EVALUATION OF ANTIOXIDANT, ANTIMICROBIAL AND POTENTIAL ANTICANCER ACTIVITIES OF PAPAYA-BASED KOMBUCHA BEVERAGES ON COLON CANCER

BY

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ABSTRACT

Traditional kombucha is prepared by fermenting sugar and tea extract with probiotic microorganisms collectively named as Symbiotic Culture of Bacteria and Yeast (SCOBY). Recently, fruits or leaves juices have become a new, favourable alternative medium for kombucha making. The employment of various starting materials and starter cultures during fermentations might result in the creation of several variable metabolites that may have various bioactivities. Thus, this study was aimed to determine the secondary metabolite content, antioxidant activities, antimicrobial effect against selected food borne pathogens and anticancer activities of the kombucha juices of papaya pulp and leaves fermented using locally isolated kombucha cultures, namely: yeast (*Dekkera bruxellensis*) and acetic acid producing bacteria (*Komagataeibacter rhaeticus*).

Tolerance of these selected cultures against low pH and different bile salt concentrations were evaluated. *D. bruxellensis* MFS1 showed tolerance to pH 2 and higher and significant tolerance to bile salt at 0.3 %, 0.5%, and 1%. In contrast, *K. rhaeticus* MFS1 was only tolerant to pH 3 and higher and exhibited poorer tolerance to bile salt. Then, the kombucha juices supernatant were subjected to the profiling of organic acids and phenolic compounds using UPLC-PDA; quantification of total phenolic content; antioxidant activity measurement using FRAP, DPPH, and antimicrobial effect by well diffusion assay. The concentrations of acetic acid, L-malic acid, kojic acid and quinic acid increased in both fermented kombucha samples, with acetic acid being the most abundant form of organic acid being produced. Besides, chlorogenic acid, ellagic acid and 2,5-dihydroxybenzoic acid were also significantly increased after fermentation. In terms of antioxidant activities, papaya leaf kombucha (PLK) revealed higher activities of antioxidant compounds in the range of 89.56% compared to papaya pulp kombucha (PPK) (54.95%) DPPH inhibition. In addition, supernatant of fermented papaya leaves kombucha were able to inhibit the growth of pathogenic strains with higher zone of inhibition on most of the pathogens when compared to non-fermented and acetate samples.

Other than that, anticancer properties of papaya-based kombucha were evaluated using *in vitro* and *in vivo* approaches. Using the MTT assay, higher concentrations of both kombucha pulp and leaves were shown to induce cellular growth suppression in two colon cancer cell lines, HT29 and SW 480. Both treated HT29 and SW480 shared a comparable cell population percentage pattern change from viable cells to early apoptotic, subsequently to the late apoptotic cell, as indicated by the Annexin V-FITC/PI assay. To determine how papaya kombucha administration affected the advancement of the cell cycles in HT29 and SW480, cell cycle analysis utilising flow cytometry was performed. Conclusively, kombucha treatment from PPK and PLK successfully inhibited the transition of the HT29 and SW480 cancer cells *in vitro*.

Next, an *in vivo* study model named the AOM/DSS-induced ICR mice model was employed to evaluate the effect of papaya kombucha on the progression of colon cancer. The colon adenocarcinoma tumourigenesis process was delayed when the mice were fed using a low dose (0.7mL/kg) of papaya-based kombucha twice a day. On the contrary, feeding the mice with a high dose (1.8mL/kg) of similar kombucha treatment two times a day hastened colon carcinogenesis. Overall, significant dysbiosis in gut microbiota was observed in the control and AOM/DSS-induced groups. As the most predominant gut microorganisms, *Bacteroidetes* and *Firmicutes* demonstrated fluctuating abundances in healthy and ill mice. For instance, *Bacteroidetes* proliferation was significantly repressed by the growing of colon adenocarcinoma. Combining treatment with kombucha juices of papaya pulp and leaves and increasing *Firmicutes* growth was shown to help restore the bacterial load of several genera to near control levels.

