Chapter-2

Fluorine is widely dispersed in nature and is a common constituent of most soils and rocks, plants and animals. It is the 13th most abundant element, averaging 650 ppm in the earth crust (Fleischer and Robinson, 1963). Being the most electro-negative of all elements and being highly reactive it does not occur in a free form in nature, but is found in the earth's crust as fluorides. Fluoride concentrations in sea waters generally range from 1.2 to 1.5 mg F/L (Dobbs, 1974). In the case of natural water, the variation in the fluoride content from region to region is dependent upon factors such as the sources of water, types of geological formation and the amount of rainfall. When water passes through and over the soil and rock formations containing fluoride, it dissolves these compounds, resulting in the small amounts of soluble fluoride present in virtually all water sources (Ozwath, 2009). The fluoride intake from consumption of drinking water and beverages is determined by factors like body size, physical activity, food habits and variations in atmospheric temperature and humidity. In tropical countries like India where average daily temperature is around 80°F in summer, the daily fluoride intake is very high. In some endemic areas of India the silt in wells may contain 0.096-0.15% of fluoride leads to high fluoride intake. The presence of particulate fluoride ions and dust in water is also held responsible for the high incidence of fluorosis in endemic areas (Nanda and Kapoor, 1972).

The problem of fluorosis has been reported in various states in India, affecting more than 150,000 villages seriously. Over 50% of the groundwater sources in India have been contaminated by fluoride (Valjavec, 1932). Fluoride toxicity may be acute or chronic. Acute toxicity is due to excess intake of certain fluoride-containing substances over a short time-period, either by ingestion or inhalation as industrial or

household accidents in humans. Even in the presence of hospital facilities, the prognosis is poor and often results in death (Schulman and Wells, 1997). Chronic toxicity is due to intermittent or continuous intake of fluorides in quantities above normal and is well described in both humans and farm animals. It may occur due to natural or industrial contamination of the environment. The former occurs in areas with rocks containing naturally high concentrations and has been reported from countries such as India (Choubisa, 1999) and New Zealand (Cronin, 2000). Industrial contamination in an agricultural context is usually due to contamination of pastures in the vicinity of processes associated with high fluoride emissions. These include aluminium smelters and brickworks (Patra *et al.*, 2000). In India, the states of Andhra Pradesh, Bihar, Chattisgarh, Haryana, Karnataka, Madhya Pradesh, Maharashtra, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal are affected by fluoride contamination in water. This involves about 9000 villages affecting 30 million people (Nawlakhe and Paramasivam, 1993 and Ozsvath, 2009).

Patients suffering from fluorosis usually experience difficulty in walking because of the progressive weakness in the lower limbs (Sharma *et al.,* 2009). Fluoride toxicity will affect all the parts of the human system leads to the altered life span. In India it is the foremost problem in different parts of the country. There is an upsurge of concern on fluoride getting entry into the animal and human system, through food chain, causing variety of health hazards. Anadromous fish species appear to be more sensitive to inorganic fluorides in freshwater (0.5 mg F/L) than in marine water where the natural concentration of inorganic fluoride averages 1.3 mg F/L (Damkaer and Dey, 1989).

Review of literature reveals wide range of structural and biochemical changes in vital tissue of experimental animal subjected to fluoride intoxication (Vani and Reddy, 2000; Choudhary and Ghar, 2001 and Achyutha and Piska, 2006). The toxicity of fluoride is due to the toxicity of the fluoride ion, a direct cellular poison that binds calcium and interferes with the activity of proteolytic and glycolytic enzymes. The symptoms of acute fluoride intoxication include lethargy, violent and erratic movement and death (Liteplo *et al.,* 2002). The weight of the juvenile prawns exposed for 60 days to 0.5, 1.0, and 1.5 mg F/L was 13, 40, and 57% less, respectively (Adhikari *et al.,* 2006). Reduced feeding intensity after fluoride exposure in fish has also been observed in different studies by Bajbai *et al.,* (2009).

