EFFECT OF LACTOBACILLI-FERMENTED JACKFRUIT SEED MILK ON COLORECTAL CANCER CELL PROLIFERATION, MIGRATION, AND INVASION

A dissertation submitted by

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In partial fulfillment of the Degree of

MASTER OF VOCATION

IN

FOOD PROCESSING TECHNOLOGY

Under the Guidance of

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Scientist-C

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Cancer Research Program

Rajiv Gandhi Centre for Biotechnology (RGCB)

Thiruvananthapuram-695014, Kerala INDIA

APRIL 2025

DECLARATION

I hereby declare that the dissertation report entitled **"Effect of lactobacilli-fermented jackfruit seed milk on colorectal cancer cell proliferation, migration, and invasion"** submitted by me in the partial fulfilment of the requirements for the award of the degree of **Master of vocation in Food Processing Technology.** The work was done under the guidance of **Dr. Ramesh Pothuraju, Scientist and Former Ramanujan fellow, Cancer Research Program, Rajiv Gandhi Research Centre for Biotechnology, Trivandrum** for 3 months.

I further declare that the result of the work has not been previously submitted for the award of any degree or diploma.

Nasrin M

Date:

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CONTENTS

LIST OF ABBREVIATIONS

ABBREVIATION	EXPANSION
LB	Lactobacillus
JFPM	Jackfruit Probiotic Mixture
CRC	Colorectal cancer
µg	microgram
PBS	Phosphate Buffered Saline
PBST	Phosphate buffered saline tween
µl	microliter
ml	milliliter
BLQ	Below the limit of quantification
CFU	Colony forming unit
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
KDa	Kilo Dalton
BSA	Bovine Serum Albumin
MTT	3- (4,5- dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazonium
SDS	Sodium Dodecyl Sulphate

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Colorectal cancer remains a mortality-leading global health issue stemming largely from gut microbiota imbalances that play havoc silently worldwide. Lactobacillus species heavily modulate gut microbiota thereby exhibiting anticancer properties and effectively inhibiting proliferation of various cancer cells suddenly nowadays. Lactobacilli-fermented jackfruit seed milk profoundly impacts CRC cell proliferation migration and invasive potential as a potentially therapeutic functional food.

Jackfruit seeds frequently end up tossed aside but they're loaded with bioactive compounds like flavonoids and saponins having pretty potent antioxidant properties. Raw jackfruit seed flour contains 82.2g of carbohydrates per 100g and has protein and hefty amounts of calcium 67.7mg. Fermentation of jackfruit seed milk with Lactobacillus bacteria significantly boosted probiotic potential overnight and enhanced nutritional value pretty substantially.

Lactobacillus proliferation was robustly demonstrated via colony-forming unit assay with the highest growth at 10^{-6} dilution showing remarkably high colony counts. JFPM's anticancer potential was assessed quite thoroughly using MTT assays and colony formation tests on CRC cell lines like HT29 HCT116 and SW480. JFPM-treated cells showed drastically reduced colony formation compared with Lactobacillus-only treatment and this highlighted potent suppressive action on vigorously proliferating cancerous cells. E-cadherin a tumor suppressor gene was upregulated significantly in JFPM-treated CRC cells mainly in HT29 cell lines and HCT116 vs LB. JFPM may bolster adhesion thereby inhibiting CRC cell migration and invasion possibly reducing metastatic potential significantly in a rather substantial manner. Additionally, JFPM treatment markedly downregulated tumor promoting Claudin 3 and Claudin 4 gene expression in HCT116, SW480 and HT29 colorectal cancer cells compared to LB treatment. This suggests that JFPM may suppress genes linked to tumor progression and metastasis apparently by some unknown mechanism fairly effectively

Lactobacilli-fermented jackfruit seed milk remarkably exhibits potent anticancer properties by significantly reducing CRC cell proliferation and boosting tumor suppressor genes. Findings highlight the potential of this food boasting therapeutic applications in CRC prevention alongside some fairly novel treatment modalities being explored lately.

CHAPTER-1

INTRODUCTION

INTRODUCTION

Colorectal cancer (CRC) is diagnosed as one of the most common malignancies worldwide, and its third most considerable incidence rates account for it as the second most common killer disease caused by cancer (Escamilla, Lane, and Maitin 2012).CRC is caused by inherited and cancerogenic changes through DNA methylation or other epigenetic mechanisms, which lead to the transformation of normal colon cells into malignant carcinoma cells. The finding showed that several factors were involved in cancer risk factors, such as dietary habits, a sedentary lifestyle, obesity, alcohol drinking, and gut microbiota composition(Ranasinghe, Maduwanthi, and Marapana 2019) .Reports from recent studies are confirming the fact that the phenomenon of gut dysbiosis¸ or an altered microbiota ecosystem, is a result of promoting chronic inflammation, immune dysregulation, and genotoxic metabolite production, which as a whole, can lead to the initiation and progression of CRC (Escamilla et al. 2012)(Santhosh and Sarkar 2022). That is exactly the reason why the issue of probiotics has become very important in the present period, taking into account their ability to bring the good flora back and to manifest anti-cancer activity, which makes them the forefront topic in the field of CRC prevention and management.

Probiotics, specifically Lactobacillus species, are thoroughly looked at for their beneficial and well-known anticancer characteristics. These friendly bacteria can do good not only by the enhancement of the intestinal barrier integrity but also by the modulation of the immune response and the production of bioactive substances such as short-chain fatty acids (SCFAs) that are the extent of anti-inflammatory and anti-proliferative talents. Several studies confirm the ability of Lactobacillus to halt the rapid growth, migration, and invasion of cancer cells through manipulation of the cell signaling pathways and triggering programmed cell death or apoptosis t(Santhosh and Sarkar 2022). Also, the antitumor actions of Lactobacillus can be linked to the prolongation of the life of the bodies including inhibiting tumor growth, by such functions as reducing oxidative stress, neutralization of carcinogenic compounds, and up-regulation of the tight junction proteins, which in the end inhibit the tumorigenesis process(Escamilla et al. 2012)

The study had to do with the creation of a high-potential probiotic based on jackfruit seed milk fermentation with Lactobacillus. Jackfruit Artocarpus heterophyllus typically thrives in South and Southeast Asian regions, greatly benefiting from nutritional and therapeutic properties. Generally,

people recklessly discard such seeds because they comprise around 10–12% of fruit weight, but are pretty nutrient-dense(Kamal et al. 2023). Nutritional analysis of jackfruit seeds revealed they contain 82.2g carbohydrates per 100g and relatively low 2.47g protein per 100g alongside 2.37g dietary fiber per 100g with calcium present at 67.7mg per 100g and iron at 2.5mg per 100g(Hajj et al. 2022). However, from the spectrum of the nutritional analysis carried out, it was concluded that the seeds contained no vitamins, as their values were found to be below the quantification limit (BLQ). Apart from the availability of the macronutrients, jackfruit seeds are also packed with bioactive compounds like flavonoids, saponins, and isoflavones, that are anti-free radical, anti-infectious, and anti-cancer both in-vitro and in-vivo agents, respectively (Tripathi et al. 2023).Furthermore, lectins are the one of the jackfruit seed's components; they reveal antibacterial activity and the potential to modulate immunity, so today they are highly demanded if one wants to develop functional foods(Escamilla et al. 2012).

The addition of Lactobacillus in jackfruit seed milk by the process of fermentation is envisioned to have the bioactivity boosted as it will increase nutrient bioavailability and give rise to the production of bioactive compounds. The fermentation is all about adding the cultures of Lactobacillus to the jackfruit seed milk, and then the mixture is covered to favor the growth of the bacteria. And thus, it will be the lactic acid that will reduce the pH and cause the sour taste which is one of the characteristics of fermented food products. A team of five evaluators was shown the answer of sensory characteristics of the fermented yogurt by its hedonic scale with the aid of which they judged attributes like taste, texture, appearance, aroma, sweetness, color, and overall acceptability. The scorecards were designed in a way that 1 was given for dislike very much and 5 for like very much. There were three sets of yogurts of which a suitable one, after the sensory evaluation, was selected.

Lactobacillus-fermented jackfruit seed milk exhibits profound anticancer effects on colorectal cancer cells in various highly complex experimental settings. The product demonstrated promising potential in inhibiting CRC cell proliferation migration and invasion by harnessing the nutritional properties of jackfruit seeds and the probiotic benefits of Lactobacillus. Favorable physicochemical properties and high sensory acceptability pretty much indicate the potential of this product as some kind of functional food. Novel approaches emerge from this research utilizing food-based interventions for preventing CRC and highlighting the therapeutic potential of

probiotic-enriched foods. Future research probably focuses on in vivo experiments validating the anticancer efficacy of this product fermented rather unusually.

Parameters like pH and titratable acidity were assessed alongside syneresis to evaluate the physicochemical properties of yogurt pretty thoroughly. pH measurements over three days showed a slight decrease from 4.5 to 4.2 reflecting a gradual increase in acidity as fermentation quickly progressed. Titratable acidity stood at 0.64% corresponding roughly to lactic acid content churned out during vigorous bacterial fermentation. Syneresis is defined as the expulsion of whey from yogurt curd measured indicating pretty moderate water holding capacity surprisingly enough. Lower syneresis is highly desirable because it yields a remarkably smoother texture enhancing mouthfeel and making products quite appealing. A colony-forming unit assay was performed quite meticulously to assess bacterial viability and proliferation capacity afterward in a rather detailed manner. Results revealed that 10^{6th} dilution boasted the highest CFU count remarkably consistent with findings reported in literature by (Escamilla et al. 2012)

Fermented yogurt exhibited remarkably high bacterial viability thus confirming huge potential as a probiotic-rich product for human consumption. Fermented jackfruit seed milk's anticancer effects were evaluated quite thoroughly using MTT assays and colony formation tests on HT29, HCT116, and SW480 colorectal cancer cell lines. MTT assay gauges cell viability somewhat crudely by quantifying metabolic activity in cells, whereas the colony formation assay probes cancer cells' ability extremely well to form colonies after treatment. Cells treated with JFPM formed significantly fewer colonies compared to *the Lactobacillus*-only group, suggesting JFPM had a rather strong inhibitory effect on CRC cell proliferation. Fermented product exhibits markedly cytotoxic and anti-proliferative properties, thereby underscoring considerable therapeutic potential quite vividly.

Western blot analysis was performed examining expression levels of E-cadherin a tumor suppressor gene associated heavily with cell adhesion and metastasis suppression. Analysis was conducted on CRC cell lines HT29 HCT116 SW480 and DLD1 pretty thoroughly in a rather elaborate experimental setup. E-cadherin expression was significantly higher in the JFPM-treated group than LB-treated group across both HT29 and HCT116 cell lines. JFPM treatment effectively upregulated E-cadherin expression which may thwart cancer cell migration quite vigorously and suppress invasion in metastasis. Lactobacillus-fermented jackfruit seed milk exhibits potent

anticancer effects on colorectal cancer cells in various experimental settings quite effectively. Additionally, qRT-PCR was performed and the JFPM treatment markedly downregulated tumor promoting Claudin 3 and Claudin 4 gene expression in HCT116, SW480 and HT29 colorectal cancer cells compared to LB treatment. Jackfruit seeds possess nutritional properties and bioactive compounds while Lactobacillus offers probiotic benefits that together inhibit CRC cell proliferation vigorously.

