobart A200T 20-quart mixer, Hobart Production Facilities, Troy, Ohio, USA), moistened to make a soft dough, and passed through an extruder machine fitted with a 3.2mm die. The resulting diets were dried in a dryer for 24-48 hr, packed in a plastic bag, and stored at 4˚C until used. The proximate composition of the diets was determined following AOAC standard protocol (AOAC 2005).

Four hundred and eighty rainbow trout (2.3±0.01 g initial weight) were randomly stocked into 24 flow-through experimental tanks (20 fish per 60 L tank), and the quadruplicate tanks were assigned to their respective dietary group. The fish were hand fed to apparent satiation twice daily, 6 days per week for 8 weeks. Tanks were flushed twice daily. The photoperiod was maintained at 14h light: 10h dark with electric timers and water temperature was held constant at 15.4ºC. Other water quality variables were monitored, such as pH (7.3 ± 0.2), DO (7.2 ± 0.3 mg/l) and ammonia (0.03 ± 0.01 mg/l). All fish handling and experimental protocols were approved by and conducted in accordance with the guidelines of the University of Idaho's Animal Use and Care Committee (IACUC-2019-78).

4.2.2 Sample collection

After 8 weeks of the feeding trial, fish were fasted for 24 hours prior to sampling. The fish were anaesthetized with tricaine methanesulfonate (MS222, 50 mg/L) and batch weighed. Three fish per tank (12 fish/group, average weight = 36 g ± 0.9) were randomly taken for blood collection through the caudal vein, dispensed in a tube coated with anticoagulant (heparin) and used for mineral load determination in the plasma. The same fish were euthanized with MS-222 (250 mg/L) and dissected for liver and intestine for the determination of the somatic indexes. Muscle was filleted for fatty acid analysis. Small portions of the liver were collected from 3 fish and kept in Bouin's solution for histological analysis. Additionally, six fish per tank were euthanized (MS-222, 250 mg/L) and the liver were taken for enzyme (3 fish/tank) and metal analysis (3 fish/tank), and samples were kept at -80°C.

Calculations

The growth response parameter was determined using the formulae listed below:

- Percentage weight gain (WG, %): [(final fish weight – initial fish weight) / initial fish weight (g)] × 100.
- Specific growth rate (SGR, %/day) = [(ln final body weight (g) – ln initial body weight (g)) /duration of feeding] × 100

- Feed conversion ratio (FCR): [dry feed weight / wet weight gain].
- Hepatosomatic index (HSI %) = [liver weight (g) / fish weight (g)] × 100.
- Intestinal somatic index (ISI) = 100 x wet weight of visceral (g)/whole body weight of fish (g)

4..3 Analytical techniques

4..3.1 Anti-oxidative enzyme assay

Superoxide dismutase (Item No. 706002), Catalase (Item No. 707002), and glutathione reductase (Item No. 703202) activities were measured in eighteen fish per treatment (3/tank) using Cayman chemical assay kits. All these assays were performed according to the manufacturer's instructions.

4.3.2 Mineral analysis

The digesta samples were dried in an oven at 60 °C for 48 h. Samples (almost 100 mg) were then mixed in a microwave digester (SP-D, CEM corporation) at 300 ° C and 250 psi for 5 min with 3 mL trace-metal-grade HNO_3 (69–70 %) and 2 mL 30 per cent H_2O_2. For achieve digestive conditions a 5-min ramp is required. For each digested solution 1 mL of internal standard (10 µg / mL, Bi, Li-6, Sc, Tb, and Y; Accustandard, Inc.) was added and then diluted to 100 mL with E-pure water. The concentration of metals (Fe, Cu, Zn) and the levels of ions (Na, K) in the digested tissues was calculated using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500cx). Calibration was carried out using a standard of multi-elements; all standard solutions and samples included an equal amount of internal standard. Calibration curves showed strong linearity ($R^2 \approx 0.99$) for all studied elements.

4.3.3 Fatty acid analysis

Muscle fatty acid composition was analyzed using modified AOAC method 991.39 (AOAC, 1995). In brief, the muscle sample was dried at 50^{o}C (OA-SYS heating system, Organomation Associates, Inc., Berlin, MA, USA) for 5-6 h under a N_2 stream. Then 2 mL of 0.5N NaOH was added sample saponification at 70^{o}C for 60 min. After

sample cooling, the free fatty acid was methylated in methanol by adding 2 mL 14 percent BF3 (Boron trifluoride) and incubated at 70^{o}C for 60 min. After allowing the samples to cool, 2 mL of hexane was added, inverted repeatedly for 60 sec and 1 ml of saturated NaCl was added. The samples were repeatedly inverted for 60 sec, and then centrifuged for 5 min at 2000 xg. For gas chromatography / mass spectroscopy (GC / MS) analysis, an aliquot (100 µL) of the clarified hexane extract was diluted in hexane (1:10) and put into autosampler vials. The injection mode with a helium flow rate and the column temperature followed the protocol stated by Overturf et al. (2013).

4.3.4 Histological analysis

After 18 hr in the fixative, tissues were transferred to 70% (v/v) ethanol for several hours before being processed at increasing concentrations of ethanol. The samples were cleared in xylene and then embedded in paraffin wax. Rotary microtomes (HM 340E, Thermo Scientific) were used to prepare 5 µm sections and stained with hematoxylin and eosin (H&E) or Schiff Periodic-Acid (PAS). Pictures were taken with a light microscope (Leica DM 2500 LED).

4.3.5 Statistical Analysis

All resulting data were subjected to one-way analysis of variance using Statistical Package for the Social Sciences (SPSS), version 16.0. Before analyzing data, homogeneity of variance and normality were validated by Levene's test and Shapiro Wilk's test, respectively. For normalization, the iron content in the liver and plasma were log-transformed. A one-way analysis of variance (ANOVA) was used to compare experimental groups, followed by Duncan multiple range tests. The main effects of iron exposure and bentonite supplementation, as well as their interactions, were studied using two-way ANOVA. A probability level was set at $p<0.05$.

4.4 Results

4.4.1 Growth performance

No mortalities occurred over 8 weeks of this study. The weight gain (WG) (%) and specific growth rate (SGR) (%) were significantly lower ($P<0.05$) in the 0.25% Fe

fed group compared to all others; however, WG and SGR showed no differences among the other dietary treatments (P>0.05, Fig. 4.1a, b). The Fe group showed significantly higher FCR compared to all other groups (P<0.05; Fig. 4.1c). The lowest HSI value was recorded in HB fed group similar to LB, Fe, and HBFe but differ significantly from LBFe and the control (P<0.05; Fig. 4.2a). The Fe group recorded the highest ISI compared to the lowest value observed in HB fed group. However, the groups fed LB, LBFe, HBFe, and the control did not differ significantly and were similar to both the HB and Fe groups (Fig. 4.2b).

4.4.2 Anti-oxidant enzymes activities in the liver

Significantly lower catalase enzyme activity was found in Fe fed group compared to other dietary treatment groups (P<0.05). The liver SOD activity was higher in HB, LB and LBFe groups (P>0.05), whereas Fe and the control groups recorded the lowest value (P<0.05; Fig. 3b). Higher activity of glutathione reductase (GR) was observed in LBFe fed trout compared to other dietary groups (P<0.05; Fig. 3c). Also, a significant interaction was seen in the antioxidant enzyme activity (CAT, SOD, GR) between Fe and bentonite (Table 4.1.5).

4.4.3 Mineral content in the liver, plasma and digesta

Dietary Fe had a distinct influence, with the Fe content in the Fe group substantially higher than the LBFe, and HBFe group in liver and plasma, respectively. The Fe load in the digesta were significantly lower in the Fe group compared to the HB, and HBFe groups.

The major electrolyte Na, K, Mg, Ca remained unaltered in the hepatic tissue. Similarly, no effect was observed in Cu and Zn content (Table 2). Furthermore, no significant differences were documented for the electrolyte Na, Mg, Ca, and K in the plasma. Significant difference was detected in the Cu content in the plasma (Table 4.1.4).

4.4.4 Histological analysis

In the Con and LB groups, the histology of rainbow trout liver appeared normal based on the sinusoid organization, circular hepatocytes with a centrally located nucleus, and no indications of inflammation; however, the liver of HB group showed some white blood cell infiltrations (Figure 5 a, b, and c). In Fe group, the liver was severely affected with infiltrations of inflammatory cells, pyknotic nuclei and necrosis that showed leaked plasma from the lysed hepatocytes (Figure 5d). The livers of trout fed the LBFe diet was less severe with some localized instances of inflammatory cells and necrosis. The liver of HBFe group appeared similar to the HB group based on localized instances of inflammatory cell infiltrations but no necrosis.

4.4.4 Fatty acid composition of Muscles

Regardless of the supplementation of bentonite, dietary Fe had no effect on any of the fatty acids analyzed (Table 4.1.2).

4.5 Discussion

In the current study, addition of bentonite in the diet improved the growth performance of rainbow trout in combination with high dietary iron. This agrees with other studies showing positive growth effects when bentonite was included in the diets, which was generally attributed to binding ammonia (Ergun et al., 2008; Yildrum et al., 2009). The reason for this, especially in the group fed Fe-based diet (LBFe and HBFe), could be due to the binding and adsorptive properties of bentonite, thereby mitigating the toxic effect of Fe. The use of calcium bentonite also reduced the toxicity of aflatoxin in Nile tilapia (*Oreochromus niloticus*) (Hussain et al., 2017). Furthermore, binders can increase the viscosity of the digesta by binding nutrients and other feed elements (Amirkolaie et al., 2005). The viscosity of the digesta has a significant impact on digestion. Through relatively slow transit of feed through the gut, an optimal viscosity of ingested feed can improve digestion. This, as suspected in this study, facilitates appropriate digestion of feed components by digestive enzymes and improves nutrient absorption and utilization, resulting in enhanced growth. This assertion was further corroborated by lower FCR value recorded in the dietary

bentonite and high dietary iron supplemented groups (LBFe and HBFe). However, the inclusion of dietary bentonite did not improve the growth when fish were fed bentonite without supplementation of iron, whereas the observations of Lanari et al. (1996) and Eya et al. (2008) reported that the dietary addition of 2.5 % and 5% zeolite, and 5% bentonite and 2.5% mordenite significantly enhanced the weight gain and feed efficiency in rainbow trout, respectively in diets without Fe inclusion. Also, Yildirim et al. (2009) documented that supplementation of zeolites at 1% or 2% in tilapia diets resulted in higher growth and feeding efficiency than those fed diets with no zeolite. The improved growth might be attributed to a reduction in ammonia accumulation in the body. Hence from these studies, it could be stated that the addition of sodium bentonite could help improve the performance of rainbow trout farming in areas impacted by high iron levels.

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as the body's initial line of defense against free radical production (Fawole et al., 2020; Yadav et al., 2020; Hossain et al., 2021). SOD-CAT safeguards the vital organs against reactive oxygen species (ROS); however, excessive production of these reactive oxygen species (ROS) often compromises the potency of antioxidant enzymes, thereby causing damage to cell membranes owing to lipid and protein oxidation (Zhang et al., 2016). Studies have shown that diets containing excessive Fe can reduce the activity of SOD, CAT and GR in different fish species, such as yellow catfish *Pelteobagrus fulvidraco* (Luo et al., 2017), grass carp *Ctenopharyngodon idellus* (Zhang et al., 2016), and stinging catfish *Heteropneustes fossilis* (Zafar and Mukhtar 2020). These authors proposed that Fe at a higher level in the feed was responsible for lowering the antioxidant defense system. Yadav et al. (2020) also observed that channel catfish exposed to Fe in the water had lower induction of SOD, CAT, and higher level of malondialdehyde (MDA) in the liver. In line with the present study, it was observed that feeding rainbow trout with elevated Fe in the plasma and liver resulted in lower SOD and CAT activity compared to the dietary bentonite fed or supplemented groups. This finding implies that high cellular levels of Fe was responsible for lowering the antioxidant enzyme system, which further reinforced earlier reports wherein Fe overload was noticed to impede antioxidant enzyme

activities (Fawole et al., 2020; Yadav et al., 2020; Hossain et al., 2021). However, dietary bentonite restored the antioxidant defense system as compared to controls. This finding indicates that Fe-mediated toxicity could be mitigated by dietary bentonite supplementation. Similarly, in sterlet (*Acipenser ruthenus*), dietary zeolite (3%) significantly increased the activity of superoxide dismutase enzyme (Abrosimova et al., 2020), which supports current study findings.

Higher accumulation of Fe was detected in the hepatic tissue of rainbow trout fed the Fe-based diet and this was found to be significantly lower in the dietary Fe groups fed with dietary bentonite. Naser (2000) also reported increased accumulation of hepatic Fe in Atlantic salmon fed with diets containing increasing amounts of dietary Fe. The observed reduction in Fe load in the liver and its subsequent significantly higher level in the digesta of bentonite fed groups, especially high dietary bentonite (HBFe), indicates that bentonite could serve as a potent adsorbent of Fe, thereby reducing it negative impact on fish hepatic cells as seen in the liver histology. Furthermore, the improved growth recorded in the present study may be attributed to the ability of dietary bentonite to reduce the Fe load in the liver and promoted its excretion via feces.

Liver cells are especially susceptible to toxins, and chemical contamination causes a variety of injuries (Oliveira Ribeiro et al., 2006). Akbary and Jahanbakhsi (2019) found blood cell aggregation, lipidosis, and hypermia in the liver of goldfish when fed diets with increasing Fe levels. Also, Singh et al. (2019) noted hepatic degeneration, vacuolization, cell boundary crumbling, intrusion with inflammatory cells, swelling or hydrotropical hepatocyte degeneration, and central hemorrhage in fish exposed to Fe. The cause of these histological modifications was due to ROS that damaged the diverse cell layer components. In this study, there were instances of adverse histopathology that included necrosis and infiltration of inflammatory cells in the Fe and LBFe groups compared to Con, LB, HB and HBFe groups. Hence, this demonstrates that dietary bentonite protects the liver from histological damage in rainbow trout, and further study could be conducted to assess the efficacy of protection from other potential toxicants

Higher dietary Fe levels can promote lipid peroxidation and thus can have an effect on the fatty acid content of fish, particularly longer chain fatty acids that are more susceptible to oxidation. In particular, high dietary Fe (1450 mg/kg) dramatically lowered the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels in the muscle of salmon (Sutton et al., 2006). High dietary levels of EPA and DHA along with low level of dietary Fe can improve the health status in Atlantic salmon (Rørvik et al., 2003). Knowing this association is crucial since omega-3 (n-3) fatty acids, particularly EPA and DHA, can help combat a variety of degenerative diseases, notably cardiovascular disease. However, in the current study, dietary Fe either alone or in combination with dietary bentonite had no effect on the muscle fatty composition of the fed fish, which correspond with previous observations in rainbow trout (Evliyaoglu et al., 2022) and channel catfish (Yadav et al., 2020).

4.6 Conclusions

Based on the parameters examined in the present study, it was clear that dietary bentonite (2% via feed) could mitigate the adverse effects of high dietary iron on the growth, antioxidant status and histopathology in the liver of rainbow trout. This was likely due to dietary bentonite adsorbing and facilitating the excretion of Fe in the digesta. Thus, dietary bentonite (2%) supplementation can be a viable approach for boosting growth as well as mitigating iron negative effect in rainbow trout. However, to validate this assertion more study is required, specifically the iron toxicity pathway needs to be elucidated further in these animals.