Using both *in vitro* and *in vivo* study designs, papaya kombucha beverages were shown to possess promising anticancer effects. This is because it contains potentially advantageous probiotic microbes, phenolic compounds, and substantial organic acid. Moreover, the acidic nature of kombucha enables it to regulate the pH balance within the intestine, helping to establish a healthy and balanced gut environment. The aforementioned positive results

demonstrate the prospective benefits of incorporating papaya kombucha into one's diet as a functional food.

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DECLARATION

I hereby confirm that:

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Date: 20th September 2023

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
AE	Acetate-ethanol
ALDH	Aldehyde dehydrogenase
AOM	Azoxymethane
APC	Polyposis tumour suppressor
BD	Bile ducts
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CFCS	Cell-free supernatant
CRC	Colorectal cancer
CV	Central vein
DSS	Dextran sodium sulphate
FAP	Adenomatous polyposis coli
FPP	Fermented papaya product
FID	Flame ionisation detector
GI	Gastrointestinal
H	Hepatocytes
HA	Hepatic artery
HAT	Hydrogen atom transfer
HDI	Human development index
H&E	Haematoxylin and eosin
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel diseases
IC50	Inhibitory Concentration at 50%
ICR	Institute of Cancer Research
JAKIM	Department of Islamic Development Malaysia
K	Kupffer cells
MARDI	Malaysian Agricultural Research and Development Institute
MRS	De Man, Rogosa, Sharpe media
MMR	DNA mismatch repair
MSI	Microsatellite instability
NFC	Non-fibre carbohydrates
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
PLK	Papaya leaves kombucha
PPK	Papaya pulp kombucha
PQQ	Pyrroloquinoline quinone
S	Sinusoids
SCOBY	Symbiotic cultures of bacteria and yeast
SET	Single electron transfer
TA	Titratable acidity
TNF-α	Tumour necrosis factor alpha
TSS	Total soluble solids
UC	Ulcerative colitis
UPLC	Ultra performance liquid chromatography
UQ	Ubiquinone
YDPA	Yeast peptone dextrose agar

LIST OF PRESENTATION AND PUBLICATION

Refereed Journal

1. Sharifudin, S. A., Ho, W. Y., Yeap, S. K., Abdullah, R., & Koh, S. P. (2021). Fermentation and characterisation of potential kombucha cultures on papaya-based substrates. *LWT*, *151*, 112060. https://doi.org/10.1016/j.lwt.2021.112060
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2. Shaiful, S.A., Koh, S.P., Razali, M., Yeap, S.K. and Ho, W.Y. (2018) Metabolite compositions, In vitro antioxidant and anti-proliferative activity on HT-29 colon cancer cell line of papaya pulp juice Kombucha. 4th Annual Postgraduate Research Symposium. 23rd July 2018. University of Nottingham, Malaysian Campus, Semenyih
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6. Shaiful Adzni Sharifudin, Koh Soo Peng, Yeap Swee Keong, Ho Wan Yong. (2021) Metabolit Minuman Kombucha Berasaskan Jus Buah Betik Melalui Fermentasi Beragitasi. ***Persidangan Buah-Buahan Kebangsaan 2021.*** Seminar atas talian. 6-8 April 2021