High sensory acceptability coupled with favorable physicochemical properties pretty much indicates its potential as quite a functional food product. Novel approaches utilizing food-based interventions for CRC prevention are provided by this research and probiotic-enriched functional foods show huge therapeutic potential. Future studies will probably focus on in vivo experiments and clinical trials validating the anticancer efficacy of this peculiar fermented product quite thoroughly.

CHAPTER-2

REVIEW OF LITERATURE

COLORECTAL CANCER(CRC)

Colorectal cancer may grow in the colon or rectum and, thus, cancer is named colon or rectal depending on the location of the tumor. Conversely, these types of cancer share the same traits and therefore they are known as colorectal cancer. The location, size, and stage of cancer where the tumor has spread to other organs of the body of the tumor can all influence the symptoms. Some of the symptoms that are general to all cancer types are the following: the feeling of vomiting, bloated stomach, and unexplained weight loss.(Granados-Romero et al. 2017).

CRC is the second most common type worldwide causes cancer-related deaths and is the third most frequent type of cancer worldwide. An inactive lifestyle, smoking, drinking, being overweight or obese, and having a significant CRC are caused by several variables, including a family history of cancer. It is a tumor of the solid type mainly found in older patients and the most common way to treat it is through surgery. However, the latest finding in research has shown that taking probiotics during the treatment of solid tumors can enhance the positive effect of the treatment (Shang et al. 2020). Distinct bacterial populations are linked with the development of CRC. It is assumed that the gut microbiota signature that defines CRC shifts as the disease progresses, starting from normal epithelium to benign adenoma stage and eventually becoming malignant carcinoma(Robson et al. 2023).

CRC occurs due to several genetic, epigenetic, and inflammatory modifications. It begins by changing a mutation in the APC gene which initiates the Wnt/β-catenin signalling pathway. This eventually leads to cell proliferation and the formation of benign adenomatous polyps. Gradually further mutations in KRAS and TP53 amplify tumors and increase genomic instability and resistance to cell death. Also, cancer cells are said to be living and multiplying because the MAPK and PI3K/AKT pathways function in such a way. Moreover, the local "running" of tumor cells eventually leads to metastasis usually are the liver and lungs ones due to EMT, an epithelial-mesenchymal transition process. Those are the links between the prognosis of a tumor, metastasis, and the inflamed state and gut microbiota(Tariq and Ghias 2016).

FACTORS AFFECTING THE COLORECTAL CANCER(CRC)

There are two types of factors that affect the CRC, they are modifiable and non-modifiable risk factors. The modifiable factors can be controlled by changing lifestyle including alcohol consumption, smoking, unhealthy diet etc. Heavy alcohol consumption may increase the risk of CRC. Heavy smoking exposes colorectal cells to carcinogens, promoting genetic mutations, while physical inactivity is associated with 25% to 50% higher CRC risk due to obesity, insulin resistance, and unhealthy intestinal function.(Roshandel, Ghasemi-Kebria, and Malekzadeh 2024)

Non-modifiable risk factors include older age, male gender, genetic susceptibility, family history, and prior radiation exposure. Age is a known risk factor for CRC; more than 90% of cases are diagnosed in persons older than 50 years. It affects more men than women, at a rate of 1.4: male: female. ~5% of cases are from hereditary CRC: germline mutations in the APC gene (familial adenomatous polyposis, FAP), DNA mismatch repair genes (Lynch syndrome).A family history of CRC in first-degree relatives appears to double or triple the risk. Likewise, cancer survivors receiving abdominopelvic radiation therapy (e.g., for prostate cancer) are at increased risk of developing subsequent CRC, which may be attributed to DNA damage caused by radiation. Therefore, awareness of these risk factors is important for screening at-risk populations and managing effective CRC prevention strategies.(Roshandel et al. 2024)

CELL ADHESION PROTEIN

E cadherin is a chief cell adhesion protein and is one of the cadherin members of the family. It interacts with calcium-dependent cells on the regulatory cell surface and is involved in the maintenance of the characteristics of one's epithelial cells. The loss of e-cadherin function in cells is associated with the development of a tumor and its transition to metastasis. Loss of E-cadherin expression in the cell, tumorigenicity is occurring and the cells begin a progression of epithelial-mesenchymal.

JACKFRUIT

Figure 1: Jackfruit

Source:(Elevitch and Manner n.d.)

Jackfruit Artocarpus heterophyllus a tropical fruit hails from south Asia and belongs mostly to Moraceae botanical family of various fruits. Fruit grows prolifically in warm humid climates typically found in tropical countries like Brazil Australia India and Pakistan under varied conditions. India ranks second globally in jackfruit production and gets touted as motherland of this rather exotic fruit somehow(Shafi Shajahan et al. 2024).Jackfruit shows a wide range of diversity, In Kerala, Karnataka and Tamil Nādu different forms of jackfruit like varikka, koozha, Navarikka etc are available. In the Northeast region of India, Jackfruit is popular among the tribals.(Sarkar n.d.) It is a non-seasonal fruit that becomes available in spring and remains in season through summer. (Shafi Shajahan et al. 2024)

Jackfruit is a tree with many benefits(Shafi Shajahan et al. 2024), All the parts of the jackfruit tree including seeds, bark, leaves, root, and latex has several uses.(Nansereko and Muyonga 2021). Jackfruit is abundant in phytochemicals, especially phenolic compounds that can produce various value-added products such as nutraceuticals to amplify health benefits(Sanam 2023).

Table 1: Taxonomic classification of jackfruit

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Hamamelidae
Order	Urticales
Family	moraceae
Genus	Artocarpus
Species	*Artocarpus heterophyllus* Lam

Source:(Jose Vazhacharickal and John Mathew 2017)

JACKFRUIT SEED

Figure 2: Jackfruit seed

Source:(Fabil et al. 2024)

Jackfruit contains edible bulbs of yellow flesh and seeds. The seeds are light brown to brown(Srivastava and Singh 2020). Jackfruit seed also has considerable nutritional benefits and can serve as a valuable component in nutraceuticals(Sanam 2023), but these seeds are

underutilized and less acknowledged by people. In various parts of south India, during the rainy season, the seeds of ripe jackfruit are collected, sun-dried, and kept appropriately for future use. (Waghmare et al. 2019).

NUTRITIONAL AND BIOACTIVE COMPOSITION OF JACKFRUIT SEED

Jackfruit seeds, which are frequently overlooked, feature lectins with antibacterial activities and compounds like flavonoids, saponins, and isoflavones that are beneficial to human health through control and protection against cancer. These seeds are helpful for digestion, have anti-cancer and anti-aging properties, and promote a healthy gut which is good for the body(Fabil et al. 2024).

Among their notable characteristics is also the fact that they are highly rich in carbohydrates and protein, at the same time they have an extensive list supplementary components like minerals, dietary fiber, antioxidant compounds etc (Kamal et al. 2023). Highy rich in minerals like sodium, potassium, magnesium, calcium, copper etc.(Hajj et al. 2022).

Jackfruit seeds are a significant carbohydrate source that carries a high amount of energy. The precise carbohydrate content can be higher or lower depending on factors like seed variety, ripeness, and size, and thus cannot be predicted accurately (Sultana 2017). The seeds also contain two interesting lectins namely jacalin and artocarpin. Among them, Jacalin serves as a good diagnostic tool for checking the immune status of HIV-1-infected patients as it specifically binds to the Thomsen-Friedenreich antigen. Recently researchers discovered lectin exerts dual effects on peripheral blood mononuclear cells inhibiting B-cell immunoglobulin synthesis while stimulating T-cell proliferation vigorously(Lavanya et al., 2022).

Jackfruit seeds carry various fatty acids that serve as the main source of nutritional value and fuel the organism's energy production processes somehow(Hajj et al. 2022). Predominant fatty acids include linolenic acid and linoleic acid along with polyunsaturated ones like erucic acid and docosahexaenoic acid recently found.(Brahma and Ray 2023).

JACKFRUIT SEED HEALTH BENEFITS

About 10% to 12% of the total weight of jackfruit is the seeds, which are also a major source of some essential nutrients. They provide the body with fiber (3.9%) and starch (22%), both of which are very useful in terms of health. Lignans, isoflavones, saponins, etc. are concrete examples of bioactive compounds found in the seeds that have resulted in the seeds' severe anti-cancer, anti-oxidant, anti-ulcer, anti-high blood, and anti-aging nature(Ranasinghe et al. 2019) .The consumption of jackfruit seeds facilitates the digestive process, potentiates the anticarcinogenic effects, and prevents wrinkles not to appear on the skin.

A few healthful products are obtained from the seeds of jackfruit such as isoflavones, lignans, and saponins (Santhosh and Sarkar 2022).There too are some non-reducing sugars present in the firm, un-dissolved part of the seeds, which are known to have prebiotic properties, and are thus able to change the flora in the intestine favorably and thus promote digestive health (Hoang, Nguyen, and Duong 2024)). From another perspective, the pyruvate and the sulphur/sulphur-related products which are also contained in the seeds are found to exert the same activity on microorganisms, especially those that cause diseases.

It is the germinated jackfruit seeds that have been tested and demonstrated to combat bacteria, boost the immunity of the host, and come up with new drugs to replace used ones thus increasing the efficacy of the existing drugs(Arpit and John 2015) . The part of quail yolk where protein and starch are found(Goh, Mamat, and Abdul Aziz 2024),the starch removed from seeds has been claimed to be a very effective disintegrant obtained from plants that are used for ultra-fast dispersed tablets and which through this mode dissolve very quickly in the mouth, without the guidance of the water(Tripathi et al. 2023)

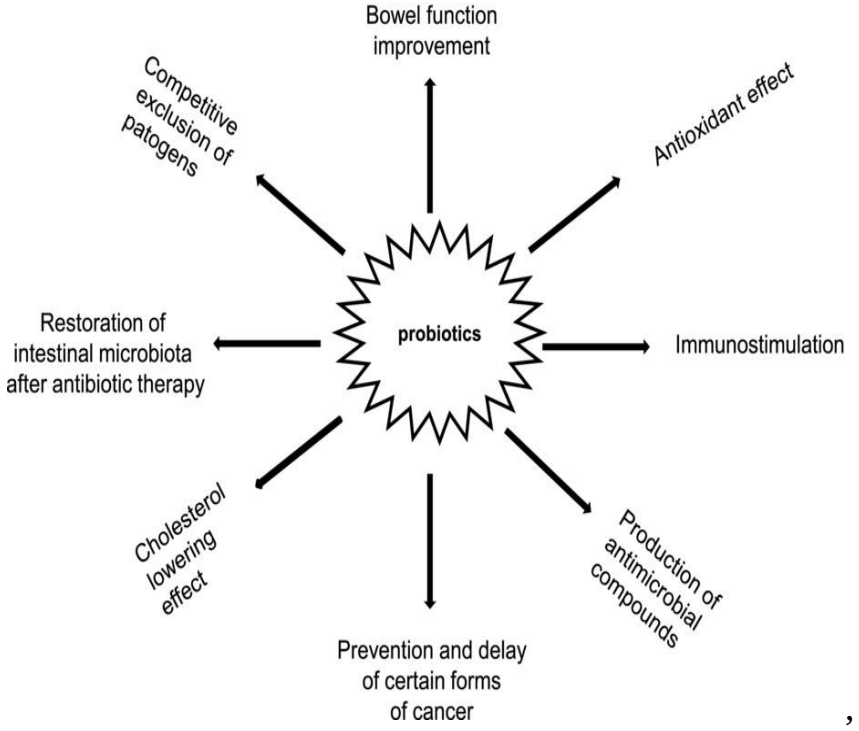
Furthermore, the seeds can assist in the treatment of dysentery and diarrhea due to the high amount of fiber present which is a major component that protects the mucous membrane of the colon from harmful substances and thus, by that, it causes their elimination(Mijin and Ding 2020)In addition, as the magnesium in jackfruit seeds is abundant, it contributes to the improvement of bone health by making calcium absorption more efficient, giving the bones sturdiness and preventing the occurrence of osteoporosis (Anon 2016).