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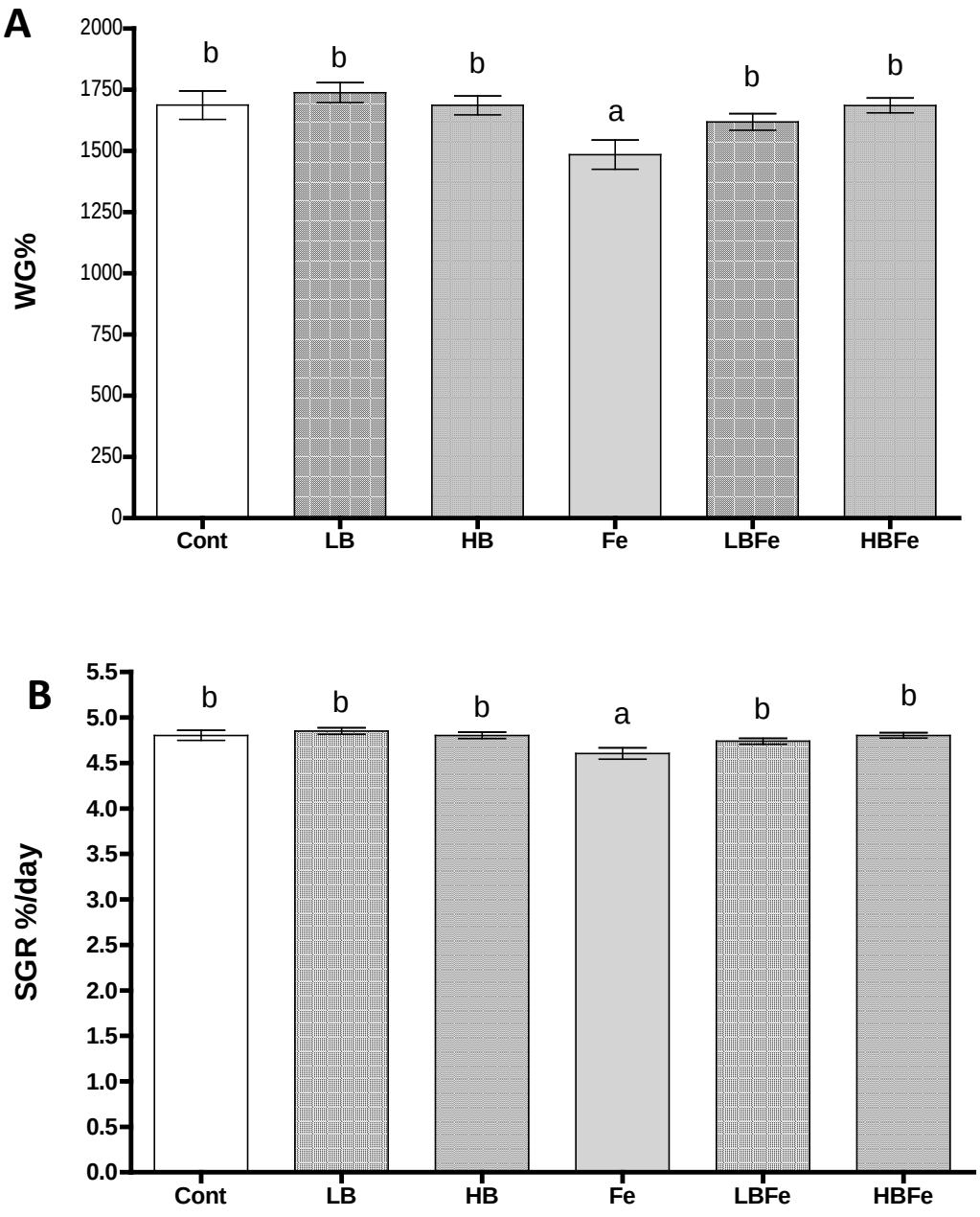
Figures


A
WG%
2000
1750
1500
1250
1000
750
500
250
0
b
b
b
a
b
b
Cont
LB
HB
Fe
LBFe
HBFe
B
SGR %/day
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
b
b
a
b
b
Cont
LB
HB
Fe
LBFe
HBFe

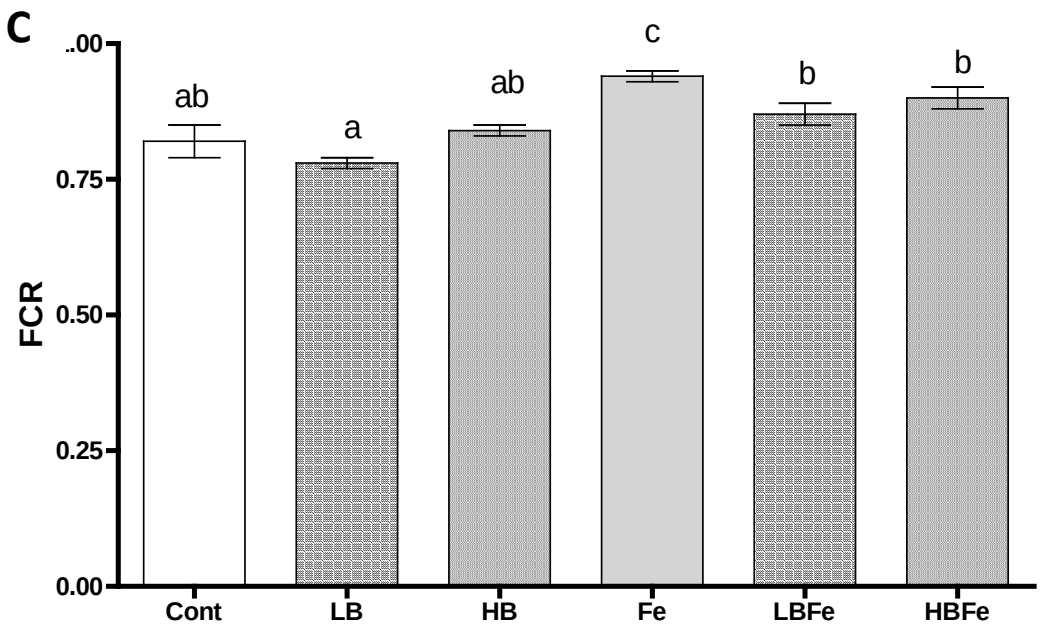


Figure 4.1. Effects of low (2%; LB) or high dietary bentonite (4%; HB) supplementation with or without dietary iron (0.25%; Fe) on (A) weight gain (%), (B) specific growth rate (SGR; %/day) and (C) feed conversion ratio (FCR) in rainbow trout after 8 weeks. Values are mean ± S.E.(n=4). small letters (a, b, c) denote significant differences ($p<0.05$) among experimental groups (Number of replicates (N)=4).

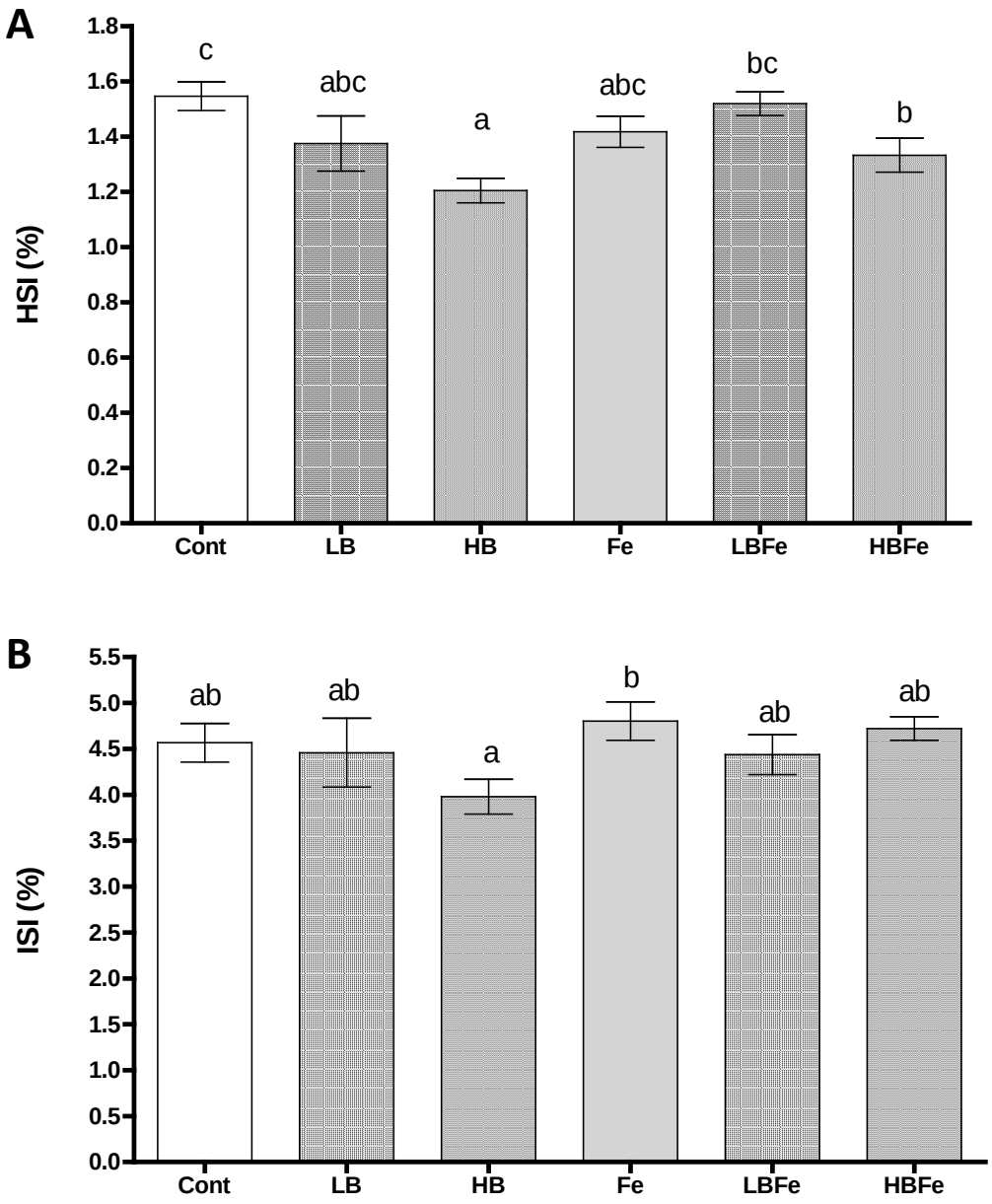
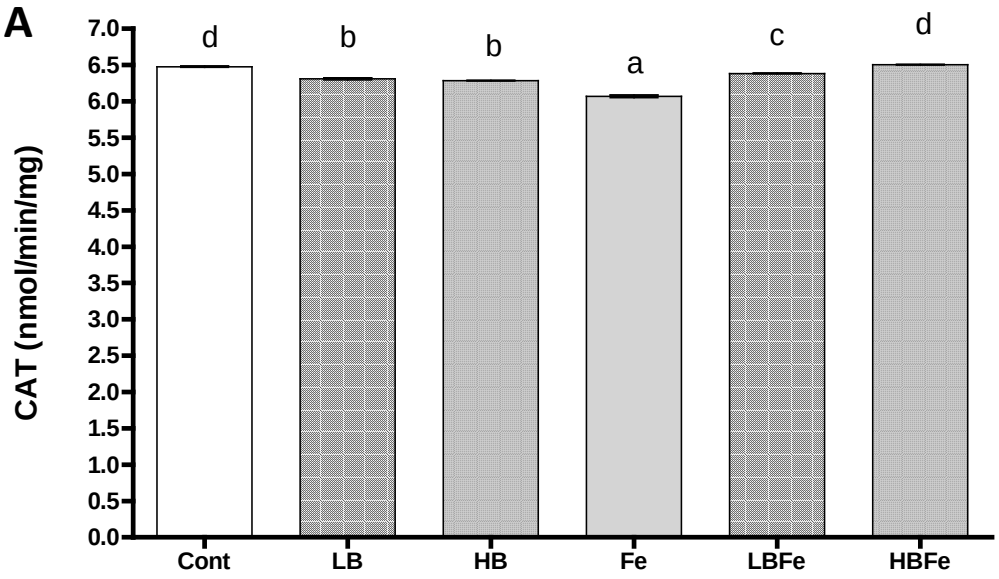


Figure 4.2. Effects of low (2%; LB) or high dietary bentonite (4%; HB) supplementation with or without dietary iron (0.25%; Fe) on the (A) HSI (%) and (B) ISI (%) in rainbow trout after 8 weeks. Values are mean ± S.E.(n=4) small letters (a, b, c) denote significant differences ($p<0.05$) among experimental groups (Number of replicates (N)=4).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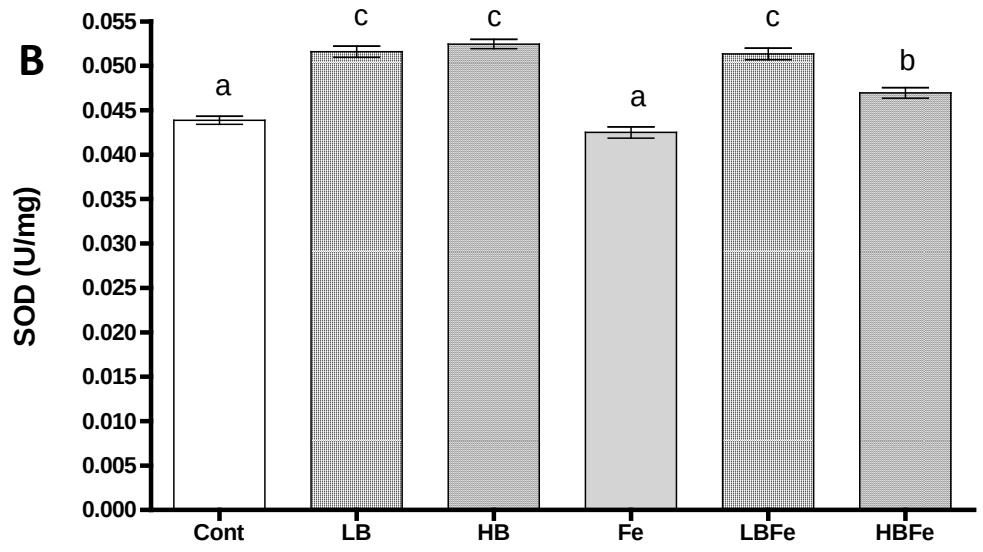
CAT (nmol/min/mg)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d
b
b
a
c
d
Cont
LB
HB
Fe
LBFe
HBFe


B
SOD (U/mg)
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
a
c
c
a
c
b
Cont
LB
HB
Fe
LBFe
HBFe

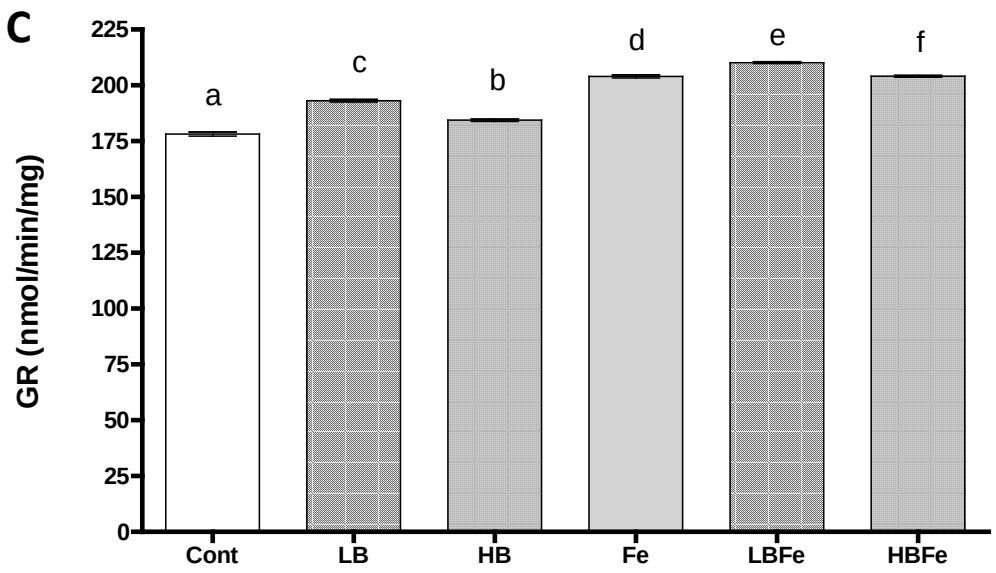
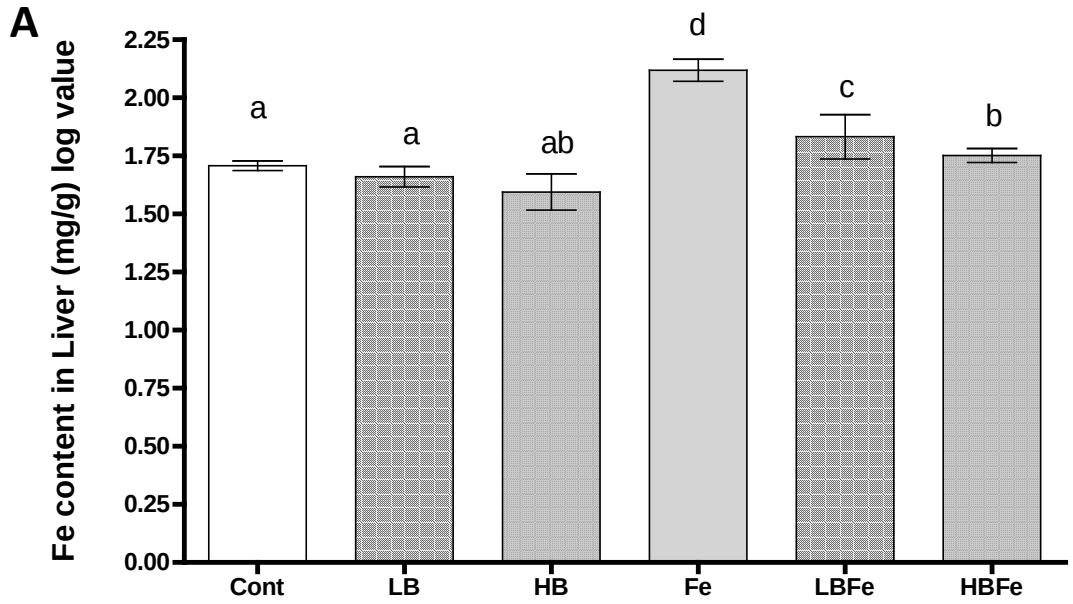
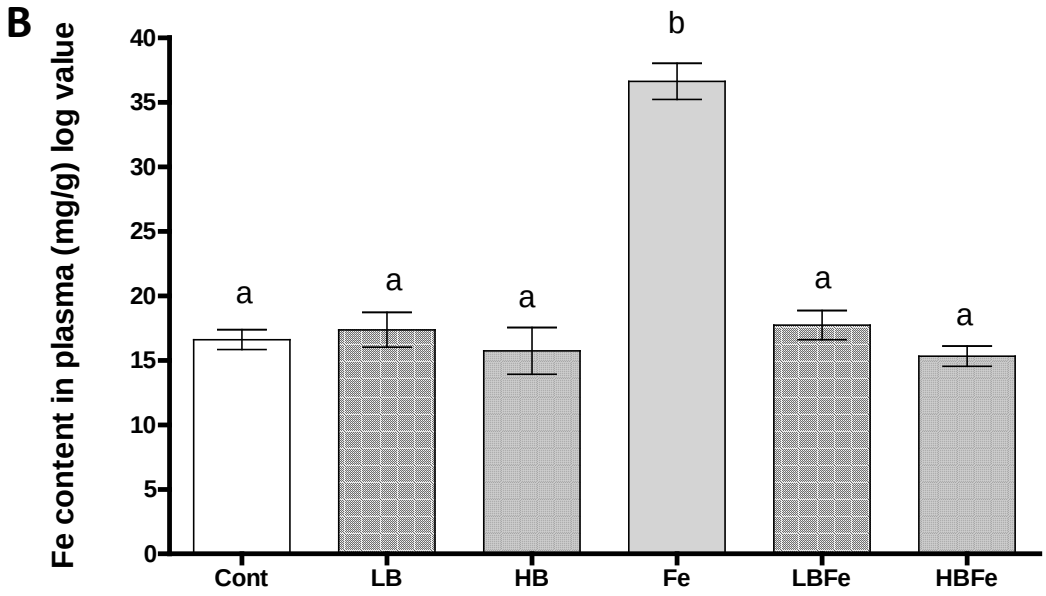


Figure 4.3. Effects of low (2%; LB) or high dietary bentonite (4%; HB) supplementation with or without dietary iron (0.25%; Fe) on the (A) catalase (CAT) activity, (B) superoxide dismutase (SOD) activity and (C) glutathione reductase (GR) activity in livers of rainbow trout after 8 weeks. Values are mean ± S.E. (n=4) Small letters (a, b, c, d, e, f) denote significant differences ($p<0.05$) among experimental groups (Number of replicates (N)=4).