CHAPTER 1

INTRODUCTION

1.1 Background of The Study

Cancer is an alarming global public health crisis as the number of cancer cases continues to rise each year. According to Sawicki et al., (2021), the prevalence of colorectal cancer (CRC) is 6.1%. With this, CRC has emerged as the third most prevalent cancer in the world. The same report also showed that this cancer is the second leading cause of cancer-related death (9.2%) across the globe. By 2035, it is estimated that the total mortality rate of colorectal cancer will increase by over 60%. In Malaysia, CRC is the second commonest cause of cancer death after breast cancer with higher occurring rates in men than women (S. Wong et al., 2021). Other than genetic predispositions, CRC is often related to environmental factors such as lifestyles and diets (Conti et al., 2020). Furthermore, different lifestyles and diets can lead to the alteration of intestinal environment and gut microbiota compositions (Artemev et al., 2022). Other factors such as gastrointestinal (GI) disorders (e.g., chronic ulcerative colitis and Crohn colitis) may also increase the risk of cancer when suffered from long duration of these disorders (Porter et al., 2021). In the case of colon cancer, treatment may be given through surgery, chemotherapy, and radiotherapy (Biller & Schrag, 2021). However, the recovery rate for CRC patients remains uncertain alongside high mortality rate and other adverse side-effects being developed (Falk et al., 2021; Lee et al., 2021). Therefore, there is a need to look for an alternative for medical solution to prevent and treat CRC.

Current trends in daily diet no longer focus on recommended nutrients intake, but also include components that can lower the risk or prevent the disease development. Natural resources including fruits, vegetables, and medicinal plants are used to make nutraceuticals, which have a significant impact on lowering the risk of CRC and slowing its advancement (Kuppusamy et al., 2014). The creation of health-promoting bioefficacy functional foods has persisted as a cutting-edge tactic to satisfy customer demand. To do so, food and beverages industries have re-look into fermentation as a crucial tool for product innovation (Shiferaw-Terefe & Augustin, 2020). Numerous advantages can be obtained by fermentation, including a distinctive flavour, improved health and nutrition, a safer product, and a 100% natural label (Leal et al., 2018; Shiby & Mishra, 2013).

The use of probiotic in CRC prevention is still new (Pino et al., 2020; Salek Farrokhi et al., 2020). Probiotics are live microorganisms that, when given in sufficient quantities, will improve the host's health (Pino et al., 2020). Potentials probiotic strain need to be able to withstand gastric acid, adapt to bile salt, and multiply and colonise the gastrointestinal system (Kerry et al., 2018; Swanson et al., 2020). Past findings have indicated the potentials of consuming probiotics namely, modulating the microbial imbalance in gastrointestinal system (Azad et al., 2018), reinforcing gut epithelial barrier (Natividad et al., 2012), and removing carcinogen in the gut by binding and inactivation of carcinogen (Guarner & Malagelada, 2003; Tuohy et al., 2003). Recent work done by Gao et al. (2017) showed that when given histamine-producing gut bacteria, the inflammation and tumour development was reduced in mice lacking the enzyme histidine decarboxylase (HDC). Furthermore, by combining probiotic strains in food fermentation as functional starter cultures, this could improve its functional

properties, food safety, nutritional and offer better health advantages (Beena Divya et al., 2012; Marco et al., 2017).

The properties of fruit and vegetable can be improved through fermentation technology (Kårlund et al., 2020). One of the straightforward, organic, and yet effective methods utilised to enhance biological activity is microbial fermentation. Through controlled fermentation process, the structure of carbohydrates, proteins, and polyphenols are further breakdown to form lower molecular weight compounds (Kårlund et al., 2020). During fermentation, many biochemical changes on plant's nutritive and anti-nutritive components are manipulated through enzymatic and metabolic reactions. These processes can enhance the digestibility and bioavailability of nutrients and enrich the bioactive components in the fermented products (Kårlund et al., 2020).

Kombucha tea is a mild vinegar drink that utilises similar genus of bacteria and yeast like vinegar in its production (Lynch et al. 2019; Kim and Adhikari, 2020). Previous studies showed that the consumptions of kombucha tea and vinegar can provide beneficial effects e.g., cancer prevention (Karabiyikli & Sengun, 2017), liver protection (Hyun et al., 2016; Wang et al. 2014), and antioxidant improvement (Beh et al., 2016; Chakravorty et al., 2019).