PROBIOTICS

Probiotic agents are one of the largest and most underestimated types of health bacteria, they are advantageous to humans in many ways, including disease prevention and faster healing. Probiotics help fight pathogens by preventing their attachment to the host, stimulating the immune system, detoxifying the organisms, reducing the risk of inflammatory bowel diseases, etc. Also, probiotics can aid in reducing cholesterol levels, prevent cancer, manufacture essential vitamins, and create antimicrobial compounds. The improvement of consumer knowledge has been the driving force of the market for new probiotic products. The addition to functional foods will also provide health benefits in addition to increasing the consumer value of products through taste and flavor when using the same nonrobotic functional food(Vijayaram et al. 2024). Probiotics are proving to be very effective in preventing CRC(Escamilla et al. 2012).

HEALTH BENEFITS OF PROBIOTICS

Figure 3: Health benefits of probiotics



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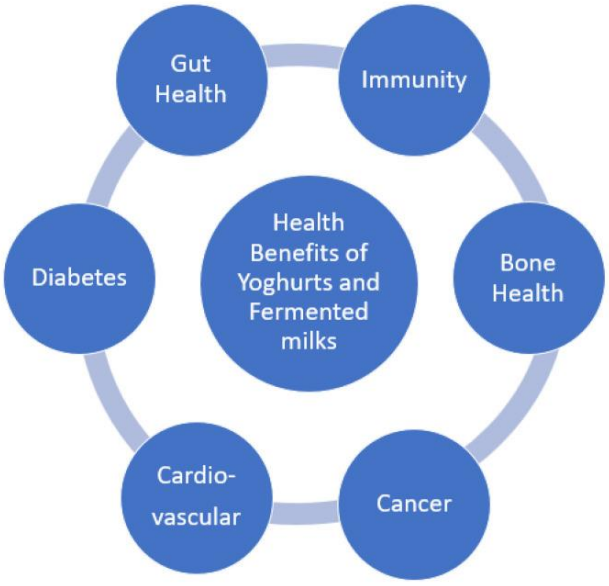
Source:(Beena Divya et al. 2012)

PROBIOTIC YOGHURT

One of the main features of yogurt is that it is an excellent source of probiotics and is therefore recommended to everyone for all their meals. The yogurt is a product that is globally known for its taste and health benefits, is usually consumed by people who consider it a very common food choice and is occasionally taken as it is as a snack. Fatty acids, such as conjugated linoleic acid (CLA), which is becoming more well-known for its immunomodulatory, anti-diabetic, anti-obesity, anti-cancer, anti-atherogenic, and bone-strengthening qualities, are abundant in yogurt and fermented milk (Mei et al. 2022)

Figure 4: Health benefits of yogurt



Source:(Hadjimbei, Botsaris, and Chrysostomou 2022)

BIO-FUNCTIONAL PROPERTIES OF PROBIOTIC LACTOBACILLUS

Starter cultures of lactic acid bacteria are very important in the fermentation process of the dairy and food industry. The use of lactic acid bacteria as probiotics introduces new functionalities to the product. Besides the already mentioned, gut-colonizing bacteria, which are probiotic, have several other beneficial health properties(O'Toole, Marchesi, and Hill 2017). Such properties include antimicrobial activity, anti-inflammatory, ACE-inhibitory, antioxidant, antidiarrheal, antiviral, immunomodulatory, hypocholesterolemia, anti-diabetic, and anti-cancer activities as

well as the most suitable research trends of lactic acid bacteria (Chandra and Vij 2018). Different strains of lactobacilli are generally used, being the common source of the probiotics. They also are capable of colonizing the gut. Occasionally, certain bacterial strains may exhibit antimicrobial activity, which further increases the potency of antibiotics.

The use of Lactobacillus to create functional meals is one of the most interesting areas of contemporary food-related science research and application.The activity of a single bacterial strain could promote the development of certain types of food with health benefits, such as functional and curative food products(Minj et al. 2021). Thus, *Lactobacillus* strains have been established in the scientific arena as probiotics and have been well-documented to possess anticancer properties. These strains showed inherent antitumor effects including apoptosis, anti-angiogenesis, and immunomodulation(Nami et al. 2024)The LAB is understood to play a part in colorectal cancer cells and have a regulating effect on them. The CFS produced by *Streptococcus thermophilus* and *Lactobacillus casei* in a co-culture system has also been found to be effective in reducing the viability of colorectal cancer cells and this, as a result, can inhibit the growth of such cells. The levels of MMP-9 were lowered, and the protein expression of ZO-1 was enhanced by the Lactobacillus. The number of bacterial units correlates with the bacterial population in the large intestine which is about 10^9 to 10^{11} CFU/mL, according to the data from the study by (Escamilla et al. 2012)

CHAPTER-3

MATERIALS AND METHODS

TOOLS AND EQUIPMENT

- Grinder
- Muslin cloth
- Spatula
- Pan
- Induction stove
- Measuring cylinder
- 1g measuring spoon
- Bottle
- Conical flask
- Funnel
- Whatman filter paper
- pH meter
- Burette
- Conical flask
- Clean glass slide
- Burner
- Microscope
- 6 Well plates
- 96 well plates
- Bench top centrifuge
- Inverted phase contrast microscope
- Neubauer Chamber
- Varioskan lux multimode microplate reader
- Pre-cool centrifuge
- Nanodrop spectrophotometer
- RT-PCR thermocycler
- Western blotting System
- Probe type Sonicator

CHEMICALS AND REAGENTS

- **REAGENTS FOR TITRATION**

- 0.1 N NaOH
- Phenolphthalein indicator

- **REAGENTS FOR BACTERIA ENUMERATION**

- Lactobacillus MRS Agar

- **REAGENTS FOR GRAM STAINING**

- Crystal violet
- Iodine solution
- Decolorizer
- 95% ethanol
- Safranin

- **REAGENTS FOR CELL CULTURE**

- Phosphate Buffer saline
- Trypsin
- Cell Culture Media: HCT116, HT29, SW480, DLD1 cell lines were cultured in Dulbecco's Modified Eagle (DMEM) medium
- Trypan Blue -Himedia (0.4 percentage in 1X PBS)

- **REAGENTS FOR MTT ASSAY**

- 3-(4,5-Dimethylthiazol-2-yl)-2,5-DiphenyltetrazoliumBromide (MTT)
- DMSO

- **REAGENTS FOR PROTEIN ESTIMATION**

- Bovine Serum Albumin (BSA)
- Radio Immunoprecipitation Assay (RIPA)Buffer
- DC protein assay reagent A-Biorad
- DC protein assay reagent S-Biorad
- DC protein assay reagent B-Biorad

- **REAGENTS FOR WESTERN BLOTING**

- 30% Acrylamide bisacrylamide solution
- 10%SDS

- 10%APS
- TEMED
- Tris HClpH8.8
- Tris HClpH6.8
- Isopropanol
- 6X Laemmli dye
- 1X Running Buffer
- Methanol
- 1X Transfer Buffer
- Skimmed milk Powder
- Primary antibody
- Secondary Antibody-Invitrogen
- Phosphate Buffered Saline Tween 20(PBST)

➢ **REAGENTS FOR cDNA SYNTHESIS**

- I script(5x)
- Nuclease Free Water

➢ **REAGENTS FOR PCR**

- Emerald GT PCR Master mix
- Forward Primer
- Reverse Primer
- Nuclease-free water

➢ **REAGENTS FOR REAL-TIME PCR**

- SYBR Green
- Forward primer
- Reverse Primer
- Nuclease-free water

RAW MATERIALS

a) Jackfruit seeds with optimum maturity (13 to 14 weeks) are collected from the farmers and used to manufacture yogurt.
b) Milk; Pasteurized, homogenized cow milk of Milma brand is purchased from the market for yoghurt production
c) Sugar; crystal form of sugar is purchased from the market. It is used as a sweetener in yogurt and it is also essential for the growth of bacteria.
d) Lactobacillus culture; mixture of lactobacillus acidophilus, lactobacillus thermophilus and lactis

PREPARATION OF JACKFRUIT SEED MILK

Raw jackfruit seeds optimally matured at 13 to 14 weeks, are collected from farmers in Trivandrum. The seeds are then preprocessed by sorting and peeling without removing the brown skin. Then the peeled seeds are sorted and washed with potable water to remove impurities. After washing, the seeds are ground with a small amount of water. Finally, the mixture is filtered to extract smooth, refined jackfruit seed milk.

Figure 5: Jackfruit seed milk

SCHEMATIC REPRESENTATION OF JACKFRUIT SEED MILK PRODUCTION

Selection of raw jackfruit seed

↓

Peeling

↓

Sorting

↓

Washing

↓

Grinding the seed

↓

Extract the milk by pressing the ground seed through the filter

↓

Jackfruit seed milk

Figure 6: Preparation of Jackfruit seed milk





NEW PRODUCT: JACKFRUIT SEED MILK YOGURT

Whole milk was measured in a measuring cylinder and poured into a pan. Then, add the required amount of sugar to the milk and heat it to the required consistency. After obtaining the required consistency, the prepared jackfruit seed milk was added to the pan.

The mixture was again heated until a suitable consistency was obtained. It was allowed cool slowly down to room temperature afterwards. A small amount of bacterial culture was added quite slowly after cooling and mixed rather thoroughly ensuring somewhat uniform distribution. The mixture was incubated overnight at 37^{o}C eventually reaching a temperature of 42°C somehow. Obtained yogurt was freeze-dried afterwards yielding lyophilized jackfruit seed milk yogurt powder. The remaining yogurt and obtained lyophilized powder were kept at 4^{o}C.

Figure 7: Jackfruit seed milk yogurt

SCHEMATIC REPRESENTATION OF YOGURT PRODUCTION

Preheat whole milk to 60*C

Add sugar and stir it.

