

STUDIES ON ALTERATIONS IN GUT MICROBIOTA OF ZEBRAFISH IN RESPONSE TO FOOD ADDITIVES

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science in Microbiology

By

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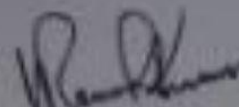
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Saranya.P (40300054) a who carried out the project entitled "STUDIES ON ALTERATIONS IN GUT MICROBIOTA OF ZEBRAFISH IN RESPONSE TO FOOD ADDITIVES" under my supervision from October 2022 to April 2023.

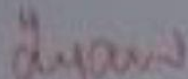

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ABSTRACT

Background of the study: Food additives have caused a sustained impact on neural development. Gut microbiota is capable of directly impacting the brain and central nervous system. Yet, there is little data on the contribution of food additives to gut microbiota hence this project's purpose is to use 16s based metagenomic approach to analyze the change of the gut microbiota of zebrafish exposed to various concentrations of food additives.

Methodology: Adult zebrafish (3 months old) were exposed to various concentrations of tartrazine (1.5ppm, 3.5ppm, and 5.5ppm) for 60 days. The toxicity effects were investigated by analyzing the survival rate, locomotor activity, and biochemical changes of the gut microbiota. The intestine of the control and tartrazine-exposed fishes were dissected, and the changes in gut microbiota were determined by the 16srRNA metagenomic approach.

Results: In control and 1.5ppm zebrafish, *Lactobacillus crispatus* was observed to be the dominant species. In contrast, the dominant species detected in 3.5ppm and 5.5ppm exposed zebrafish was *E. coli*.

Conclusion: This study for the first time demonstrated that exposure to food additives viz., Tartrazine causes intestinal dysbiosis.

Keywords: Food additives, Tartrazine, Zebrafish, Gut microbiota, locomotor activity, 16S rRNA,

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ABBREVIATIONS

IBD- INFLAMMATORY BOWEL DISEASE

CD- COHN'S DISEASE

1XPBS- PHOSPHATE BUFFER SALINE

TTZ- TARTRAZINE

MTP- MICEOSOMAL TRIGLYCERIDE TRANSFER PROTEIN

NAFLD- NON -ALCOHOLIC FATTY LIVER DISEASE

MP- MORTALITY PERCENTAGE

16s rRNA- 16s RIBOSOMAL RIBONUCLEIC ACID

FD-FOOD ADDITIVES

ADI-ACCEPTABLE DAILY INTAKE

NAFLD-NON-ALCOHOLIC FATTY LIVER DISEASE

CNS- CENTRAL NERVOUS SYSTEM

NAD-NICOTINE AMINO DINUCLEOTIDE

FAD- FLAVIN MONO NUCLEOTIDE

CHAPTER-1

1. INTRODUCTION

Microbiota is defined as the assemblage of living microorganisms present in an environment. The gut microbiome codes for biochemical functions that host genomes cannot encode, such as the breakdown of otherwise indigestible macromolecules into products that their host can utilize. The microbiome has been implicated in neural development and function, and consequently, perturbation of the microbiota is implicated in neurological disease. It is known that metabolites act as communication signals between the host and microbiome in the form of neuromodulators or neurotransmitters. Both neural and circulatory routes have been proposed as a means of gut-brain signaling, including the vagus nerve and enteric nervous system, and direct absorption from the intestinal lumen into the bloodstream. Here, we review the literature on the intestinal microbiota of the human host with a focus on bacteria and archaea and their potential importance for human metabolism. In the literature, there is no consensus on how to define metabolic health. Yet a tentative definition is to have an overall metabolism that in representative epidemiological studies linked with both desirable life quality and longevity. (Yong Fan et al., 2020).

1.1. Gut microbiota

The gut microbiota has a symbiotic relationship with the host, taking part in functions such as host metabolism and immunity. Microbiota also regulates the function of the intestinal barrier because they have a highly specific composition; however, this relationship can be altered either by intrinsic factors of the host or by the surrounding environment. Dysbiosis is caused by an imbalance in the gut microbiota, which causes an alteration in the host's homeostasis. Zebrafish is an omnivorous freshwater fish that belongs to the small carp family. It has been widely used for research purposes on embryology and tissue regeneration, molecular genetics, and reproductive biology. The microbiome has been recognized to profoundly affect the neurochemistry and central nervous system in zebrafish, the GI tract limits exposure of the host immune system to gut microbiota by recruitment of a multifactorial and dynamic intestinal barrier.

Microbiota composition is subject to shaping by host and environment selective pressures. To protect from injury and maintain homeo importantly microbial colonization is required for normal development. This study aims to investigate zebrafish microbiota alterations after exposure to commonly used food additives.

Through a variety of physiological processes, such as preserving gut integrity or reshaping the intestinal epithelium, the microbiota confers numerous advantages on the host. A role for the microbiota in many intestinal and extra intestinal diseases has steadily emerged as more advanced methods to profile and characterize complex ecosystems are developed. The development and makeup of the human GI microbiota, as well as its effects on gut integrity and host health, are discussed.

Ancient bacteria that lived in the guts of our common ancestors gave rise to the bacteria that now reside in the intestines of modern apes and humans. In order to compare rapidly evolving *gyrB* gene sequences in fecal samples from people and wild chimpanzees, bonobos, and gorillas, Moeller et al. developed a method (see the Perspective by Segre and Salafsky). A comparison of the major bacterial lineages' *gyrB* phylogenies shows that, with the exception of a few infrequent symbiont transfers between primate species, they largely match the ape hominid phylogeny. Therefore, gut bacteria are not just acquired from the environment; they have been coevolving for millions of years with hominids to influence the development of our immune systems.

The host genome, method of delivery at birth, geography, stage of life, use of antibiotics, and diet continuously influence the make-up and function of the dynamic microbial community that makes up the gut microbiome. One of the most important factors in determining the integrity of the microbiome is diet. Early life diet appears to have a significant impact on the risk of later health or disease. For instance, eating ultra-processed foods as a child or adolescent may increase the risk of developing inflammatory bowel disease or colorectal cancer later in life. This effect is thought to be mediated by modulation of the gut microbiota. When gut diseases are already present, dietary factors can have an impact on symptoms and disease activity, act as a risk factor for future disease, or be used as a treatment to lessen disease activity.

1.2 FOOD ADDITIVES

Many substances are incorporated into foods for functional purposes, and in many cases, these ingredients can also be found occurring naturally in some food. However, when they are used in processed foods, these chemicals have beyond me known as "food additives." From a regulatory standpoint, each of the food additives must provide some useful and acceptable function or attribute to justify its usage. Generally, improved keeping quality, enhanced nutritional value, functional property provision and improvement, processing facilitation, and enhanced consumer acceptance are considered acceptable functions for food additives. One of the technologies driving the so-called Industry 4.0 paradigm shift is an additive manufacturing (AM). Scholars continue to debate the actual and potential effects of this technology on manufacturing, logistics, and operations strategy as well as on perspectives on sustainability and the overall business strategy. 53.8% of food items contained at least one food additive, and 11.3% had at least five. Artificially sweetened beverages, ice cream, processed sandwiches, biscuits, and cakes were the food categories most likely to contain food additives (in more than 85% of food items).

Citric acid, lecithins, and modified starches were the most widely used food additives (>10,000 products each). Some food additives with suspected health effects also pertain to the top 50: sodium nitrite, potassium nitrate, carrageen an, monosodium glutamate, sulfite ammonia caramel, acesulfame K, sucralose, (di/tri/poly) phosphates, mono- and triglycerides of fatty acids, potassium sorbet, cochineal, potassium metabisulphite, sodium alginate, and bixin (>800 food products each). Six groups of food additives that frequently co-occur in food products were identified by us. Food additives are frequently used in commercials. Substances that are of little or no nutritive value, but are used in the processing or storage of foods or animal feed, especially in developed countries; include antioxidants; food preservatives; food coloring agents; flavoring agents; anti-infective agents; vehicles; excipients and other similarly used substances. Many of the same substances are pharmaceutical aids when added to

pharmaceuticals rather than to foods. Food additives are substances added to food to preserve flavor or enhance its taste and appearance. Some additives have been used for centuries; for example, preserving food by pickling with vinegar, salting, as with bacon, preserving sweets, or using sulfur dioxide as in some wines. With the advent of processed foods in the second half of the 20th century, many more additives have been introduced, of both natural and artificial origins. It is sometimes wrongly thought that food additives are a recent development.