Ralio and Nikinmaa, (1985) reported that the blood parameters of diagnostic importance are erythrocytes and haemogobin, haematocrit and leucocytes differential count would readily respond to incidental factor such as physical stress and environmental stress due to water contaminants. In water, inorganic fluorides remain dissolved in solution under acidic conditions, low hardness and the presence of ion-exchange material (e.g. bentonite clays and humic acid) and of calcium or aluminium ions (Coker and Shilts, 1979; Pickering *et al.,* 1988 and Sahu and Karim, 1989). Fluoride inhibits oxygen consumption and blood clotting and diminishes erythrocyte glycolysis (McIvor, 1990). Fluoride interferes with normal functioning of the liver and causes destruction of hepatocytes, degenerative and inflammatory changes (Kapoor *et al.,* 1993). Bajbai and Tripathy (2010) reported that fluoride diminishes protein and lipid metabolism in the gill, liver, kidney and muscle tissues of fish, most probably by inactivating enzymes and hormones that regulate their synthesis and by

increasing their utilization in cell repair, tissue reorganization and to meet high energy demand during stress caused by fluoride exposure.

In India an estimated 60 million people have been poisoned by well water contaminated by excessive fluoride, which is dissolved from the granite rocks. The Effect of fluoride on human health has long been of interest to medical researchers. Fluorosis is an important clinical and public health problem in several parts of the worlds (Singh and Jolly, 1970). Global prevalence of fluorosis is reported to be about 32% (Mella *et al.,* 1994). There are several million people in India who are exposed to drinking water sources that represent a definite risk of developing fluorosis. Skeletal fluorosis in village drinking water containing 2.4 ppm of fluoride and crippling fluorosis in villages with mean fluoride levels of 3.00 ppm has been observed (Jolly *et al.,* 1969). Teotia and Teotia, (1994) have reported dental and skeletal fluorosis in residents of rural areas consuming water containing 0.6 ppm fluoride. Fluoride concentration of up to 38.5 ppm in drinking water has been reported (Susheela and Ghosh, 1990) in several states of India. Fluoride is omnipresent in our environment and has been added to drinking water supplies for cariostatic purposes as a prophylactic agent in dental caries with a recommended dose between 0.7 and 1.2 mg/L (Leverett *et al.,* 1997). Excess fluoride ingestion is a major health problem, 15 of the 30 states and Union territories in India being endemic for fluorosis (Susheela, 1993). Fluoride toxicity is increasingly becoming a matter of grave concern as many countries have been declared endemic for fluorosis. This makes it imperative for scientists to focus on the precise toxic effects of fluoride on various soft tissues, so that therapeutic agents can be effectively used.

More than 90% of the total body burden of fluoride is retained in bones and teeth, because of its profound affinity for calcified tissues, where most of the remaining portion is distributed in highly vascularized soft tissues and blood (Fawell *et al.*, 2006). Therefore, the most obvious early toxic effects of fluoride in humans are skeletal fluorosis, leading to a variable degree of combined locomotors disability and neurological impairment (Reddy, 2009). Individuals affected with skeletal fluorosis revealed joints pain in limbs, numbing, cramping and tingling of extremities accompanied with back pain with difficulties during walking (Shashi *et al.*, 2008). Since animal studies and human clinical trials indicated that fluoride can reduce bone strength even before skeletal fluorosis is present, a heavy and tired feeling in the legs with frequent falling or "a foot slapping gait" is also a commonly noticeable manifestation (Mousny *et al.*, 2006). In advanced stages, "crippling skeletal fluorosis" characterized by damage of musculoskeletal and nervous systems is then observed. The later disorder results in mal shaping of bones, muscles wasting and arthritic pain with restricted joints motion. Neurological complications of skeletal fluorosis, namely paralysis of limbs, vertigo, spasticity in extremities, arise primarily from mechanical compression of the spinal cord and nerve roots from sclerosed vertebral column and ossified ligaments (Reddy, 2009).

Alarm was then given about an increased fluoride toxicity inflicted risk on physical activity and motor health. In addition to the effect on hard tissues, fluoride also manifests its toxicity on soft tissues, where it is known to cross the cell membranes and to enter soft tissues, impairing its function (Vani and Reddy, 2000). Amongst soft tissues, muscles and brain have been reported to retain the ingested fluoride which may in turn interfere with their physiological functions. Despite the