In this project, kombucha cultures or symbiotic cultures of bacteria and yeast strains (SCOBY) are combined with papaya juices (pulp and leaves) to enhance their phytonutrients through fermentation. It is hypothesized that fermented products produced via a controlled fermentation process will contain more functional metabolites than its original form. The SCOBY strains used in this study were isolated

from the traditional starter culture used in kombucha tea production. The SCOBY strains isolated are then evaluated for their probiotic potential. When fermented together with papaya pulp and leaves, the phytonutrients produced may enhance the modulation of imbalanced microorganisms in GI system and also provide a therapeutic and preventive effect by inhibiting the genotoxicity of carcinogen in the gut. Subsequently, this study focuses on identifying the possibility of using the fermented papaya as a substance to curb colon cancer. By proving the mentioned postulates, papaya fermented with SCOBY may have great potential as nutraceutical ingredients and be part of functional beverages.

1.2 Problem Statements

The leading cause of cancer-related death worldwide is colorectal cancer. (Xie, Chen and Fang, 2020). There are numerous colorectal cancer risk factors, such as age, lifestyle, diet, family history, and ethnic background. Typically, colon cancer is discovered after it is already advanced and typically not identified until symptoms appear (Biller & Schrag, 2021). Plant phytochemicals and microbial metabolites as nutraceutical and functional foods have shown to exert anticancer activities, thanks to numerous bioactivities such as antioxidant, antidiabetic, anti-obesity, and anti-inflammatory (Katz, Nisani & Chamovitz, 2018; Omara et al., 2020). These natural functional foods may provide preventive actions or better treatment when combined with existing therapeutics to exhibit fewer adverse effects on the patients.

Purified bioactive ingredients from food known as nutraceuticals have been shown to provide health advantages, including the ability to treat and/or prevent disease. Meanwhile, functional foods can be defined as foods that contain bioactive components

in a regular diet to provide health benefits over normal foods. Both products are sometimes difficult to differentiate due to their compositions, applications, and marketing strategies. Nevertheless, both products must demonstrate protective actions against chronic diseases or any physiological benefits (Katz, Nisani & Chamovitz, 2018; Omara et al., 2020). The drive behind the growth of this sector is the increasing consumer's demands wanting for healthy food and drinks. Taking advantage of today's healthy eating habits, interest in and impressions of traditional fermented and natural foods are rising (Katz, Nisani & Chamovitz, 2018; Omara et al., 2020). As a result, there is constantly a need to create and invent healthy functional foods to satisfy the needs of these consumers.

1.3 Research Objectives

The objectives of this research are as follows:

1) To isolate, identify and characterise the yeast and bacteria species present in traditional kombucha culture for their probiotic properties.
2) To determine the presence of metabolites in isolated kombucha strains' fermented papaya pulp and leaves.
3) To determine the antioxidant and antimicrobial properties of isolated kombucha strains' fermented papaya pulp and leaves.
4) To evaluate the effects of fermented papaya pulp and leaves on the proliferation of colon cancer cell lines (HT-29 and SW 480) *in vitro.*
5) To assess the effects of fermented papaya pulp and leaves on the chemoprevention, immune biomarker expression, and gut microbiome on colon carcinogenesis in azoxymethane-induced ICR mice.

1.4 Significance of The Study

The consumption of plant-based foods rich in antioxidation and anti-inflammatory components can reduce the risk of chronic diseases. Besides, this also has carcinogenic and mutagenic protection effects. Phytochemical analysis revealed that C. papaya contains compounds including alkaloids, glycosides, flavonoids, saponins, tannins, phenols, steroids, and enzymes that have therapeutic potential. These basic materials can be fermented to create products with value added by microbial fermentation, leading to the creation of novel functional foods. Due to the lack of knowledge on the chemical makeup of the kombucha based on papaya pulp and leaf juices, this study can establish the basis of the development of new kombucha beverages by profiling their biochemical compositions. On the other hand, the therapeutic values of *C. papaya* fruit, rind, leaves, roots, and seed extracts have been confirmed in many published pieces of literature. Besides increasing its flavor and bioactive components, fermentation may enhance the nutrient bioavailability, digestibility, and bioactivities of papaya. Various substrates can impact kombucha beverage produced by influencing with its microbial growth, and metabolite synthesis, thereby affecting the functionality of the beverage. In this study, we aim to evaluate the effect of fermenting papaya pulp and leaf (kombucha) on the potential antioxidant properties, antimicrobial activities, and antiproliferative effects against colon cancer cell lines and colon carcinogenesis *in vivo*.

