

FABRICATION OF A PLANT BASED FOOD TO ENHANCE THE GUT MICROBIOME - BRAIN INTERACTIONS AND VALIDATION OF THE EFFECTS ON *DANIO RERIO* (ZEBRA FISH)

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Technology in Biotechnology.

By

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This is to certify that this Project Report is the bonafide work of

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**FABRICATION OF A PLANT BASED FOOD TO ENHANCE THE GUT
MICROBIOME - BRAIN INTERACTIONS AND VALIDATION OF THE EFFECTS
ON *DANIO RERIO* (ZEBRA FISH)"** under our supervision from

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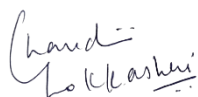
External Examiner

DECLARATION

I CHANDINI LOKKASHRI S B hereby declare that the Project Report entitled FABRICATION OF A PLANT BASED FOOD TO ENHANCE THE GUT MICROBIOME - BRAIN INTERACTIONS AND VALIDATION OF THE EFFECTS ON DANIO RERIO (ZEBRA FISH) done by me under the guidance of Dr. R. THYAGARAJAN (Internal) and Dr. JERRINE JOSEPH (External) at CENTRE FOR DRUG DISCOVERY AND DEVELOPMENT, Col. Dr. Jeppiaar Research park, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 21-04-2022

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A handwritten signature in blue ink, reading "Chandini Lokkashri S B". The signature is written in a cursive style with a horizontal line under the last name.

SIGNATURE OF THE CANDIDATE

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ABSTRACT

The global pandemic and the fear of contracting with the virus has led to the community living in an aseptic environment, to facilitate sterility, which irrespective of providing hygiene, completely disrupts the gut microbiome which is of immense importance for our survival. Also, other modern day problems like immense stress and irregular consumption of food leads to various health problems, one of which is possession of a dysfunctional gut. The fabricated nutritional bar aims at enhancing the gut microbiome of the consumer, with an additional benefit of improving mental health, which is achieved by using cost effective, plant based ingredients with high nutritional value. Various commercial sources of the ingredients were identified and the most economical option was finalized, from which the ingredients were obtained. The ingredients, namely, rolled oats, quinoa, Kehwa leaves, spinach powder, dates and Triphala powder, were added in an appropriate ratio and order and baked at 180 ° C for 15 minutes. The resultant bar underwent nutritional, physical and microbial analysis which yielded satisfactory results, proving that the nutritional bar possesses satisfactory levels of the tested parameters. Then, to demonstrate the enhancement of the gut microbiome and mental health, the nutritional bar was subjected zebra fish (*Danio rerio*) as modified pellets in varying ratios to understand the most efficient dosage. After observing the eating patterns, the gut microbiome of the fish was extracted and cultured to identify certain colonies of bacteria which were proven to improve the transmission of certain positive mood triggering neurotransmitters, justifying the improvement of gut and mental health.

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(II)

ABBREVIATIONS USED

ACRONYM	EXPANSION
GF	Germ free
MRS	deMan, Rogosa and Sharpe Agar
CNS	Central nervous system
ANS	autonomic nervous system
ENS	enteric nervous system
HPA	Hypothalamic pituitary adrenaline axis
LDL	Low density Lipoprotein
GPCR	G-protein-coupled receptors
ML	Millilitre
WT	Weight
KCal	Kilo Calories
DNA	Deoxyribo-Nucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
PCR	Polymerase Chain Reaction
RPM	Revolutions Per Minute
UV	Ultra Violet
TAE	Tris Acetate EDTA
EtBr	Ethidium Bromide
Taq	Thermus aquaticus
dNTP	Deoxy ribo Nucleotide Tri Phosphate
Ng	Nano gram
Kb	Kilo byte
V	Volt
Mg	milli gram
ml	milli litre
mM	milli molar
SDS	Sodium Dodecyl Sulfate

CHAPTER 1 – INTRODUCTION

1.1 INTRODUCTION TO THE GUT MICROBIOME –

The microbiome is a consolidation of protozoa, viruses, eukaryotes, archaea and various bacteria that exist symbiotically on and within several locations of the body. A few examples of occupied sites of are the oral cavity, respiratory tract, gastrointestinal system, genital organs and the skin (Lloyd-Price et al., 2016). However, the diverse microbiome in the gastrointestinal tract majorly consists of three phyla, namely *Bacteroidetes*, *Actinobacteria* and *Firmicutes* (Tap et al., 2009). This complex and diverse microbiome enables functional expression of host genomes. It is estimated to possess 50 to 100 more genes, when compared with the host and these genes add several non-encoded enzymatic proteins and therefore play a vital and crucial role in host metabolism ultimately regulating physiology (Kho & Lal, 2018).

The Human microbiome project (HMP) has been carried out in two phases over the period of ten years to establish and link interactions between humans and microbes and provide resources that facilitate discoveries of the same (Consortium et al., 2019). Thanks to the development and application of advanced molecular and metagenomic technologies and we now have access to precise and new insights on the role of the microbiota (MH et al., 2012). These contributions have provided direction towards the several patho-physiological roles played by the gut microbiota in various disorders and fields. The interaction of the gut microbiota with other factors like diet and stress provide a foundation for highlighting the gut microbiota as a measure of possible prevention and treatment (Dash et al., 2015).

1.2 FUNCTIONS AND SIGNIFICANCE OF THE GUT MICROBIOME –

The significance of the gut microbiome extends over various physiological domains. The relationship between the host and the gut microbiota is established by a network of various interactions. These interactions constitute metabolic structures and metabolism, Immune system and the neuroendocrine system and signaling between them (Kho & Lal, 2018).

1.2.1 **Metabolic functions –**

Analyzing the functional activities of the microbiome is of immense importance to the host physiology. The gut microbiota is essential for digestion and nutrition for the host.

A few metabolites and their functions are briefly tabulated in the figure below

Metabolites	Role and function	Example
Short chain fatty acids (SCFA)	Host metabolic pathway regulation	acetate, propionate, butyrate, valerate, hexanoate.
Indole derivatives	Significant antioxidant, Inhibits formation of amyloid-beta fibril exhibits cryoprotective and neuroprotective effects against antioxidants.	Indole-3-propionic acid, indole and indoxyl sulphate.
Vitamins	Production of vitamins, nucleotides and amino acids and sustains the high proliferation rate of the epithelial cells of the intestines. Also enhances immune functioning, energy production. Aids in DNA repair and methylation.	riboflavin, thiamine, niacin, pyridoxine, menaquinone, cobalamin, biotin, folate, etc
Phenolic derivatives	Protection against oxidative stress, antimicrobial effects, Impacts gut microbiota composition, maintains intestinal health, modulates estrogen, anti-inflammatory and chemopreventive effects	Equol, urolithins, enterodiol, enterolactone, etc
Choline metabolites	Aids in lipid metabolism, glucose homeostasis. Causes fatty liver, cardiovascular diseases.	choline, trimethylamine N-oxide (TMAO) and betaine
Polyamines	enhances maturation rate of adaptive immunity and intestinal barrier integrity, proinflammatory, sustains high proliferation rate if epithelial cells of intestine.	Spermidine, spermine and putrescine,
Bile acid metabolites	Activates nuclear receptor of host and cell signalling pathways, regulates cholesterol, bile acid, glucose, energy and lipids, antimicrobial effects.	deoxycholic acid (DCA), lithocholic acid (LCA)

Fig 1.1 – Metabolites and their functions

1.2.2 Interactions with the immune system –

There are numerous bidirectional complex interactions between the host and the microbiota. The involvement of the gut microbiota in the development of host immunity and response regulation at systematic and host levels had been demonstrated. But, the reversal of the differentiation-suppressed conditions of lymphoid and myeloid progenitor cells in germ-free mice (GF mice) after colonization resulted in a normal microbiota indicating that it facilitates maturation of innate immunity (hematopoiesis) and adaptive immunity (lymphocytopoiesis) (Khosravi et al., 2014).