The business models, strategies, and operations of companies are changing as a result of this emerging technology, which has enormous advantages for the sustainability of their social, economic, and environmental systems (Niaki and Nonino, 2018b). Food products may contain a variety of additives to achieve desired effects like flavor, preservation, and sweetness. The European Food Safety Authority, the Organization for Economic Cooperation and Development, the International Conferences on Harmonization, and the United States Food and Drug Administration all publish lists of approved food additives along with guidelines for safe use. By using a variety of techniques, the food additives are specifically screened for cytotoxicity, genotoxicity, mutagenicity, and hepatotoxicity. Cytogenetic evaluation is used to conduct food additive safety studies, which include gene mutation assays, long-term carcinogenicity tests, reproductive and developmental toxicology tests, and activity tests for hyperactivity, anxiety, and depression. Inappropriate *in vivo* follow-up assays and subject specialization are these studies' limitations.

1.2.1 Importance and disadvantages of food additives

Numerous people regularly consume a variety of food additives, which have both benefits and drawbacks. Food additives play a significant role in today's plentiful and nutrient-dense food sources, enabling people to enjoy a variety of wholesome, delectable, and secure foods throughout the year. Different positive effects of food additives on foods exist. However, some metabolites found to be carcinogens, like monosodium glutamate and nitrous compounds, may be present in food additives. Whether a food component is toxic or beneficial depends on how much it interferes with

digestion, elimination, or metabolism. The interaction between various substances further complicates the description of the appropriate safety limits for human ingestion. The likelihood that chemical compounds will be toxic means that all new compounds should be thought of as toxic in the context of nutrition.

Due to the possibility of chemical compounds being toxic, all novel compounds in nutrition should initially be thought of as toxic until their safety has been established. Food additives can occasionally destroy vitamins in food (adding caramel to food has been shown to result in a vitamin B6 deficiency), are used to make poor-quality food look good, and can cause allergies in many people, including diarrhea, skin irritation, stomach disorders, vomiting, or an increase in body temperature. Additionally, it might reduce food's nutritional value. A number of food colorings have been prohibited because of their propensity to cause tissue damage and cancer. People who consume tartrazine as a food additive have been shown to experience a wide range of negative side effects and allergic reactions. These include migraines, anxiety, and attacks of asthma. (Kamal A et al., 2007)

1.3 TARTRAZINE

Artificial azo dyes are playing a very important role in the appearance and enhancement of the quality of foods. Azo dyes are making products more attractive to consumers, particularly children (Hofer and Jenewein, 1997). Azo dyes have been in use for a long time in the food industry worldwide, but their uses are controversial in light of its adverse effects on health (Eman et al., 2000). Azo dyes contain at least one group in the molecule with two to three aromatic rings. The European Union has permitted some azo dyes to be used in food products such as tartrazine (TTZ) (E102); Quinoline Yellow (E104) and Sunset Yellow (E110). TTZ is an orange-colored, water-soluble dye with inclusive applications in food industries, cosmetics, textiles, drugs, and pharmaceuticals (Merinas-Amo et al., 2019). TTZ is an anionic molecule having a benzene ring also shown in Fig. 1A. Food Additives (JECFA) reported the data of more than 300 studies on laboratory animals and clinical trials on human beings on toxicological concerns in 1964. From the report, it was found that TTZ has no cancer-causing potential while having little adverse effect on reproduction and development (Collins et al.,

1990, Tanaka et al., 2008). Thus, intake of low concentrations of TTZ doesn't cause any genotoxicity in humans (Poul et al., 2009). It will be interesting to check the effects of Tartrazine on proteins found in milk.

Tartrazine is a synthetic lemon-yellow azo dye primarily used as a colorant in food products and cosmetics. Tartrazine is a monoazo pyrazolone dye used as a colorant in bakery products, flavoring cheese, carbonate toothpaste, drugs, and cosmetics. In addition, this dye is also used as a substitute for saffron in tropical cuisine. Although the acceptable daily intake of tartrazine for humans is 7.5 mg/kg body weight, prolonged use of this dye at higher concentrations is reported to increase the number of lymphocytes and eosinophils in gastric mucosal cells. Produce abnormal reproductive cells. In recent years, the zebrafish has emerged as a versatile vertebrate model for studying. Tartrazine is the best-known and one of the most commonly used food additives. Food colorants are also used in many medications as well as foods. There is no evidence that tartrazine makes asthma worse or that avoiding it makes asthma patients any better.

The use of tartrazine yellow additives in human food products is permitted within limits. However, studies have described this as a toxic component. The dye, which is intensively used in industry and commerce, enters the aquatic environment through releases of non-treated or inadequately treated effluents. However, further ecotoxicological research is needed. Tartrazine e-number 102 Tartrazine usage ranged from 12.5 to 1092 mg/kg³. Another study from Uttar Pradesh reported that 59% of food items contained more than the maximum limit, with average quantities crossing the threshold of 100 mg/kg in most food commodities.

Tartrazine has been found by numerous researchers who have been examining the quality and safety of food products. We highlight various tartrazine detection and extraction techniques in this review paper. High-performance liquid chromatography, electrochemical sensors, thin-layer chromatography, spectro photometry, capillary electrophoresis, and liquid chromatography-tandem mass spectrometry are a few of the analytical techniques that are available. Additionally, we go over the extraction procedures known as liquid-liquid extraction, solid-phase extraction, membrane

filtration, cloud point extraction, and others. Additionally, a succinct overview is provided explaining tartrazine's synthesis, metabolism, and the highest levels allowed in various nations.

Tartrazine excretion and metabolism have been investigated in rats, rabbits, and humans. The route of administration affects the type of excretory products. The same color is excreted after intraperitoneal injection. Sulphanilic acid that is partially conjugated is excreted in equivalent amounts as a result of oral dosage.

The spectroscopic, microscopic and computational results support that the electrostatic, as well as hydrophobic interactions, played a very important role in TTZ-induced BLG fibrillation.

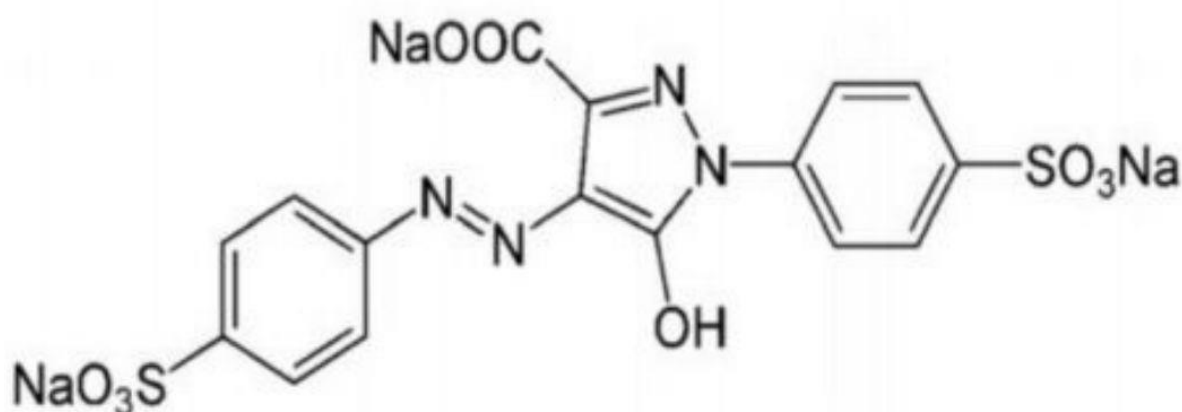


Figure 1.1 Structure of Tartrazine

1.3.1. EFFECTS OF FOOD ADDITIVES ON GUT-MICROBIOTA

Disturbance of the microbiota –host balance is associated with numerous chronic inflammatory diseases, including inflammatory bowel diseases and a group of obesity associated diseases collectively referred to as metabolic syndrome and insulin resistance. Such as nonalcoholic liver disease (NAFLD). Recent research has demonstrated the role of gut microbiota in energy storage (Luxiu Zang et.al. 2019). Inflammatory bowel diseases (IBD), which included Crohn's disease (CD) and ulcerative colitis, are featured as chronically recurrent inflammatory disorders of the digestive tract.