CHAPTER 2

LITERATURE REVIEW

2.1 Chemical Composition of Papaya

Papaya (*Carica papaya* Linn*)* also known as paw paw, babaya, papailer, melonenbraum, papeeta, also known as betik to Malaysians, is a tropical American fruit that is indigenous to Mexico and South America (Sharma et al., 2020). It is cultivated in tropical and sub-tropical areas with estimation of more than 100 tonnes per hectare of fruits have been produced in over 60 countries in 2020 (Cabrera-Mederos et al., 2020).

Researchers have concluded that the chemical composition of papaya varies based on several factors, e.g., cultivars, sunlight exposure, growing location, ripeness, agricultural practices, and postharvest handling (Khor et al., 2021; Nakhate et al., 2019; Santana et al., 2019). Apparently, as the fruit progresses through different stages of maturity, the changes in biochemical and physiological lead to modification of its consumption and composition (Behera, Rath & Sethy, 2021).

Papaya pulp is a great source of ascorbic acid and vitamins (Fernandez et al., 2019). It also contains carotenoid compounds such as β-cryptoxanthin and β-carotene that produces yellow and orange color in the pulp, respectively. While lycopene in papaya is significantly higher in red flesh cultivar than yellowish flesh cultivars and often lycopene content in yellowish cultivars is either small or does not contain any lycopene (Sancho et al., 2011).

Meanwhile, the amounts of polyphenolic compounds in papaya differ based on the types of plant tissues, cultivars, ripening stages, and extraction techniques (Abdel-Halim et al., 2021; Vallejo-Castillo et al., 2020; Yap et al., 2020;). Caffeic acid, para coumaric acid, protocatechuic acid, quercetin, kaempferol, and tannin have all been found and quantified as polyphenolic chemicals in papaya (Ikram, 2019; Rivera-Pastrana et al. 2010; Saeed et al., 2014).

2.2 Nutraceuticals and Functional Foods

In recent years, nutraceuticals and functional food products have become an important topic that is being discussed to meet the needs of health-conscious consumers globally. The word nutraceuticals are coined in 1989 by combining 'nutrition' and 'pharmaceutical'. Nutraceutical products are really refined bioactive ingredients acquired from food that have health or medical advantages, such as the ability to prevent or treat disease (Ek, 2003). While functional food can be defined as food that is eaten in a normal diet but can provide health benefits over normal foods and contain bioactive compounds. Both products are sometimes difficult to differentiate due to its compositions, applications, and marketing strategies. However, both products must demonstrate protective actions against chronic diseases or any physiological benefits (Foster, Arnason & Briggs, 2005; Nobili et al., 2009).

Example of nutraceuticals and functional food products which claimed to have activity towards colon cancer cells and preventive role were resveratrol from grapes (Schaafsma et al., 2016), sulforaphane from broccoli (Erzinger et al. 2016), yellow mustard (Eskin, Raju & Bird, 2007), catechins in tea preparations (Haratifar, Meckling & Corredig, 2014) and lycopene from tomato (Cha et al., 2017). Apart from vegetable and fruits as

part of nutraceuticals and functional food, the beverages and food industries are also rediscovering fermented product as an innovation in providing unique taste, improved nutrition and new functional properties under the label of 100% natural (Hugenholtz, 2013). Fermented products are produced using either using dairy or non-dairy with single cultures (lactic acid bacteria or probiotics, e.g., yogurt) or mixed culture (within the similar bacterial genus, e.g., yogurt, cheese, sauerkraut or symbiotic cultures of bacteria and yeast, e.g., Kefir and kombucha tea).