1.2.3 Gut-Brain axis and the nervous system –

The gut brain axis is influential with respect to the central nervous system (CNS) and its health (Sharon et al., 2016). The prime components of the gut-brain axis as illustrated in the figure, includes the central nervous system (CNS), the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic pituitary adrenaline axis (HPA) (Carabotti et al., 2015a).

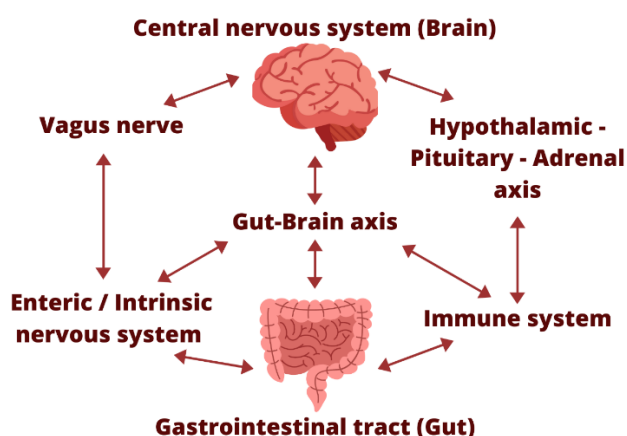


Fig 1.2 – The gut brain axis depicting the gut-brain interactions

The gut microbiome can metabolize neurotoxicants directly through reduction and de-conjugation/hydrolysis reactions (Claus et al., 2017; Lu et al., 2015). The microbiome can possibly communicate with the liver by enterohepatic circulation of primary and secondary metabolites which, which in turn alters the predominant functions of the liver like nutrient homeostasis and hepatic xenobiotic biotransformation (Klaassen & Cui, 2015; Spanogiannopoulos et al., 2016; Swanson, 2015; Visschers et al., 2013).

Chemical exposure leads to intestinal dysbiosis which may lead to leaky gut or local inflammation, eventually increasing levels of cytokines which are pro-inflammatory in the systematic circulation finally contributing to neuroinflammation (CN et al., 2018; Janakiraman & Krishnamoorthy, 2018; Lin et al., 2018; Rea et al., 2016; Sampson et al., 2016).

The gut microbiota can produce various neuro-reactive microbial metabolites which have the potential to enter the systemic circulation and eventually reach

the molecular targets present in the brain to modulate several types of cognitive functions (Carabotti et al., 2015b).

1.3 INTERDEPENDENCY BETWEEN THE GUT MICROBIOME AND DIET

Recent studies postulated the significance and impact of the gut microbiome on our health and the relationship between the diet consumed on the gut microbiome health and ultimately on our health (Singh et al., 2017). The gut microbiome is vital for metabolism and breaking down of dietary components resulting in energy generation (M et al., 2014). Different food nutrients have different impacts on health and are discussed individually, followed by the relevance of polyphenols and the significance of probiotics. The relevance of certain nutrients and their significance in the enhancement of the gut microbiome are enumerated.

1.3.1 Protein

A culture-based study portrayed a decrease in number of *Bifidobacterium* adolescents and a rise in the counts of *Bacteroides* and *Clostridia* in participants consuming a diet rich in beef in comparison to participants consuming a meatless diet (Hentges et al., 1977). Various studies have confirmed the impact of dietary protein on the gut microbial composition where participants were given different forms of protein across these studies. It was observed that protein consumption correlates with the overall microbial diversity (David et al., n.d.).

Consumption of pea protein extract and whey extract has proven to increase *Bifidobacterium* and *Lactobacillus*, while whey additionally decreases the amount of *Clostridium perfringens* and *Bacteroides fragilis* (David et al., 2014). Pea protein has been observed to facilitate the rise in intestinal SCFA levels, which possess anti-inflammatory properties and are essential for the maintenance of the mucosal barrier (Kim et al., 2014). The number of bile-tolerant anaerobes such as *Bilophila*, *Bacteroides* and *Alistipes*, and were observed to increase with consumption of animal-based protein (David et al., n.d.).

1.3.2 Carbohydrate

1.3.2.1 Digestible carbohydrates

Enzymatically degraded molecules, which are degraded in the small intestine are termed Digestible carbohydrates and include starches and sugars, such as

glucose, fructose, sucrose, and lactose. These compounds release glucose into the bloodstream and stimulate an insulin response upon degradation (Singh et al., 2017). Human subjects who fed on high levels of glucose, fructose, and sucrose in the form of dates and fruits had a reduced *Bacteroides* and an increased relative abundance of *Bifidobacteria* (Parvin, 2015).

1.3.2.2 *Non-Digestible carbohydrates and prebiotics*

Non - digestible carbohydrates such as resistant starch and fiber are not enzymatically degraded in the small intestine, and instead they travel to the large intestine where they undergo fermentation by microorganisms during which they modify the intestinal environment. Dietary fiber is proven to be a good source of microbiota accessible carbohydrates (MACs), which can be used by microorganisms to release energy and a carbon source (Pistollato, 2016).

Prebiotics which are non-digestible dietary fibers that facilitate the host health with the help of selective stimulation of the growth, synthesis and activity of certain microorganisms (Singh et al., 2017). Prebiotics have various sources, a few being, unrefined wheat, barley, soybeans, raw oats, inulin and several non-digestible oligosaccharides such as polydextrose, fructans, galactooligosaccharides (GOS), fructooligosaccharides (FOS), arabinooligosaccharides (AOS) and xylooligosaccharides (XOS) (KR Pandey, 2015). A diet deficit in these substances has proven to reduce the total bacterial abundance (Halmos et al., 2015).

1.3.3 *Fat*

The day-to-day consumption of high saturated and trans-fat diets increases the risk of cardiovascular diseases through the synthesis and regulation of blood total and LDL cholesterol (Singh et al., 2017). Advantageous fats, such as mono and polyunsaturated fats, are of immense importance in reducing the risk of chronic diseases.

Various human studies have proposed that a diet rich in fats increases total anaerobic microflora and amount of *Bacteroides* (F Fava, 2013). A high saturated fat diet is known to increase the relative proportion of the *Faecalibacterium prausnitzii* whereas participants with high monounsaturated fat consumption did

not experience variation in the relative abundance, but did have reduced overall bacterial and plasma total and LDL cholesterol (F Fava, 2013).

1.3.4 Probiotics

Fermented foods possessing lactic acid bacteria and represent a source of consumable microorganisms that may benefit and regulate intestinal health are termed probiotics (Shen et al., n.d.). Participants have reported an increased total bacterial load after regular utilization of fermented milk (K Matsumoto, 2010; Singh et al., 2017). A significant increase in beneficial gut *Lactobacilli* and *Bifidobacteria* have been observed consistently with different types of probiotics (S Wang, 2012; YJ Yang, 2012).

The benefits of consuming fermented dairy products include accelerated intestinal transit time [96], reduction of GI intolerance symptoms (H Yoon, 2015; Kang et al., 2019; R del Campo, 2014), inhibition of pathogen adhesion to intestinal mucosa, an increase in the total serum IgA to potentiate the humoral immune response (H Akatsu, 2013; YJ Yang, 2012), and ultimately, decreased abdominal distention and ascites observed in chronic liver disease patients (Singh et al., 2017).

1.4 RELATIONSHIP BETWEEN THE GUT MICROBIOME AND MENTAL HEALTH –

The significance and effect of the gut microbiome on mental health and the central nervous system (CNS) and their relationship can be understood by discussing them based on their categories namely immunological, biochemical and neuroendocrine mechanisms (Cryan et al., 2012).