A
Fe content in Liver (mg/g) log value
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
a
ab
d
c
b
Cont
LB
HB
Fe
LBFe
HBFe


B
Fe content in plasma (mg/g) log value
40
35
30
25
20
15
10
5
0
a
a
a
b
a
a
Cont
LB
HB
Fe
LBFe
HBFe

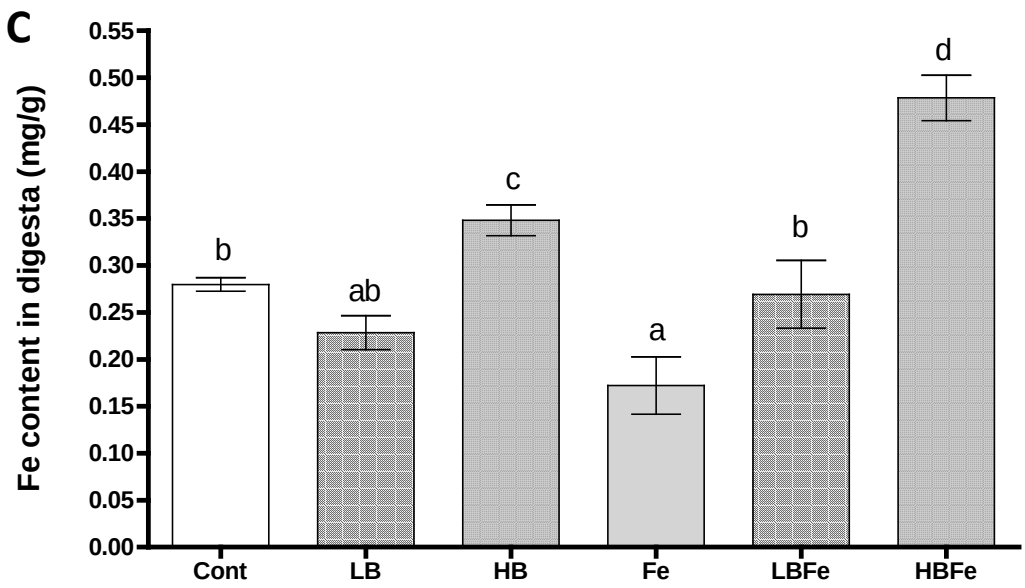


Figure 4.4. Iron (Fe) accumulation in the (A) Liver, (B) plasma, and (C) Fe in the digesta of rainbow trout following low (2%; LB) or high dietary bentonite (4%; HB) supplementation with or without dietary Fe (0.25%). Values are mean ± S.E.(n=4) small letters (a, b, c, d) denote significant differences ($p<0.05$) among experimental groups (Number of replicates (N)=4).

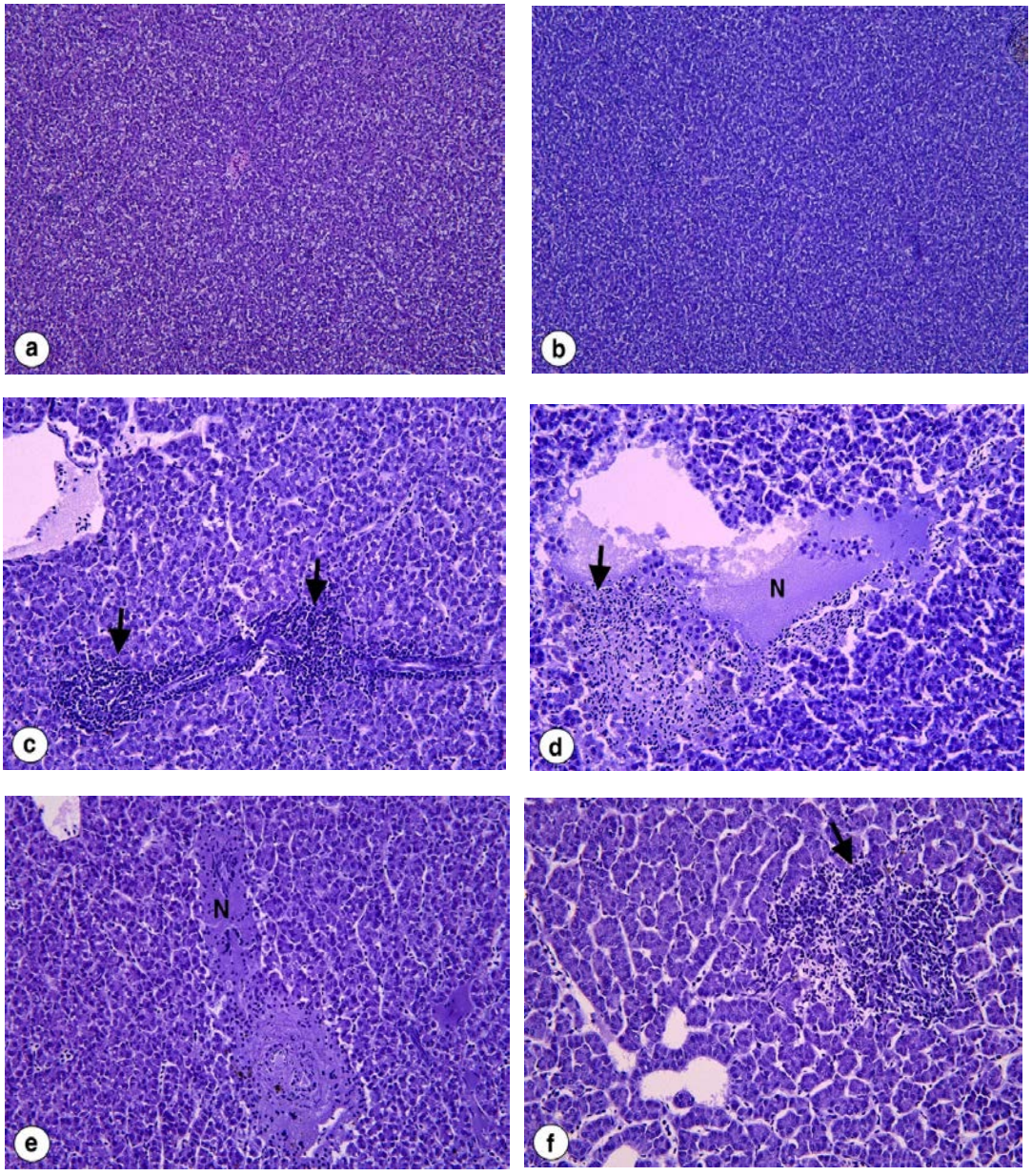


Fig 4.5. Liver histology of rainbow trout fed different combinations of dietary bentonite (2-4%), and iron (Fe) (0.25%). In the (a) Control and (b) low bentonite (LB) treatments, liver showed normal hepatic structure and organization with no evidence of inflammation. Infiltrations of white blood cells (arrowhead) was observed in (c) HB, as well as necrosis (N) in (d) the Fe treatment. Necrosis was observed in (e) the LBFe treatment while infiltrations of white blood cells were observed in (f) HBFe. x 100 magnification (a, b) and x 200 magnification (c-f). Hematoxylin and eosin staining.

Table 4.1.1: Formulation (%) of the experimental diets

Ingredients	Con	LB	HB	Fe	LBFe	HBFe
Fish meal	27	27	27	27	27	27
Soybean meal	5	5	5	5	5	5
Poultry by-product meal	9	9	9	9	9	9
Soy potrien concntrate	9	9	9	9	9	9
Wheat glutein	8	8	8	8	8	8
Corn Protein Conc	9	9	9	9	9	9
Wheat flour	10.5	10.5	10.5	10.5	10.5	10.5
Fish oil	14	14	14	14	14	14
Dicalcium phosphate	1.2	1.2	1.2	1.2	1.2	1.2
Choline chloride (60%)	0.6	0.6	0.6	0.6	0.6	0.6
Vitamin premix	0.8	0.8	0.8	0.8	0.8	0.8
Trace Mineral mixture, Trouw nutr	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin C, Stay C-35)	0.2	0.2	0.2	0.2	0.2	0.2
α-cellulose	5.6	3.6	1.6	5.35	3.35	1.35
Bentonite	0	2	4	0	2	4
Ferrous Sulphate (Chemical formula)	0	0	0	0.25	0.25	0.25
Proximate Composition						
Total Protein (%)	47.12	47.07	47.04	47.08	47.34	47.05
Total Lipid (%)	18.76	18.74	18.71	18.68	18.58	18.72
Ash Content (%)	7.7	8.3	8.5	7.7	8.4	8.6
Fatty acid Composition (% of total fatty acids)						
C14:0 Myristic acid	3.39	3.61	2.90	3.11	3.01	3.17
C16:0 Palmitic acid	15.58	15.68	13.84	15.09	15.31	15.46
C16:1 Palmitoleic acid	4.80	4.93	4.33	4.39	4.40	4.51
C18:0 Stearic acid	3.22	3.07	3.28	3.14	3.17	3.26
C18:1n-9 Oleic acid	38.78	39.08	37.78	37.69	38.26	38.64
C18:2n-6 linoleic acid	7.79	7.60	7.63	7.42	7.63	7.49
C18:3n-3 α-Linolenic acid	1.16	1.08	0.95	0.91	0.89	0.98
C20:1n-9 11-Eicosenoic acid or Gondoic acid	10.77	10.62	11.37	10.35	9.96	10.81

C20:5n-3	Eicosapentaenoic acid	6.81	6.74	5.51	3.71	4.21	6.51
C22:6n-3	Docosahexaenoic acid	6.48	6.43	7.16	6.55	6.67	6.73
ΣSFA		22.19	22.36	20.02	21.33	21.49	21.89
ΣMUFA		54.35	54.63	57.42	58.81	57.94	55.23
ΣPUFA		23.45	23.01	22.56	19.86	20.57	22.87
Σn-3 PUFA		15.03	14.82	14.31	11.82	12.37	14.75
Σn-6 PUFA		9.59	9.27	9.19	8.95	9.09	9.10
EPA/DHA		1.05	1.05	0.77	0.57	0.63	0.97
DHA/EPA		0.95	0.95	1.30	1.77	1.58	1.03

Sources

[a]Rangen Inc., Buhl, ID, USA
[b]Profine VF, The Solae Company, St. Louis, MO, USA.
[c]Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[d]US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as $ZnSO_4 \cdot 7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4 \cdot 5H_2O$), 1.54; I (as KIO_3), 10.
[e]Skretting USA, Tooele, UT, USA.

Table 4.1.2: Muscle fatty acid composition of rainbow trout with supplementation of bentonite and iron at different concentration

Fatty acids		Treatments group					
Type		Con	LB	HB	Fe	LBFe	HBFe
Saturates							
C14:0	Myristic acid	2.93 ± 0.05	2.82 ± 0.08	2.71 ± 0.39	3.09 ± 0.07	3.03 ± 0.07	3.01 ± 0.11
C16:0	Palmitic acid	11.93± 0.66	11.95 ± 0.63	9.70± 1.44	12.03 ± 0.57	12.14 ± 0.67	11.90 ± 0.66
C18:0	Stearic acid	4.27 ± 0.10	4.27 ± 0.11	4.33 ± 0.19	4.13 ± 0.07	4.22 ± 0.10	4.14 ± 0.11
ΣSFA		19.14± 0.65	19.05 ± 0.54	16.75 ± 1.63	19.25 ± 0.48	19.39± 0.59	19.06 ± 0.60
Monoenes							
C16:1n-7	Palmitoleic acid	4.82 ± 0.15	4.64 ± 0.18	5.19 ± 0.13	4.9 ± 0.15	4.99 ± 0.15	5.00 ± 0.15
C18:1n-9	Oleic acid	38.29 ± 0.68	38.63 ± 80	41.23 ± 0.91	39.11 ± 0.57	39.38 ±0.64	39.98 ± 0.47
C20:1n-9	11-Eicosenoic acid or Gondoic acid	10.88 ± 0.63	10.47 ± 0.63	11.95 ± 0.62	10.40 ± 0.55	10.44 ± 0.64	10.65 ± 0.66
C22:1n-9	Erucic acid	2.83 ± 0.24	1.99 ± 0.19	2.28 ± 0.08	2.37 ± 0.21	2.64 ± 0.23	2.35 ± 0.08
ΣMUFA		56.84 ± 1.31	55.74 ± 1.68	60.66 ± 1.56	56.81 ± 1.19	57.47 ± 1.13	58.00 ± 1.09
Polyunsaturate							
C18:2n-6	linoleic acid	6.83 ± 0.19	6.47 ± 0.19	7.21 ± 0.21	6.73 ± 0.17	6.64 ± 0.17	6.76 ± 0.22
C18:3n-3	α-Linolenic acid	1.42 ± 0.14	1.38 ± 0.08	1.07 ± 0.23	1.32 ± 0.11	0.98 ± 0.26	1.41 ± 0.10
C20:2n-6	Eicosadienoic acid	0.45 ± 0.07	0.44 ± 0.06	0.51 ± 0.04	0.41 ± 0.03	0.42 ± 0.04	0.31 ± 0.08
C20:3n-6	Dihomo-γ-linolenic acid	0.23 ± 0.01	0.20 ± 0.03	0.21 ± 0.03	0.17 ± 0.02	0.20 ± 0.01	0.15 ± 0.04
C20:4n-6	Arachidonic acid	0.67 ± 0.01	0.65 ± 0.01	0.55 ± 0.08	0.63 ± 0.01	0.64 ± 0.02	0.56 ± 0.09

C20:5n-3	Eicosapentaenoic acid (EPA)	3.23 ± 0.24	3.69 ± 0.20	3.31 ± 0.08	3.26 ± 0.14	3.01 ± 0.08	3.10 ± 0.15
C22:5n-3	Docosapentaenoic acid	1.69 ± 0.08	1.77 ± 0.06	1.75 ± 0.10	1.58 ± 0.06	1.55 ± 0.07	1.48 ± 0.06
C22:6n-3	Docosahexaenoic acid (DHA)	9.13 ± 1.03	10.19 ± 1.42	7.32 ± 0.31	9.40 ± 1.10	9.17 ± 1.13	8.65 ± 0.91
ΣPUFA		24.01 ± 0.69	25.19 ± 1.20	22.58 ± 0.54	23.92 ± 0.72	23.12 ± 0.63	22.93 ± 0.84
EPA + DHA		12.36 ± 1.26	13.89 ± 1.58	10.63 ± 0.38	12.66 ± 1.17	12.18 ± 1.11	11.76 ± 1.02
EPA/DHA		0.36 ± 0.01	2.69 ± 0.03	0.45 ± 0.01	0.37 ± 0.03	0.35 ± 0.03	0.37 ± 0.02
DHA/EPA		2.77 ± 0.14	6.23 ± 0.26	2.20 ± 0.06	2.88 ± 0.32	3.08 ± 0.44	2.76 ± 0.21
Σn-3 PUFA		15.48 ± 1.04	17.05 ± 1.47	13.47 ± 0.47	15.57 ± 1.01	14.72 ± 0.88	14.65 ± 0.93
Σn-6 PUFA		9.61 ± 0.41	9.15 ± 0.29	9.58 ± 0.31	9.28 ± 0.29	8.90 ± 0.47	9.21 ± 0.42
n-3/n-6 ratio		1.66 ± 0.17	1.90 ± 0.21	1.41 ± 0.06	1.71 ± 0.17	1.71 ± 0.19	1.62 ± 0.15

Data expressed as Mean ± SE, n = 8 per treatment; values along row with different superscript differ significantly ($P < 0.05$). SFA=saturated fatty acid, MUFA=Mono-unsaturated fatty acid, PUFA=Poly-unsaturated fatty acid, EPA= Eicosapentaenoic acid, DHA= Docosahexaenoic acid.