Fermented foods can provide good health benefits as they contain functional microorganisms which carry characteristic such as probiotic, anti-microbes, antioxidant, and functional peptides, when combined with good properties of food itself can help to impart various health benefits such as prevention of cancer, metabolic disorders, allergy and gastrointestinal disorders (Tamang et al., 2019). Nearly 50% of anti-cancer medications on the market are made from natural substances or their derivatives (Newman & Cragg, 2016). Nutraceuticals, functional foods and natural active compounds had exhibited anti proliferation effects by suppressing wide range of colon cancer cell line such as Caco-2 (Youns & Hegazy, 2017), HT29 (Jaganathan et al., 2014), SW 620 (Gu et al., 2014), HCT 116 (Khan & Kang, 2017) and SW 480 (Mao et al., 2011).

2.3 Nutraceuticals Potential of Carica Papaya

Given papaya's versatility in being used for making jams, cocktails, and syrup cans, the fruit is also commonly enjoyed in its ripe state as well. In Asian cultures, it is common to prepare unripe fruits and leaves as a salad or as a standalone dish. (Ezike et al., 2009; Ikram, 2019). Apart from its culinary aspects, papaya in Malaysia has been traditionally

used to cure various ailments. For the treatment of diabetes, jaundice, high blood pressure, and dengue fever, papaya leaves are boiled, and their decoctions are employed (Ahmad et al., 2011; Gunjan et al., 2012; Ong & Norzalina, 1999). In other countries, different parts of papaya such as barks, roots, latex, and seeds have various applications such as remedies for eczema, psoriasis, cancer, fever and beri beri (Nguyen et al., 2013). Furthermore, many of these uses have been validated to demonstrate their properties of anticancer, antimicrobial (Muhamad et al., 2017), antioxidant (Somanah et al., 2017), anti-inflammatory and cytotoxic effect (Sagnia et al., 2014).

In addition, *C. papaya* extracts has been experimented on various cancer cell lines showing cytotoxicity activity, for instance, colon cancer, breast cancer, stomach cancer, lymphoma cancer, leukaemia and pancreatic adenocarcinoma (Fauziya & Krishnamurthy, 2013; Jayakumar & Kanthimathi, 2011; Nguyen et al., 2013; Otsuki et al. 2010; Vuong et al., 2015). While most of these studies focused on assessing the cytotoxic activities of the native papaya extracts against cancer cell lines, more recent publication demonstrated increasing trend of the evaluation of the cytotoxicity of fermented papaya preparations against cancers (Gupta et al., 2020; Heung et al., 2021) . However, little to none have reported the anticancer potential of kombucha beverages from papaya against cancer cell lines. However, this niche of study is still expanding with more explorations needed to confirm the findings. Researchers also intend to fully utilize the plant parts of papaya and their modifications to obtain satisfactory results in this attempt of treating and combatting cancer diseases.

2.4 Fermentation of Papaya

Traditionally, food fermentation aims to preserve food economically so that the food can last longer to be consumed. Notably, food preservation now not only improves the composition of nutrients and sensory attributes, but it also inhibits the growth of pathogens in the products (Kårlund et al., 2020; Shiferaw Terefe & Augustin, 2020; Vinicius et al., 2020). Amylases, lipases, and proteases, which are created by edible microorganisms (bacteria, yeast, and moulds) present in the goods, are used or digested by enzymes during fermentation (Sharma et al., 2020). The enzymes convert polysaccharides, lipids, and proteins into non-toxic molecules with enticing flavour, aroma, and texture, therefore enhancing their ability to give customers more appealing sensory qualities (Tangyu et al., 2019). The products may contain ethanol, carbon dioxide, and organic acids that may prevent the growth of harmful microbes (Cao et al., 2019). Consequently, the shelf life of fermented goods is generally longer than that of their original goods (Behera, Ray & Zdolec, 2018; Singh, 2018).