1.4.1 Immunological mechanisms

The structural components of bacteria like lipopolysaccharides provide a low-grade tonic stimulation of the innate immune system and excessive stimulation may cause central nervous system inflammation. Various bacterial proteins can cross-react with host antigens to stimulate several dysfunctional responses of the adaptive immune system.

1.4.2 Innate immune system

The structural components of the cell wall of the microbes, stimulate the innate immune system to give rise to cytokines, creating a state of basal immune activation which begins at the mucosal surface of the intestines and benefits the entire body (Galland, 2014).

The human gut of the adult is believed to possess one gram of LPS. IL-1b, TNFa, and IL-18 are inducers of nREM or nonrapid eye movement sleep (Galland, 2014). Cytokine secretion which is induced by the low-level exposure of the immune cells to cell wall components of the bacteria, impacts normal sleep patterns and excessive cytokine levels can be associated with disrupted sleep (Galland, 2014).

1.4.3 Adaptive immunity

The adaptive immune system provides a response to very specific microbes with antibodies or antigen-specific cellular immune responses and can lead to the dysfunction of the CNS through the reactions which are auto immune which are caused by the molecular mimicry between the bacterial and self-proteins.

Currently there is very little evidence to establish a link between the adaptive immune system, the gut microbiome, autoimmunity, and disorders of the CNS in humans (Berer et al., n.d.; rheumatology and 2013, n.d.). However, the Celiac disease (CD) is an exception, irrespective of the mechanism being indirect. The modifications in the gut microbiome play a primary role in the pathogenesis of this gluten-sensitive disease in which the adaptive immune system causes damage, not only the gut but also the brain (Pozo-Rubio et al., n.d.).

Researchers have observed significant differences between children affected with CD and healthy children and in the duodenal and oral populations (Cheng et al., 2013; Francavilla et al., 2014).

1.4.4 Biochemical mechanisms

1.4.4.1 D-lactic acid

D-lactic acid is a product of microbial fermentation of carbohydrate, and is generally produced in excess when small bowel resection facilitates delivery of a high load of carbohydrate to the colon. An increase of D-lactate in the plasma may occur after abdominal surgery of various types and results in elevated intestinal permeability and bacterial translocation across the mucosal barrier of the

intestines. Nonsurgical causes of intestinal hyperpermeability also increase absorption of D-lactate from the intestinal lumen (Ying et al., n.d.; Zhao et al., 2011).

Studies suggest that an increased intestinal permeability or SIBO in CFS patients may enable overabsorption of D-lactate and other similar compounds produced by the gut flora which have direct or indirect neurotoxic effects, and contribute to chronic fatigue. Prebiotics and probiotics limit production of D-lactic acid in the gut and should be picked carefully. Some *Lactobacillus* species are producers of D-lactate and high-dose beta-glucan can rise intestinal permeability (Ewaschuk et al., n.d.).

1.4.4.2 Ammonia –

Ammonia is a neurotoxin that is produced by the action of bacterial ureases from urea by the action of bacterial ureases in the intestinal tract. Gut-derived ammonia is absorbed by the liver and consumed in the urea cycle. Cirrhosis allows the taken-up ammonia to escape hepatic metabolism by the creation of portosystemic shunts, eventually increasing the blood ammonia which aids in the pathogenesis of hepatic encephalopathy (HE) (Qureshi et al., n.d.).

Apart from direct neurotoxic injury, ammonia also alters the function of the blood–brain barrier, therefore leading to the impaired intracerebral synthesis of dopamine and serotonin, producing abnormal neurotransmitters like octopamine (Skowrońska and Albrecht, 2012). Altering the gut microbiome with the application of synbiotics has shown to alleviate cognitive dysfunction in cirrhosis patients. Administration of synbiotics has been suggested for cirrhosis patients as a preventive measure for MHE (Galland, 2014).

1.4.4.3 Short Chain Fatty Acids

Short-chain fatty acids are volatile fatty acids with a length of two to four carbon atoms per chain (acetate, propionate, and butyrate) and are produced in abundance through fermentation of indigestible carbohydrate in the colon by bacteria. SCFA are significant for at least two systems of molecular signaling and have impactful regulatory effects in the body, a few being, G-protein-coupled receptors (GPCRs) and histone deacetylation (HDAC) (Tan et al).

SCFA are activators of specific GPCRs and enable natural inhibition of histone deacetylases. GPCRs can be termed transmembrane proteins as they recognize molecules in the extracellular milieu and transfer information within cells regulating cell behavior. SCFAs also activate two specific GPCRs (GPR41 and GPR43) that do not have other known ligands (Wauson et al.). Despite proven to possess anti-inflammatory properties of propionic acid, the increasing rate of propionic acid synthesis in the natural colon may be of therapeutic benefit for metabolic disorders (Puertollano et al.).

1.4.4.4 Neuroendocrine mechanisms

Different bacteria can synthesize and respond differently to neurotransmitters.

Human studies have revealed impactful differences in the microbial composition in the gut that depend on genetic background, environmental factors, diet, age, microbial interactions and physiological state. The application of laboratory and clinical research on the health effects of modifying the microbiome has to be individual specific (Galland, 2014).

CHAPTER 2 – LITERATURE SURVEY

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CHAPTER 3 - AIM AND OBJECTIVE

1.1 AIM –

To fabricate a plant-based nutrition bar to improve the gut microbiome brain interactions and to validate the results on *Danio rerio* (Zebrafish).

1.2 OBJECTIVES –

- Identification and procurement of raw materials from efficient and cost-effective sources.
- Fabrication and curation of the nutritional bar by appropriate addition of the ingredients and baking at 180 ° C for 15 minutes.
- Performing nutritional analysis to justify nutritional value. Antimicrobial and physical analysis was also performed to determine the quality of the product developed.
- Comparison and identification of bacteria which are present in the gut microbiome of zebra fish and humans, that trigger the synthesis of neurotransmitters which lead to enhanced mood and well-being.
- The fabricated product is converted to suit fish consumption and subjected to zebra fish (*Danio rerio*) in different ratios to understand the most suitable dosage.
- Extraction of the gut and overnight culture of the microbiome followed by transfer to Petri plates and incubation.
- Identification of the bacterial colonies which aid in the synthesis of positive feelings triggering hormones and subcultures.
- Colony counting of the morphologically different colonies and subcultures followed by bacterial DNA isolation and 16s rRNA amplification by PCR (polymerase chain reaction) and sequencing was carried out.

CHAPTER 4 - MATERIALS AND METHODS

4.1 IDENTIFICATION AND PROCUREMENT OF RAW MATERIALS

4.1.1 Identification of feasible raw materials

A total of 30 ingredients were identified and then filtered out based on the following parameters – taste, cost, nutritional value and composition. The composition of each raw material was researched and its ability to improve gut health was taken as the most important factor.

After carefully examining the nutritional values of the ingredients, 6 ingredients, namely, rolled oats, quinoa, triphala, spinach, dates, and kahwa leaves were finalized.

4.1.1.1 Regular consumption of *oats* has proven to improve gut health and reduce the risk of cardiovascular disease. The health benefits of wholegrains might be due to effects on inflammation, fasting blood glucose, and markers of lipid metabolism (Kristek et al., 2018).

4.1.1.2 *Quinoa* is widely considered a nutraceutical due to its significant nutritional value and its bioactive components and contains a lot of phytochemicals, including saponins, phytoecdysteroids, phytosterols, phenolic compounds, bioactive peptides, and polysaccharides (Zeyneb et al., 2021) making it a favourable ingredient for the improvement of gut health.