Cool to 40 - 45*C

↓

Add jackfruit milk to whole milk and stir well

↓

Heat mixture to the required consistency

↓

Cool to 37-40*C

↓

culture of bacteria was inoculated and mixed uniformly

↓

Keep it overnight at 37 to 42*C

↓

Yogurt

↓

Freeze drying at -40^{o}C to 40*C

↓

Lyophilized jackfruit seed milk yogurt powder

Figure 8: Preparation of lyophized powder of JFPM

TRAILS DONE FOR STANDARDISATION

Table 2: List of trial samples

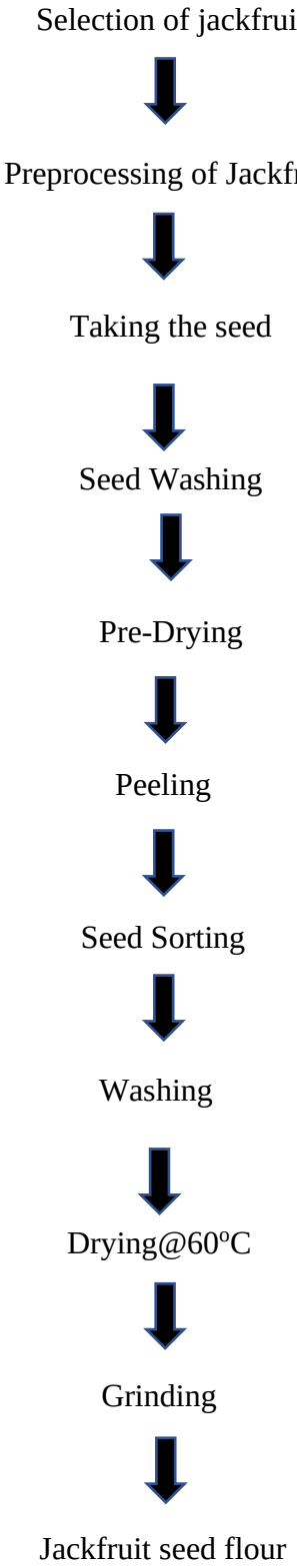
SI no	Trial no	Cow milk (ml)	Jackfruit seed milk(ml)	Sugar(g)	Bacteria culture(g)
1	T1	250	250	3	1
2	T2	500	250	5	2
3	T3	250	500	5	1

PREPARATION OF JACKFRUIT SEED FLOUR

Raw jackfruit with an optimum maturity of 13 to 14 weeks is collected from farmers in Trivandrum. The jackfruit is preprocessed and the seeds are collected. Then the seeds undergo preprocessing, beginning with sorting, washing, and peeling without removing the brown skin. These seeds are again followed by sorting and washing with potable water to remove impurities. Seeds are dried at 60°C pretty slowly until optimum dryness level is achieved after being washed thoroughly. Dried seeds get ground into fine powder afterward vigorously.

Figure 9: Jackfruit seed flour

SCHEMATIC REPRESENTATION OF JACKFRUIT SEED FLOUR



SENSORY EVALUATION

A panel consisting of five members has been formed for the purpose of conducting a sensory evaluation. Three females and two males make this group, all falling within the age range of 22 to 40 years. They used a hedonic scale to evaluate the sense of yogurt prepared. The most important sensory attributes for the evaluators included general acceptability, flavor and taste in addition to color, aroma, and texture as well as the appearance of the yogurt. The evaluators were given prepared yogurt samples and instructed to indicate whether or not they preferred certain sensory traits the yogurt possessed.

A five-point hedonic scale was used for scoring. With this assessment, the features of yogurt samples were tested for sensory features and marketability.

Table 3: Hedonic rating scale

Scale	**Hedonic rating**
5	Like very much
4	Like moderately
3	Neither nor dislike
2	Dislike moderately
1	Dislike very much

Score Card

Product name: Jackfruit seed Milk Yoghurt Date:

Analyzer name:

Characteristics	Score		
	T_1	T_2	T_3
Appearance			
Colour			

Odour			
Flavour			
Texture			
Taste			
Overall acceptability			

QUALITY ANALYSIS

SYNERESIS

Syneresis of undisturbed yogurt was estimated using a drainage test according to Atmer and Sezgin (1986). 25 g of yogurt was weighed and filtered at 4^{o}C and after 3 hr of drainage. the volume of filtrate collected in a graduated cylinder was measured and used as an index of syneresis.

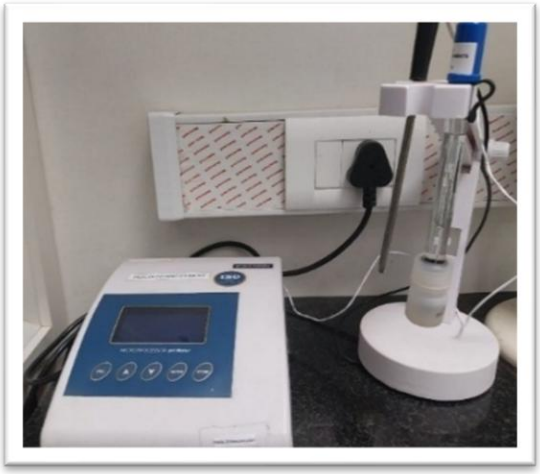
This was done for three continuous days. The syneresis can be affected by various factors like low solid content, use of high temperatures during incubation, inadequate storage temperatures, high acidity, etc.(Matela, Pillai, and Thamae 2019)

Figure 10: Syneresis

pH

A pH meter was used to measure the yogurt's pH. 20 ml of distilled water was added to 10g of prepared yogurt, weighed in a beaker. The pH is then determined using a pH meter. The estimation was done for three continuous days.

Figure 11: pH Meter



TITRATABLE ACIDITY

The Hooi et al. (2004) method was used to determine the titratable acidity. 20 ml of distilled water was added to a beaker containing a 10g prepared yogurt. This combination was then titrated against 0.1 N NaOH solution taken in a burette, using phenolphthalein as an indicator. The endpoint of the titration was identified by a color change from colorless to pale pink, indicating the endpoint of the reaction. The end values are noted, and the percentage of acidity is expressed using the formula. This was done for three continuous days.

$$TA = \frac{\text{Vol of 0.1 N NaOH used(ml)} \times 0.009 \times 100}{\text{Weight of the sample(g)}}$$

Figure 12: Titration

BACTERIAL ENUMERATION

Media preparation

9.9 g of Lactobacillus MRS Agar (based on 65.13 g per 1000 mL) was weighed and added to 150 ml of distilled water taken in a beaker. Then stir it gently until gets dissolved. This is then autoclaved at 121 °C for 15 minutes, then allowed to cool to room temperature for further use.

Sample preparation

The sample was produced at a 10% w/v concentration. Initially, 5 g of lyophilized jackfruit seed milk-fermented yogurt powder was weighed. Then, 50 mL of Milli-Q water was taken in a beaker and pre-heated in a water bath at 38°C for 1 minute. Then this was poured into a Falcon tube, and then the weighed lyophilized powder was added to it and was mixed thoroughly. Then the prepared solution was mixed thoroughly to ensure complete dissolution. From the completely dissolved solution, 10 ml was transferred to a centrifuge tube and it is considered as sample 1, while another 10 ml of dissolved solution was subjected to centrifugation at 3000g, 4°C for 30 minutes and this is considered as sample 2.

Serial dilution

Serial dilution was done in a Laminar Air Flow (LAF) cabinet. Initially, using 70% ethanol the workspace was cleaned. Then the test tubes were labeled with the appropriate dilution factors like 10^{-1}, 10^{-2}, $10^{-3,}$ etc. up to $10^{-7.}$ 9ml of autoclaved distilled water was added to each test tube. After that 1ml of the prepared sample was transferred into the 10^{-1} test tube, representing the first dilution. Then 1 ml from the 10^{-1} test tube was transferred to the 10^{-2} test tube and this was repeated up to the 10^{-7} dilution.

Figure 13: Serial Dilution

Pour plating technique

A 100 µl sample was pipetted and transferred into a sterile Petri dish. After that, a mixture of 15 -20 ml of molten agar and the samples was added to the dish. The compound was carefully rotated in a circular motion to make sure that the mixture was uniformly distributed.Then the Petri plates were allowed to remain at room temperature until the agar solidified. After the solidification, plates were incubated at 37°C for 48 hours. After incubation, the number of colonies formed was counted and calculated by the formula ie,

$$\text{No: of bacteria} = \frac{\text{Number of bacterial colonies}}{\text{Volume x Dilution factor}}$$

Figure 14: Pour Plating

Gram staining technique

On a clean glass slide, a thin smear of the bacterial sample was prepared and heat-fixed by passing it quickly through a flame one to three times. Then the crystal violet was applied to the heat-fixed smear and left for one minute before being washed off under running water. Then gram's iodine solution was applied to the smear and rest for one minute, followed by rinsing under running water. Later, 95% ethanol was applied and immediately rinsed off with distilled water. Then the final reagent safranin was added to the smear and allowed to rest for 30 seconds before a final rinse with distilled water. Then the slide was allowed to air dry and observed under a fluorescent microscope.

CELL CULTURE

Cell culturing

CRC cell lines like HT29, SW480, DLD1, and HCT116 were cultured in Dulbecco's Modified Eagle medium containing 10% FBS and penicillin-streptomycin antibiotics inside a 60mm petri dish. To facilitate cell detachment the old media was removed. After those dead cells were removed from the cells by washing them with phosphate-buffered saline (PBS). To detach the cells,2ml of 0.5%trypsin was added after washing, and the dish was incubated at 37º C for 4 minutes. Then 5 mL of complete culture media was added, and the mixture was carefully swirled to inhibit trypsin activity. After that, the cell suspension was then transferred to a Falcon tube and centrifuged for 3 minutes at 2000 rpm at 26°C. The supernatant was discarded after the centrifugation and the pellet was dissolved in 1ml media and allowed to mix. To a new flask, 3ml of media and 100 µl of resuspended cells were added. The cells were then placed in the incubator at 37ºC with 95%air and 5%CO2.

Counting the Cells

Cells were counted using a Neubauer Chamber with Trypan Blue staining, which works based on the exclusion principle of cell viability. The following protocol was followed:

Initially, the chamber and coverslip were wiped with 70% ethanol.190 µl of Trypan Blue was added to 10 µl cell suspension and mixed thoroughly creating a dilution factor of 20. The dilution factor (Df) was calculated using the formula:

$$Df = \text{Total volume of the sample} / \text{Total volume of cell}$$

The coverslip was carefully placed on the chamber, and 10 µl of the Trypan Blue-cell suspension mixture was loaded to the gap between the coverslip and the chamber. An inverted microscope was then used to focus the counting chamber grid under a 10x objective. Cell counting was performed in four corner squares of the grid. Dead cells that appeared in blue, were eliminated from the count, while viable cells, which appeared in white, were counted. Then the total number of cells was calculated using the formula:

Cell count (No. of cells/ml) =Average cell count from 4 quadrants×Df×10^4

Once the cells were counted, a suitable volume of the cell suspension was seeded into 96-well and 6-well plates for various experiments.

Preparation of plates and seeding of cells

For the western blot experiments, a 6 cm petri plate was labeled accordingly: HT29 Control, HT29 JPBM, HCT116 Control, HCT116 JPBM, SW480 Control, SW480 JPBM, DLD1 Control, and DL1 JPBM. Each petri plate was then filled with 3 mL of prepared DMEM media, followed by the addition of 70 µL of the centrifuged cells. For the MTT Assay,2000 cells were seeded in each well in 96 well plates and 1ml media was added to spread the cells. For the colony formation assay,2000 cells were seeded in 6 well plates, The plates were incubated overnight in a CO_2 incubator (5% CO_2) to allow for cell growth and differentiation.