Fermentation of papaya includes the innovation of fermented pulp and leaves of papaya that has been investigated to bring certain health benefits (Leitão et al., 2022). It has been established that fermented papaya preparation (FPP) is a food supplement product in white granular form produced by yeast fermentation (Alara, Abdurahman & Alara, 2020). This project was initiated in Japan and has widely marketed in other countries. FPP products have high content of carbohydrates and amino acids that are good for health (Alara, Abdurahman & Alara, 2020). The fermentation process has allowed enhancement of nutrients as can be seen in fermented papaya seed that has improvement of amino acids, protein, and lipids. Interestingly, fermentation also manages to reduce

the antinutrients in papaya such as oxalate, phytic acid, tannin, and typsin inhibitor at 80.9%, 33.3%, 46.5%, and 97.4%, respectively (Ikram, 2019).

2.5 Kombucha Beverages

One form of well-liked health beverage is kombucha tea. It is produced through the fermentation of tea leaves that have been infused utilising a symbiotic mix of bacteria and yeast (Villarreal-Soto et al. 2018). When developed on sweet tea broth, kombucha tea, commonly referred to as tea fungus, is produced. It has a flavour that is somewhat acidic and reminiscent of cider. Although kombucha tea has a long history, it is thought to have come from Tibet, Manchuria, or Japan. This fermented beverage has become popular and acceptable throughout Europe around the 20th century (Coelho et al., 2020). Today, kombucha tea is sold worldwide in different flavours.

2.6 Microbiology of Kombucha

The microbiology of kombucha cultures or SCOBY has been investigated to reveal its composition of diverse bacteria and yeast genera (Amarasekara, Wang & Grady, 2020; Laavanya, Shirkole & Balasubramanian, 2021; Soares, de Lima & Schmidt, 2021). Because starter cultures differ from one another, it is impossible to pinpoint the precise composition of kombucha cultures. *Acetobacter* and *Gluconobacter* genera make up most of the recovered bacteria from kombucha cultures. *Acetobacter aceti, Acetobacter pasteurianus, Acetobacter xylinum,* and *Gluconobacter oxydans* are the four bacteria that were isolated with the highest frequency (Sievers et al. 1995; Liu et al. 1996). Dutta and Gachhui (2007) identified the nitrogen-fixing bacterium *Acetobacter nitrogenifigens* sp.nov and *Gluconobacter kombuchae* sp.nov. Other than acetic acid bacteria, kombucha cultures contain unusual combinations of microorganisms that

include a variety of yeast species, including *Saccharomyces, Saccharomycoides, Schizosaccharomyces, Brettanomyces/Dekkera, Candida*, and *Pichia* (Jayabalan et al., 2014; Marsh et al., 2014; Teoh et al., 2004). Figure 2.2 illustrates kombucha colony as viewed under scanning electron microscope.