4.1.1.3 *Triphala* is rich in Polyphenols modulate the human gut microbiome and promotes the growth of beneficial Bifidobacteria and Lactobacillus and inhibits the growth of undesirable gut microbes (Peterson et al., 2017).

4.1.1.4 *Spinach* is a great source of nutrients and its leaves contain carotenoids, polyphenols, flavonoids, and ascorbic acid making it ideal for the improvement of gut health and increases the nutritional value.

4.1.1.5 *Dates* are extremely nutritious and are rich in many compounds that enhance food safety and promote the health of human gut microbiota (Ayad et al., 2020).

4.1.1.6 *Green tea* has a positive influence on the gut microbiota by stimulating the growth of specific species and by triggering their development (Pérez-Burillo et al., 2021).

4.2 FABRICATION OF THE NUTRITION BAR –

The afore mentioned ingredients were modified to fit the fabrication and formulated into a nutrition bar. The acquirement of the ingredients was done by comparing the cost from multiple sources and the most efficient source was finalized. Then on a trial-and-error basis, the nutrition bar was fabricated.

4.2.1 Materials –

- Rolled Oats
- Quinoa
- Triphala
- Spinach
- Dates
- Water
- Measuring cups
- Weighing machine
- Baking Equipment (OTG)

4.2.2 Preparation of ingredients for fabrication.

Each ingredient was modified to achieve a favourable texture. The fabrication process begins with baking 50 grams of quinoa seeds for 3 minutes at 180 ° C and grinding them into a semi granulated powdered form for 30 seconds in a blender-mixer. The quinoa is added to 90 grams of rolled oats and this is followed by the addition of 5 grams of spinach powder. 15 grams of kehwa leaves are added to 250ml of water which is boiled for 3 minutes and is allowed to settle for 10 minutes and the leaves is filtered and extract is added. 1.5 grams of Triphala powder is mixed in 60ml of lukewarm water and added. 350grams of deseeded dates are blended in a blender-mixer to form a thick paste and is added. All the ingredients are mixed to provide a holding consistency and is laid on a baking sheet.

Ingredients used	Image	Ingredients used	Image
Quinoa		Kehwa Leaves	
Rolled Oats		Triphala Powder	
Dates		Spinach Powder	

Fig 4.1 - The ingredients used to fabricate the nutrition bar

4.2.3 Baking of the nutrition bar

The OTG is preheated for 5 minutes at 180 ° C followed by baking at 180 ° C for 15 minutes, after which the baking tray is taken out and the bars are cut into pieces weighing 50 grams each.



Fig 4.2 - The fabricated nutrition bar

4.3 ANALYSIS TO JUSTIFY QUALITY AND NUTRITIONAL VALUE –

The fabricated nutrition bar should be analysed for various physical, nutritional and microbial parameters. The physical parameters to be analysed are texture and colour. The nutritional parameter to be tested include Protein, Fiber, Carbohydrate, Sugar, Energy, Ash and Fat. Iron is also tested as it is a prevalent deficiency. Microbial analysis was performed for *Bacillus*, *Salmonella* and *Coliforms*.

The materials and methodology employed are on par with the food safety and standards authority of India and the test method used for each parameter can be found tabulated below

Table 4.1 Standard methodology employed for analysis

S.NO	TEST PARAMETER	TEST METHOD
1	Texture	Physical Observation
2	Colour	Physical Observation
3	Protein	IS 7219 (Kjeldahl Method)
4	Crude fiber	FSSAI manual of methods of analysis of foods (Cereal and Cereal products) Crude fiber % by wt. = $(W_1 - W_2) \times 100 / W$
5	Carbohydrate	Total solids – (proteins + fat + ash)
6	Total Sugar	FSSAI manual of methods of analysis of foods (Cereal and Cereal products)
7	Energy	Energy (in Kcal) = 4x (Proteins and carbohydrates mass in grams) + 9 x mass of fat in grams.
8	Ash	AOAC 21 st edition: 950.49 (Ignition followed by Repeatability and Reproducibility)
9	Iron as Fe	Microwave digestion and measurement
10	Fat	AOAC 21 st edition: 922.06
11	Bacillus	IS 5887-6: Microbiology of Food and Animal Feeding Stuffs – Horizontal

		Method for the Enumeration of Presumptive Bacillus Cereus
12	Salmonella	IS 5887-3: Methods for Detection of Bacteria Responsible for Food Poisoning, Part 3: General Guidance on Methods for the Detection of Salmonella
13	Coliform	IS 5401 covers general guidance for enumeration of coliforms by colony count technique

4.3.1 Texture and color –

Examination is performed by physically viewing and observing the characteristics.

4.3.2 Protein –

The sample is oxidized in the presence of nitrogenous compounds and sulphuric acid and are converted into ammonium sulphate. Mercury is added to the digestion mixture as a catalyst and alkali sulphate as a boiling point elevator. Ammonia is liberated by adding an excess of alkali and is quantitatively distilled into a measured volume of standard hydrochloric or sulphuric acid. The acid not neutralized by ammonia is back titrated with standard alkali.

4.3.3 Crude Fiber –

Weigh accurately about 2.5-3 gm sample and transferred and Soxhlet extraction is performed. The sample is Air dried and transferred to 1 L conical flask and connect the flask with a water-cooled reflux condenser and boil for exactly 30 minutes. Remove the flask and filter through fine linen (about 18 threads to a cm) held in a funnel and wash with boiling water until the washings are no longer acid. Bring to boil some quantity of Sodium hydroxide solution. Wash the residue on the linen into the flask with 200 mL of boiling Sodium hydroxide solution. Immediately connect the flask to the reflux condenser and boil for exactly 30 minutes. Remove the flask and immediately filter through the filtering cloth. Thoroughly wash the residue with boiling water and transfer to a Gooch crucible prepared with a thin compact layer of ignited asbestos. Wash the residue thoroughly first with hot water and then with about 15 mL of ethyl alcohol. Dry the Gooch crucible and contents at $105 \pm 2^{\circ}\text{C}$ in an air oven until constant weight is achieved. Cool and weigh.

Incinerate the contents of the Gooch crucible in a muffle furnace until all carbonaceous matter is burnt. Cool the Gooch crucible containing ash in a desiccator and weigh (Dry the crucible with its residues in an oven at 130°C for 2 hours).

Crude fiber % by wt. = $((W_1 - W_2) \times 100) / W$

W₁ = wt. in gm of Gooch crucible and contents before ashing

W₂ = wt. in gm of Gooch crucible containing asbestos and ash

W = wt. in gm of the dried material taken for the test

4.3.4 Carbohydrate –

The values of proteins, fat and ash are added and are together subtracted from the total solids to give the carbohydrate content including sucrose, dextrins, maltose or lactose by difference.

4.3.5 Total Ash –

Ignite the dried sample in a dish with a burner until charred and transfer to a muffle furnace until a grey ash is obtained. Cool this ash and weigh it. Repeat this process at half hour intervals till the difference is less than 1 mg.

Total ash on dry basis (% by weight) = $(W_2 - W) \times 100 / W_1 - W$

Where, W₂ = Weight in gm of the dish with the ash

W = Weight in gm of empty dish

W₁ = Weight in gm of the dish with the dried material taken for test

4.3.6 Bacillus species –

The sample is inoculated and spread using a spreader and the plates are incubated for 18 hours to 24 hours in an incubator at 30 ° C. Select the plates after 2 dilutions and perform colony counting. Compare the results with the template and perform confirmatory tests on sheep blood agar.

4.3.7. Coliforms –

Preparation of two poured plates, using a solid selective culture medium, and using a specified quantity of the test sample of the initial product is liquid, or using a specified quantity of an initial suspension in the case of other products.

Preparation of other pairs of poured plates under the same conditions, using decimal dilutions of the test sample or of the initial suspension. Incubation of the plates at 30 ° C followed by calculation of the number of coliforms.