In recent decades IBD has affected about 150_200 cases per 100,000 in western countries. Moreover, its prevalence in many newly industrialized countries presents a trend that increases fleetly, indicating the crucial player of environmental factors as the disease progresses. Gut microbiota is a aberrant in obesity. The community structure and functionality of the human gut microbiota are significantly influenced by dietary factors. Little research has been done on the effects of different food additives on gut microbiota, despite the fact that many dietary components, including carbohydrates, fats, proteins, and phytochemicals, have been extensively studied for their effects. The incidence of obesity and its metabolic co-morbidities in developed countries has increased dramatically since the mid-twentieth century. Sedentary lifestyles and increased food consumption in combination with a widespread polygenic susceptibility are considered to be the major cause of the obesity epidemic. The gut microbiota has been confirmed as an important part of human health, and is even taken as an 'organ'. The interaction between the gut microbiota and the host intestinal environment plays a key role in digestion, metabolism, immunity, inflammation, and diseases. The dietary component is a major factor that affects the composition and function of gut microbiota. A number of allergic and autoimmune diseases are pathogenic by impaired intestinal barrier function in addition to genetic factors and antigen exposure. Thus, it's crucial to lessen the effects of everyday life that harm the barrier and promote the absorption of harmful agents. Even the most frequently consumed foods, like bakery goods, heavily employ synthetic surfactants to the overuse of food surfactant additives,

Recently, increased attention has been given to the benefits and toxicity of synthetic food additives in food, particularly for children. Tartrazine, an artificial azo dye, is one of these additives. Outline the current research on the positive and negative effects of food additives, paying particular attention to tartrazine's effects on the health of various organs. The procedures include a current search of the pertinent databases. The studies focused on the effects of tartrazine on the nervous system, liver, kidney function, lipid profile, oxidative stress biomarkers, hyperactivity, behavior, cancer, reproductive and developmental toxicity, and some element levels of tartrazine. They also provided a description of the different types of food additives and products

containing tartrazine. Numerous studies were found, some of which looked into tartrazine's benefits and drawbacks.

The gut microbiota contributes to diverse aspects of host physiology, ranging from immune modulation to drug metabolism. Changes in the gut microbiota composition are associated with various diseases as well as with the response to medications. It is therefore important to understand how different lifestyle and environmental factors shape gut microbiota composition. Beyond the commonly considered factor of diet, small-molecule drugs have recently been identified as major effectors of the microbiota composition.

1.3.2. Food additives cause disease

Tartrazine is reduced inside an organism to an aromatic amine, which is greatly sensitized since it is a nitrous derivative (azo class). The chief metabolite recognized so far is sulfanilic acid. Tartrazine is identified to cause allergies such as urticaria and asthma, besides the emphasis of studies on its carcinogenesis and mutagenesis because of its metabolic conversion into an aromatic amine (sulfanilic acid) via the gut microflora (Moutinho et al., 2007) and possibly by mammalian azo reductase in the hepatic or intestinal wall after consumption (Chequer et al., 2011). When these azo dyes are totally reduced into aromatic amines, they are oxidized to N-hydroxy derivatives by the enzymatic system of P450 (Demirkol et al., 2012). This mechanism of biotransformation takes place in many species, including humans (Chequer et al., 2011), and is responsible for various disorders, including anemia, pathological lesions in the brain, liver, kidney, and spleen, aside from allergic reactions, tumors, and cancer. However, tartrazine has no possibility to induce malignant or benign neoplasias (Kamal A et al., 2007). In China, food-safety issues that are related to food additives, such as their misuse or overuse due to anthropogenic stressors, or the reckless use of nonedible chemical substances, have become increasingly prominent. Studies have shown that, between 2006 and 2015, a total of 253,617 food-safety incidents were reported in China, of which 75.5 percent were caused by anthropogenic factors.

Moreover, the highest number of food-safety incidents was caused by the illegal use of food additives, which accounted for 34.36 percent of the total.

Gut bacteria play an important role in several metabolic processes and human diseases, such as obesity and accompanying co-morbidities, such as fatty liver disease, insulin resistance/diabetes, and cardiovascular events. Among other factors, dietary patterns, probiotics, probiotics, symbiotic, antibiotics, and non-dietary factors, such as stress, age, exercise, and climatic conditions, can dramatically impact the human gut microbiota equilibrium and diversity. However, the effect of minor food constituents, including food additives and trace contaminants, on human gut microbiota has received less attention.

1.4 ZEBRAFISH: ANIMAL MODEL

The zebrafish is a freshwater fish belonging to the minnow family of the order Cypriniformes, native to South Asia. It is a popular aquarium fish frequently sold under the trade name "zebra danio." It is also found in private ponds. The zebrafish has become known as an excellent model organism for studies of vertebrate biology. Vertebrate genetics, embryonic development for use in genetic studies as an animal model, the zebrafish was initially introduced by Streisinger and colleagues in the early 1980s. Zebrafish compared to 80% of human genes Zebrafish for understanding human genetic diseases Zebrafish possess several advantages over rodent models in the study of vertebrate development and diseases. One such popular animal model is that of the Zebrafish (*Danio rerio*), a teleost found in freshwater systems, which has been at the forefront of several research fields since the 1980s. When zebrafish models started to be used in research, the primary focus was to contribute to the field of developmental biology, with thousands of zebrafish mutant models being identified with highly conserved disease genes among different species. This finding made researchers aware of the importance of the zebrafish model in other research fields and subsequently led to gene cloning through rigorous expansion of gene mapping resources. Today, zebrafish are being seen as a powerful model of acquired and genetic human diseases with increasing utility in translational research.

The freshwater teleost is a popular animal model choice because of its high embryo yield, and rapid reproduction rate. The necessity of model organisms to recapitulate humans has led to the exploitation of several animal species, among which rodents have been widely employed. Host-pathogen interactions are complex but the zebrafish can also be used to investigate pathogenic mechanisms. Such approach is feasible due to the conservation of innate immunity mechanisms in the fish, including mucus secretion, synthesis of piscidins and defensin enzymes, and the inflammatory response triggered by bacterial components (LPS, peptidoglycan, flagellin) interacting with Toll-like receptors. Furthermore, the zebrafish also has an adaptive immune system sharing. Arguably one of the most exciting aspects of the gut microbiota is its role in brain development and function. In that context, one of the limitations of the rodent models has been the use of relatively invasive methods to monitor the live development and activity of the brain. The transparency of the zebrafish, either in larvae or adult stages, allows live brain imaging, without the need for implanting a probe or a cranial window to perform two-photon imaging or light-sheet microscopy. Animal models are used extensively in medical research to better understand the causes of both human and animal diseases as well as to facilitate the creation of novel treatments. Though rodents are still the most popular research model used globally, zebrafish (*Danio rerio*) models have rapidly gained popularity among scientists in recent years. This is due to the fact that a tiny tropical freshwater teleost fish shares significant physiological, anatomical, and genetic similarities with mammals. In order to better understand the genetic, toxicological, and behavioral aspects of various human diseases, zebrafish serve as an excellent experimental model. Testing novel therapeutics, such as a new vaccine's safety, is also beneficial.