Table 4.1.3: Electrolyte and metal load in the hepatic tissue of rainbow trout following bentonite supplementation (2-4%), iron exposure (0.25%) and their combination.

Treatment	Na (mg/g)	K (mg/g)	Ca (µg/g)	Mg (mg/g)	Cu (µg/g)	Zn (µg/g)
Control	0.92 ± 0.17	3.31±0.39	4.32±0.99	0.14±0.01	20.09±2.37	44.17±4.08
LB	1.68±0.47	2.92±0.34	10.97±2.95	0.23±0.04	30.09±9.34	38.39±8.54
HB	1.14±0.04	3.57±0.06	13.49±1.36	0.22±0.01	30.65±2.8	38.22±3.16
Fe	1.51±0.32	3.57±0.13	13.32±0.79	0.18±0.03	32.37±6.17	38.67±8.07
LBFe	1.32±0.10	3.57±0.05	13.51±0.38	0.17±0.04	25.48±6.12	31.11±9.04
HBFe	1.26±0.03	3.75±0.04	15.61±1.11	0.22±0.01	29.79±0.92	37.49±4.21

Table 4.1.4: Electrolyte and metal load in the plasma of rainbow trout following bentonite supplementation (2-4%), iron exposure (0.25%) and their combination.

Treatment	Na (mg/g)	K (mg/g)	Ca (µg/g)	Mg (mg/g)	Cu (µg/g)	Zn (µg/g)
Control	3.46 ± 0.17	0.30±0.02	19.51±1.28	0.03±0.01	0.06±0.01^{ab}	22.14±1.20
LB	4.09±0.13	0.19±0.02	18.15±0.89	0.02±0.02	0.07±0.01^{ab}	19.04±1.18
HB	4.14±0.35	0.18±0.02	17.73±0.69	0.03±0.01	0.08±0.01^{bc}	20.05±1.39
Fe	4.00±0.30	0.18±0.03	17.23±0.75	0.02±0.03	0.06±0.02^{a}	20.82±1.95
LBFe	4.47±0.25	0.15±0.01	15.84±0.43	0.01±0.02	0.09±0.03^{c}	21.31±1.55
HBFe	4.24±0.15	0.15±0.02	16.15±0.65	0.01±0.01	0.06±0.01^{ab}	24.01±1.83

Table 4.1.5: Effects of iron exposure, Bentonite supplementations and their interaction on growth, oxidative index and antioxidant enzymes, metals/electrolyte parameters and fatty acids in the rainbow trout after 8 weeks of the experimentation

Treatment	Iron		Bentonite		Iron × Bentonite	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*
Production						
WG%	8.807	0.011	0.904	0.427	3.007	**0.082**
SGR	9.561	0.008	1.201	0.331	3.324	0.066
FCR	10.066	0.077	1.449	0.268	0.028	**0.973**
Antioxidant Enzyme						
CAT	345.481	0	926.92	0	27.596	**0**
SOD	24.923	0	111.147	0	11.097	**0.001**
GR	1621	0	146.052	0	24.852	**0**
Metals and electrolyte (Plasma)						
Fe	32.065	0	27.598	0	27.518	**0**
Cu	0.617	0.446	1.041	0.381	6.161	**0.013**
Zn	1.921	0.191	0.274	0.764	0.749	0.492
Ca	7.201	0.019	0.121	0.887	0.444	0.651
Mg	4.889	0.046	3.111	0.079	2.908	0.092
Na	4.837	0.047	3.885	0.048	0.308	0.741
K	17.132	0.001	7.511	0.007	1.991	0.176
Metals and electrolyte (Liver)						
Fe	355.651	0	75.673	0	182.858	**0**
Cu	0.253	0.621	2.139	0.143	0.246	0.784
Zn	0.339	0.566	0.143	0.867	1.589	0.228
Ca	12.419	0.002	9.067	0.001	3.867	**0.037**
Mg	0.017	0.897	1.116	0.346	1.418	0.264
Na	0.636	0.434	6.081	0.008	2.744	0.087
K	0.111	0.742	1.146	0.337	0.715	0.501
Fatty acids (Muscle)						
ΣSFA	1.713	0.198	1.521	0.235	0.956	0.393
ΣMUFA	0.081	0.778	2.407	0.103	1.295	0.285
ΣPUFA	0.852	0.361	1.74	0.188	1.29	0.286
EPA	4.732	0.035	0.416	0.662	2.516	0.093
DHA	0.051	0.822	1.387	0.261	0.625	0.54
EPA+DHA	0.011	0.919	1.318	0.279	0.791	0.46
Σn-6 PUFA	1.088	0.303	0.747	0.48	0.014	0.986
Σn-3 PUFA	0.179	0.675	1.758	0.185	1.538	0.227

EPA/DHA ratio	2.497	0.122	1.422	0.253	1.127	0.334
DHA/EPA ratio	2.45	0.125	1.238	0.301	0.351	0.706
n-3/n-6 ratio	0.034	0.855	1.438	0.249	0.67	0.517

CHAPTER 5

THE DIETARY SUPPLEMENTATION OF VITAMIN C AND BENTONITE IMPROVE THE FISH GROWTH AND ALLEVIATE IRON TOXICITY IN RAINBOW TROUT, *Onchorhynchus mykiss*

5.1 Introduction

Iron is a trace element that plays a vital role in several physiological processes, including fish, like, oxygen transfer, energy production, and DNA synthesis (Bury and Grosell, 2003; NRC, 2011; Luo et al., 2017; Ganz and Nemeth, 2015; Tarifeno - Saldivia et al., 2018). While iron is a vital element for a variety of physiological processes in an animal's body, (Aisen et al. 2001) excessive iron accumulation in tissue over the threshold limit can induce toxic consequences, leading to cell and organ damage (Aisen et al. 2001; Lebster, 2014). In the Fenton reaction (Fenton, 1894), iron serves as a catalytic component, promoting the production of free radical species (ROS) (Li et al., 2009; Baker at al., 1997). ROS buildup can damage lipids, proteins, and DNA, lowering fish physiology, biochemistry, and metabolism, and even causing cell death in extreme circumstances (Bagnyukova et al., 2006; Bresgen and Eckl, 2015; Li et al. 2009; Papanikolaou and Panto, 2005). Fish are able to absorb soluble forms of iron from natural water through the gill membrane and intestinal mucosa (Roedar and Roedar 1966; Sealey et al., 1997), however, the concentration of soluble iron in natural water is low and passage of iron across the gills is minimal. Hence, feed is regarded to be a primary source of iron for fish (NRC 1993; Bury et al., 2003).

In both mammals (Knutson et al., 2000) and fish (Wang et al., 2009; Zhang et al., 2016; Luo et al., 2017; Guo et al., 2018), iron deficiency has been found to alter oxidative stress. There are so many studies on the effects of iron on the fish immunological response (Lim et al., 2000; Rigos et al., 2010; Behera et al., 2014; Zhang et al., 2016), growth performance, changes in hematological parameters, disease susceptibility, and microcytic anemia (Tacon 1992; Andersen et al., 1996; Sakamoto and Yone 1978). As a result, the importance of iron in aquatic species' diets

for optimal growth and avoidance of various deficient symptoms has been highlighted. Excess iron in the feed is also detrimental to fish because it slows growth, increases mortality, causes diarrhea, and impairs hepatic function (NRC 2011). Iron has a toxic effect on rainbow trout when it is consumed at a greater rate (1380 mg/kg) in their diet (Desjardins. 1985).

The involvement of free or loosely chelated iron in the uncontrollable generation of ROS and tissue damage is still a common aspect of iron overload circumstances. As a result, effective scavenging of excess iron could be a potential strategy to limit and alleviate tissue damage caused by free radicals. Iron chelation is becoming more popular as a treatment for a wide range of iron-related illnesses. Chelators not only remove iron from the body, but they also scavenge and bind free iron to prevent reactive oxygen species (ROS) from being produced (Hatcher et al., 2009; Hershko 2010). Dietary chelator supplementation, which is less expensive and more widely available, could be an alternate source for reducing feed-borne iron toxicity. Dietary supplementation of bentonite and vitamin C could play an important in this regard.

Bentonite has superior binding (adsorption) properties, high cation exchange capacity, natural occurrence, and relatively low cost. In the contemporary setting, bentonite's importance as a feed supplement for aquaculture methods is progressively increasing. A varying level of bentonite supplementation (1-5%) in the diet of rainbow trout (*Oncorhynchus mykiss*) enhanced their growth performance and feed conversion ratio (Obradovic et al., 2006; Mumpton 1999; Eye et al., 2008). Evidence suggests that tilapia (Tilapia zillii) fed 1.0 percent and 2.0 percent bentonite reduced ammonia and ammonia output significantly compared to those fed a control diet without bentonite (Yildirim et al., 2009). In a similar study, Ergün et al. (2008) found that giving dietary trout mixed with 2.5 percent zeolite (bentonite) reduced ammonia discharge by 24 percent when compared to the control group.

Vitamin C has been found to have an antioxidant protective effect against the toxicity of waterborne metals (such as lead, iron) in a variety of fish species (Nourian et al., 20190; Shahsavani et al., 2017; Yadav et al., 2020). Also, it functions as a

chelating agent for some of the metals (like arsenic and lead; Flora et al., 2012; Kalia and Flora, 2005; Patrick, 2006) which helps in limiting the metal bioaccumulation in the tissues. Furthermore, existing evidence suggests that vitamin C aids iron absorption from the intestine by converting Fe3+ to a more soluble Fe2+ form state (Chen et al., 2015; Lim et al., 2000). Due to the lack of L-gluconolactone oxidase in most teleosts (Fracalossi et al., 2001), exogenous vitamin C supplementation in fish diets is required. A higher dose of vitamin C is often used in aquatic animals to enhance their resistance to pathogens (Anbarasu and Chandran 2001; Liang et al., 2017; Lim et al., 2000) and to mitigate environmental stressors such as trace elements (lead, copper, zinc, cadmium, iron; Fox et al., 1980; Jiraungkoorskul and Shaphong, 2007), elevated ammonia (Mazik et al., 1987; Wang et al., 2005), salinity stress (Merchie et al., 1996), hypoxia (Mazik et al., 1987) and thermal shock (Falcon et al., 2007).

One of our recent research findings suggests that supplementation of vitamin C (143 or 573 mg/kg diet) or bentonite (2%, unpublished, Yadav et al.,) in the diet of catfish and trout have (Yadav et al., 2020) positive effects to reduce feed-borne iron toxicity. However, the combined effect of bentonite and vitamin C for reducing iron toxicity hasn't been studied so far. In these circumstances, the current study was designed to determine whether feed-borne iron (0.25%) mediated toxicity in rainbow trout can be mitigated via dietary supplementation of vitamin C (500, 1500, 3000 ppm), 2% bentonite with or without in combination. To meet our objectives, the current study looked at the effects of dietary bentonite and vitamin C supplementation and its ability to reduce the iron toxicity in rainbow trout by measuring: (i) weight gain (%), ii) iron and mineral load on the liver, and digesta, (iii) hepatic MDA level and antioxidant enzyme gene expression, (iv) histopathological examinations of liver and gills; and (v) gene expression of iron metabolism.

5.2 Materials and methods

5.2.1 Fish and Experimental Settings

A total of 600 rainbow trout fingerlings (average weight: 1.0 ± 0.02 g) were collected from a clear spring near Buhl, Idaho, and transferred to the Aquaculture Research Institute's cold-water facility in Moscow, Idaho. Fish were placed in two 360-L fiberglass aquariums when they arrived. The fish were fed skretting oncore trout diet (protein: 52%, oil: 16%) twice daily during the acclimatization stage until they reached a size of 2-3 g. Water quality parameters such as temperature and pH is maintained around 15.3ºC and 7.3, respectively. Ammonia level was around 0.03 mg/l. This experiment was approved by the University of Idaho's Institutional Animal Care and Use Committee including experimental protocols for fish handling and sampling in advance (IACUC-2020-40).

560 rainbow trout (6.41±0.06 g start weight) were randomly stocked into 28 flow-through experimental tanks (20 fish per 60 L tank), with each tank assigned to a different treatment group.

5.2.2 Experimental Diets and Feeding

Fish fed seven isonitrogenous (45% crude protein) and isolipidic (20% lipid) diets at three different levels (500 ppm, 1500 ppm, and 3000 ppm) of vit. C with or without 2% bentonite and/or 0.25% iron. The diets are designated as follows: (i) Con (Control; Basal diet acts as a positive control), (ii) ConFe (control diet supplemented with 0.25% iron as $FeSo_4$: acts as a negative control), (iii) BenFe (Control diet supplemented with an optimum dose of vit. C, 500 ppm, bentonite 2%, and iron 0.25%), (iv) MVCFe (medium higher dose of vit. C, 1500 ppm and iron 0.25%), (V) MVCFeBen (medium higher dose of vit. C, 1500 ppm, iron 0.25%, and bentonite 2%), (VI)HVCFe (high dose of vit. C, 3000 ppm and iron 0.25%) and (VII) HVCFeBen (high dose of vit. C, 3000 ppm, iron 0.25%, and bentonite 2%), respectively. The inclusion levels of Vitamin C are 500, 1500, and 3000 mg/kg of Vitamin C in the form of Stay-C (35% equivalent, L-ascorbyl-2-poly phosphate). The feed was formulated to meet the requirement of rainbow trout (NRC, 2011). Supplementation of Vitamin C at a dose of 1500 mg/kg was based on the wound healing and disease resistance (Halver et al., 1969; Wahli et al., 2003) whereas the 3000 mg/kg is based on the enhancement of immune response and enhancing growth followed by copper toxicity (Liu et al., 1989;

Lanno et al., 1985). The level of bentonite at 2g/kg was chosen for enhancing growth and alleviating dietary iron toxicity in a previous study (Unpublished Yadav et al). The composition of experimental diets is summarized in table 1. Fish meal (FM), poultry by-product meal (PBM), and soy protein concentrate were the three most common sources of dietary protein. Fish oil (FO) was used as a source of lipid. Briefly, the feed ingredients were fully mixed in an industrial mixer (Hobart A200T 20-quart mixer, Hobart Production Facilities, Troy, Ohio, USA), hydrated to form a soft dough, and extruded using a 3.2mm die. The diets were then dried in a dryer for 24-48 hours, sealed in a plastic bag, and kept at 4°C until needed. The diets' proximate makeup was determined according to AOAC guidelines (AOAC 2005).

For 10 weeks, the fish were hand-fed to apparent satiation twice a day, six days a week. To avoid iron blockage, the tanks were cleaned twice a day. The photoperiod was kept at 14 hours of light and 10 hours of darkness using electric timers, and the water temperature was kept at 15.40 degrees Celsius. pH (7.3), DO (7.2 mg/l), and ammonia (0.03 mg/l) were among the other water quality characteristics evaluated.

5.3 Sampling Procedure

Fish were fasted for 24 hours before sampling after a 10-week feeding trial. The fish were batch weighed after being anesthetized with tricaine methanesulfonate (MS 222, 50 mg/L). Two fish per tank (8 fish per group) were selected at random, were sacrificed and the liver was taken for stress enzyme activity. For histological investigation, additional 2 fish per tank were sacrificed, and a little part of the liver and gill were taken and stored in Bouin's solution and phosphate-buffered formalin, respectively. Also, the liver was taken from additional two fish for the analysis of gene expression (antioxidant and iron metabolism).

5.4. Analytical techniques

5.4.1 Histological procedure and analysis

The liver, gills, and intestine were placed in 10% (V/V) phosphate-buffered formalin and Bouin’s solution, respectively. The samples were transferred to 70%

(V/V) ethanol after 18 hours. The dehydration of the samples was carried out in a graded level of alcohol and xylene was used to cleanse the alcohol in a Tissue-Tek Vacuum Infiltration Process (Sakura Finetek Inc., Torrance, CA). After that, the samples were embedded in a paraffin wax. 5µm sections were prepared by using Rotary microtome (HM 340E, Thermo Scientific) and stained with hematoxylin and eosin (H&E). A light microscope (Leica DM 2500 LED) was then used to take pictures.

5.4.2 Determination of iron concentration in liver, muscle and digesta

Liver, muscle, and digesta samples were placed in an oven at 48ºC for 48 h for drying. After that, digestion step was carried out in a heat block (Environmental Express, SC, USA) at 115ºC for 30 min in a mixture of 4.0ml trace-metal-grade HNO_3 (69%) and 0.1 ml of H_2O_2. Digested solutions were then diluted to 50 ml with Mili-Q-water. Iron determination in the liver, muscle and digesta was performed with an AAS (iCE 3000 series, Thermo Scientific, USA) with deuterium lamp background correction and calibration using iron standard solutions (CPI International, CA, USA).

5.4.3 Molecular analysis

Total RNA was isolated from liver samples using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Isolated RNA samples were treated with DNA-free (DNase) treatment to avoid genomic contamination. Quantification of RNA was done using a NanoDrop 2000 (Thermo Fisher, Waltham, MA). Also, quality was assessed by denaturing gel electrophoresis (1% agarose gel) and purity by OD_{260}/OD_{280} nm absorption ratio >1.95.