4.4 EXPLORATION OF ZEBRAFISH GUT MICROBIOME CONFIRMATORY STUDIES

A zebra fish (*Danio Rerio*) facility is maintained and the fish feed on commercially available fish feed for the adaptation period of 10 days. A total of 20 fishes were obtained from which 8 were used as control and continued to feed on commercial feed.

4.4.1 Conversion and subjection of nutrition bar to zebrafish

The nutrition bar ingredients were separately ground and formulated and finally scraped into a particulate powder to visually resemble the commercially available fish feed. The granulate nutrition bar is subjected to zebra fish in different ratios, 20g, 40g and 60g respectively. Figure 8 represents the zebra fish tanks in which the nutrition bar is fed.



Fig 4.3 – The Established Zebrafish Facility

4.4.2 Zebrafish gut extraction and culture

Post 3 weeks of subjecting the feed to the zebrafish, one fish was selected at random from each tank and was euthanized and dissected with sterile instruments.

4.4.2.1 Preparation of Nutrient Broth

Materials –

- 13 grams Nutrient broth powder
- 1 litre distilled water
- Sterile (Autoclaved) 250ml containers
- Autoclave
- Weighing balance

13 grams of nutrient broth powder is weighed and 1 litre of distilled water is added. The mixture is shaken until the powder dissolves and is autoclaved at 121 ° C for 15 minutes. This broth is later transferred into 4 sterile 250ml containers.

4.4.2.2 Zebrafish Dissection

Materials –

- Cultured Zebrafish
- Sterile dissection blades
- Sterile forceps
- Petri plates
- Distilled water
- Discard Bin
- Prepared Nutrient Broth
- Incubator (37 ° C)

One Zebrafish was selected at random from each tank and euthanized. This was followed by dissection which was done by making small and sharp slit on the ventral side of the zebra fish under the fin and extracting the gut using sterile forceps. Each gut sample which was obtained gut was crushed separately on Petri plates using forceps and added to 250ml nutrient broth and was allowed to incubate for 24 hours at 37 ° C. The fat deposits and fecal matter surrounding the gut was discarded by spraying distilled water (Gupta & Mullins, 2010)

4.5 BACTERIA ISOLATION AND IDENTIFICATION

4.5.1 Preparation of media for subculture

Materials –

- Nutrient Agar (7 grams)
- MRS Agar (De Man, Rogosa and Sharpe) (16.78 grams)

- LB Agar (Luria Bertani) (10 grams)
- Distilled water (750 ml)
- Autoclave
- Sterile Petri plates
- Laminar air flow

Three different types of media, namely MRS Agar (De Man, Rogosa and Sharpe), LB Agar (Luria Bertani) and Nutrient Agar were chosen to subculture to increase the possibility of morphologically different colonies to be present. Also, MRS Agar (De Man, Rogosa and Sharpe) is a selective media for the growth of Lactobacillus and Bacillus species and therefore facilitates a more convenient method of identification and isolation of bacterial colonies. The media are prepared by adding appropriate amounts of each media powder into a conical flask and adding 250 ml distilled water into each flask. This is followed by autoclaving the media at 121 ° C for 15 minutes and pouring them into Petri plates in a laminar air flow.

4.5.2 Serial Dilution and Subculture

Materials –

- Broth Culture
- Sterile Test Tubes
- Test tube rack
- Micropipette (1ml)
- Distilled water
- L Rod
- Laminar Air Flow
- Petri Plates with Media
- Incubator

A five-fold serial dilution is performed in the following method. 10 ml of broth culture is transferred into a labelled test tube, from which, 1 ml of sample is transferred into the next test tube, to which, 9 ml of distilled water is added. Then, 1 ml is obtained from the second test tube and is added to the third test tube followed by the addition of 9 ml of distilled water. This process is repeated for 5 test tubes for the 4 samples after which 1 ml from the 3rd 4th and 5th fold of each

sample is spread on the petri plates with 3 different media each. The plates were allowed to incubate for 24 hours at 3 Figures represent the serial dilution test tubes and the Petri plates with the inoculum respectively

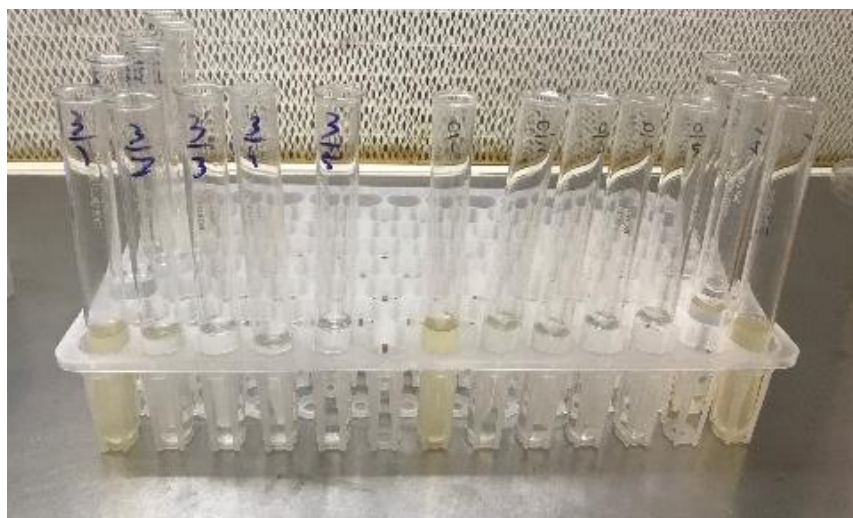


Fig 4.4 - Testubes of the 5-fold serial dilution

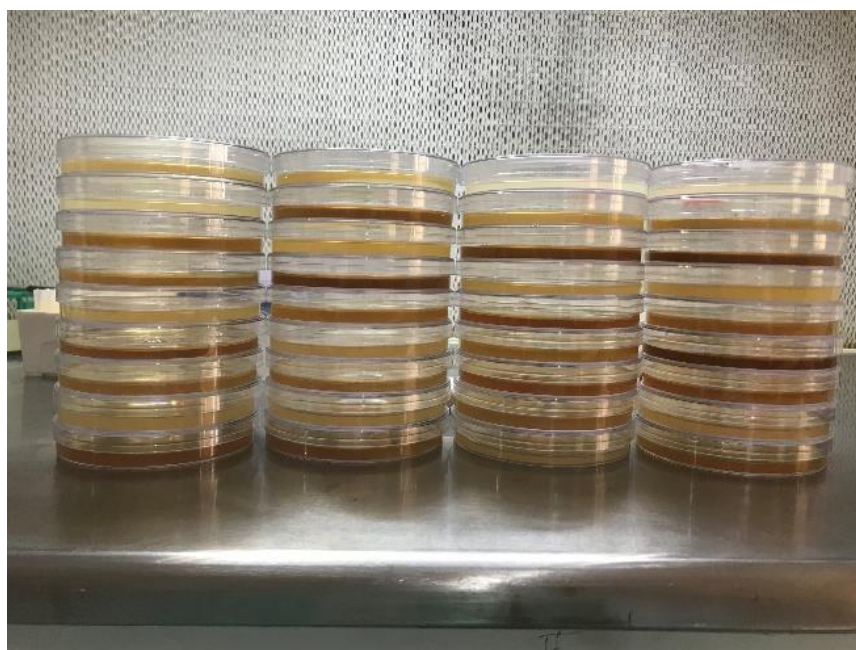


Fig 4.5 – Prepared testubes with different media of 4 samples

4.5.3 Colony Counting

Materials –

- Marker
- Petri Plates with Culture

Morphologically different colonies on each plate were observed and counted manually and documented.