Table 1.1 Taxonomy classification of zebrafish (Danio Rerio)

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Cypriniformes
Family	Cyprinidae
Sub family	Danioniane
Genus	Danio
Species	Danio Rerio

1.4.1. 16S rRNA USED IN ZEBRAFISH

The use of a 16S rRNA gene sequence to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. There reasons, include (i) its presence in almost all bacteria often existing as a multi gene family, or operons; sequence changes are a more accurate measure of time (evolution); and (iii) The 16S rRNA gene (1500 bp) is large enough for informatics purposes (MichaelJanda et.al., 2007). Bacterial 16S rRNA amplicon sequencing was performed on DNA isolated from homogenates of the larvae revealing distinct microbiota variations between the two groups. The dataset described here is also related to the research article entitled "Microbial. The 16S rRNA gene has been used extensively in culture-independent molecular surveys for the past 25 years to characterize the structure of microbial communities. Metagenomic, which offers comprehensive view of a community's functional potential rather than just an inventory of its members, has recently eclipsed this strategy. However, a number of methodological developments, including higher resolution (more sequences), analysis of numerous related samples (e.g., spatial and temporal series), improved metadata, and the use of metadata, are helping the groundbreaking 16S rRNA gene make a comeback in its own right. Now, detailed insights into microbial ecology and evolution based solely on this one historically significant marker gene are replacing the

conventional conclusion that microbial ecosystems are remarkably complex and diverse.

Understanding that libraries of PCR-amplified 16S rRNA and 16S rRNA genes may not provide a precise or comprehensive representation of the bacterial community is crucial. First off, there are so many different species (28, 46, 109) that libraries of 400 cloned sequences must only be a partial sampling. Even the sum of all currently published sequences would appear to be an incomplete count of all 16S rRNA genes on the planet (98). The contributions of the various bacterial groups to libraries might also be biased. Different bacteria may have different nucleic acid extraction efficiencies, different numbers of copies of 16S rRNA or 16S rRNA genes per cell, and preferential amplification of specific sequences. Modulation of behavior and stress responses in zebrafish. 16S- based metagenomic approach to analyze the changes of gut microbiota of adult zebrafish we exposed to the food additives. Data presented here represent results of 16S rRNA sequencing of V4 region amplicons, generated using the Illumina MiSeq platform. Data are presented at the taxonomic levels of phylum, family, and the operational taxonomic unit, and represent an average coverage of 4235 reads per sample. We report here 16S rRNA gene amplicon sequence analysis of the gut microbiota in three species of deep-sea fish collected from Suruga Bay, Japan. 16S rRNA gene-based MTP pipeline was subjected to the taxonomic assignment using the sequence identity thresholds proposed previously. Sample and sequence data are summarized. Recently, our ability to survey the breath of the gut microbiota has greatly improved due to the advent of culture-independent approaches such as high-throughput and low-cost sequencing methods. Targeting the bacterial 16S ribosomal RNA (rRNA) gene is a popular approach since this gene is present in all bacteria and archaea and contains nine highly variable regions, which allow species to be easily distinguished. Former techniques concentrated on sequencing the entire 16S rRNA gene. We compared the gut bacterial communities of recently collected zebrafish from their natural habitat with those that had been raised in lab facilities for generations in various geographical locations using 16S rRNA gene sequences. The extreme insensitivity and bias of culturing methods were highlighted since 76% of the rRNA sequences obtained from an adult male fecal sample belonged to novel and uncharacterized species.

(Elizabeth Thorsby et al., (2017). Raw sequences from 16S rRNA gene profiling are available in the NCBI SRA database with access numbers.

CHAPTER-2

2. LITERATURE SURVEY

In an average life time, around 60 tons of food pass through the human GI tract, along with an abundance of microorganisms from the environment which impose a huge threat on gut integrity. The collection of bacteria, archaea and eukarya colonising the GI tract is termed the 'gut microbiota' and has co-evolved with the host over thousands of years to form an intricate and mutually beneficial relationship. The number of microorganisms inhabiting the GI tract has been estimated to exceed 10^{14} , which encompasses ~10 times more bacterial cells than the number of human cells and over 100 times the amount of genomic content (microbiome) as the human genome. However, a recently revised estimate has suggested that the ratio of human: bacterial cells are actually closer to 1:1. As a result of the vast number of bacterial cells in the body, the host and the microorganisms inhabiting it are often referred to as a 'super organism' (Thursby et al., 2017).

Gut bacteria play an important role in several metabolic processes and human diseases, such as obesity and accompanying co-morbidities, such as fatty liver disease, insulin resistance/diabetes, and cardiovascular events. Among other factors, dietary patterns, probiotics, prebiotics, symbiotic, antibiotics, and non-dietary factors, such as stress, age, exercise, and climatic conditions, can dramatically impact the human gut microbiota equilibrium and diversity. However, the effect of minor food constituents, including food additives and trace contaminants, on human gut microbiota has received less attention (Roca-Saavedra P, et al., 2018).

The gut microbiota has been confirmed as an important part in human health, and is even taken as an 'organ'. The interaction between the gut microbiota and host intestinal environment plays a key role in digestion, metabolism, immunity, inflammation, and diseases. The dietary component is a major factor that affects the composition and function of gut microbiota. Food additives have been widely used to improve the color, taste, aroma, texture, and nutritional quality of processed food. The increasing variety and quantity of processed food in diets lead to increased frequency and dose of food

additives exposure, especially artificial food additives, which have become a concern of consumers. There are studies focusing on the impact of food additives on the gut microbiota, as long-term exposure to food additives could induce changes in the microbes, and the gut microbiota is related to human health and disease (Xueweizhou et al.; Yuyuzhang et al., 2023)

Dietary factors substantially influence the community structure and function of human gut microbiota. Although many dietary components, such as carbohydrates, fats, proteins and phytochemicals, have been extensively explored for their impact on gut microbiota, little attention has been paid to the influences of various food additives on gut microbiota. In vitro studies, animal models, and human clinical trials all indicate that different types of food additives could modify gut microbiota and health status (Yucan et al., 2020).

Food additives are chemical substances which are added intentionally to food to upgrade its quality. Allura red (food color) and sodium benzoate (food preservative) are the most commonly used food additives in food industries, pharmacies as well as in cosmetics for different purposes like preserving and coloring. Experimental data from the last ten years on Allura red and sodium benzoate caused liver, kidney, reproductive, and brain toxicity in various animal models have been reviewed. Allura red and sodium benzoate cause histological changes in the liver, kidney and, stomach, DNA damage, behavioral changes, biochemical changes, hematological alterations and oxidative stress at various dose levels (Shahani L And Sharma 2021).

The use of food additives in food product is inevitable in this modern world. Safe amount of food additives is approved but safety has been questioned. In fath Gultekin et al., (2020) review, artificial sweeteners are highly likely to destroy glucose tolerance and support weight gain by affecting microbiota negatively. Most of the sugar alcohols are fermentable by bacteria and may show similar properties to prebiotics.

Aqua feed companies aim to provide solutions to the various challenges related to nutrition and health in aquaculture. Solutions to promote feed efficiency and growth, as well as improving the fish health or protect the fish gut from inflammation may include dietary additives such as probiotics and prebiotics. The general assumption is that feed

additives can alter the fish microbiota which, in turn, interacts with the host immune system. However, the exact mechanisms by which feed influences host-microbe-immune interactions in fish still remain largely unexplored. Zebrafish rapidly have become a well-recognized animal model to study host-microbe-immune interactions because of the diverse set of research tools available for these small cyprinids. Genome editing technologies can create specific gene-deficient zebrafish that may contribute to our understanding of immune functions. Zebrafish larvae are optically transparent, which allows for in vivo imaging of specific (immune) cell populations in whole transgenic organisms. Germ-free individuals can be reared to study host-microbe interactions. Altogether, these unique zebrafish features may help shed light on the mechanisms by which feed influences host-microbe-immune interactions and ultimately fish health (Adria Lopez Nada et al., 2020)

In the past few decades, zebrafish models have proven their value by becoming such potent models of human disease that their use has been expanded to various research fields to understand the underlying mechanisms of the diseases. Due to the 70% genetic homology between human and zebrafish genes, zebrafish are being studied as model animals in understanding the molecular basis and genetics of many diseases and fascinating facts about the diseases are emerging, establishing it as a very powerful tool for upcoming research. In the near future, these potential research areas can be investigated using zebrafish as a model (Adhish et al., 2023). They are used to determine effect of gut microbiota on the growth of the brain. (Stagaman et al., 2022).