Using a High-Capacity cDNA Reverse Transcription Kit, 0.5 g RNA was reverse transcribed to cDNA for quantitative real-time PCR (qPCR) (Applied Biosystems, Foster City, CA, USA). The specific primers for HAMP, HO1, GPX1, CAT, and reference gene β-actin, EF1-α are listed in the table 5.1.2. qPCR analyses were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to Egnew et al., 2019.

Melting curve analysis was used to confirm the specificity of PCR reactions for target and reference genes, yielding in single products with particular melting temperatures. Additionally, for each set of genes, ‘no-template' controls (i.e., with water sample) were measured to ensure that reagent contamination and primer–dimer formation were not a problem. The primer efficiency is shown in table 5.1.2.

5.5. Statistical analysis

The data is presented as mean ± standard error. The Shaprio Wilk test was used to check for normality, while the Levene test was used to check for variance homogeneity. A one-way analysis of variance (ANOVA) was used to compare the experimental groups, followed by Duncan multiple range tests. The data for iron concentration in muscle were log transformed for the normalization.

A significance level (P value) <0.05 was assessed significant. SPSS software version 20.0 was used for all analyses.

5.6. Results

5.6.1 Growth Performance

Following 10 weeks of the experiment, the weight gain (%) was significantly higher in the positive control (Con) group compared to the negative control group (ConFe). Also, weight gain was significantly ($P<0.05$) augmented in BenFe, MVCBenFe, HVCFe, and HVCBenFe group compared to the negative control group, ConFe. No significant differences ($P>0.05$) were seen in BenFe, MVCFe, MVCBenFe, and HVCFe groups, however, the HVCBenFe group showed higher weight gain (%) are significant with those groups.

5.6.2. Histological analysis

Gills. The gills of the Con group were normal in structure with well-defined primary and secondary lamellae, and cartilage. However, aneurisms, excessive mucus secretion, intralamellar cell mass deposition were observed in the ConFe group. Deposition cell masses in the intralamellar spaces, mucus secretion, increased

in size of goblet cells were seen in the rest groups, however, it was less pronounced in the HVCBenFe group compared to the Confe group.

Liver. Likewise, normal sinusoid organization, uncongested central veins in Cn, and BenFe groups were observed. Similarly, MVCFeBen, HVCFe, and HVCBenFe group some congestions, and no signs of inflammation were observed that could show signs of injury. However, hemorrhage, necrosis, hydropic vacuolization, and congestion was observed with the inclusion of iron in the diet in the ConFe and MVCFe group.

5.6.3. Iron load in the liver and digesta

Liver. Figure 5.2 (A, B) depicts the trend of iron build up in the liver and digesta. Higher dietary iron had a significant impact ($P<0.05$), with the iron concentration in the liver of the ConFe group significantly higher compared to Con, and other groups.

Similarly, the efflux of iron was significantly lower in the ConFe, and MVCFe groups compared to the Con, MVCFe, HVCFe, and HVCBenFe groups. A higher efflux of iron was seen in the BenFe group. No significant difference in the efflux of iron was seen in ConFe and MVCFe groups.

5.6.4. Effect of higher dietary iron on MDA activity in Liver

The stress enzyme i.e. malonaldehyde (MDA) activity was significantly higher in ConFe group compared to the Con and rest of the groups (Fig 5.3). No significant differences were seen in BenFe, MVCFe, HVCFe, and HVCBenFe groups. No differences were seen in Con and MVCBenFe groups in MDA activity.

5.6.5 Effect of higher dietary iron on gene expression

The result of the qPCR showed that higher dietary iron inclusion caused significant downregulation of the expression of all the antioxidant genes in the ConFe group compared to all other groups at the end of 10 weeks of experiment (Fig 5.4). The supplementation of vitamin C (Medium, high), bentonite (2g/kg), or in combination

significantly increased the expression of SOD and CAT in all groups compared to the ConFe group. The expression of SOD and CAT were higher in HVCBenFe among all the groups. However, significant upregulation of GPX1b was seen in the ConFe group compared to the Con group. Also, a higher expression of GPX1b was seen in the HVCFe group compared to all other groups. Hepcidin (HAMP), a key regulatory iron hormone, was upregulated in the ConFe group compared to the Con group. Also, HAMP was upregulated in BenFe, MVCFe group compared to Con, MVCBenFe, HVCFe, and HVCBenFe groups. Heme oxygenase (HO1) was upregulated in the ConFe group and is significant ($P<0.05$) with the rest of the groups. HO 1 activities were downregulated in BenFe, MVCBenFe group but are non-significant ($P>0.05$). No significant differences were seen in Con and MVCBenFe group, however, the further downregulation of H01 were seen in HVCFe, HVCBenFe groups, and were significant ($P<0.05$) compared to Con, MVCFe, and MVCBenFe groups.

Also, transferrin (Trf) were upregulated in ConFe, BenFe, and MVCFe group, and are non-significant ($P>0.05$). However, all these groups were statistically significant ($P<0.05$) compared to the Con, MVCBenFe, HVCFe, and HVCBenFe groups.

5.6. Discussion

In the present study, higher dietary iron supplementation retarded the weight gain (%), which is in accordance with the result for rainbow trout, grass carp, and African catfish (Desjardin et al., 1987; Zhang et al., 2016; Baker et al., 1997). Similar results were seen with the higher supplementation of dietary iron in poultry (Vahl and Van., 1987). Desjardin et al., (1987) stated that >1250 mg/kg of iron results in toxic effects on trout, which is consistent with our findings, suggesting the higher dietary iron exert a toxic effect on rainbow trout. There are reports that suggest that dietary iron toxicity can be mitigated by dietary vitamin C in Atlantic salmon (Anderson et al., 1998; Maage et al.,) as well as channel catfish (Lim et al., 2000). Also, a dose of 143 or 573 mg/kg of vitamin C can be used to enhance the growth and alleviate iron toxicity in catfish (Yadav et al., 2020), but in this study the medium dose of vitamin C (1500 mg/kg) doesn't improve their weight gain (%) in MVCFe group, suggesting that

medium dose is not able to ameliorate the toxic effect of iron in rainbow trout. However, a higher dose of vitamin C (3000 mg/kg) significantly enhance the WG % in the HVCFe group. This indicates that a higher dose of vitamin C can be used to improve the growth performance of rainbow trout.

Similarly, there are several studies indicating that the addition of zeolites in the fish diet enhances their growth performance. For example, the inclusion of clinoptilolite (2%), bentonite, and mordenite (5 and 2.5%) in the diet of trout increase their weight gain, feed efficiency, respectively (Leonard, D.W., 1979; Eye et al., 2008). Likewise, the addition of zeolite in rainbow trout diets (2.5-5%) promoted their weight gain and feed efficiency (Lanari et al., 1996). Also, the inclusion of montmorillonite about 0.5% in the diet of carp and tilapia has shown to alleviate the dietary borne cadmium and lead toxicity, respectively (Dai et al., 2010; Kim et al., 2011). However, to the best of our knowledge, this is our first study to observe the combined effect of vitamin C (medium and high doses) and bentonite (2%) at higher dietary iron inclusion in their growth performance. Based on the growth results, it can be said that it has a synergistic effect when there is a higher dietary iron.

Iron toxicity is typically attributed to its ability to cause lipid peroxidation in cellular membranes. As a result, increased iron content in the hepatic tissue in the ConFe group could explain the overproduction of MDA, which is an end product of lipid peroxidation and a sign of oxidative damage (Lushchak, 2011). This might be a cause of higher dietary iron inclusion suppressing the growth and physiological capacity of fish in the ConFe group. A histopathology is an important tool for investigating the effects of chemicals (Pollutants) on the critical process including reproduction and growth (Adams, 2002). Fish organs like the liver, gills, kidney, blood parameters are sensitive markers for the aquatic pollutant, with exposure to the chemical’s materials causes several injuries to the liver including gills (Oliveira Ribeiro et al., 2002, 2006). The liver plays a vital role in the detoxification process, high enzymatic activity in the degradation of toxic compounds, however, it might affect adversely itself at higher concentrations (Brusle et al., 1996). Histopathological alterations like hemorrhage, congestion, necrosis, vacuolization are reported in

several studies (Onita et al., 2021; Mela et al., 2007; Akbary and Jahanbakhshi 2019; Ostaszewska et al., 2016; Hao et al., 2009; Al-bairuty et al., 2013). These findings are in accordance with our findings for the ConFe group.

Hepatic vacuolations could be related to lipid and/or glycogen accumulation (Myers et al., 1987; Abalaka 2015), which could indicate metabolic problems as a result of hazardous chemical exposure (Pacheco and Santos 2002). Cellular infiltrations in the ConFe, MVCBenFe group damaged liver suggested that the affected liver were indicative of inflammatory reactions. The observed hemorrhage could be attributable to toxic damage to the fish liver, whereas necrotic lesions in the damaged liver were caused by metal toxic effects (Mohammed 2008; Abalaka 2015).

Likewise, in response to higher dietary iron inclusions, the gills showed several structural changes, which include formation and deposition of interlamellar cell masses at the interlamellar gap, aneurysm, mucus secretion, and goblet cells. According to Martinez et al (2004), aneurysm results from blood leakage with the lamellae and rupture of the pillar cell, and dilation of blood vessel, which reflects a harmful effect of xenobiotics on branchial tissue (Simonato et al., 2008). Mucus plays vital role in fish gills (Saboaia-Moraes et al., 2005), primarily helps in lubrication and protection against pathogenic microorganism (Zaccone et al., 1989; Whitear and Mittal 2006), ion regulation and diffusion in fish (Handy et al., 1989). These anomalies are in accordance with the previous findings (Li et al., 2009; Singh et al., 2019; Mohamed 2008; Abalaka 2015). These anomalies were lessened with the increment in vitamin C dose in combination with the bentonite, indicating that vitamin C in combination with bentonite can mitigate the negative effect of higher dietary iron.

Dietary vitamin C has been shown to reduce dietary iron toxicity in Atlantic salmon (Andersen et al., 1998; Maage et al., 1990) and channel catfish (Lim et al., 2000; Yadav et al., 2019). To our knowledge, this is the first study to look at the effects of dietary vitamin C, bentonite, and/or in combination. In mammalian models, Lynch and Stoltzfus (2003) claimed that dietary vitamin C supplementation provides some protection against iron toxicity by chelating Fe3+ in the liver. Indeed, in the current investigation, the hepatic iron burden was significantly reduced in higher dietary iron

fish provided vitamin C and bentonite (BenFe, MVCFe, MVCBenFe, HVCFe, HVCBenFe groups) compared to higher dietary iron fish fed a vitamin C at basal level (ConFe group). On the basis of the oxidative stress/antioxidant gene expression, vitamin C, bentonite, and/or in combination provided protection against iron toxicity. At both doses, vitamin C, bentonite, and in combination reduced the hepatic MDA concentration of iron-exposed fish compared to the ConFe group, as well as up-regulating SOD and CAT activity above the higher dietary iron fed fish. These data show that any excess MDA produced by iron exposure was rapidly cleared by vitamin C and bentonite-mediated increases in the antioxidant defense systems SOD, CAT, and GPX1.

Hepcidin (HAMP), a key regulator of iron hemostasis, has a strong influence on erythrocyte formation. Hepcidin is normally produced in the hepatocytes of the liver, although it has been found in other cells and organs (Yang et al., 2013). The liver is thought to play a major role in the regulation of systemic iron metabolism, as it produces and releases large amounts of hepcidin, which inhibits ferroportin-mediated iron release from hepatocytes, macrophages, and enterocytes (Ganz 2005; Ramey et al., 2010). High hepcidin levels cause anemia and iron restriction erythropoiesis, impeding intestinal iron absorption and macrophage recycling. The HAMP genes encode hepcidin in mammals, including some teleost (Douglas et al., 2003), like rainbow trout (Alvarez et al., 2013). In the present study, the trout fed iron without supplementation of vitamin C and bentonite had higher expression of HAMP compared to the rest of the groups which are in accordance with a zebrafish and sea bass experiment, indicating that hepcidin expression increases in whole embryos after iron dextran injections (Frankel et al., 2005) and iron overload conditions (Rodrigues et al., 2006), respectively. This suggests that with the supplementation of vitamin c and bentonite helps in antagonizing the inhibitory effect of hepcidin.

Heme oxygenase 1 (HO1) enzyme helps in the conversion of free heme into iron, biliverdin, and carbon monoxide (Ferris et al., 1999; Immenschuh et al., 2010), upregulated by substrate heme and various oxidative stress stimuli. Increased concentration of heme cause induction of HO 1, helping in reduction of free heme.

Raval and Lee (2010) stated that excess heme acts as a pro-inflammatory and pro-oxidant agent. In the present study, we found that HO 1 is highly increased in the liver of ConFe group trout, implying that HO 1 is more sensitive to oxidative stress caused by intracellular iron. We believe that an increase in HO 1 and HAMP (negative regulation of iron export), may result in the rapid accumulation of potentially damaging free irons in the liver contributing to the infiltration of inflammatory cells in the liver and higher MDA production, which was also found in the experiment conducted by the McDonald et al. (2011) in the mouse liver fed with higher dietary iron. The additional release of ROS in inflammatory conditions worsens the pathophysiological conditions as indicated by Immenschuh et al. (2010). Also, in the present study, we found that the supplementation of vitamin C, bentonite in combination is able to reduce the expression of both HAMP and HO, ultimately reducing the MDA production, infiltration of inflammatory cells, resulting in decreased iron load in the liver.

On the other hand, in the liver, transferrin in trout seems to act as a positive acute-phase protein, with increased expression with the supplementation of higher iron in the diet. Transferrin act as a negative acute-phase protein in humans (Ritchie et al., 1999), whereas in mammals and fish (Bayne and Gerwick 2001; Congleton and wagner 1991; Schreiber et al., 1989) can act as a positive or negative acute-phase protein. Transferrin acts as a positive acute-phase protein in Channel Catfish and was upregulated and magnified with the injection of iron dextran along with infected with the bacterial infection (Liu et al., 2010). Neves et al (2009) stated the basal level of transferrin is sufficient to deal with the excess of iron introduced in the system, however, in our experiment, this was not the case. The basal level of transferrin were not sufficient to deal with the excess iron but the combination of bentonite and vitamin C helped in the regaining the transferrin level compared to the control group which were not statistically significant.

5.7 Conclusions

Results showed that a high level of feed borne Fe caused deleterious effects on growth, which was likely related to hepatic oxidative stress, Fe load in the liver as well as adverse histopathological changes in the liver and gills. The changes in the

gene expression of heme oxygenase, transferrin, hepcidin further suggest that higher levels of Fe in the feed are deleterious to fish health at the molecular level. It was obvious, however, that dietary supplementation of bentonite (2%), vitamin C (1500 or 3000 mg/Kg diet) and in their combination suppressed the deleterious effects of Fe exposure on growth, a protective effect against gill/liver damage likely by minimizing lipid peroxidation (as indicated by lower MDA levels) by upregulating the gene expression of SOD2, CAT, and GPX1. This was also complemented with the downregulation of genes responsible for maintaining Fe homeostasis. In summary, dietary supplementation of vitamin C at medium and higher doses alone or in combination with bentonite appears to be a promising treatment to mitigate the harmful effects of feed borne Fe in rainbow trout.

5.8 References

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Figures

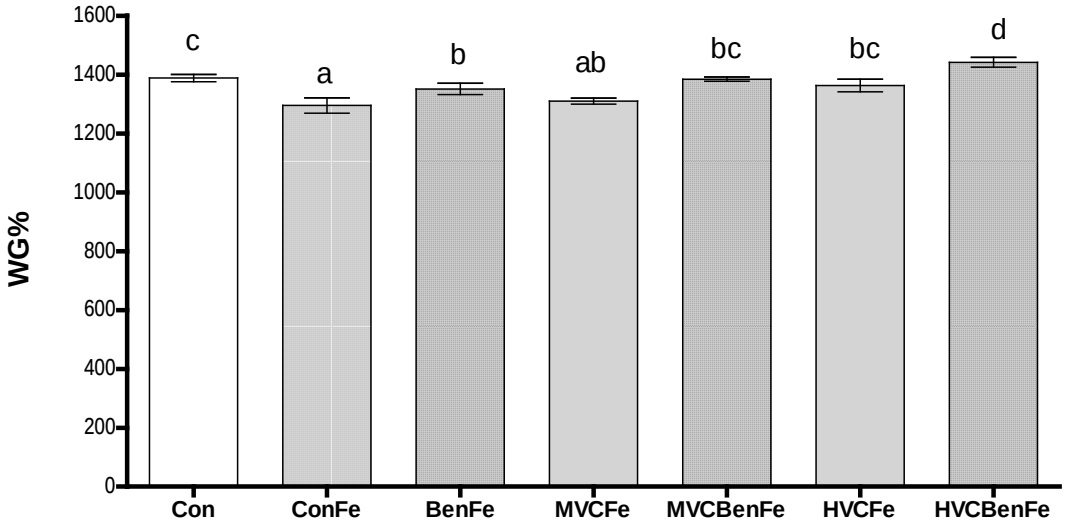


Figure 5.1. The effect of dietary vitamin C, bentonite supplementation (2%), iron exposure (0.25%) and their combination on weight gain (%), (B) in rainbow trout during 10-week trial. Values are mean ± S.E. smalll letters (a, b, c, d) denote significant difference between experimental groups (Number of replicates (N)=4,).