4.5.4. Colony isolation and subculture

Materials –

- Marker
- Petri Plates with culture
- Petri Plates with Nutrient Agar
- Conical flask
- Inoculation Loop
- Laminar Air Flow
- Bunsen Burner
- Autoclave
- Incubator

4.5.5.1 Preparation of media for subculture

250 ml of Nutrient Agar was prepared by adding 7 grams of nutrient agar powder into a conical flask after which 250 ml of distilled water was added. This mixture was shaken until the powder dissolved. The conical flask was autoclaved at 121 °C for 15 minutes.

4.5.5.2 Isolation of colonies for subculture

A distantly located morphologically different colony without overlap is marked using a marker and is inoculated with an inoculation loop and smeared onto the

nutrient agar petri plated to obtained pure culture. This is performed in a laminar air flow and after which the plates are incubated at 37 ° C for 24 hours

4.6 BACTERIAL IDENTIFICATION AND CONFIRMATION

4.6.1 DNA isolation

The subculture plates with pinhead sized colonies visible are used to perform DNA isolation (Zoetendal et al., 2006).

Materials –

- Nutrient Broth
- Pipette
- Centrifuge
- Sterile water
- LE buffer
- Lysozyme
- 10 % sodium dodecyl sulphate
- Proteinase K
- 5 M NaCl
- Chloroform
- 70 % ethanol
- RNase

The isolate of interest is grown in 5 ml nutrient broth at 30°C, with shaking and 1.5 ml is pipetted in 1.5 ml tube. This is ccentrifuged for 2 min at 14,000 rpm at RT. The supernatant is discarded and the pellet is washed 3 times with sterile water. 550µl of TE buffer and lysozyme is added and is resuspend by pipetting. This is incubated for 30 min at 37 C. 76 µl of 10 % SDS is added along with proteinase K and the mixture is flipped and incubated at 65 ° C. 100 µl of 5 M NaCl is added followed by 80 µl of CTAB/NaCl. The mixture is flipped an incubated for 10 minutes at 65 ° C. 700 µl of Chloroform is added and centrifuged for 5 min at 14,000 rpm at RT. Transfer the aqueous layer (without disturbing or carrying over any of the white middle layer) to new 1.5 ml tube. This step can be repeated several times when proteins are taken up with the aqueous layer. Add equal volume of Isopropanol and invert several times to mix and centrifuge for 15 min at

4 C. Gently drain the supernatant and carefully add approximately 1 ml ice cold 70 % ethanol. Collect DNA by centrifugation for several minutes at 14,000 rpm at RT. Carefully remove the supernatant and evaporate the remaining ethanol in the laminar flow cabinet. Dissolve DNA in 50-100 μ l 10mM Tris (pH 8.0). Dissolve the DNA by incubating over night at 4 C. To remove contaminating RNA from preparation, add 6 μ l of RNase to DNA solution, incubate for 30 min at 37 C. Store the DNA at -20°C.

4.6.2 Agarose gel electrophoresis and spectrophotometer –

Quantification of DNA is performed using agarose gel electrophoresis(Lopez-Canovas et al., 2019).

- Check the quality of genomic DNA in 0.7% agarose gel. Boil 0.28g of Agarose in 40ml of 1x TAE in a 150 ml flask by heating the flask in a microwave oven
- Cool molten agarose to 50°C and add 40 μ l of EtBr (1.0mg/ml). Cast a gel in a gel-casting tray with properly placed combs
- Mix 10 μ l of DNA and 2 μ l of gel loading buffer and load the mix to each well without spill over
- Subject the gel to 3V/cm electricity for 1 hour in 1X TAE buffer
- Visualize the gel in multi-image™ light cabinet (Alpha Imager 2200) and photograph the gel images by Charge-Coupled Device (CCD) camera. Store the images as JPEG/TIFF extension file format for analysis
- For quantitation, dilute DNA samples tenfold times and then quantified in UV-VIS spectrophotometer. Calculate A260/A280 ratio to check the purity of the DNA preparation (Reading between 1.8-2.0 indicates its good quality)

4.6.3 PCR Amplification and Sequencing –

The 16S rRNA gene was amplified using genomic DNA as a template in the Polymerase chain reaction. The components of PCR reaction apart from the template were: 25 μ l EmeraldAmp® GT PCR Master Mix, 2.5 μ l of forward primer, 2.5 μ l of reverse primer, PCR water to make up the reaction volume to 50 μ l. The universal primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1100R (5'-

GGGTTGCGCTCGTTG-3') were used to amplify the 16S rRNA region of the genomic DNA. The following PCR protocol was used: initial denaturation at 94°C, 5 min; again for 34 cycles denaturation at 94°C for 45 sec, annealing at 55°C for 60 sec, and extension at 72°C for 1:30 min then followed by a final extension at 72°C for 10 min and hold at 12°C, using a thermocycler. The amplified fragment of genomic DNA was electrophoresed in 1.0% agarose gel prepared in 1X TAE (pH 8.0). 0.5 µg/ml gel red dye was added and allowed to polymerize after casting in the gel tray. The aliquot of PCR reaction was mixed with 5 µl of 6X loading dye and loaded onto the well of gel. Along with a 1kb marker in another well of the gel. The electrophoresis was carried out in 1X TAE buffer at 60 volts. The gel was observed under the transmitted UV light of Gel documentation system (Abram et al., 2020).

4.6.4. Purification using the PEG-NaCl method

PCR product (45 µl) was mixed with an equal volume of PEG NaCl by vigorous pipetting in 0.5 ml vial and incubated at 37°C for 30 minutes. After incubation, the tubes are centrifuged at 13,000 x g for 25 min. The supernatant was discarded, and the pellet was washed with 125µl of 70% chilled ethanol and centrifuged at 1000 x g for 20 minutes. Again, the supernatant was removed, and the pellet was dried using a speed vacuum and dissolved in 7-8 µl of TE buffer. Sequencing. Universal Primers were used for automated sequencing. Sanger sequencing (dideoxy chain termination) was carried out. Sequencing was carried out in 96 well microtiter plate filled with sample mix containing purified PCR product (50 to 100 ng/µl), 0.4 µl primer, and molecular grade water to adjust the volume to 7.75 µl. In that, 2 µl of big dye was added to each well. PCR programmed by using the following conditions: 90°C for 5 min, followed by 25 cycles of 90°C for 10 sec, 50°C for 20 sec, and 60°C for 4 min, followed by the final hold at 20°C. After PCR, 12 µl of master mix I containing 10 µl PCR water + 2 µl 125 mM EDTA and 52 µl of master mix II containing 2 µl 3 M sodium acetate + 50 µl 100% ethanol added to each well. Then the tubes were incubated at room temperature for 15 minutes and centrifuged at 3000 g for 30 min at room temperature. Then the plates were inverted on a tissue paper and kept in a centrifuge in invert position only at 180 g and stopped by adding 100 µl 70% ethanol in each well and centrifuged at 3000 g for 5 min. Again, the plates were

inverted on a tissue towel and centrifuge in an inverted position at up to 180 g. The ethanol wash was again repeated with 70% ethanol. Then the ethanol was removed from each well, added 10 µl of Hi-Di formamide, heated at 95°C for 5 min, and snap chilled the plate. Finally, the samples were run in a 3500x genetic analyzer (Invitrogen)(Berdimuratova et al., 2020) .

4.6.5. Contig formation and BLAST analysis –

The obtained sequence length of the amplified fragment from the forward and reverse primers required the removal of the overlapping region to become a single contig. Thus, the sequences were merged manually, and the obtained contig sequences were performed to NCBI-BLAST analysis for sequence similarity.