PREPARATION OF CONTROL AND DRUG FOR THE TREATMENT

1. Preparation of control

In this research, bacteria served as control at a rather high concentration of 10% w/v made pretty carefully.1 g of Lactobacillus culture was weighed out and added rapidly to preheated Milli-Q water in a beaker at 38°C after 1 minute.1 ml was subsequently taken from the combined solution and serial dilution was performed up to the 6th dilution where in 10^8 CFU/ml was actually confirmed. Then the 6th dilution was subjected to centrifugation for 30 minutes at 3000g at 4°C yielding the Cell Free Supernatant (CFS). Then the CFS was filtered using a 0.2µm syringe filter to make it free from bacteria and other unwanted factors. This Bacteria-free CFS is stored at -20^{O}C for further use.

2. Preparation of Drug

In this research, Lyophilized powder of jackfruit seed milk-fermented yogurt served as a drug made at a fairly high 10% w/v concentration.50 mL of Milli-Q water taken in a beaker is pre-heated at 38°C for 1 minute in a water bath and afterward, 5 g of lyophilized jackfruit seed milk-fermented yogurt powder is weighed out and added to preheated water and mixed thoroughly ensuring complete dissolution.1 ml was subsequently taken from the combined solution and serial

dilution was performed up to the 6th dilution wherein 10^8 CFU/ml was ultimately confirmed. Then the 6th dilution was subjected to centrifugation for 30 minutes at 3000g at 4°C yielding the Cell Free Supernatant (CFS). Then the CFS was filtered using a 0.2µm syringe filter to make it free from bacteria and other unwanted factors. This Bacteria-free CFS is stored at -20^{O}C for further use.

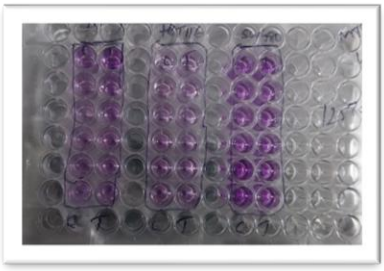
TREATMENT OF CELLS WITH DRUG

After 24 hours of incubation, the cultured cells were treated with Cell Free Supernatant (CFS) as follows: CFS from *Lactobacillus* bacteria was used as the control, while CFS from jackfruit seed milk yogurt (JFPM) was used as the experimental treatment. Both treatments were prepared at a 50% v/v concentration in a DMEM medium. Only 5 ml medium was mixed with the 5 ml of CFS from the lactobacillus bacteria for the control treatment and 5 ml DMEM medium was mixed with 5ml of CFS from JFPM for the experimental treatment. 1 ml from the solution was used for the protein extraction of petri plates.,1 ml was used in 6 well plates for the colony formation assay and 100 µl of the solution was used for the MTT assay in 96-well plates as per the labeling information given. The plates were then incubated for 24 hours in a CO_2 incubator.

MTT ASSAY/CELL VIABILITY

Cell viability was measured using the MTT assay after 24 hours of treatment with LB and JFPM. Old medium was carefully aspirated after the treatment period, and cells were incubated with 10 µl MTT solution at 37°C for 3–4 hours, allowing the formation of formazan crystals. A 100µl of DMSO was added after four hours of incubation to dissolve formazan crystals. Absorbance was measured subsequently at 570 nm wavelength using a 630 nm wavelength as a reference. Cell viability was calculated based on the absorbance data obtained.

Figure 15: 96 well plates used for MTT Assay

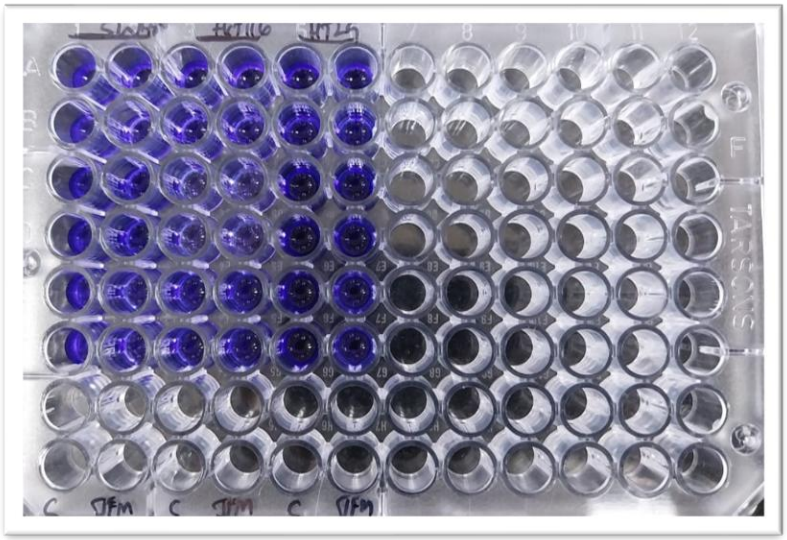


COLONY FORMATION ASSAY

Colony forming o assay is an in vitro quantitative technique to examine the capability of a single cell to grow into a large colony through clonal expansion.The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo “unlimited” division. A colony is considered to be made up of at least 50 cells. The assay evaluates each cell in the population for its potential to undergo continuous division(Franken et al. 2006).

HT29, HCT116, and SW480 cells were seeded at a quantity of 2000 cells per well. After 24 hours of incubation, the cells were treated with filtered CFS from *Lactobacillus* bacteria was used as the control treatment, and filtered CFS from jackfruit seed milk yogurt (JFPM) was used as the experimental treatment. After 48 hours, the old media was removed and new media was added. After 15 days of incubation, the media was removed and the cells were washed with PBS. Colonies were fixed with 1 ml of methanol. Then the cells were stained with 0.2%crystal violet. Then excess stain was removed by washing. After staining, 600µl of glacial acetic acid was added to each well to dissolve the crystal violet. Then the plates were incubated overnight in a shaker at 80 rpm. The next day, 150 µl of the colonies formed in a 6-well plate were transferred into a 96-well plate. For each cell line, triplicates were prepared along with two identical sets. The absorbance was then measured at 590 nm using a Varioskan Multilux plate reader.

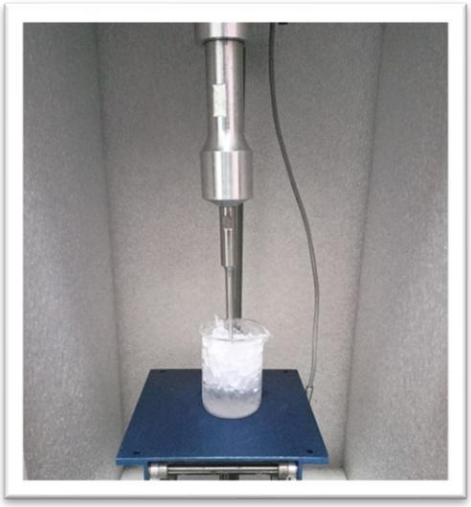
Figure 16: 96 well plates used for colony formation assay



PROTEIN EXTRACTION

The media was discarded and the cells were washed with to remove any media components. Then 300 µl 1X RIPA buffer was added subsequently cells were scraped vigorously and disruption was carried out using rather intense pulsator sonication effectively breaking down cellular components. Cell lysate obtained after sonication was spin at 1600 rpm for thirty minutes in a centrifuge at 4°C. The supernatant was carefully collected in an eppendorf tube after centrifugation and stored at -20°C for later experimentation.

Figure 17: Sonicator



PROTEIN ESTIMATION

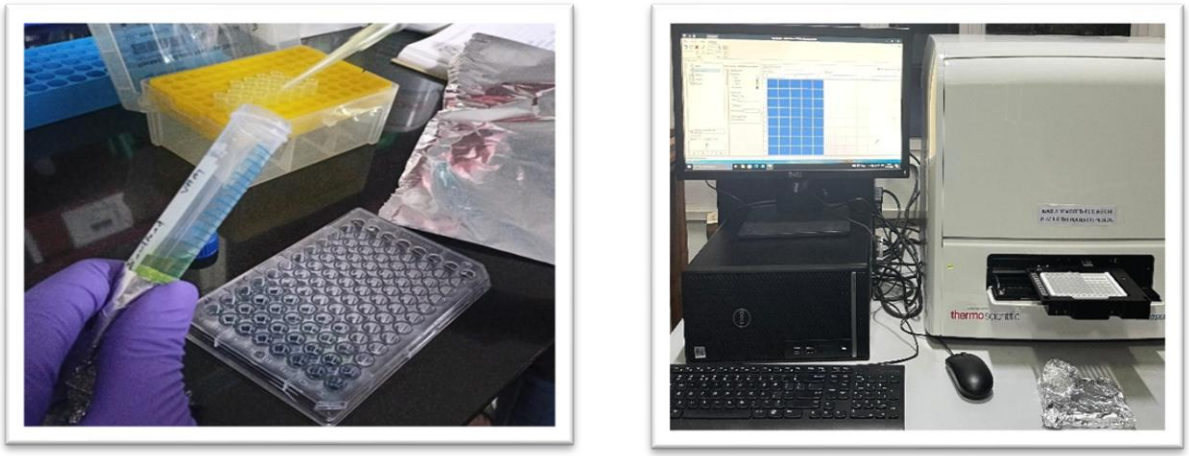
For protein estimation, 96-well plates were used, with the first and second rows designated for the preparation of standards. The protein standards were prepared as follows:

Table 4: Protein standard preparation

BSA	RIPA(µL)	CONCENTRATION
1	6.5	0.2
1.5	6	0.3
2.5	5	0.5
3.5	4	0.7
4.5	3	0.9
5	2.5	1
6.5	1	1.3
7.5	0	1.5

In the remaining wells, 2 µl of the sample was mixed with 8 µl of 1X RIPA buffer. A blank control was prepared using 10 µl of 1X RIPA buffer. To all wells, 25 µl of Reagent A and Reagent S, and 200 µl of Reagent B were added. The absorbance was then measured at 750 nm using the Varioskan Multilux Reader.

Figure 18: Protein estimation



WESTERN BLOTTING

Ready-to-load sample preparation

Protein samples were diluted to a concentration of 1.5mg/mL based on the absorbance values obtained from protein estimation. The final ready-to-load samples were prepared by mixing the diluted protein with 6X loading dye (Laemmli buffer) and 1X RIPA buffer.

Table 5: Loading samples preparation

Ready to Load	1.5 mg/ml	dye	RIPA
HT29 LB	53.48435814	16.6	29.91564186
HT29 JFPM	44.80120482	16.6	38.59879518
HCT116 LB	78.61522199	16.6	4.784778013
HCT116 J	32.66857017	16.6	50.73142983
SW480 LB	55.41728763	16.6	27.98271237
SW480 JFPM	65.40897098	16.6	17.99102902
DLD1 LB	45.41679389	16.6	37.98320611
DLD1 JFPM	48.8312541	16.6	34.5687459

Gel Preparation

There are two types of gel, resolving gel and stacking gel.

10% Gel

Table 6: 10% Resolving gel preparation

Resolving Gel	×1	×2	×4	×6	×8
Acrylamide (30%)	1.66mL	3.32	8	12	16
Tris pH 8.8	1.25mL	2.5	5	7.5	10
SDS (10%)	50µL	100	200	300	400
TEMED	3.5µL	7	14	21	28
APS (10%)	50µL	100	200	300	400
Water	1979.8µL	3959.6	4939.5	9879	13172

Table 7: 10% stacking gel preparation

Stacking Gel	×1	×2	×4	×6	×8
Acrylamide (30%)	0.5mL	1	2	3	4
Tris pH 6.8	0.5mL	1	2	3	4
SDS (10%)	25µL	50	100	150	200
TEMED	2.5µL	5	10	15	20
APS (10%)	25µL	50	100	150	200
Water	1447.5µL	2895	5790	8685	11580

Electrophoresis

The SDS-PAGE setup was first cleaned with ethanol and securely placed in the gel casket. To check for any leakage, water was added between the glass plates. If no leakage was observed, the water was removed, and any remaining droplets were carefully wiped with filter paper.