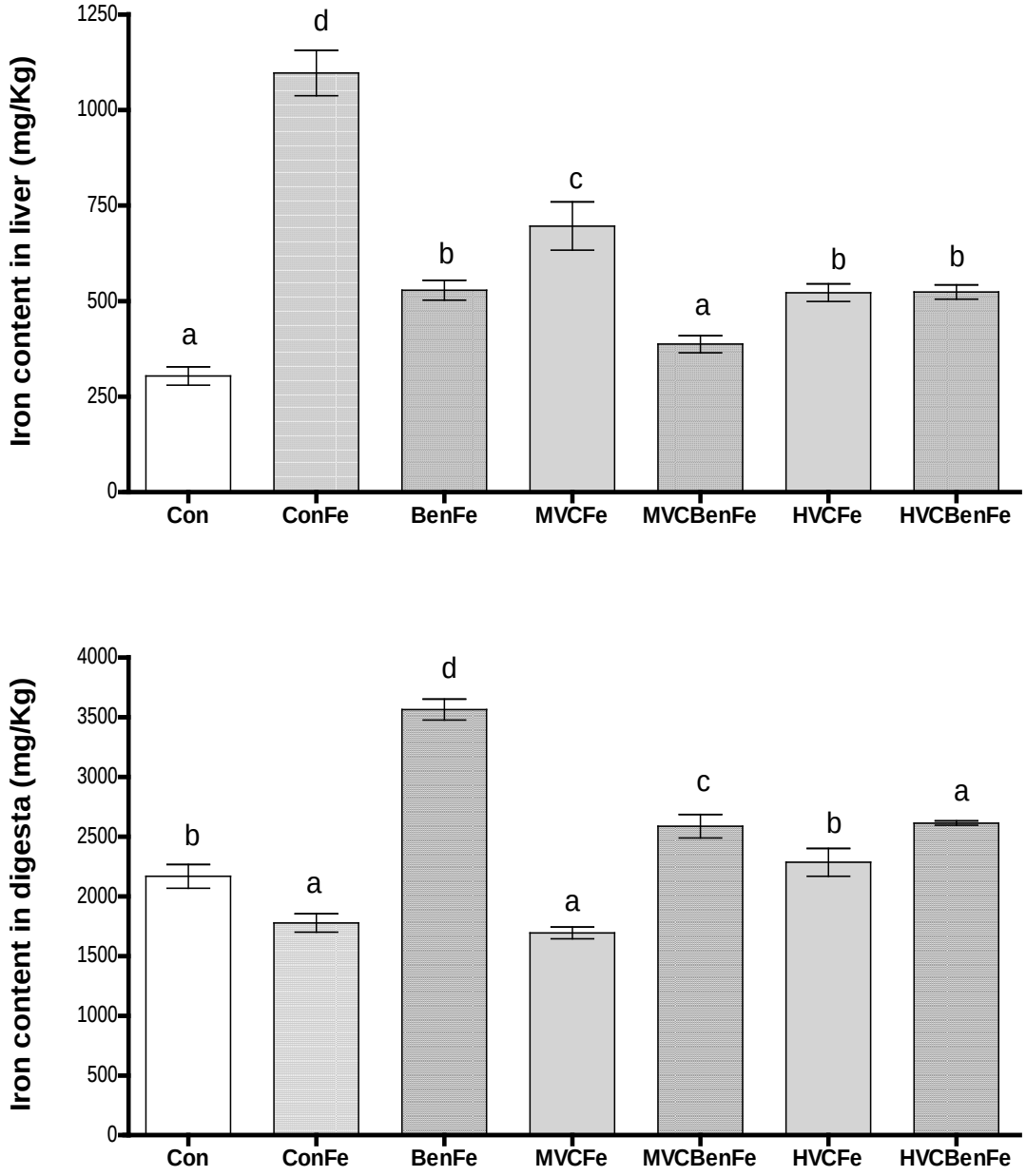


Figure 5.2. Iron accumulation in the hepatic tissue and excreted iron in the digesta of rainbow trout following vitamin C, bentonite supplementation (2%), iron exposure (0.25%) and their combination. Values are mean ± S.E. small letters (a, b, c, d) denote significant difference between experimental groups (Number of replicates (N)=4).

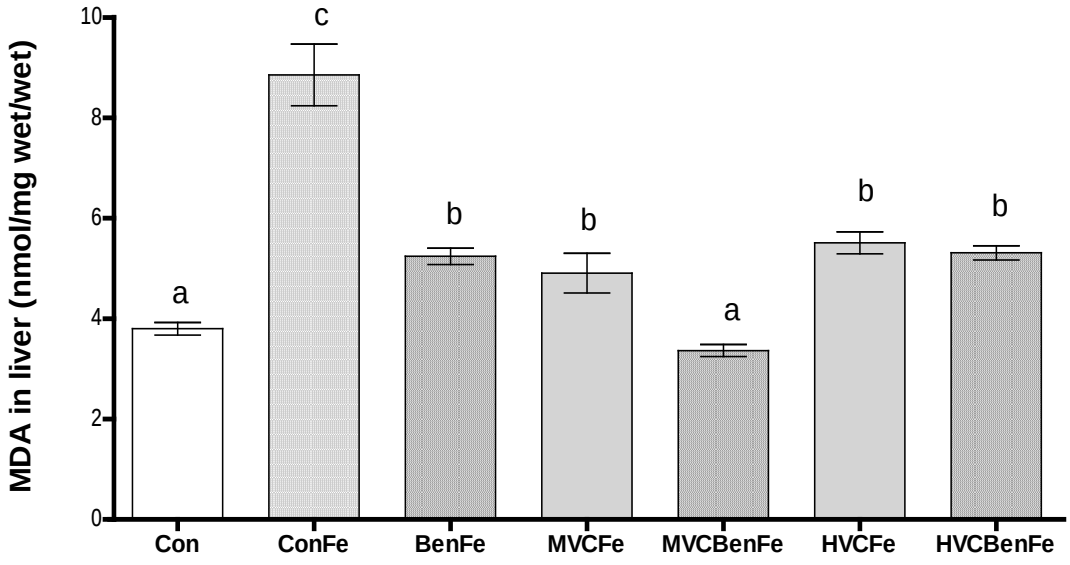


Figure 5.3. Effect of iron exposure (0.25%), vitamin C, bentonite supplementations (2%) and their combination on malondialdehyde (MDA) content, activity in liver of rainbow trout during 10 weeks' time interval. Values are mean ± S.E. Small letters (a, b) denote significant difference between experimental groups (Number of replicates (N)=4).

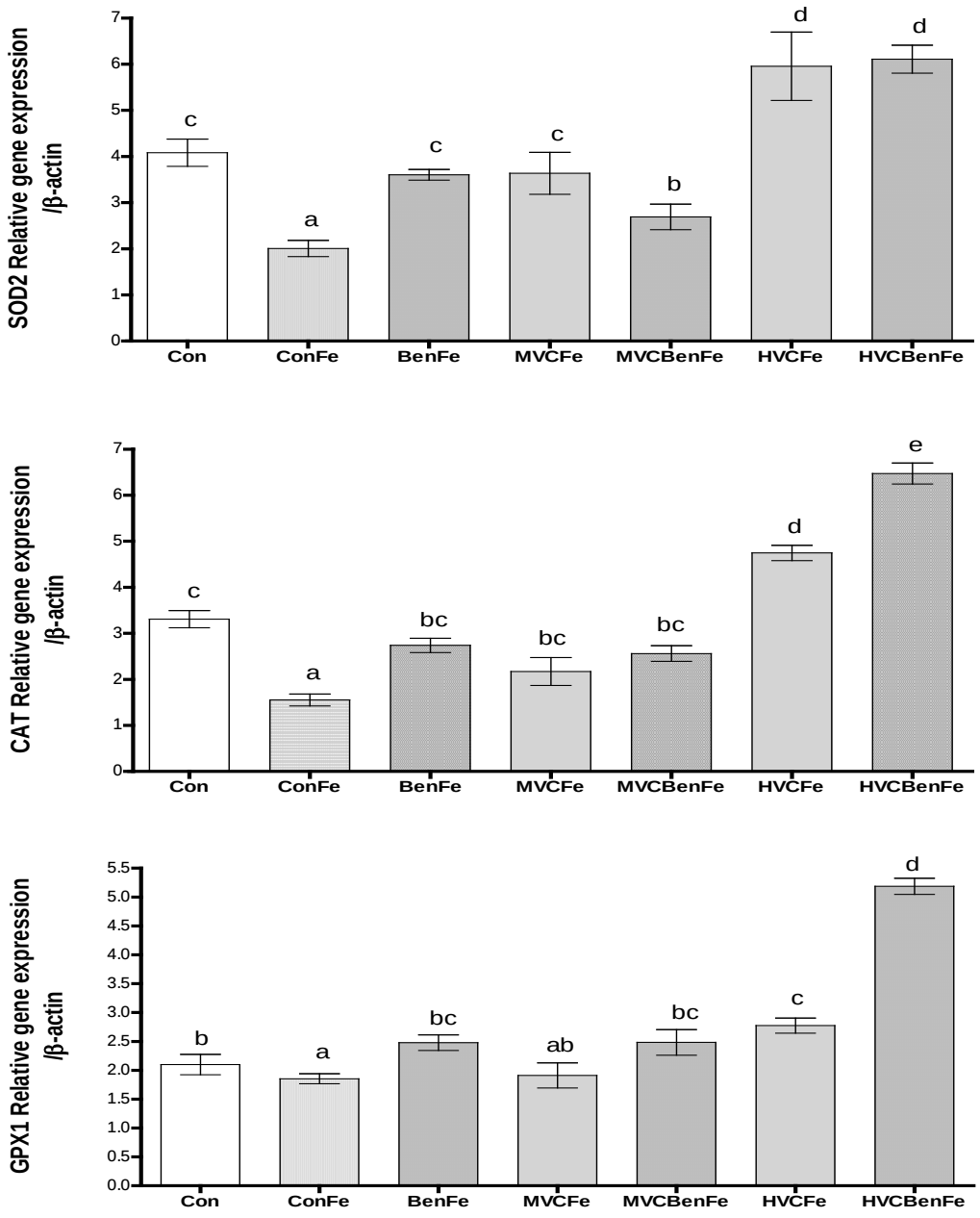


Figure 5.4. Effect of iron exposure (0.25%), vitamin C, bentonite supplementations (2%) and their combination on gene expression of antioxidant (A) superoxide dismutase (SOD) activity, (B) catalase (CAT) activity and (C) glutathione peroxidase 1 (GPX1) activity in liver of rainbow trout during 10 weeks' time interval. Values are mean ± S.E. Small letters (a, b, c, d) denote significant difference between experimental groups (Number of replicates (N)=4).

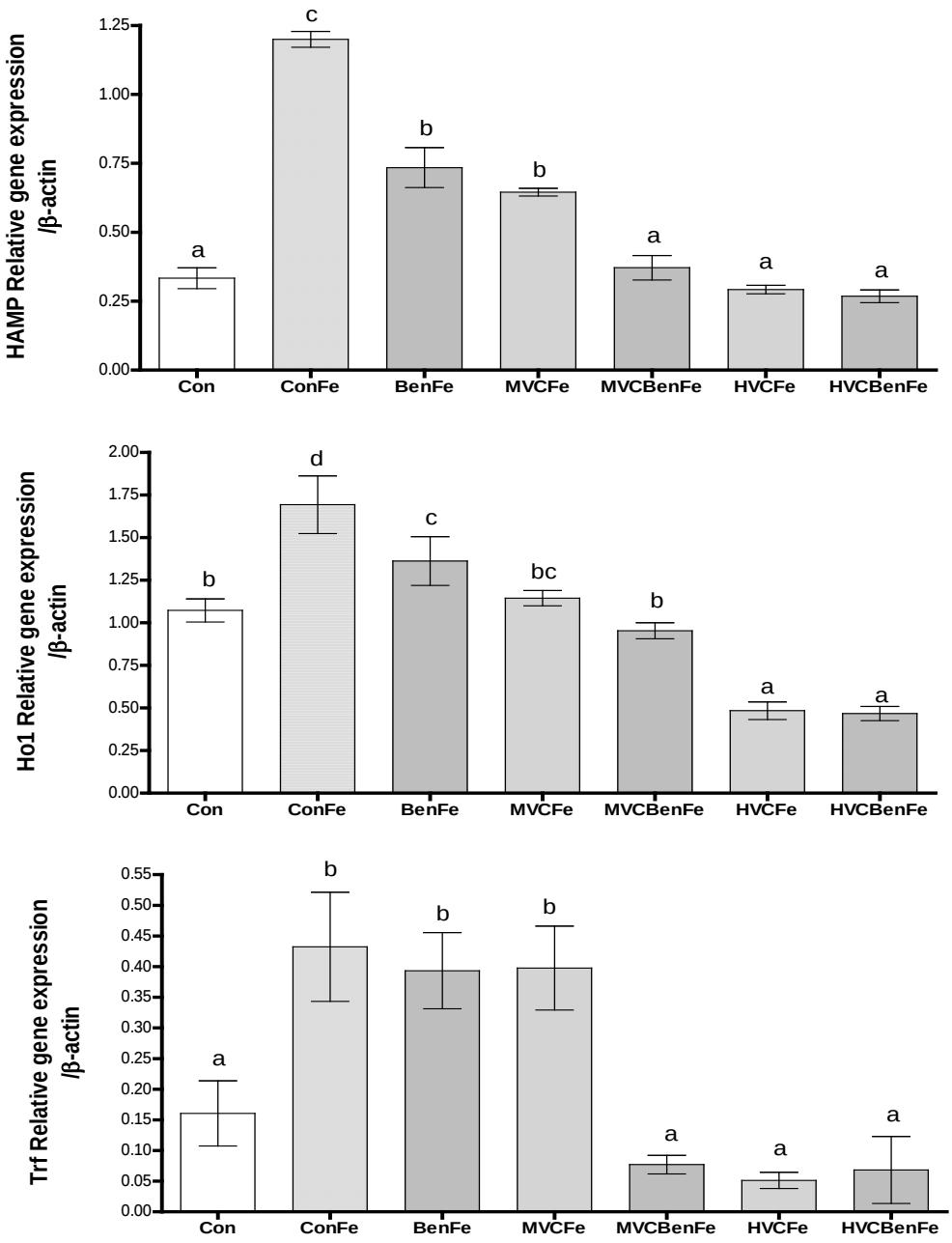
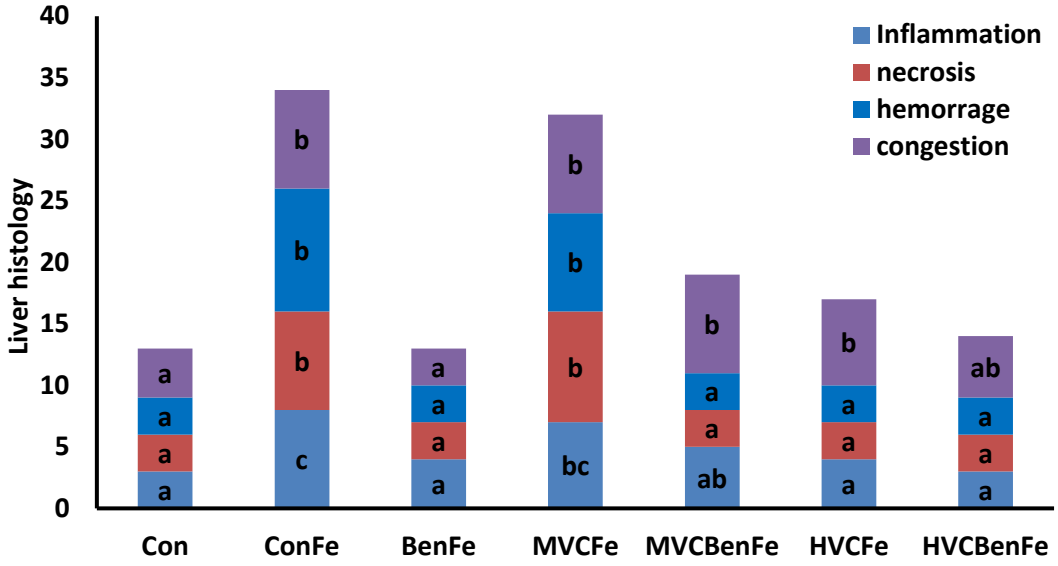