Experimental analysis of gut microbial communities and their interactions with vertebrate hosts has been conducted predominantly in domesticated animals and zebrafish intestinal habitats selected for specific bacterial taxa despite radical differences in host provenance and domestication status (Guus Roeselers et al., 2011).

Gut microbiota plays a critical role in the maintenance of host health. As a low-cost and genetically tractable vertebrate model, Zebrafish and humans share some similarities in intestinal physiology and function, and this allows zebrafish to be a surrogate model for investigating the crosstalk between the gut microbiota and host. Zebrafish is an attractive research tool to understand mechanistic insights into host-microbe interaction (Hui Xia et al.; Comualt et al., 2022).

According to Manuele Rinninella et.al. (2021) study, titanium dioxide (TiO₂), a food additive in pastries, sweets, and sauces was exposed to 18 animals (10 mice, 5 rats, 2 fruit flies, and 1 silkworm) and caused variations in abundance in specific bacterial species and lead to gut dysfunctions.

Carlos et.al. (2020) have exposed WAF and CEWAF of a light crude oil to the adult zebrafish and analyzed the changes in the gut microbiota using a 16S-based metagenomic approach and observed a dysbiosis in the gut microbiota of *Danio rerio*.

Abnormal gut microbiota may contribute to various common metabolic disorders including obesity, type 2 diabetes, non-alcoholic liver disease, cardio-metabolic diseases, and malnutrition (Yong Fan et.al. 2021).

An authorised azo dye called tartrazine (FD&C Yellow No. 5) can be found in lots of medications and food items. There were numerous reports of tartrazine sensitivity in the 1970s. As a result, new laws were passed requiring the disclosure of azo dyes on food and drug package inserts. Urticaria and asthma are the most frequent side effects of tartrazine sensitivity. Tartrazine is not regarded as a violator, despite the fact that azo dyes have been linked to the amplification of hyperkinetic syndromes. Rarely, tartrazine sensitivity will manifest as contact dermatitis, purpura, or vacuities. The cause of sensitivity is unknown and is thought to be pseudo allergic. Avoiding medications and food products that contain tartrazine is the mainstay of management (J R Diploma, 1990).

Vani Joshi et al., (2017) study evaluates the impacts of TTZ on the embryonic development of zebrafish (*Danio rerio*). The embryos were exposed from the gastrulation stage (5.25 hours post fertilization [hpf]) until hatching and developmental trajectory was traced up to day 7 and concluded TTZ is not embryo toxic or teratogenic for zebrafish embryos.

The effects of tartrazine consumption on health were evaluated using a crucian carp (*Carassius auratus*) as the experimental fish. The results indicate that tartrazine consumption, even at a low concentration causes severe cellular alterations, oxidative stress and inflammatory response in crucian carp. They could also induce gut microbiota dysbiosis (Limin Wu et.al., 2021).

Athira N and Jaya DS (2020) studied the effects of tartrazine on the growth and brain biochemistry of Indian major carp. The results indicate impairment in bimolecular production and cellular destruction and the variation in antioxidant enzymes indicates the stress associated with the toxicity of tartrazine. The biochemical alterations in the brain resulted in the behavioral pattern and growth of the experimental fishes. The scientist confirmed the cytotoxicity and neurotoxicity of the azo dye.

A higher intake of erythrosine and tartrazine is shown to exhibit adverse effects in mammalian models, and is thus a matter of public health concern. Radhika Gupta et al., (2021) studied the effects of erythrosine and tartrazine on causing oxidative stress in zebrafish embryos. Erythrosine-treated zebrafish embryos hatch much slower when compared to tartrazine-treated embryos and control embryos. While erythrosine affects yolk utilization, tartrazine exhibits teratogenic effects on early zebrafish embryos. The SOD mRNA expression as well as the biochemical analysis indicates that exposure to food colorants induces cyto plasmic SOD transcription to combat the ROS toxicity in zebrafish embryos.

CHAPTER-3

3. AIM AND SCOPE OF THE PRESENT INVESTIGATION

3.1 AIM

The aim of the study is to investigate how food additives tartrazine alter the zebra fish's gut microbiota.

3.2. SCOPE

This study using zebrafish as an animal model investigates the effects of the daily acceptable intake of food additives on gut microbiota.

CHAPTER-4

4. MATERIALS AND METHODS

4.1. MATERIALS

4.1.1. EQUIPMENTS AND GLASSWARE

Cooling centrifuge, tissue culture flasks, autoclave, stereo zoom microscope, centrifuge tubes, measuring cylinders, NanoDrop, dissection needles, gloves, Personal Protective Equipment and Fish tank, Needle, Blade, Scissors, Ice cubes, dissection mat. All the glassware were thoroughly cleaned and sterilized as per the standard procedures. For DNA work plastic wares were autoclaved (121°C for 20 minutes at 15psi) and then used.

4.1.2. Chemicals

Tartrazine (C.I. 19140, CAS No 1934-21-0, Mw 534,37, synonyms: E 102, Food Yellow 4, Acid Yellow 23, FD &C Yellow No.5) is an azo dye with the chemical formula 4,5-Dihydro-5-oxo-1-(4-sulfophenyl)-4-((4-sulfophenyl) azo)- 1H-pyrazole-3-carboxylic acid, TRIZOL Reagent, Chloroform, Methanol, Ethanol, Iso-Propanol, 1x PBS, Sodium Citrate and tri-sodium salt (95%) was purchased from TCI chemicals (Tokyo Chemical Industries), India. All other chemicals and solvents used in this study were of analytical grade.

4.2 Fish Husbandry and Exposure

Six-month-old healthy male adult wild-type zebrafish (*Danio rerio*) were raised under a standard laboratory condition (28 °C with 14/10 h light/dark cycle). The fish were fed in the morning and evening during the exposure. The randomly selected male adult zebrafish were exposed to 1.5ppm, 3.5ppm, and 5.5ppm concentrations of Tartrazine for 60 days respectively. In each treatment, 20 male zebrafish were reared in a glass tank with 3 L water (pH 7.0–7.5 and hardness 40–60 mg CaCO₃/L), and the exposure

solutions were changed every other day. All the zebrafish were exposed to the same laboratory conditions, and the samples in all fish were collected correspondingly.

4.3 Sample Collection

After 60 days exposure, the fish were firstly anesthetized on ice and killed by cutting off the spine before being dissected. Then the whole gut in each fish was immediately excised. Every four whole gut was collected as one sample. During the sample collection process, all samples were washed in 1X PBS buffer and distilled water. The gut samples in each group were used for DNA extraction using the Trizole reagent.



Figure 4.1 Zebrafish intestine dissected from control male zebrafish

4.4 DNA Extraction

4.4.1 Principle

The basic principle of DNA isolation is disruption of the cell wall, cell membrane, and nuclear membrane to release the highly intact DNA into solution followed by precipitation of DNA and removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols, and other secondary metabolites by enzymatic or chemical methods. TRIZOL is a reagent that solubilizes biological materials and denatures protein. It is used to disrupt cells and dissolves their cellular components. TRIZOL also acts as a strong protein denaturant, not just breaking the cell wall and membrane but also inactivating RNases or DNases, whereas the chloroform helps in the separation of RNA. The additional advantages are that all analyses can be

performed on the same cell mass; this facilitates direct comparisons of alterations in the genome, transcriptome, and proteome.