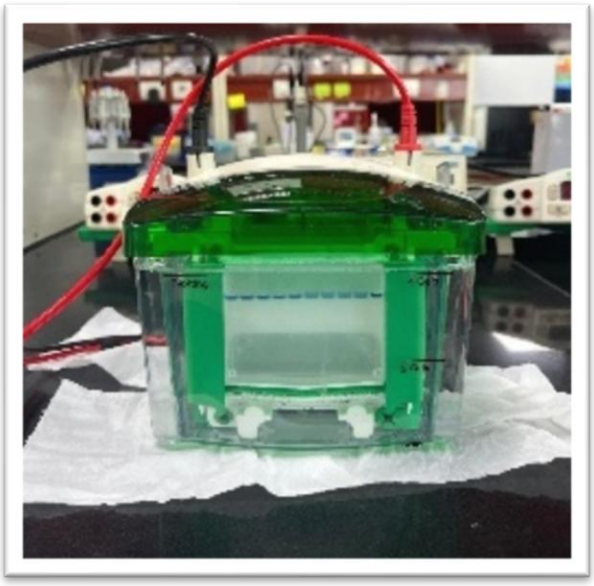
A resolving gel was prepared based on the molecular weight of the protein of interest. Lower-percentage gel is used for high molecular-weight proteins, whereas for lower-molecular-

weight proteins a higher percentage gel is used. In this experiment, the protein of interest, E-cadherin, has a molecular weight of 135 kDa and for that, we used 10% gel.

8 ml of resolving gel solution was poured into the glass plate setup for the gel preparation, followed by the addition of 1 mL of isopropanol on top to ensure a smooth gel surface. After the solidification of the resolving gel, the isopropanol was removed, and 2 mL of stacking gel was poured above the resolving gel. Then the comb was carefully placed to create wells for sample loading.

Once the gel had solidified, the comb was gently removed, and excess acrylamide was removed. The gel casting was then positioned in the electrophoresis tray filled with 1X running buffer. A molecular weight marker was loaded in the first well. Protein samples were denatured by heating at 95℃ for 3 minutes, and from the prepared protein sample,40µg was loaded into the wells. Electrophoresis was begun at 80V until the bands entered the resolving gel, after entering the resolving gel the voltage was increased to 100V. The run continued until the green band of the protein ladder reached the bottom of the resolving gel.

Figure 19: Western Blotting



Protein transfer to PVDF membrane

A PVDF membrane of the required size was prepared. The gel was removed from the electrophoresis setup and the membrane was activated using methanol, followed by immersion in transfer buffer along with the sponge, filter paper, and gel. The transfer stack was assembled in the following order:

Anode cascade → Sponge → Filter paper → Gel → PVDF Membrane → Filter paper → Sponge → Cathode cascade

The transfer apparatus was placed in the electrophoresis tank filled with transfer buffer, and protein transfer was carried out at 100V for 90 minutes.

Blocking and antibody incubation

To avoid non-specific binding, the membrane was treated in 3% skimmed milk for 1 hour on a rocker at room temperature. Following blocking, the membrane was washed with 1X PBST solution 3 times, each for 5 minutes. Then the required primary antibody was added and the blot was stored at 4°C for the entire night. Then the next day, the primary antibody was discarded and washed with 1XPBST for 4 times, each for 15 minutes. After that, the appropriate secondary antibody is added to the blot and kept at a rocker for 1 hour, after the secondary antibody is discarded and again washed with 1X PBST 4 times, each for 15 minutes.

Detection

A 1:1 ratio of luminol and H2O2 was prepared in an amber tube. It was added to the blot in the dark and the membrane was developed in x-ray for visualization

RNA ISOLATION

In 6 well plates, SW480, HCT116, and HT29 cells were seeded. After the cells attained confluency, they were treated with LB and JFPM. Media was aspirated after 48 hours of incubation and cells were washed gently with PBS subsequently. Cells were scraped subsequently after 300 µL of RLT buffer was added quite liberally

RNA isolation was performed using the Qiagen kit protocol as follows:

1. **Lysis and Binding:**

- To lysate,600 µL of 70% ethanol was added rapidly .
- To the RNeasy mini spin column,300 µL of the sample was transferred and centrifuged at 8,000g for 1 minute.
- The flow-through was discarded, and for 1 minute the sample remaining was centrifuged at 8,000g.

2**. Washing Steps:**

- 700 µL of wash buffer was added down the column and centrifuged at 8000g for 1 minute quickly afterwards.
- 500 µL of RPE buffer was added to the column and centrifuged at 8,000g for 3 minutes.

3. Elution:

- To the column,15 µL of RNase-free water was added .
- To elute the RNA.,the column was centrifuged at 8,000g for 1 minute .

The concentration and purity of total RNA were measured using a NanoDrop spectrophotometer. 1.5 µL of the sample was placed on the detection probe, and the RNA concentration was quantified. Nuclease-free water was used as a blank.

cDNA Synthesis

Using the Qiagen kit protocol the RNA which was isolated is subjected to cDNA synthesis. The reaction mixture included:

- RNA isolated-1 µg
- iScript Reverse Transcription Supermix- 4 µL
- Nuclease-free water

The iScript Supermix contained RNaseH, MMLV reverse transcriptase, RNase inhibitors, dNTPs, oligo(dT), random primers, buffers, and stabilizers. In a thermocycler, the reaction was set with the following conditions

Table 8: cDNA synthesis temperature condition

Stages	Temperature	Time
Stage 1	25°C	5 min
Stage 2	46°C	20 min
Stage 3	95°C	1 min

qRT-PCRz

In a 96-well plate, quantification of gene expression was performed using quantitative real-time PCR (qRT-PCR).In this research, claudin3 and claudin 4 primers were used, which have a tumor-promoting role. The reaction mixture per well contained:

- cDNA-1.5 µL
- Forward primer (FP)- 0.5 µL
- Reverse primer (RP)- 0.5 µL
- SYBR Green- 5 µL
- Nuclease-free water- 2.5 µL

Each sample was loaded in triplicates, with β-actin as the housekeeping control gene. A master mix cocktail was prepared based on the total number of wells and mixed thoroughly. The qRT-PCR plate was sealed with a transparent adhesive sheet to prevent evaporation.

qRT-PCR Cycling Conditions

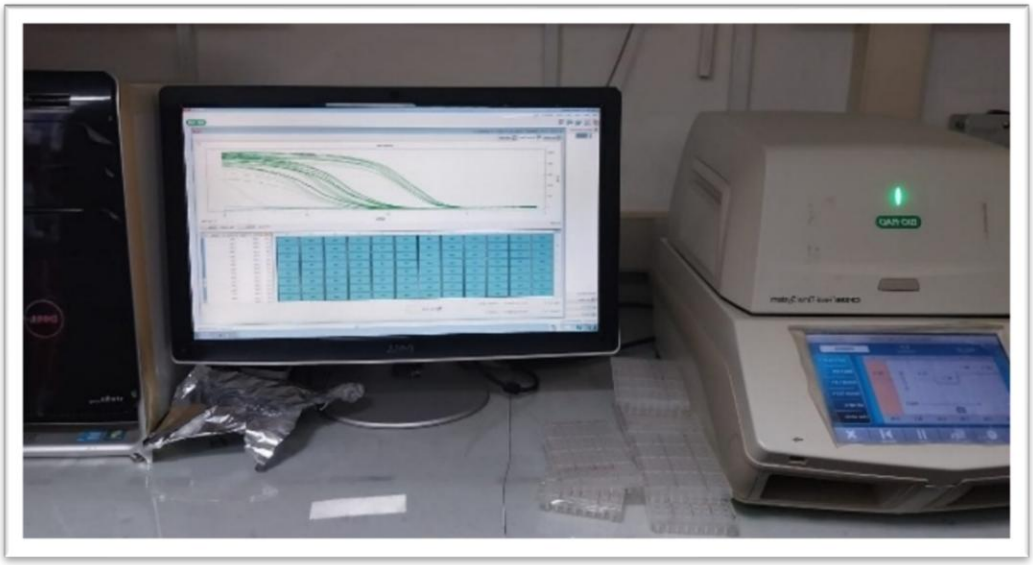
The qRT-PCR was run under the following conditions:

Table 9: RT-PCR cycle temperature

Step	Temperature	Time
Initial Denaturation	95°C	5 min
Denaturation	95°C	30 sec
Annealing	58°C	30 sec
Extension	72°C	30 sec
Final Extension	72°C	5 min

Following the reaction, the results were analyzed, and fold changes in gene expression were calculated using appropriate data normalization methods.

Figure 20: qRT-PCR



CHAPTER-4

RESULTS

SENSORY ANALYSIS

Sensory evaluation of jackfruit seed milk-based yogurt was conducted and findings were meticulously tabulated in tables ,10, 11, and 12 afterwards. Evaluation centered on various sensory attributes such as appearance, colour ,odour, flavour ,texture .taste and overall acceptability pretty thoroughly.

Table 10: Scorecard of Jackfruit seed milk yogurt (Trail no 1)

Characteristics	Panelist1	Panelist2	Panelist3	Panelist4	Panelist5	Total score (TS)	Average TS
Appearance	5	5	4.8	5	5	24.8	4.1333333
Colour	4.6	4.5	4.8	5	4.7	23.6	3.9333333
Odour	4.5	4.5	4.5	4.3	4.5	22.3	3.7166667
flavour	4.3	4.2	4.5	4.6	4.5	22.1	3.6833333
Texture	4.5	4.6	4.5	4.3	4.5	22.4	3.7333333
Taste	4.2	4.4	4.5	4.5	4.3	21.9	3.65
OVERALL PERCENTAGE OF ACCEPTANCE: 76.166%							

Table 11: Scorecard of Jackfruit seed milk yogurt (Trail no 2)

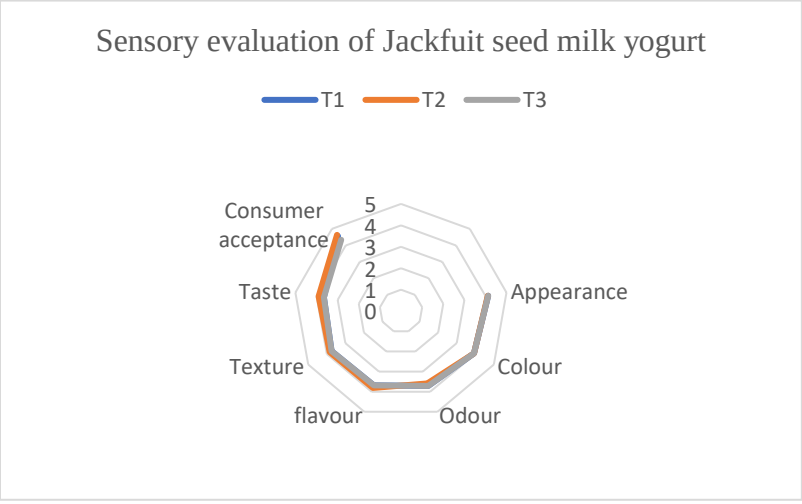
Characteristics	Panelist1	Panelist2	Panelist3	Panelist4	Panelist5	Total score (TS)	Average TS
Appearance	4.8	4.9	5	5	5	24.7	4.1166667
Colour	4.6	4.5	4.8	5	4.7	23.6	3.9333333
Odour	4.4	4.3	4.3	4.3	4.3	21.6	3.6
flavour	4.5	4.5	4.5	4.8	4.6	22.9	3.8166667
Texture	4.5	4.7	4.5	4.6	4.7	23	3.8333333
Taste	4.7	4.7	4.8	4.5	4.7	23.4	3.9
OVERALL PERCENTAGE OF ACCEPTANCE: 77.33%							