Figure 5.5. Effect of iron exposure (0.25%), vitamin C, bentonite supplementations (2%) and their combination on gene expression of iron metabolism (A) hepcidin (HAMP), (B) hemeoxygenase 1 (HO1), and (C) transferrin (Trf) in liver of rainbow trout during 10 weeks' time interval. Values are mean ± S.E. Small letters (a, b,) denote significant difference between experimental groups (Number of replicates (N)=4).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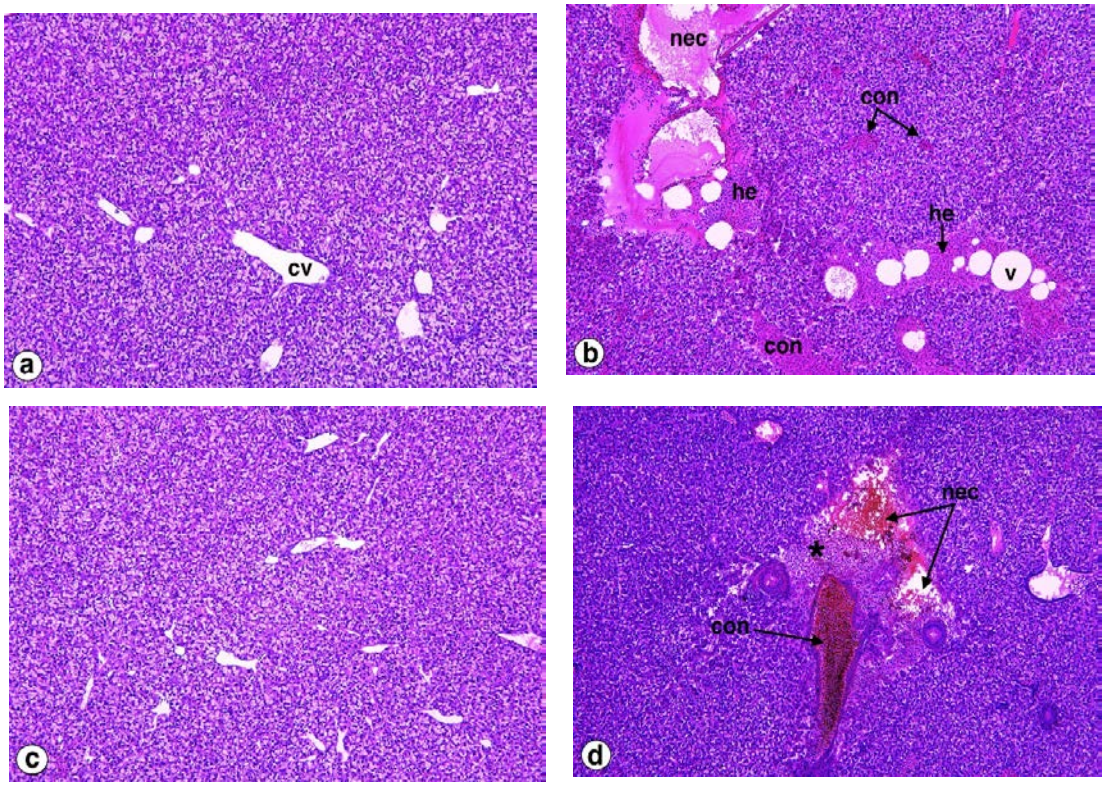
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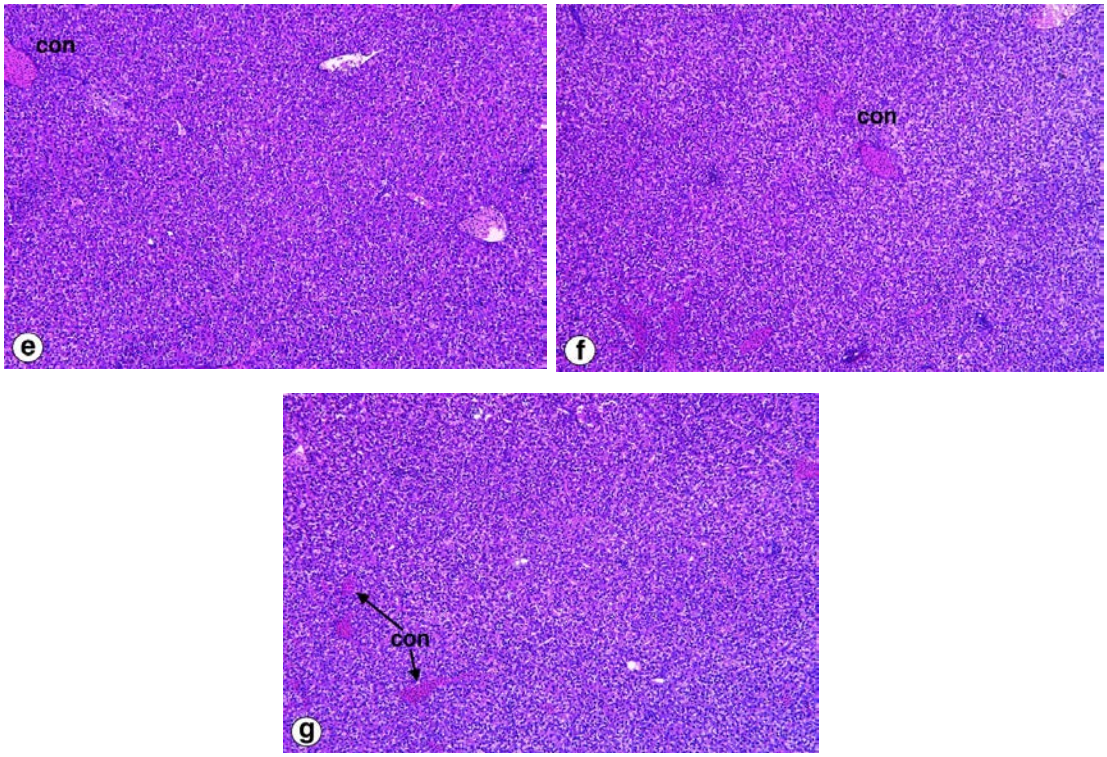
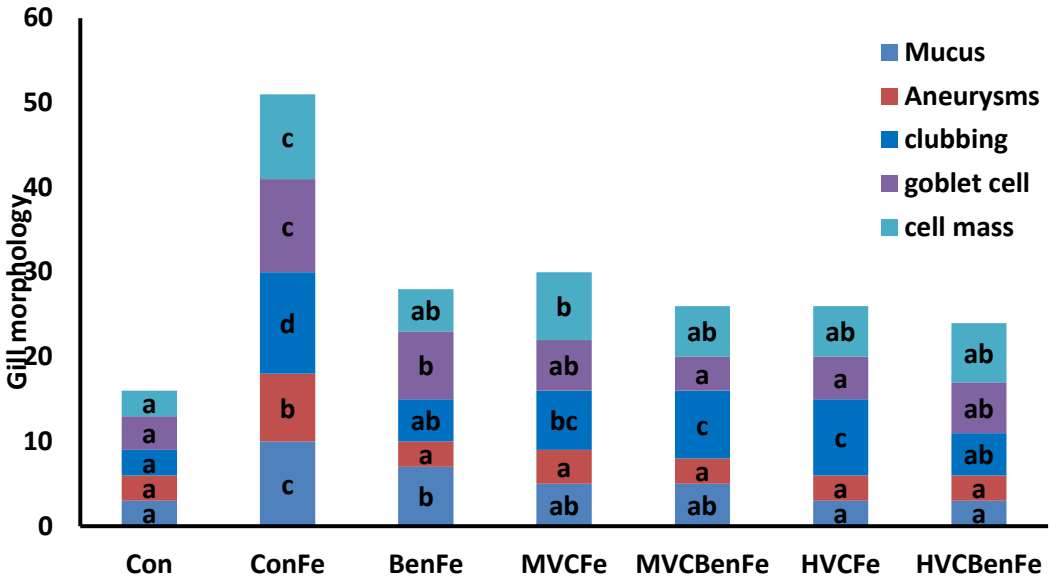


Fig. 5.6. Hepatic histological assay of rainbow trout under experimental conditions of iron exposure (0.25%) and vitamin C, bentonite (2%) supplementations. Normal structure of hepatic lobules found in (a) Control, (Con), (c) BenFe groups, and congestion (Con), necrosis (nec), hemorrage (he), vacuolization (V) in (b) ConFe and (d) MVC group, congestion (con) in (e)MVCBenFe, (f) HVCFe, and (g) HVCBen groups.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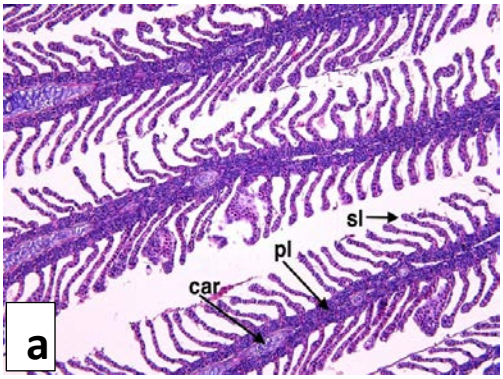
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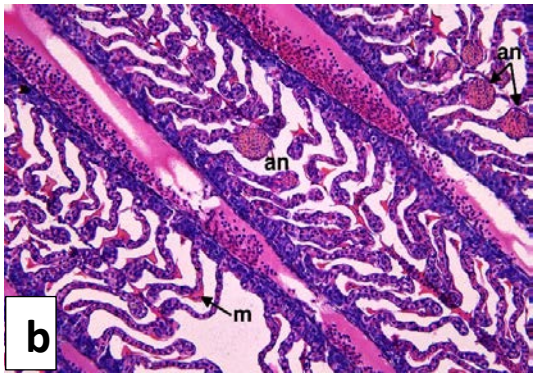
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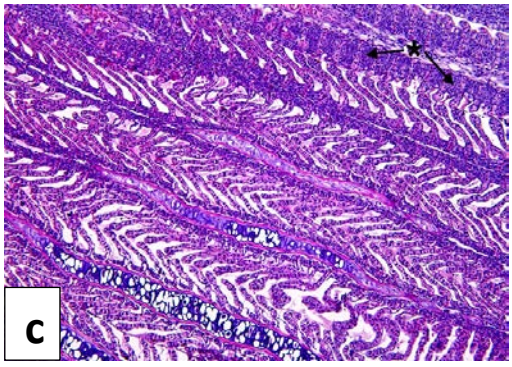
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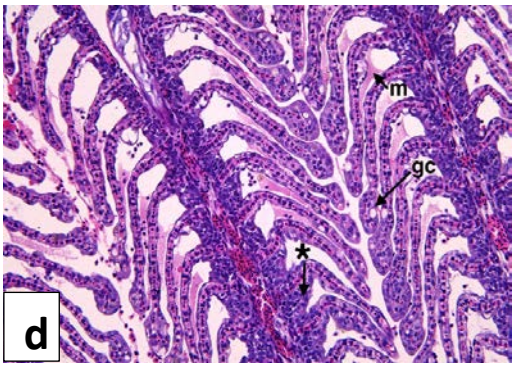
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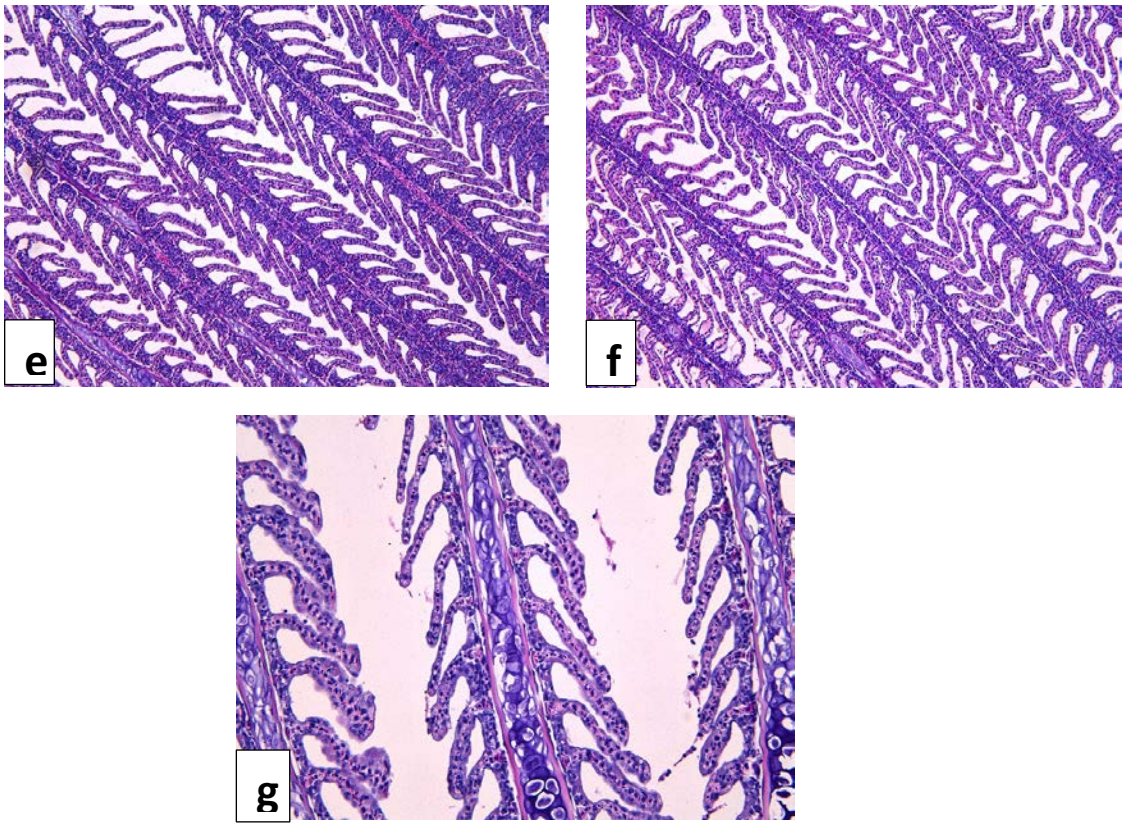


Fig. 5.7. Gills histological assay of rainbow trout under experimental conditions of iron exposure (0.25%) and vitamin C, bentonite (2%) supplementations. Normal structure of gills found in (A) Control, (Con), (e) MVCBenFe, (f) HVCFe, (g) HVCBenFe groups, mucus (m) secretion, aneurysm (an) in (b) Confe, intralamellar cell mass in (c) BenFe, mucus (m), goblet cells (gc) secretion in (d) MVCFe groups.

Table 5.1.1: Formulation of the experimental diets

Ingredients	Con	ConFe	BenFe	MVCFe	MVCBenFe	HVCFe	HVCBenFe
Fish meal[a]	19.00	19.00	19.00	19.00	19.00	19.00	19.00
Soybean meal[c]	14.00	14.00	14.00	14.00	14.00	14.00	14.00
Poultry by-product meal[a]	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Soy potrien concntrate[b]	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Wheat glutein[a]	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Corn Protein Conc[a]	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Wheat flour[a]	15.40	15.40	15.40	15.40	15.40	15.40	15.40
Fish oil[a]	15.70	15.70	15.70	15.70	15.70	15.70	15.70
Dicalcium phosphate[a]	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Choline chloride (60%)[a]	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Vitamin premix,ARS 702a	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Trace Mineral mixture, Trouw nutrition[d]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin C, Stay C-35)[e]	0.20	0.20	0.20	0.43	0.43	0.86	0.86
α-cellulose[a]	3.00	2.75	0.75	2.40	0.47	2.05	0.05
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Bentonite	0.00	0.00	2.00	0.00	2.00	0.00	2.00
Ferrous Sulphate	0.00	0.25	0.25	0.25	0.25	0.25	0.25
Proximate Composition							
Total Protein (%)	41.52	41.42	41.22	41.41	41.52	41.61	41.35
Total Lipid (%)	18.78	18.56	18.54	18.65	18.72	18.52	18.63
Ash Content (%)	7.7	8.3	8.5	7.7	8.4	8.6	8.7
Minerals (mg/Kg)							
Fe	232.30	2477.30	2593.25	2411.04	2330.01	2281.397	2444.85
Cu	9.77	23.80	22.12	25.66	20.01	24.27	13.10
Zn	90.73	104.85	74.14	113.62	85.68	93.78	64.95
Na	2804.15	3356.58	4598.33	5148.32	4360.645	5004.61	4526.00
K	10057.51	11786.07	12106.39	13932.98	10656.83	12293.79	10971.69
Mg	2959.80	3444.46	3286.64	3981.09	3175.55	3589.26	3138.66

[a]Rangen Inc., Buhl, ID, USA
[b]Profine VF, The Solae Company, St. Louis, MO, USA.
[c]Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[d]US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as $ZnSO_4 \cdot 7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4 \cdot 5H_2O$), 1.54; I (as KIO_3), 10.
[e]Skretting USA, Tooele, UT, USA.

Table 5.1.2: qPCR primer list of target and reference, and efficiency of the PCR reactions

Gene	Reference	Sequence of primer (5'-3')	Calculated efficiency
Reference gene			
B-actin	Cleveland et al., 2018; Lee et al., 2020	F: GCCGGCCGCGACCTCACAGACTAC R: CGGCCGTGGTGGTGAAGCTGTAAC	94.54
Target gene			
SOD2	Prabhu et al., 2016	F: TCCCTGACCTGACCTACGAC R: GGCCTCCTCCATTAAACCTC	105.71
CAT	Prabhu et al., 2016	F: TGATGTCACACAGGTGCGTA R: GTGGGCTCAGTGTTGTTGAG	104.43
GPX1b1	Prabhu et al., 2016	F: CGAGCTCCATGAACGGTACG R: TGCTTCCCGTTCACATCCAC	103.09
HAMP	Prabhu et al., 2016	F: GGAGGAGGTTGGAAGCATTG R: GATGGTTTTAGTGCAGGCAGG	104.80
HO1	Prabhu et al., 2016	F: ACTCTTCCGCAGTACAAGCT R: CTGTGTGTTGCAGCAGGAAT	98.43
trf	NM_001124552	F: ATCCACCGCTATGGCATCTG R: AAGCACCTGACTGTACCTGC	117.86

Table 5.1.3: Electrolyte and metal load in the hepatic tissue of rainbow trout following vitamin C, bentonite supplementation (2%), iron exposure (0.25%) and their combination.