4.4.2 Extraction procedure

1. The sample was homogenized in 500 μ l of TRIzol reagent and incubated for 15 minutes.
2. Following this 200 μ l of chloroform was added and mixed and incubated for 15 minutes. After the incubation, the sample was centrifuged at 12,000 rpm for 10 minutes at 4°C.
3. The aqueous upper layer was used for RNA extraction. The bottom layer from the RNA isolation process was taken and treated with 150 μ l of 100% ethanol.
4. The sample was inverted and incubated at room temperature for 2-3 minutes and centrifuged at 2000 rpm for 5 min. at 4°C.
5. The pellet was resuspended in 1 μ l of 0.1M sodium citrate solution and was incubated for 30 minutes.
6. The incubated sample was centrifuged for 5min for 2000rpm at 4°C.
7. The supernatant was discarded and fifth step was repeated.
8. The pellet was resuspended in 2ml of 75% of ethanol and incubated for 10-20mins and was centrifuge at 2000rpm for 5 minutes at 4°C.
9. The pellet was dried and was stored with 30 μ l of DEPC water.
10. Quantification of isolated DNA was done using the NanoDrop (Thermo USA). The OD 260/280 value of ~1.8 was considered good quality and used for further studies.

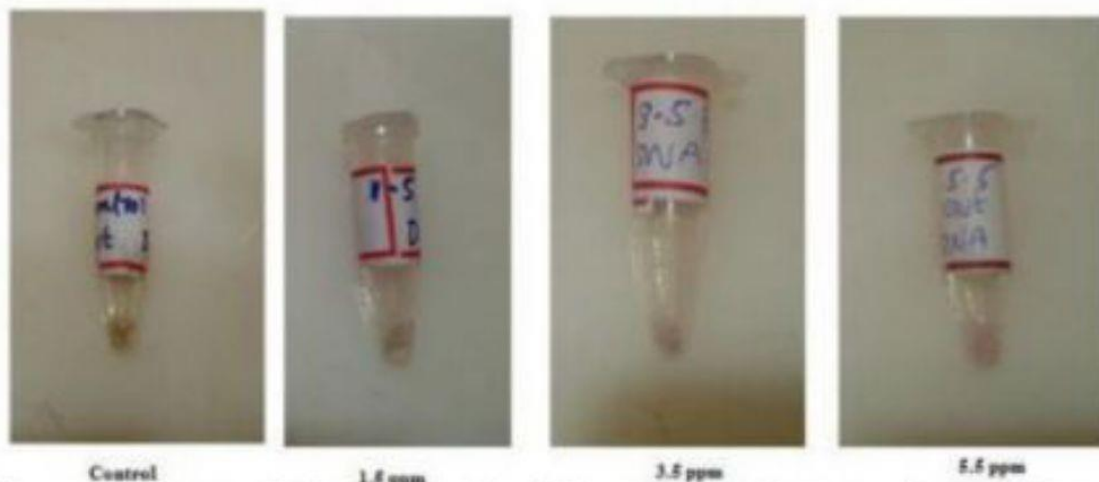


Figure 4.2 Extracted DNA of the zebrafish gut exposed to tartrazine at different concentrations (1.5ppm, 3.5ppm and 5.5ppm)

4.5 Locomotor activity

Locomotor activity assays were performed as described with minor modifications (Miklosi A and Andrew R.J., 2006). Locomotor activity of the zebrafish was studied in a fish tank marked with graph lines of 1 cm distance. Fishes were allowed to swim for 1 minute and the total distance crossed was noted. This was performed at 12, 18, 24, 30, 36, 42, and 50 and 60 days respectively for both control and treated (1.5ppm, 3.5ppm and 5.5ppm) groups. The results were documented and compared.

4.6 16S rRNA Gene Amplification

The V3-V4 region of the 16S rRNA bacterial gene was amplified by PCR from the gDNA of each sample. For PCR amplification, we used the 16S rRNA Forward primer: 5'TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGG GNGGCWGCAG and 16S rRNA Reverse primer: 5'GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC, which amplify a region of ~550 bp (35). PCRs were performed in a volume of 25 μ L containing 12.5 μ L of Dream Taq Green PCR Master Mix (2X) (Thermo Scientific®), 0.5 μ L of gDNA (equivalent to 328 ± 23 ng/ μ L of gDNA), and 0.115 μ L of each primer (125 nm). All reactions were performed in a Thermal cycler C1000 Touch TM (Bio-Rad Laboratories®) using a cycling program as follows: initial denaturation of 3 min at 95°C

followed by 36 cycles of denaturation for 30 s at 95°C, annealing of 30 s at 53°C, an extension of 60 s at 72°C, and a final extension of 5 min at 72°C. PCR for no-template control was included to guarantee that no cross-tube reactions occurred ().

4.7 Statistical Analysis

The total weigh and length of each fish were measured at the beginning and at the end of the experiment (Day 1 and Day 60, respectively), and analyzed by a two-tailed Student's t-test. The locomotor activity was analyzed by One Way ANOVA. SPSS 15ver software was used to perform One Way ANOVA and two-tailed Student's t-test in order to determine the significance of difference in the studies between the Control groups and Test groups. All data are reported as a mean $\pm$ standard error (SEM) and * $p < 0.05$ and ** $p < 0.01$ were considered statistically significant

CHAPTER-5

5. RESULTS

5.1 Effect of Tartrazine in locomotor activity

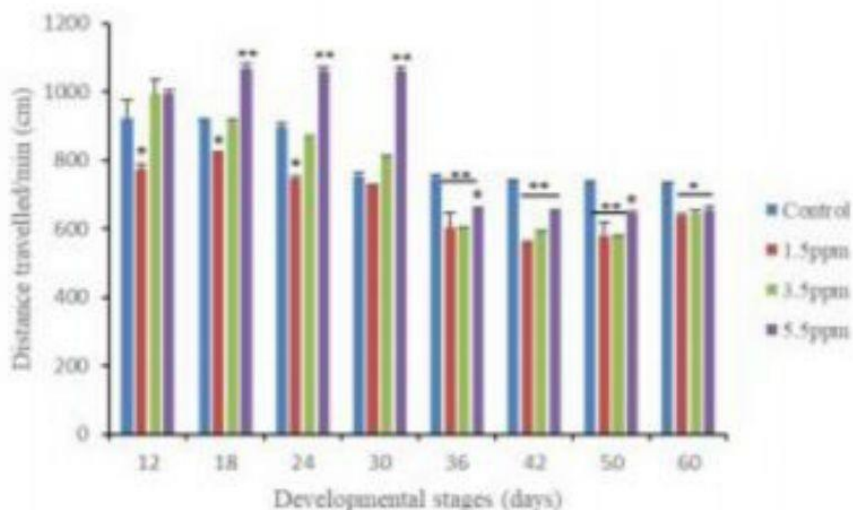


Figure 5.1 Locomotor activity of zebrafish exposed to tartrazine at different concentrations for 60 days. Locomotor activity measured by distance traveled per min. Data are presented as Mean $\pm$ SEM (ANOVA, * $p \leq 0.05$, ** $p \leq 0.001$ relative to control) $n=5$

From Figure 5.1, a significantly higher locomotor activity in 5.5ppm test group from 18-30 days and a significant decrease from 12-30days in 1.5ppm exposed group were observed while in 3.5ppm concentration no significant changes were observed when compared to control. In contrast, from 36-60 days significant reduction in the locomotor activity was observed in all three treated groups when compared to control.

5.2 Mortality rate

The mortality rate of treated zebrafish after exposing to tartrazine was calculated and compared with control. A slight change in the mortality rate in the treated groups comparing to control was observed from 30 days to 60 days. At 60th day, the mortality

percentage (MP) in 1.5ppm, 3.5ppm and 5.5ppm was 15%, 20% and 20% respectively was found (Table 5.1).