CHAPTER 5 - RESULTS AND DISCUSSION

5.1 NUTRITIONAL AND PHYSICAL ANALYSIS OF THE FABRICATED NUTRIITION BAR –

The nutrition bar proved to possess highly satisfactory levels of energy and protein. The bar was curated with no added preservatives of any sort including salt which is beneficial comparatively to commercially available nutrition bars which have harmful additives. The healthy levels of all the parameters indicate that the selection of raw materials was successful and that they contribute satisfactorily to the nutritional value of the bar.

One of the additional aims of the nutrition bar was to improve the levels of iron in the body as it is a deficiency which leads to anaemic condition.

Table 5.1 - Results of Physical and Nutritional analysis of formulated nutrition bar

SL.NO	TEST PARAMETER(S)	TEST METHOD	UNIT	RESULTS
1	Texture	CVR/FD/SOP/002	-	Soft, Moist, Chewy texture with visible Grains
2	Color	CVR/FD/SOP/002	-	Dark Brown
3	Protein(N*6.25)	IS 7219	g/100g	5.74
4	Crude Fiber	FSSAI manual of methods of analysis of foods (Cereal & Cereal Products)	g/100g	1.07
5	Carbohydrate	CVR/FD/SOP/001	g/100g	63.46
6	Total Sugar	FSSAI manual of methods of analysis of foods (Cereal & Cereal Products)	g/100g	29.09
7	Energy	CVR/FD/SOP/001	Kcal/100g	292.64
8	Ash	AOAC 21st Edition : 950.49	g/100g	1.53
9	Iron as Fe	CVR/INS/SOP ICPMS/003	mg/100g	4.20
10	Fat	AOAC 21st Edition : 922.06	g/100g	1.76

With respect to the physical analysis, a soft and chewy texture is suitable for people of all age groups and the dark brown colour facilitates a visual appeal.

5.2 MICROBIOLOGICAL ANALYSIS TO INDICATE SHELF LIFE AND LONGEVITY –

Any food product needs to be curated in a way in which the shelf life is reasonably long to facilitate a feasible consumption period. The presence of Bacillus,

Salmonella and coliforms was checked for in the nutrition bar and the results indicate that the bar has a safe consumption period.

Table 5.2 - Results of Microbiological Analysis of formulated nutrition bar

SL.NO	TEST PARAMETER(S)	TEST METHOD	UNIT	RESULTS
1.	Bacillus	IS:5887 (Part-6)	Cfu/g	< 10
2.	Salmonella	IS:5887 (Part-3)	-	Absent/25g
3.	Coliform	IS:5401 (Part-1)	Cfu/g	< 10

Note:

< 10 cfu/g can be taken as absent in 1:10 Dilution.

5.3 COLONY COUNTING AND IDENTIFICATION –

Colony counting was performed manually for all the 4 samples on 3 media by performing a 5-fold serial dilution and enumerating the number of colonies on each plate.

It was visually understandable that the samples 1, 2 and 3 had a more diverse and a greater number of bacterial colonies when compared to sample 0 which consumed commercial feed.

Table 5.3 - Results of Colony Counting indicating enhanced growth in tanks in which nutrition bar was suspended

Sample Number	Fold Number	Media	Number of colonies	Sample Number	Fold Number	Media	Number of colonies
0	3	LB Agar	TNTC	2	3	LB Agar	TNTC
0	3	MRS Agar	60	2	3	MRS Agar	TNTC
0	3	Nutrient Agar	TNTC	2	3	Nutrient Agar	TNTC
0	4	LB Agar	102	2	4	LB Agar	210
0	4	MRS Agar	42	2	4	MRS Agar	90
0	4	Nutrient Agar	310	2	4	Nutrient Agar	195
0	5	LB Agar	86	2	5	LB Agar	100
0	5	MRS Agar	12	2	5	MRS Agar	52
0	5	Nutrient Agar	200	2	5	Nutrient Agar	160
1	3	LB Agar	TNTC	3	3	LB Agar	TNTC
1	3	MRS Agar	TNTC	3	3	MRS Agar	TNTC
1	3	Nutrient Agar	TNTC	3	3	Nutrient Agar	TNTC
1	4	LB Agar	120	3	4	LB Agar	280
1	4	MRS Agar	78	3	4	MRS Agar	112
1	4	Nutrient Agar	280	3	4	Nutrient Agar	170
1	5	LB Agar	93	3	5	LB Agar	113
1	5	MRS Agar	45	3	5	MRS Agar	71
1	5	Nutrient Agar	185	3	5	Nutrient Agar	143

There are certain observations that can be made from the colony counting which can be put forth in the following graph

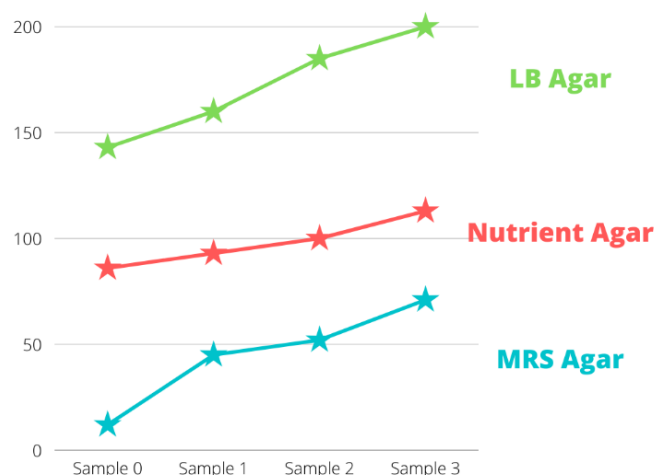


Fig 5.1 – Graphical representation of colony counting

The number of bacterial colonies in sample 3 is observed to be higher than the number of bacterial colonies in sample 0. From this statement, we can infer that the consumption of the nutrition bar is positively increasing the number of bacterial colonies. It can also be inferred that more the quantity of nutrition bar consumed, more the bacterial growth.

Furthermore, Luria Bertani Agar is identified as the most effective media for zebra fish gut microbiome extract culture as it portrayed maximum bacterial growth.

5.4 DNA SEQUENCING RESULTS -

After obtaining pure cultures of the morphologically different colonies and filtering them out and processing with multiple methods to obtain the sequence, the following colonies have been obtained.

Table 5.4 – Morphology of the different bacterial colonies which have been sequenced

S.NO	MEDIA IDENTIFIED ON	Whole colony	Edge / Margin	Surface	Elevation
1	Nutrient Agar	Circular	Entire	Smooth & Glistening	Pulvinate
2	Nutrient Agar	Punctiform	Entire	Smooth & Glistening	Raised
2	Nutrient Agar	Irregular	Entire	Smooth & Glistening	Raised

3	MRS Agar	Rhizoid	Filamentous	Wrinkled	Raised
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5.4.1 *Klebsiella quasipneumoniae* -

CATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGAT
 GTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGAC
 GATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGAAGACACG
 GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCA
 AGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAG
 CACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGT
 TACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
 GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG
 GTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTGCG
 AAAGTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCG
 GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTG
 GACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA
 TACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTG
 AGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGG
 CCGCAAGGTTAAAGTCAAATGAATTGACGGGGGCCCCGCACAAGCGGTGGA
 GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCC
 ACAGAACTTGCCAGAGATGGCTTGGTGCCTTCGGGAACTGTGAGACAGGTG
 CTGCATGGC

Klebsiella Pneumoniae is proven to produce reasonably high amounts of histamine production. The neurotransmitters serotonin, dopamine and histamine are proven to enhance eventually leading to the improvement of the gut microbiome brain interactions (Özoğul, 2004b; P Lin, 2017; Strandwitz, 2018)

Klebsiella species have proven to portray increased metabolization of Tryptophan which is a precursor of indole, melatonin and serotonin levels (O'Mahony et al., 2015; Skonieczna-żydecka et al., 2020)