fact that muscles were more affected than brain, probably due to the protective role of the blood brain barrier, there is a paucity of studies on the effect of fluoride intoxication on motor function and coordination performance in rats (Vani and Reddy, 2000). Human and animal studies have shown that fluoride can cross the placenta. There is a direct relationship between the serum fluoride concentration of the mother and that of the fetus. Fluoride is then readily taken up from fetal blood to bones and teeth (IPCS, 2002 and ATSDR, 2003). Fluoride increased generation of free radicals, lipid peroxidation and depleted antioxidant defense systems shifting the oxidant/antioxidant balance towards oxidative stress are proposed to mediate the toxic effects of fluoride on soft tissues (Shivarajashankara *et al.,* 2002 and Trivedi *et al.,* 2008). Intact cerebellum has been reported primarily to be indispensable for successful coordination of voluntary motor function and to make an important contribution to control of muscle tone, equilibrium, gait and posture (Konarski *et al.,* 2005; Koros *et al.,* 2007 and Baldacara *et al.,* 2008). Cerebellar cortex has been evidenced to be particularly susceptible to sodium fluoride-induced oxidative stress and could contribute to the development of neurodegenerative diseases (Saad El-Dien *et al.,* 2010).

Nevertheless, it is expected that factors such as water hardness and temperature, the presence of cations such as calcium, magnesium (Pimentel and Bulkley, 1983 and Smith *et al.,* 1985) and potentially, selenium (Pang *et al.,* 1996) or anions such as chloride (Neuhold and Sigler, 1962) can reduce the toxicity of inorganic fluorides on a site-specific basis. Vitamins C and E act as antioxidants scavengers of free radicals and peroxides, which accumulate after fluoride exposure. Vitamin D is known to promote intestinal absorption of Ca^{++} and phosphate. Co^{-}

treatment with Vitamin C, D and E ameliorates NaF induced reduction in serum Ca^{++} and phosphorus (Guna Sherlin and Verma, 2000). Oral administration of Vitamin C (50mg/kg body weight/day) and Vitamin E (2mg/0.2ml olive oil/day) from days 6-19 of gestation along with NaF (40mg/kg body weight) significantly ameliorates NaF induced total percentage of skeletal and visceral abnormalities (Verma and Sherlin, 2001). Ekambaram and Paul, (2002) reported that calcium carbonate prevents not only fluoride induced hypocalcaemia and also the locomotion behavior and dental toxicities of fluoride by decreasing the bio availability of fluoride in rats. Calcium (Ca) and ascorbic acid (vitamin C) can counteract with fluoride toxicity (Rathore, 2009; Goyal and Sharma, 2009; San *et al.,* 2009 and Khan, 2009) and alleviate fluorotoxicosis. Toxic effects of fluoride were reversible if its exposure was withdrawn for two months. Intra-arterial administration of 1.8mM $CaCl_2$/kg reduced the risk of death by 35% in a mouse lethal model of fluoride poisoning (Heard *et al.*, 2001). However, all these findings have been reported from mammalian systems and studies on fishes in this regard are very scarce.

When fluoride is removed from the body, a detoxification reaction can occur in some people. To help combat the lack of mental clarity that occurs during this detoxification reaction, phosphatidylserine, glycerophosphocholine (GPC) and acetyl-L-carnitine (found in Brain Vibrance™) can be used to energize the brain and eliminate foggy thinking. Phosphatidyl serine has been shown to improve cognitive impairment in elderly subjects as well as improve mental or physical performance under stress in younger subjects (Benton *et al.,* 2001). GPC increases levels in the brain of choline and the neurotransmitter acetylcholine, which is involved specifically in learning and memory and has been shown to support cognitive function (De Jesus

Moreno, 2003). Therefore, nutritional intervention with antioxidant-rich substances is the ultimate goal as antidotes for combating with the health complaints arising from fluorosis (Chinoy and Menon, 2001 and Susheela and Bhatnagar, 2002). Acetyl L-carnitine arginate, acetyl L-carnitine, Gotu kola (*Centella asiatica*), *Ginkgo biloba* and uridine (found in Neuron Growth Factors-NGF™) support the regeneration of neurites and dendrites in the brain (Pooler *et al.,* 2005) and can thus counteract the damaging effects of fluoride on brain cells. In addition, a special bio-available form of curcumin called Longvida®, green tea, vitamin D3, niacin and serrapeptase (all found in DejaVida™), support healthy cognition and fight the free radical damage (Lee *et al.,* 2009) that occurs in the brain after fluoride exposure. Dr. Susheela, a pioneer in fluorosis, along with her coworkers has set a trend in preventive, curative and rehabilitative research in the area (Susheela, 2007b). Probiotic strategies are part of the continuing evolution of the treatment of oral infection that produces the clinical manifestations of dental caries. Science is providing the tools to diagnose and treat the infection before it causes damage. The application of probiotic strategies may, in the not-distant future, provide the end of new cavities in treated populations (Anderson and Shi, 2006).