Table 2: Scorecard of Jackfruit seed milk yogurt (Trail no 3)

Characteristics	Panelist1	Panelist2	Panelist3	Panelist4	Panelist5	Total score (TS)	Average TS
Appearance	4.8	4.9	4.8	4.7	4.5	23.7	3.95
Colour	4.6	4.5	4.8	4.7	4.7	23.3	3.8833333
Odour	4.2	4	4.2	4.1	4.2	20.7	3.45
flavour	4.1	4	3.9	4.2	4.1	20.3	3.3833333
Texture	4.2	4.1	4.1	4	4.3	20.7	3.45
Taste	4.1	4	4.2	4.5	4.7	21.5	3.5833333
OVERALL PERCENTAGE OF ACCEPTANCE: 72.33%							

For further experiments both the quantity of jackfruit seed milk and overall acceptability of the product is an important factor. Based on the scorecard of 3 trials, we choose trial no 1 for further experiments. It is because that trail no 1 has an overall acceptance of 76.1% with the 50% of jackfruit seed milk. In trial 2, overall acceptance of the yogurt was high, but the amount of jackfruit seed milk was low also in trial no 3, even though it had a high amount of jackfruit seed milk, the overall acceptance was low compared to the other 2 trials. The figure 21 shows a visual representation of the sensory evaluation of jackfruit seed milk yogurt

Figure 21: Sensory evaluation of Jackfruit seed milk Yogurt



TITRATABLE ACIDITY

The acidity of the yogurt increased during the storage time due to the production of lactic acid. The table 13 show the titratable percentage of prepared yogurt.

Table 13: Titratable Acidity

Sample weight	Titratable acidity percentage		
	Day 1	Day 2	Day 3
10g	0.64%	0.66%	0.67%

EFFECT OF pH

pH is an essential yogurt characteristic determinant. The pH of the yogurt measured is an immediate indicator of lactic acid brought about by bacterial fermentation. In this research, yogurt's PH is the range from 4.2 to 4.6. According to Lee and Lucey (Anon n.d.), the typical pH of yogurt is 4.6. The pH of the product is affected by the storage period. pH decreases during storage may be due to the increase in the acidity of the sample

Table 14: pH value

Sample weight	PH Value		
	Day 1	Day 2	Day 3
10.19g	4.6	4.5	4.2

SYNERESIS

Syneresis is an important factor that indicates the quality of yogurt. The amount of whey collected from yogurt was used to measure the syneresis. Here the syneresis value was lesser in the 1st day and its value increased in the next 2 days. By increasing the time, the amount of whey also

increases. The texture and basic quality of yogurt can be affected by the high syneresis value, which indicate the low water-holding capacity of yogurt.

Table 15: Syneresis Data

SAMPLE WEIGHT	Volume of whey (ml)		
	DAY 1	DAY 2	DAY 3
25g	1	1.9	2.9

NUTRITIONAL VALUE OF RAW JACKFRUIT SEED FLOUR

The nutritional analysis of raw jackfruit seed flour was conducted in an external laboratory, where the AOAC method was applied for the analysis. The test results come out to confirm that jackfruit seed flour is quite rich in carbohydrates, protein, calcium, and Iron. However, it was discovered that no vitamins were included in the sample.

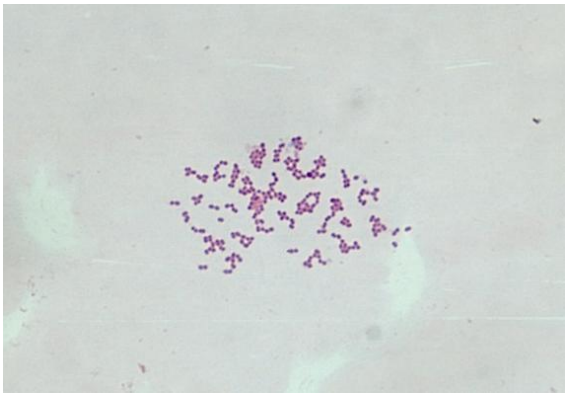
Table 16: Nutritional data of jackfruit seed flour

Sample	Carbohydrate g/100g	Protein g/100g	Total fat g/100g	Dietary fiber g/100g	Ca mg/100g	Fe mg/100g	Vitamin A mcg/100g
Jackfruit seed flour	82.2	2.47	1.00	2.37	67.7	2.5	BLQ

GRAM STAINING

The prepared samples were subjected to Gram staining and was observed under a bright light fluorescent microscope. The observed bacteria have a rod shape structure, and retained the crystal violet stain lead them to appear in purple colour. These results confirm the presence of Gram-positive bacteria, *lactobacillus* that added during the yogurt production process to facilitate fermentation

Figure 22: Microscope images of *Lactobacillus*



COLONY FORMING UNITS

The proliferation and differentiation capacity as well as the number of viable bacterial cells suitable for growth and differentiation were tested by a colony-forming unit Assay. The results were obtained that the 10^{-6} dilution had the highest number of colony-forming units, which was consistent with findings according to the literature(Escamilla et al. 2012).

Figure 23: Colony growth in the sample

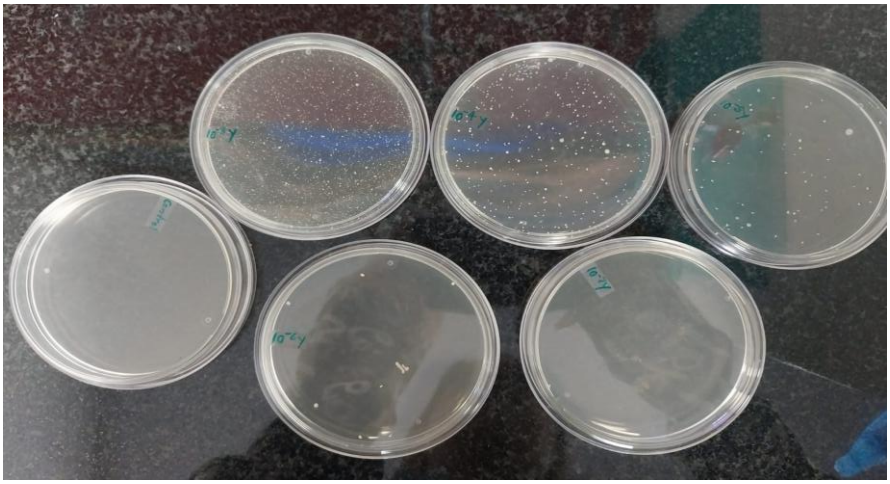


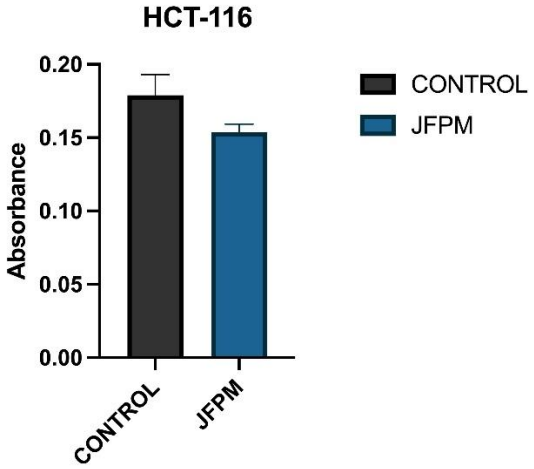
Table 17: CFU Data

Dilution Factor	No: of colonies formed	CFU
10^{-3}	110	110 X 10^4 cfu/ml
10^{-4}	69	69 x 10^5 cfu/ml
10^{-5}	17	17 x 10^6cfu/ml
10^{-6}	10	10 x 10^7 cfu/ml
10^{-7}	NIL	

MTT ASSAY RESULTS

The cytotoxic effect of JFPM and LB was monitored by the MTT Assay.A greater number of viable cells was suggested by absorbance value around 0.18 exhibited by control group. JFPM-treated group showed markedly reduced absorbance roughly 0.15 indicating some decrease in cell viability after treatment very effectively. Bioactive compounds present in JFPM likely exhibit potent cytotoxic properties or anti-proliferative effects severely impairing metabolic activity of HCT-116 cells drastically.

Figure 24: Graphic representation of MTT Assay



COLONY FORMATION ASSAY RESULTS IN POST-TREATMENT CANCER CELLS

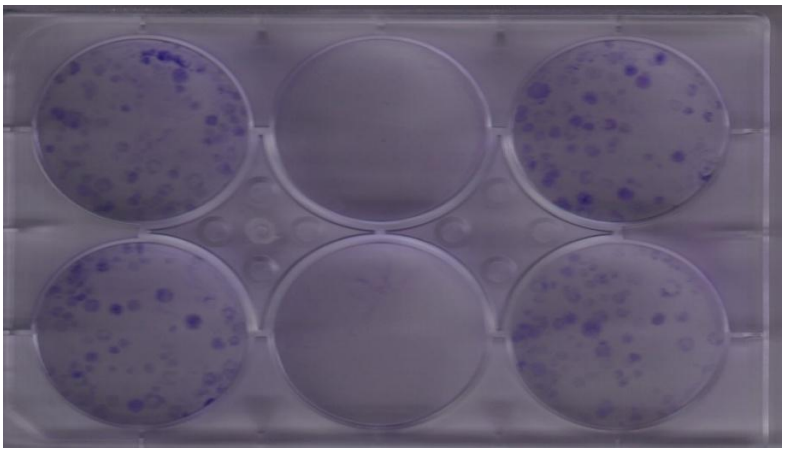
A colony formation assay on three kinds of colorectal carcinoma cells, HT29, HCT16, and SW480 cell lines was done to investigate the influence of LB on cancer cell proliferation**.** From the figure below we can identify that, the cells treated with JFPM form fewer colonies compared to LB, indicating that LB and JFPM in colorectal cancer cells are capable of impairing colony formation.