Days	Initial No. of zebrafish in each group	No. of zebrafish dead and Mortality Percentage (MP)							
		Control	MP (%)	1.5ppm	MP (%)	3.5ppm	MP (%)	5.5ppm	MP (%)
12	20	0	0	0	0	0	0	0	0
18	20	0	0	0	0	0	0	0	0
24	20	0	0	0	0	0	0	0	0
30	20	0	0	1	5	2	10	1	5
36	20	0	0	2	10	3	15	2	10
42	20	1	5	2	10	3	15	3	15
50	20	1	5	3	15	4	20	4	20
60	20	1	5	3	15	4	20	4	20

Table 5.1 Mortality rate and percentage of zebrafish at different stages

5.3 Tartrazine effects on weight

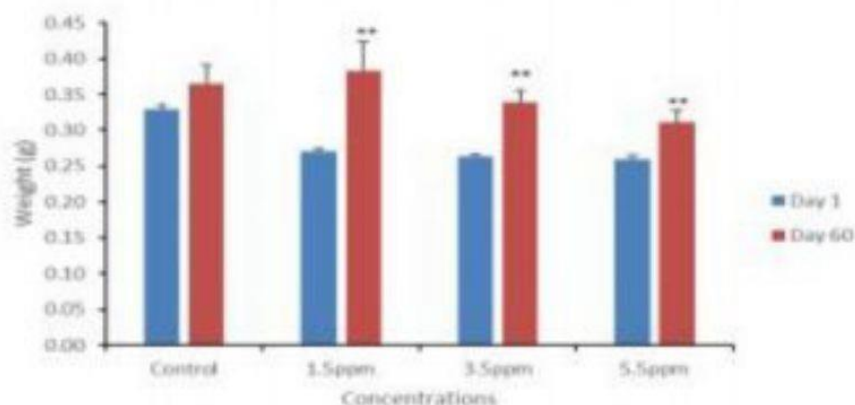


Figure 5.2 Comparison of zebrafish weight during 60 days of treatment with tartrazine. Results are shown as Mean $\pm$ SEM (Two-tailed Student's t-test, **p $\leq$ 0.001 relative to control) n=5

Figure 5.3 shows the comparison of body weight evolution in day 1 and day 60. In control, there is no significant change in 60th day when compared to day 1. But in the treated groups, at 60th day, zebrafish has gained weight with the significant value of 0.001.

5.4 Quantification OF DNA

The DNA samples of zebrafish gut from different concentrations of treated and control groups at 60th day were measured using Nano drop to check the absorbance ratio of 260nm/280nm in order to assess its purity.

Groups	DNA Purity (260/280)	Concentration (ng/ μ l)
Control	1.78	1744.4
1.5 ppm	1.83	1697.5
3.5 ppm	1.81	1517.5
5.5ppm	1.80	1555.1

Table 5.2. Quantification of DNA using Nano drop

5.5 Effect of tartrazine on the Composition of Gut Microbiota of zebrafish

A	Name	Bit-Score =	Grade	Hit s...	Hit end	Description	% Pairwise L...	Sequence L...	% GC	Topology
	MT510321	1,792.37	97.1%	1,459	282	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT510281	1,792.37	97.1%	1,460	283	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT464263	1,792.37	97.1%	1,462	286	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT464261	1,792.37	97.1%	1,458	281	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT463797	1,792.37	97.1%	1,458	281	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT463549	1,792.37	97.1%	1,459	282	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT459392	1,792.37	97.1%	1,458	281	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	MT459387	1,792.37	97.1%	1,465	288	Lactobacillus crispatus...	95.1%	1,179	53.4%	linear
	OP728785	1,790.52	97.0%	1,502	326	Lactobacillus sp. strain...	95.1%	1,178	53.4%	linear
	OP102580	1,790.52	97.0%	1,438	260	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
	OM265437	1,790.52	97.0%	1,458	282	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
	OM265436	1,790.52	97.0%	1,458	283	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
	OM265434	1,790.52	97.0%	1,456	280	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
	OM265433	1,790.52	97.0%	1,457	281	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
	OM265432	1,790.52	97.0%	1,460	284	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
	OM265431	1,790.52	97.0%	1,457	281	Lactobacillus crispatus...	95.1%	1,178	53.4%	linear
B	Name	Bit-Score =	Grade	Hit s...	Hit end	Description	% Pairwise L...	Sequence L...	% GC	Topology
	OM265433	1,703.73	96.6%	18	1,179	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT516168	1,703.73	96.6%	1	1,162	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT516139	1,703.73	96.6%	2	1,163	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT510321	1,703.73	96.6%	19	1,180	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT510284	1,703.73	96.6%	23	1,184	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT510281	1,703.73	96.6%	20	1,181	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT510276	1,703.73	96.6%	21	1,182	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT510268	1,703.73	96.6%	22	1,183	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT464262	1,703.73	96.6%	21	1,182	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT459393	1,703.73	96.6%	22	1,183	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	MT459392	1,703.73	96.6%	18	1,179	Lactobacillus crispatus...	94.1%	1,171	52.1%	linear
	OP102580	1,701.88	96.4%	1	1,158	Lactobacillus crispatus...	94.2%	1,167	52.2%	linear
	MT463549	1,701.88	96.4%	19	1,180	Lactobacillus crispatus...	94.1%	1,171	52.0%	linear
	MN473351	1,701.88	96.4%	1	1,164	Bacterium strain JMA...	94.0%	1,173	52.1%	linear
	KX674035	1,701.88	96.4%	1	1,158	Lactobacillus crispatus...	94.2%	1,167	52.2%	linear
	KX674034	1,701.88	96.4%	1	1,158	Lactobacillus crispatus...	94.2%	1,167	52.2%	linear
C	Name	Bit-Score =	Grade	Hit s...	Hit end	Description	% Pairwise L...	Sequence L...	% GC	Topology
	OP985050	1,993.66	98.1%	1,467	326	Escherichia coli strain ...	98.3%	1,144	54.9%	linear
	CP099886	1,993.66	98.1%	2,197...	2,196,2...	Escherichia alberti str...	98.3%	1,144	54.8%	linear
	OP051137	1,989.96	98.1%	1,434	291	Escherichia sp. strain ...	98.3%	1,146	54.8%	linear
	KX349997	1,989.96	98.1%	1,442	299	Escherichia coli strain ...	98.3%	1,146	54.8%	linear
	KU682213	1,989.96	98.1%	1,383	240	Escherichia coli strain ...	98.3%	1,146	54.8%	linear
	KT260398	1,989.96	98.1%	1,422	279	Escherichia coli strain ...	98.3%	1,146	54.8%	linear
	OQ381129	1,988.12	98.1%	1,345	204	Escherichia coli strain ...	98.3%	1,144	54.7%	linear
	OP986844	1,988.12	98.1%	1,472	330	Escherichia coli strain ...	98.3%	1,145	54.9%	linear
	OP986837	1,988.12	98.1%	1,472	330	Escherichia sp. strain ...	98.3%	1,145	54.9%	linear
	OP986834	1,988.12	98.1%	1,472	330	Escherichia sp. strain ...	98.3%	1,145	54.9%	linear
	OP986833	1,988.12	98.1%	1,472	330	Escherichia sp. strain ...	98.3%	1,145	54.9%	linear
	OP986594	1,988.12	98.1%	1,472	330	Escherichia fergusonii ...	98.3%	1,145	54.9%	linear
	OP975787	1,988.12	98.1%	1,445	303	Escherichia coli strain ...	98.3%	1,145	54.9%	linear
	CP119120	1,988.12	98.1%	980,469	979,327	Escherichia coli strain ...	98.3%	1,145	54.9%	linear
	CP119116	1,988.12	98.0%	4,969...	4,970,7...	Escherichia coli strain ...	98.3%	1,142	54.7%	linear
	CP118558	1,988.12	98.0%	2,035...	2,036,1...	Escherichia coli strain ...	98.3%	1,142	54.7%	linear