Probiotic is a live microbial food supplements that are consumed with the aim of providing health benefit to the host by contributing to an improved microbial balance within the intestinal microbiota (Crittenden *et al.,* 2005) are biologically active components or single or mixed cultures of microorganisms capable of improving the health of the host (Ochoa-Solano *et al.,* 2006) live microorganisms and/or disease resistance (Tacon, 2002) live microorganisms administered in adequate amounts that confer a health effect on the host (Gomez *et al.,* 2007).

Mechanisms for the benefits of probiotics are incompletely understood. According to Sartor, (2004) an efficient probiont should have the pre-requisite such as,

- Adherence and colonization of the gut
- Suppression of growth or epithelial binding/invasion by pathogenic bacteria and production of antimicrobial substances
- Improvement of intestinal barrier function
- Controlled transfer of dietary antigens
- Stimulation of mucosal and systemic host immunity

Historically, *Lactobacilli* and *Bifidobacteria* associated with food have been considered to be safe (Adams and Marteau, 1995). Although the exact mode of action of probiotics is not fully understood (Irianto and Austin, 2002 and Balcazar *et al.*, 2006), it is widely accepted that probiotics generally work by preventing pathogens from proliferating in the intestinal tract, on the superficial structures or in the culture environment; securing optimal use of the feed by aiding in its digestion; improving water quality; or stimulating the immune system of the host. Probiotics have also been shown to act as a source of nutrients and enzymatic contribution to digestion (Burr *et al.*, 2005). Bacteria isolated from fish digestive systems have been shown to digest chitin (Hamid *et al.*, 1979), starch (MacDonald *et al.*, 1986 and Gatesoupe *et al.*, 1997), protein (Gatesoupe *et al.*, 1997), cellulose (Erasmus *et al.*, 1997 and Bairagi *et al.*, 2002) and lipids (Gatesoupe *et al.*, 1997) *in vitro*.

Aquaculture has become an important economic activity in many countries (Balcazar *et al.*, 2006). However, this aquaculture develop show many problems as widespread epizootics, feed efficiency and growth performance (Fegan, 2001 and

Gaiotto, 2005). This is principal caused by the large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to disease, inadequate balance of nutrient in the artificial diets an deterioration of environmental conditions, since the physiological stress is one of the primary contributing factors of aquatic organisms disease, poor growth and mortality in aquaculture (El-Haroun *et al.*, 2006 and Rollo *et al.*, 2006).

Functional additive, as probiotics, is a new concept on aquaculture (Li and Gatlin III, 2004), where the additions of microorganisms on diets show a positive effect on growth caused by the best use of carbohydrates, protein, and energy (Chang and Liu, 2002 and Irianto and Austin, 2002), diminishing mortality by disease, antagonism to pathogen, better microbial intestinal balance and in the environment (Holmstrom *et al.*, 2003). In some case this beneficial effect had attribute to the capacity of the probiotic to stimulate and/or produce some enzymes on the intestinal tract. For example, Lara-Flores *et al.*, (2003) observed a high activity of alkaline phosphatase in Nile tilapia (*Oreochromis niloticus*) when administered probiotics in the diet, the result show a high activity reflected a possible development of brush border membranes of enterocytes that can be stimulated by the probiotic and this it can be a indicator of carbohydrate and lipid absorption and explain the higher weight gain and the best feed conversion. The most likely explanation of the effective role of probiotics is their effect in suppressing pathogenic coli forms in the stomach and intestine and improving the absorption of nutrients by reducing the thickness of intestinal epithelium (Venkat *et al.*, 2004).