Treatment	Na (mg/kg)	K (mg/Kg)	Mg (mg/Kg)	Cu (mg/Kg)	Zn (mg/Kg)
Con	853.75 ± 35.78^{a}	3881.50 ± 237.85^{a}	123.53 ± 12.40^{a}	375.17 ± 27.13^{b}	74.52 ± 5.19^{a}
ConFe	1503.50 ±151.28^{c}	4473.30 ± 296.84^{ab}	395.62 ± 15.03^{b}	413.55 ± 31.31^{b}	123.66 ± 15.38^{b}
BenFe	1024.80 ± 62.79^{ab}	4096.10 ± 153.92^{ab}	382.03 ± 30.32^{b}	393.08 ± 46.74^{b}	76.842 ± 4.54^{a}
MVCFe	1041.30 ± 115.69^{ab}	4282.50 ± 206.85^{ab}	393.34 ± 42.93^{b}	362.39 ± 19.03^{b}	86.40 ± 12.47^{a}
MVCBenFe	1215.70 ± 101.51^{b}	5573.10 ± 346.98^{c}	425.05 ± 47.78^{b}	192.01 ± 10.37^{a}	100.60 ± 11.43^{ab}
HVCFe	1102.50 ± 72.68^{ab}	4804.70 ± 262.91^{b}	318.54 ± 19.29^{b}	215.41 ± 24.65^{a}	86.31 ± 10.48^{a}
HVCBenFe	968 ± 44.37^{ab}	3907.70 ± 264.64^{a}	338.31 ± 48.83^{b}	187.67 ± 17.20^{a}	84.12 ± 10.58^{a}

Table 5.1.4: Electrolyte and metal load in the digesta of rainbow trout following vitamin C, bentonite supplementation (2%), iron exposure (0.25%) and their combination.

Treatment	Na (mg/kg)	K (mg/kg)	Mg (mg/kg)	Cu (mg/kg)	Zn (mg/kg)
Con	5457.81 ± 300.04^{a}	3468.33 ± 116.22^{ab}	1817.90 ± 95.83^{a}	108.18 ± 0.38^{a}	111.17 ± 4.15^{a}
ConFe	5728.15 ± 207.52^{a}	3904.58 ± 148.82^{b}	2545.70 ± 211.37^{b}	114.72 ± 1.65^{a}	109.80 ± 3.45^{a}
BenFe	6746.23 ± 342.82^{b}	4531.52 ± 218.98^{c}	4064.40 ± 437.64^{c}	153.65 ± 2.12^{cd}	153.98 ± 4.00^{b}
MVCFe	5466.18 ± 90.99^{a}	3420.02 ± 95.31^{a}	3361.20 ± 93.67^{c}	159.08 ± 6.48^{d}	141.13 ± 3.93^{b}
MVCBenFe	5527.86 ± 171.05^{a}	3461.81 ± 123.37^{ab}	3491.10 ± 101.03^{c}	132.80 ± 3.84^{b}	154.89 ± 10.53^{b}
HVCFe	6071.78 ± 524.25^{a}	3785.09 ± 140.25^{ab}	3687.50 ± 133.39^{c}	144.43 ± 7.04^{bc}	139.36 ± 9.13^{b}
HVCBenFe	5568.53 ± 90.43^{a}	3526.69 ± 85.14^{ab}	3711.70 ± 326.25^{c}	133.69 ± 4.97^{b}	144.49 ± 3.25^{b}

CHAPTER 6

Summary and Recommendations

6.1 Introduction

Aquaculture is one of the fastest-growing food-producing sub-sectors in the world, as evidenced by a steady increase in overall production over the last decade or more, notably in a number of developing nations. Channel catfish and rainbow trout are importantl species for aquaculture development. They are in high demand among customers, and food fish aquaculturists prefer them as prospective species. This is owing to the species' optimal traits, which include a fast growth rate at high stocking densities, a high food conversion rate, and high meat quality.

Intensive finfish culture in a recirculating aquaculture system (RAS) is a production method that reuses fish culture water multiple times, reducing the amount of space and water needed for fish culture. This technology has been effectively applied to the culture of catfish and trout all over the world. RASs were created in response to growing environmental rules in countries with limited land and water availability. Despite the benefits of using RAS, researchers have noticed an issue with the accumulation of inorganic nitrogen compounds and solids, especially minerals load. Because of its significant toxicity to fish health, rising minerals (especially iron) is a growing problem.

Several solutions for managing iron levels in fish culture systems have been proposed, including the use of permanganate; however, it has been reported to be hazardous to phytoplankton and other aquatic life. In places with high iron burdens, other tactics such as vigorous agitation, spraying through water towers, filtration, and microbubble generators are frequently utilized, although this is considered a partial solution because only a fraction of the iron is removed. Due to these limitations,

research into a therapeutic method to directly alleviate iron-induced toxicity in fish is required.

Bentonite (NB) is an aluminosilicate-based natural clay having features such as binding, swelling, and cation exchange capacity. To improve the physical qualities of aquafeeds, clay and its acid-activated form (AB) were utilized as feed additives. Physical feed integrity, with minimum disintegration and nutrients leaching into the water, is a critical management tool for ensuring production success. Also, vitamin C is an essential micronutrient for most of the teleost because they are not capable of synthesizing vitamin C (due to the lack of L-gulonolactone oxidase), therefore, an exogenous supplementation of vitamin C is requisite in fish diets.

The hypothesis was that feeding a diet containing vitamin C, bentonite or in combination to catfish or trout at different inclusion levels would improve growth, feed efficiency, and minimize the toxic effects of iron.

6.2 Potential amelioration of waterborne iron toxicity in Channel catfish through dietary supplementation of Vitamin C

The study evaluated the effect of vitamin C (143 or 573 mg/kg diet) on mitigating the toxic effects of iron by measuring the growth performance, hematology, oxidative stress and antioxidant enzyme activity, mineral load in liver and digesta, histology of liver, and gills. The experimental diets were fed ad libitum to channel catfish for 8-weeks. The weight gain (%), HIS, RBCs, hemoglobin level, MDA, and antioxidant enzyme activity were significantly ($p<0.05$) affected by the treatment diets. Vitamin C appears to have a powerful protective impact against cellular lipid peroxidation by increasing antioxidant capacity of ROS scavenging SOD and CAT enzyme activities, hence reducing iron toxicity. This was accompanied by fewer negative effects on the liver. Based on our findings, we found both dose of vitamin C (143 or 573 mg/kg diet) are equally effective, the lower tested dose (143 mg/kg diet) can be recommended from an economical point of view.

6.3 Dietary bentonite supplementation enhances growth and mitigate iron toxicity in rainbow trout.

The study investigated the dose of bentonite (2-4%) to mitigate the foodborne iron toxicity in rainbow trout by exposing feed to iron (2500 mg/kg diet) by measuring the weight gain %, SGR, FCR, antioxidant activity, histology of liver, mineral load on liver, plasma, and digesta. All the measured parameters were significantly ($p<0.05$) affected by the dietary treatments. Because of iron toxicity, the Fe group's growth was stunted. Additionally, bentonite supplementation increased the antioxidant capacity of the fish, reduced liver disturbance, and improved iron excretion by raising the iron level in the digesta. The finding that supplemented bentonite (2-4 percent) groups had increased iron efflux in their digesta suggests that bentonite aids in the removal of iron excess in the body via the digestive system. As a result, bentonite (2%) supplementation may be a promising strategy for increasing growth and reducing the detrimental effects of iron in rainbow trout.

6.4 The dietary supplementation of vitamin C and bentonite improve the fish growth and alleviate iron toxicity in rainbow trout.

In this study, vitamin C and bentonite are included as a feed additive to mitigate the feed borne iron toxicity in rainbow trout by dietary iron (2500 mg/kg diet). This experiment was carried out for 10 weeks. Seven treatment diets were fed twice daily, and water quality were maintained during the experiment period. The weight gain % were significantly retarded ($p<0.05$) with exposure of iron compared to rest of the groups. Higher levels of feed-borne iron harmed growth, which was likely due to hepatic oxidative stress, iron overload in the liver, and severe histopathological alterations that reduced surface area and hence function. Changes in heme oxygenase, transferrin, and hepcidin gene expression also imply that increased levels of iron in diet are detrimental to fish health at the molecular level. However, it was clear that a combination of bentonite (2%) and vitamin C (1500 or 3000 mg/Kg diet) supplementation reduced the negative effects of iron exposure on growth. By upregulating SOD2, CAT, and GPX1 gene expression and lowering MDA levels, vitamin C and bentonite appear to have a potent protective effect against cellular lipid

peroxidation, thus minimizing iron toxicity. This was also accompanied by a decrease in the expression of a gene involved in iron homeostasis. The negative effects on the liver and gills were also reduced when dietary bentonite and vitamin C were supplemented together. In conclusion, dietary supplementation with medium and higher doses of vitamin C, alone or in combination with bentonite, appears to be a promising therapeutic option for rainbow trout to minimize the detrimental effects of feed borne iron.

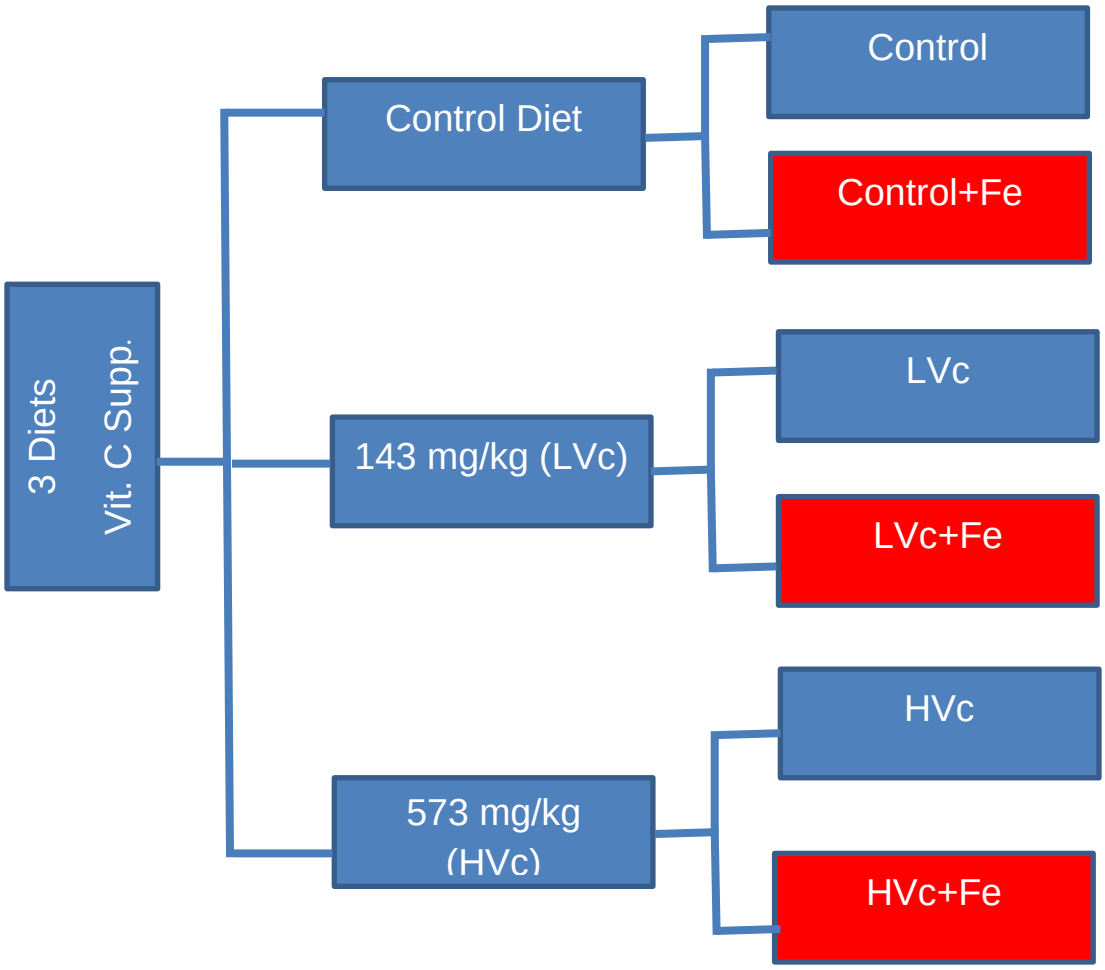
6.5 Recommendations

The applications of dietary bentonite and vitamin C as a feed additive indicated significant effects on growth, production performance, and mitigating negative effects of iron on catfish, and trout. Based on the available literature, this study investigated the inclusion levels of vitamin C and bentonite to mitigate the toxic effects of iron in both catfish, as well as in trout. This study will give future prospects that require more research for better improvement. Hence, the associated research on the mitigation of iron toxicity will insight into more further studies. The following aspects are recommended:

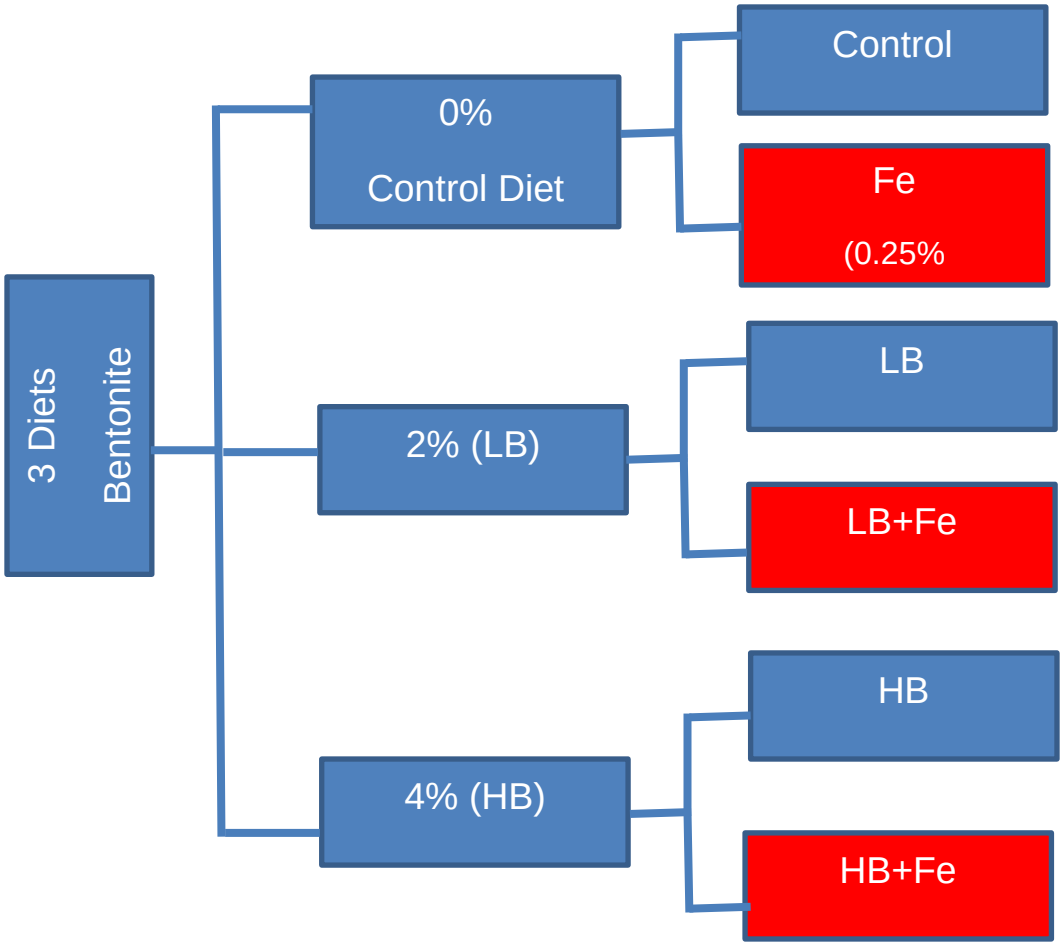
- Evaluating the binding effects of bentonite on micronutrients (vitamin and minerals) in feed and body of fish after feeding the feed.
- Evaluating the effects of vitamin C and bentonite in the mitigation of toxic effects of iron on other freshwater fish species.
- Evaluate the effects of bentonite as feed additives on marine species growth performance along with water quality since cation exchange properties of bentonite can limit its scrubber efficiency.
- Evaluating the effects of dietary bentonite on physical characteristics of aquafeeds.
- Evaluating the effects of bentonite against the disease perspective in both catfish and trout.
- Evaluating the synergistic effect of vitamin C and bentonite requires further study.

Appendices

Chapter 3 Experimental design: Potential amelioration of waterborne iron toxicity in channel catfish through dietary supplementation of Vitamin C



Chapter 4 Experimental design: Dietary bentonite supplementation enhances growth and mitigates iron toxicity in rainbow trout



Chapter 5 Experimental Design: The dietary supplementation of vitamin C and bentonite improve the fish growth and alleviate iron toxicity in rainbow trout