D

Name	Bit-Score	Grade	Hit s...	Hit end	Description	% Pairwise I...	Sequence L...	% GC	Topology
OP355452	1,864.39	97.8%	9	1,174	Escherichia sp. strain ...	96.2%	1,166	55.1%	linear
MZ289014	1,864.39	97.7%	10	1,171	Escherichia coli strain ...	96.3%	1,162	55.0%	linear
MT453882	1,864.39	97.6%	4	1,169	Escherichia coli strain ...	96.2%	1,166	55.1%	linear
MN094130	1,864.39	97.7%	10	1,171	Escherichia coli strain ...	96.3%	1,162	55.0%	linear
MF511712	1,864.39	97.9%	3	1,173	Escherichia coli strain ...	96.1%	1,171	55.1%	linear
KP789326	1,864.39	97.8%	9	1,174	Escherichia coli strain ...	96.2%	1,166	55.1%	linear
OP355282	1,862.54	97.8%	6	1,173	Escherichia sp. strain ...	96.1%	1,168	55.1%	linear
KP789334	1,862.54	97.8%	6	1,171	Escherichia coli strain ...	96.2%	1,166	55.4%	linear
KP789325	1,862.54	97.8%	9	1,176	Escherichia coli strain ...	96.1%	1,168	55.1%	linear
OP762972	1,860.7	97.8%	10	1,176	Escherichia coli strain ...	96.1%	1,167	55.3%	linear
MW629931	1,860.7	97.8%	11	1,177	Escherichia coli strain ...	96.1%	1,167	55.1%	linear
MN094133	1,860.7	97.8%	7	1,173	Escherichia coli strain ...	96.1%	1,167	55.4%	linear
MN094132	1,860.7	97.8%	7	1,172	Escherichia coli strain ...	96.1%	1,166	55.3%	linear
MN094128	1,860.7	97.7%	11	1,173	Escherichia coli strain ...	96.2%	1,163	55.3%	linear
KP789333	1,860.7	97.8%	8	1,174	Escherichia coli strain ...	96.1%	1,167	55.1%	linear
OP762960	1,858.85	97.7%	15	1,177	Escherichia coli strain ...	96.2%	1,163	55.1%	linear

Figure 5.4. Bacterial genera obtained from the sequencing of the 16S RNA gene A) Control, B) 1.5ppm, C) 3.5ppm and D) 5.5ppm

A total of two bacterial genera was identified from the 16s rRNA gene amplification (Fig. 5.3). The two major phyla were *Lactobacillus crispatus* (Control: 97%; 1.5ppm: 96.6%) and *Escherichia coli* (3.5ppm: 98.1%; 5.5ppm: 97.8%). These two bacteria are not harmful to humans and some are even beneficial. These species are capable of degrading the azo dyes (Seesuriyachan et. al., 2007; Feng et. al., 2010).

CHAPTER-6

SUMMARY AND CONCLUSIONS

SUMMARY

Food additives have a long-lasting effect on neural growth. The brain and central nervous system are susceptible to the direct effects of the gut microbiota. However, there is little information on how food additives affect the gut microbiota, so the goal of this project is to use a 16S rRNA-based metagenomic approach to examine how the gut microbiota of zebrafish exposed to different levels of food additives.

CONCLUSION

Food additives (FAs) are substances/chemicals supplemented during the processing of food either to enhance the nutritive value or quality or to enhance taste, flavor, freshness, and appearance. Among the food colorant azo dyes, tartrazine (TTZ) is the most widely used food additive. Although the acceptable daily intake (ADI) of TTZ for humans is 7.5 mg/kg body weight, prolonged use of this dye at higher concentrations is reported to increase the number of lymphocytes and eosinophils in gastric mucosal cells, produce abnormal reproductive cells, cause hepatic/renal toxicity, and neurobehavioral alterations in laboratory rodents (Vani Joshi and Pancharatna Katti, 2018). Some food additives have demonstrated to induce dysbiosis leading to the development of gut and gastrointestinal diseases. In this present study, we propose to use 16S based metagenomic approach to analyze the effect of tartrazine in the gut microbiota of six months old adult male zebrafish which is exposed to different concentrations (1.5ppm, 3.5ppm, and 5.5ppm) for 60 days. Dominant species of *Lactobacillus crispatus* was found in the control and 1.5ppm group and the dominant species of *Escherichia coli* was observed in the 3.5ppm and 5.5ppm exposed groups. *Lactobacillus crispatus* is an obligately homofermentative, catalase-negative, Gram-positive bacterium. Individuals with non-alcoholic fatty liver disease (NAFLD) have an increased abundance of *Escherichia* and *Lactobacillus* species. NAFLD frequently regarded as the hepatic manifestation of the metabolic syndrome, occurs in many

countries with a prevalence of 20-40% of the adult population. *L. crispatus* is a species of bacteria that is marketed as a probiotics (good bacteria). They can inhibit vaginal pathogenic microorganisms, and maintain the health of the female reproductive system (Wang S. et.al., 2017; Antonio M.A. et.al., 2013; Li T, et.al., 2019). *E. coli* is a member of the intestinal microbiome of over 90% of individuals and is interested in the role of digestion and intestinal disease. *E. coli* is a bacterial pathogen that can disrupt CNS function, especially in neonates (Lieblein-Boff et. al., 2013). Some studies have shown that normal gut microbiota modulates brain development and behavior of the host including mice (Diaz Heijtz et al., 2011) and zebrafish (Davis et al., 2016). In the present behavioral study, an increase in the locomotor activity was observed till 30 days in the higher concentration, and a significant reduction in the activity was observed in all test groups in the further exposure days. A slight change in the mortality rate was also observed from 30 days to 60 days. In the Lieblein-Boff et al., (2013) study, *E. coli* infection led to reduced motor coordination and increased locomotor activity in male juveniles and adult mice. *E. coli* and *Lactobacillus* sp. reduces hyperactivity behavior and has some influence on the survival rate, nutrient metabolism, stress response, and neurodevelopment suggesting microbial colonization during early life is required for normal neuro behavior (Fang Tan et.al., 2019). In general, our results showed that the two bacteria species at different concentrations caused some effects on the behavioral changes and mortality rate.

The effects of tartrazine consumption on health were evaluated using a crucian carp (*Carassius auratus*) as the experimental fish. The results indicate that tartrazine consumption, even at a low concentration causes severe cellular alterations, oxidative stress and inflammatory response in crucian carp. They could also induce gut microbiota dysbiosis (Limin Wu et al., 2021). The increase in body weight was found to be 12.12% (control), 40.74% (1.5ppm), 30.70% (3.5ppm) and 19.23% at 5.5ppm group respectively at day 60 when compared to the 1st day of exposure. Hence, it can be inferred from the view of the above observation that the dye somehow, raised the body weight and caused obesity in the experimental animals. In the Gautama et al., (2010) study, the administration of tartrazine in Swiss albino mice caused a significant increase in body weight. Similar results have also been reported by Chakra arty et al., in mice fed

with malachite green; Sharma et al., in mice fed with orange-red; Sharma et al., in mice fed with apple green. Food additives such as food coloring agents trigger some anxiety disorders and hyperactivity behavior. Tartrazine has been associated with memory, learning and behavioral changes, such as attention deficit and hyperactivity (Sambu et al., 2022). The brain is the center of all coordination and control of any animal and any type of destruction in the organ affects the entire well-being. The effects of tartrazine in this study resulted in the behavioral pattern and growth of the experimental fishes. The study also confirmed the neurotoxicity of the tartrazine in zebrafish.

Some bacterial species can metabolize "azo" bonds by enzymes, degrading toxic or mutagenic components. Bacteria composing the intestinal microbiota are able to degrade "azo" bonds by azo-reductase using Nicotine Amino Dinucleotide (NAD), Flavin Mono Nucleotide (FMN) and flavin adenine dinucleotide (FAD) as cofactors. *E. coli* and *Lactobacillus sp.* is capable of increasing the reduction of azo dyes (Seesuriyachan et. al., 2007; Feng et. al., 2010) which proves that they may have degraded the azo dye in the tartrazine we used in this study.

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