5.4.2 *Aeromonas hydrophila*

CCCCAGGCGGTTCGATTAAACGCGTTAGCTCCGGAAGCCACGTCTCAAGGAC
 ACAGCCTCCAAATCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATC
 CTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGC

CGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTACCGCTACACCT
GGAATTCTACCCCCCTCTACAAGACTCTAGCTGGACAGTTTTAAATGCAATTC
CCAGGTTGAGCCCGGGGCTTTCACATCTAACTTATCCAACCGCCTGCGTGCG
CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCT
GCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCACAGCCAGC
AGATATTAGCTAATGACCTTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAG
GCCTTCTTCACACACGCGGCATGGCTGCATCAGGGTTTCCCCCATTGTGCAA
TATTCCCAATGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGT
GTGGCTGATCATCCTCTCAGACCAGCTAGGGATCGTCGCCTTGGTGAGCCAT
TACCTCACCAACTAGCTAATCCCACCTGGGCATATCCGATCGCGCAAGGCCC
GAAGGTCCCCTGCTTTCCCCCGTAGGGCGTTTGCGGTATTAGCGGTCGTTTC
CAACTGTTATCCCCCTCGACTGGGCAATTTCCCAGGCATTACTACCCATCC
GCCGCTCGCCGGCAAAGTAGCAAGCTAGTTTCCCGCTGCCGCTCGACNCG
C

5.4.3 *Aeromonas hydrophila*

Aeromonas hydrophila

CCCCAGGCGGTGCGATTTAACGCGTTAGCTCCGGAAGCCACGTCTCAAGGAC
ACAGCCTCCAAATCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATC
CTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGC
CGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTACCGCTACACCT
GGAATTCTACCCCCCTCTACAAGACTCTAGCTGGACAGTTTTAAATGCAATTC
CCAGGTTGAGCCCGGGGCTTTCACATCTAACTTATCCAACCGCCTGCGTGCG
CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCT
GCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCACAGCCAGC
AGATATTAGCTACTGACCTTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAG
GCCTTCTTCACACACGCGGCATGGCTGCATCAGGGTTTCCCCCATTGTGCAA
TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGT
GTGGCTGATCATCCTCTCAGACCAGCTAGGGATCGTCGCCTTGGTGAGCCAT
TACCTCACCAACTAGCTAATCCCACCTGGGCATATCCAATCGCGCAAGGCCC
GAAGGTCCCCTGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCAGTCGTTTC
CAACTGTTATCCCCCTCGACTGGGCAATTTCCCAGGCATTACTACCCGTCC
GCCGCTCGCCGGCAAATTAGC

5.4.2 and 5.4.3 *Aeromonas hydrophila* which is a bacteria proven to synthesize norepinephrine which is also a positive mood triggering neurotransmitter (Clark & Mach, 2016)

5.4.4 *Staphylococcus epidermidis*

CGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTAC
TCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAAC
CCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAAT
CCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAG
TCGCCTTCGCCACTGGTGTTCTCCATATCTCTGCGCATTTCACCGCTACACA
TGGAATTCCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTC
CACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCG
CTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCT
GCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACGTGCA
TAGTTACTTACACATTTGTTCTTCCCTAATAACAGAGTTTTACGATCCGAAGAC
CTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTCGCGGAAGA
TTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGT
GGCCGATCACCTCTCAGGTCTGGCTACGCATCGTTGCCTTGGTAAGCCGTTA
CCTTACCAACTAGCTAATGCGGCGCGGATCCATCTATAAGTGACAGCAAAAC
CGTCTTTCACTATTGAACCATGCGGTTCAATATATTATCCGGTATTAGCTCCG
GTTTCCCGAAGTTATCCCAGTCTTATAGGTAGGTTATCCACGTGTTACTCACC
CGTCCGCGCTAACGTCAGAGGAGCAAGCTCCTCGTCTGTTCGCTCGACTTG
CA

Staphylococcus epidermidis is of vital importance for the natural microbiome, especially the skin. It has various beneficial properties which contribute to its significance(Cogen et al., 2008; *Cogen: Staphylococcus Epidermidis Functions as a...* - Google Scholar, n.d.).

The bacterial colonies which have been obtained from sequencing are of immense interdisciplinary importance but the positive neurotransmitter triggering properties have been highlighted.

The gut is the source for the synthesis of 90% of the body's serotonin out of which almost 50% of the body's dopamine (Gershon & Tack, 2007; Terry & Margolis, 2017). The levels of serotonin and dopamine is regulated by the gut microbiota (Liu et al., 2016; Özoğul, 2004a; Sjögren et al., 2012; Yano et al., 2015). Various other neurotransmitters of immense significance like histamine, acetyl-choline, gamma-aminobutyric acid are also produced in the gut by the bacteria of the microbiome (Asano et al., 2012a; Bravo et al., 2011; Gezginc et al., 2013; Stanaszek et al., 1977).

Dopamine, the reward motivated behaviour mediating neurotransmitter, is a precursor of noradrenalin and adrenaline and is of significance. (Asano et al., 2012b) It is also responsible for learning and motivation (Berke, 2018). Histamine is significant with respect to sleep regulation and the sleep wake cycle (circadian rhythm) (Scammell et al., 2019). Serotonin is also a positive mood triggering neurotransmitter with various other benefits (Strasser et al., 2016).

CHAPTER 6 – SUMMARY

The COVID-19 pandemic took over the world and completely altered the society's lifestyle by increasing the levels of stress, anxiety, etc. which are prime disrupting factors of the gut microbiome – an extremely vital ecosystem situated in our gastrointestinal tract.

Apart from this, the continuous sanitization of various body parts and our environment, makes it aseptic which eventually prevents the entry of certain beneficial microbes into our body. Therefore, our microbiome is affected negatively and is in dire need of an enhancement factor. Considering how stress has affected several people, mental health is also a field that needs attention. The gut feeling or simply the interdependency of the gut microbiome and mental health is the principle behind fabricating a comfort food which is the most convenient form of mood enhancer. There rises the need of a plant based fabricated food as the society is slowly gleaning toward sustainable food products.

The nutrition bars were curated with a comparatively low carbon footprint, releasing almost no waste making it environment friendly. The ingredients underwent crucial investigation and were compared based on several parameters amidst which the best of the best was chosen and delivered in the form of a nutrition bar filled with nutritious and taste enhancing, completely plant-based ingredients.

The nutritional, physical and microbial analysis yielded a very positive response for a product with absolutely no added preservatives, the shelf life was also ensured. The fish which consumed the nutrition bar in the established zebra fish facility seemed visually more active when compared to the fish which consumed commercially available feed.

Finally, the microbiology analysis and the molecular biology experimentation yielded positive responses demonstrating that the nutrition bar not only improves gut health and mental health, but also ensured a general state of well-being.

CHAPTER 7 – CONCLUSION

Thus the results of the study of the fabricated nutrition bar which is vegan and completely plant-based proves that it possess significantly high nutritional values. Consuming the bar enhances the gut which is confirmed by the high gut bacterial growth numbers. A total of 7 morphologically different colonies have been obtained out of which 4 colonies underwent DNA sequencing and yielded 3 bacteria which have been scientifically proven to trigger the synthesis of certain important neurotransmitters which enhance positive mood and ensure overall well-being. Also this bar will be a welcome approach and palatable for kids and adults. Thus the target is to provide suitable sustainable nutritional food alternatives in the form of bar rather than chemical based pills and synthetic dietary supplements. Collaboration across the disciplines of nutrition science and microbiology is crucial for high-quality diet–microbiome research. Improvements in our understanding of the interaction between nutrient intake and microbial metabolism, as well as continued methodological innovation, will facilitate development of effective evidence-based personalised dietary treatments in the future.

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