Ogunshe and Olabode (2009) evaluated the ability of *Lactobacillus fermentum* LbFF4 isolated from Nigerian fermented food (fufu) and *L. plantarum* LbOGI from a

beverage (Ogi) to induce immunity in *Clarias gariepinus* (Burchell) against some selected fish bacterial pathogens. A different strain (JCM 1136) of *Lactobacilus rhamnosus* was also used by Panigrahi, (2005) to test this immune response induced by the bacteria. Rainbow trout (*O. mykiss*) was fed a diet incorporated with different forms (heat-killed, live-sprayed or freeze-dried) of *L. rhamnosus* twice daily for 30 days, after which the diet was withdrawn and replaced with diet used for the control fish for up to 45 days. At the end of the study, fish fed the viable form (live-sprayed) of *L. rhamnosus* showed better phagocytic and complement activities. The plasma immunoglobulin levels also showed an increasing trend in those fish fed the live-sprayed diet. Probiotics in aquaculture can be effectively employed to help fish protect themselves and promote safe farming that would be less dependent on chemotherapy and vaccine for disease prevention (Panigrahi *et al.*, 2004). It seems likely that the use of probiotics will gradually increase and that success of aquaculture in future may be synonymous with the success of probiotics, which, if validated through rigorous scientific investigation and used wisely, may prove to be a boon for the aquaculture industry.

Microorganisms are commonly used in aquaculture to control microbiological ecosystems, especially to treat the water in tanks and ponds. This is known as the bio-remediation concept. The advantage of probiotic over bioremediation is a direct effect of the micro-organism in the gut, due to its ingestion, as well as a better control of the number of microorganism delivered to the animal and the environment. Probiotics colonize the gastrointestinal tract. In aquatic animals, the intestinal microflora change rapidly due to the constant influx of microbes from food and water, creating a transient microbial community. This transience makes it possible to use live microbial

preparations in ponds. It can therefore be difficult to distinguish between probiotics and bioremediators in aquaculture (Karunasagar, 2001).

Enzymatic reduction of Cr (VI) by hexavalent Cr-tolerant *Pseudomonas ambigua* G-1 isolated from activated sludge has been reported. The intracellular reducing enzyme required NADH as a hydrogen donor (Silver and Misra, 1998). A membrane associated chromate reductase activity from *Enterobacter cloacae* isolated from activated sludge has also been documented (Wang *et al.,* 1990). The ability of microorganisms to alter their chemical physiology in order to compensate for potentially traumatic changes in their external environment represents a built in factor of safety for biological survival. Growth of *Escherichia coli*, *Micrococcus luteus* and *Azotobactor* sps, in the presence of lead and growth of *Chlamydomonas reinhardi* in the presence of mercury are examples of biological accommodation (Cervantes *et al.,* 2001).

The term "probiotics" is used to describe the kind of non-pathogenic microorganisms that are used medicinally as lactic acid bacteria (LA) (Alm *et al.,* 1982; Duggan *et al.,* 2002 and Bengmark, 2005). It has been found that probiotic microorganisms can effectively trap reactive forms of oxygen. The experiment with vitamin E-deficient rats has revealed that the intracellular extract from *Lactobacillus* sp. recovers this deficiency (Kaizu *et al.,* 1993). It has been reported that *Propionibacterium freudenreichii* produces extracellular growth stimulator(s) for bifidobacteria that effectively suppress the production of peroxide under anaerobic conditions (Mori *et al.*, 1997). The classical yoghurt bacteria *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* inhibit peroxidation of

lipids through scavenging the reactive oxygen radicals, such as hydroxyl radical or hydrogen peroxide (Ling and Yen, 1999). The dietary supplementation with lactic acid bacteria (LA) modulates immunity, release antioxidants and improve gastrointestinal functions (Madsen *et al.,* 1999). Lactic acid bacteria may help in the maintenance of pH and protection against pathological changes in the colon (Salminen, 2001). Commensal bacteria affect a variety of complex behaviors, including social, emotional and anxiety-like behaviors and contribute to brain development and function in mice (Collins *et al.*, 2012) and humans (Tillisch *et al.*, 2013). Hsiao *et al.*, (2013) reported that treatment with *Bacillus fragilis* corrects intestinal permeability defects, as well as altered levels of tight junction proteins and cytokines in mice displaying gastrointestinal and neurological symptoms related to Autism spectrum disorder. Knowledge is accumulating that microbiota modulates gut physiology, immunological functions and may produce other beneficial effects. This has led scientists to investigate the efficacy of probiotics in the treatment of diseases and toxicities. Based on the foregoing literature evidences the present research work is being designed to determine fluoride toxicity in fish *Mystus montanus* and Albino rat *Rattus norvegicus* and bioremedial measures using probiotic bacteria.