Figure 25: 6 well plates of Colony Formation Assay

HCTT116

LB

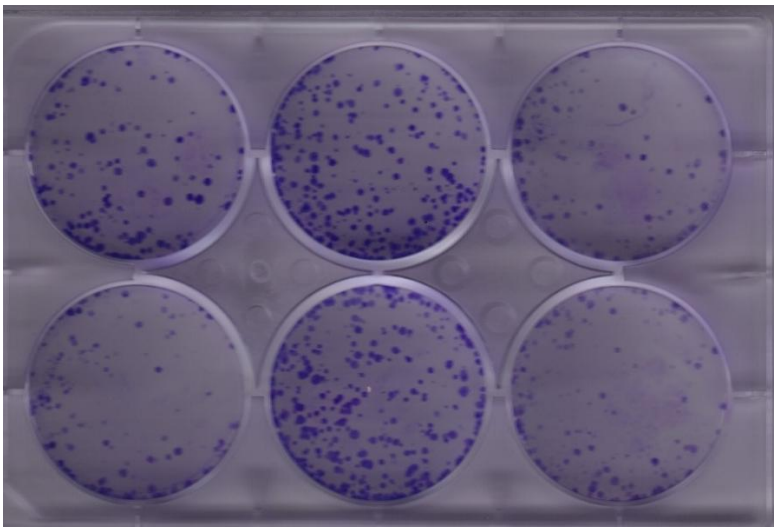
JFPM



HT29

LB

JFPM



SW480

LB

JFPM

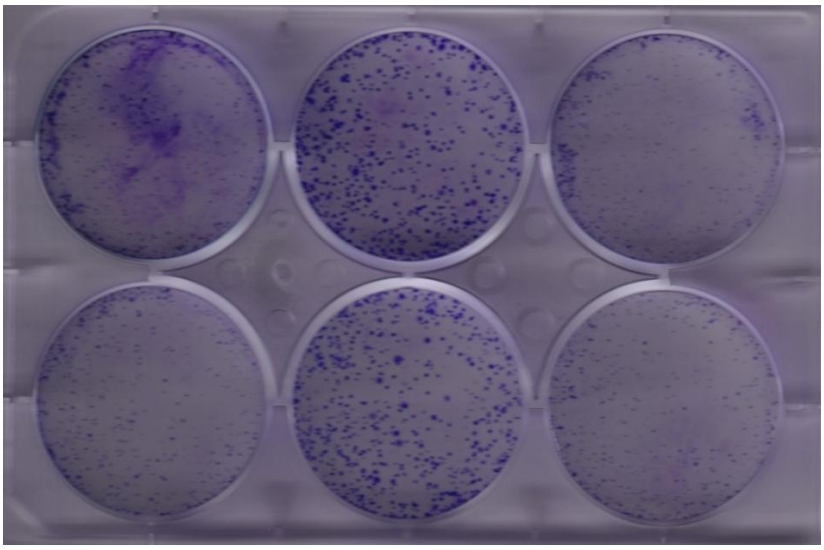
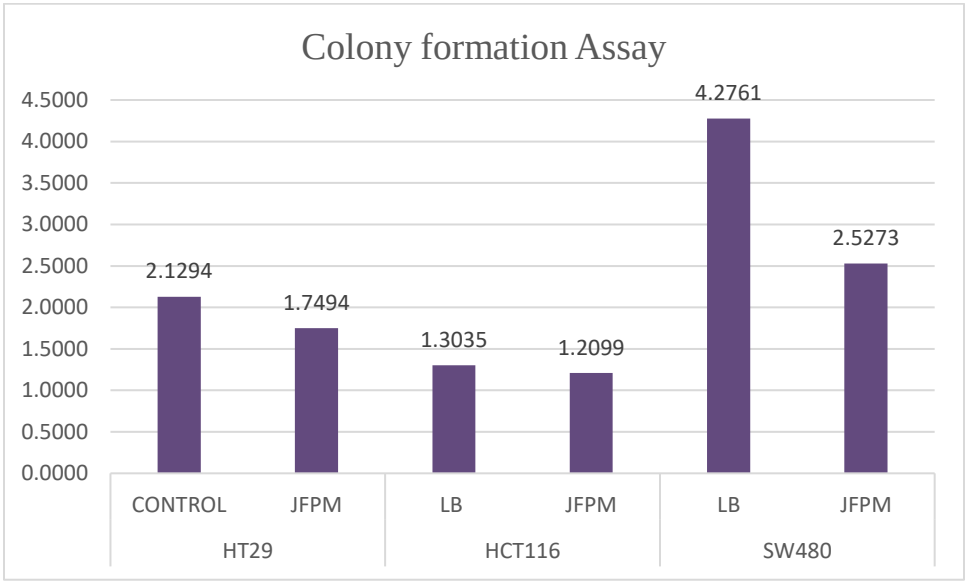
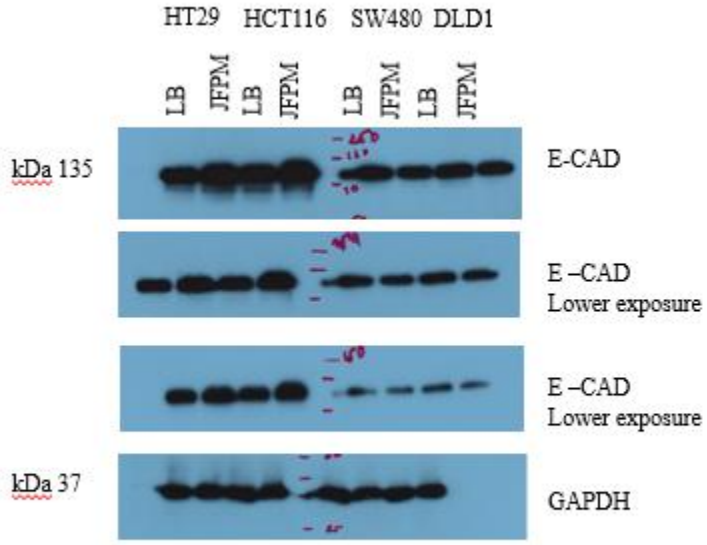


Figure 26: Graphic representation of Colony Formation Assay



WESTERN BLOTTING

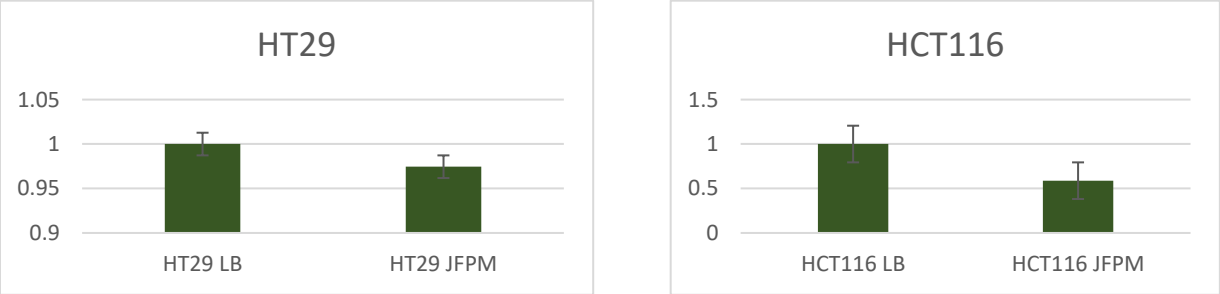
Figure 27: Western Blotting Data



Expression levels of tumor suppressor gene E-cadherin were examined in colorectal cancer cell lines HT29 HCT116 SW480 and DLD1 during Western Blot. HT29 cells and HCT116 treated with JFPM exhibit markedly diminished E-cadherin expression relative to cells subjected to LB treatment. JFPM therapy successfully controlled expression of colorectal cancer cells.

qPCR

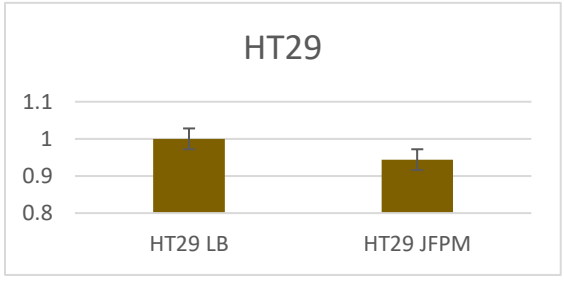
Figure 28: Graphical representation of qPCR with Claudin 3

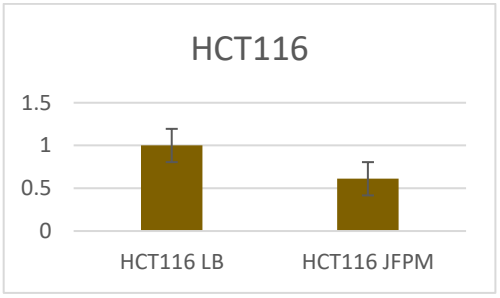


Effect of JFPM on Claudin 3 Expression

Quantitative real-time PCR analysis showed significant downregulation of Claudin 3 mRNA expression in HT29, HCT116 cells, and SW480 colorectal cancer cells treated with JFPM rather than control LB. JFPM may modulate transcriptional regulation of Claudin 3 a key marker intricately linked with tumorigenic progression significantly.

Figure 29: Graphical representation of qPCR with Claudin 4





Effect of JFPM on Claudin 4 Expression

Quantitative real-time PCR analysis showed significant downregulation of Claudin 4 mRNA expression in HCT116 cells and SW480 colorectal cancer cells treated with JFPM rather than control LB. JFPM may modulate transcriptional regulation of Claudin 4 a key marker intricately linked with tumorigenic progression, significantly.

CHAPTER- 5

CONCLUSION

CONCLUSION

The study highlights the potential of lactobacillus fermented jackfruit seed milk yogurt as a nutritious functional food with anticancer properties, specifically targeting colorectal cancer. The prepared yogurt was characterized through sensory evaluation, physicochemical analyses, microbial assessments, and in vitro cellular assays using colorectal cancer cell lines.

One among the prepared JFPM yogurts was chosen for additional biological tests after sensory analysis. Trail no 1, with 50% jackfruit seed milk, was used for further biological evaluation. Based on the physicochemical evaluations, the yogurt exhibited a reasonably good texture over the continued 3 days of storage, including titratable acidity (0.64–0.65%), an acceptable pH (4.2–4.5), and moderate syneresis levels.

Microbiological tests confirmed the existence of live gram-positive lactobacillus strains with the 10^{6th} dilution showing the highest CFU, indicating its probiotic viability which is an important factor in this research.

Importantly, the MTT assay and colony formation assay show the apoptosis effect of treatment against colorectal cancer cell lines such as SW480, HT29, AND HCT116.JFPM treatment much decreased the Cell viability and colony formation compared to the Control treatment which is lactobacillus alone, indicating that the combination of jackfruit seed compounds and probiotics has more effect in preventing cancer cells. JFPM-treated cancer cells, particularly HT29 and HCTT116 showed an upregulation of the tumor suppressor protein E cadherin in western blotting data, confirming that the JFPM may improve cell adhesion and prevent the potential for metastasis. In qRT PCR, JFPM treatment markedly downregulated Claudin 3 and Claudin 4 gene expression in HCT116, SW480 and HT29 colorectal cancer cells compared to LB treatment. This suggests that JFPM may suppress genes linked to tumor progression and metastasis apparently by some unknown mechanism fairly effectively.

In conclusion, Jackfruit seed milk fermented yogurt is a good functional food option that poses both nutritional benefits and anticancer properties, especially with regard to colorectal cancer. From this research, it is very clear about its therapeutic potential. Future studies can explore its effects in vivo, identify active components, and evaluate its use in clinical settings as a dietary supplement for cancer prevention.

CHAPTER-6
REFERENCE

REFERENCE

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