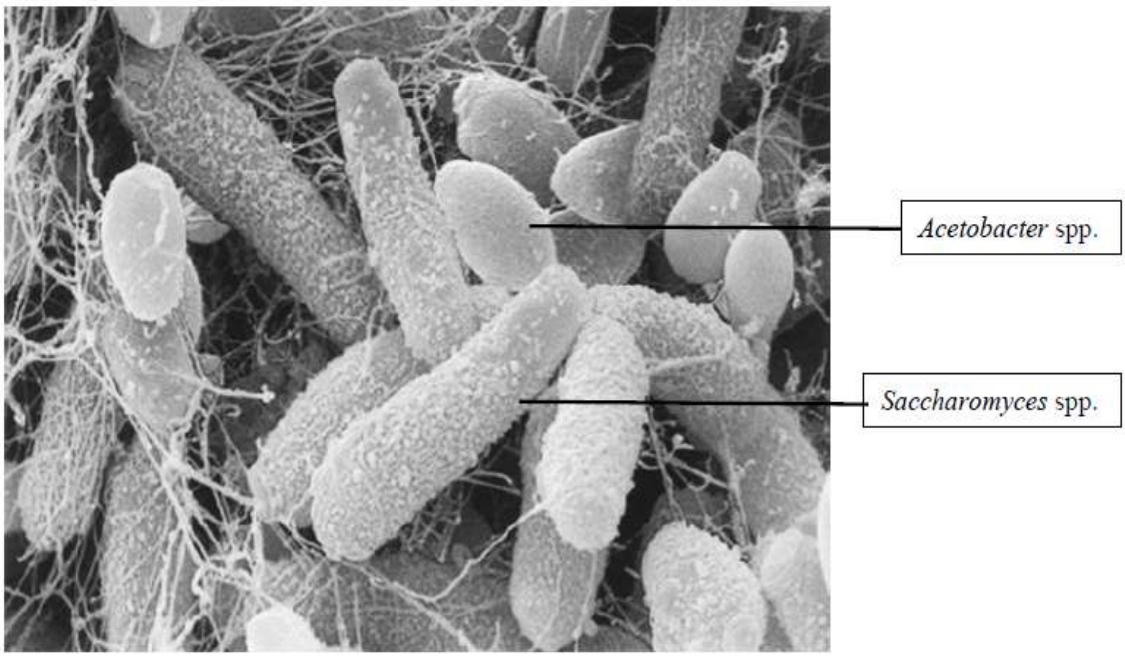


Figure 2.1: Micrograph of kombucha colony. Source: Greenwalt, Steinkraus and Ledford (2000)

The sources or types of kombucha culture/tea will determine the microbiological profile of their exact composition of microorganisms. The compositions of kombucha cultures are not similar worldwide. Nonetheless, a symbiotic kombucha cultures surely will contain predominantly yeast and acetic acid bacteria (Jayabalan et al. 2014).

2.7 Bioavailability of Kombucha

Microorganisms that are both functional and non-functional may be present in fermented foods and beverages. The functional microorganisms will improve the nutritional and functionalities of the raw material through fermentation. These microbes may act by enhancing the bioavaibilities of nutritive components, producing antimicrobial and antioxidant compounds, improving food safety, producing organic acids as preservatives, fortifying with some health promoting component and may also impart potential probiotic characteristic (Tamang et al., 2019). Among the functional microbes associated in fermented beverages are as below: (A) lactic acid bacteria such as *Lactobacillus*, *Lactococcus*, *Leuconostoc, Bifidobacterium,* etc. mostly in fermented milk (Heller, 2001), (B) acetic acid bacteria (*Acetobacter* and *Gluconobacter* sp) in kombucha tea (Greenwalt et al., 2000), (C) several genera of yeast (*Candida, Saccharomyces, Pichia, Saccharomycopsis, Brettanomyces/Dekkera*) in fermented food and alcoholic beverages (Arslan, 2015) and other fermented food and beverages such as species of *Amylomyces, Aspergilus, Monascus, mucor* and *Rhizopus* (Tamang et al., 2019). During fermentation, transformation of raw materials occurs, thereby enhancing the bioavailability of the nutrient (Rekha & Vijayalakshmi, 2010), biopresevative effect (Nath et al., 2020), degradation of anti-nutritive and toxic compounds (Sharath et al., 2014), antioxidant (Shumoy et al., 2017), antimicrobial activity (N'Tcha et al. 2017) and fortification of food with health-related bioactive compounds (Fujita et al., 2017; Saubade et al., 2016).

In kombucha tea, the biological activity and health benefits are due to the relationship between the tea itself which are rich in phenolic compounds and metabolite produced from the fermentation such as acetic acid, glucuronic acid, ethanol and other organic

acids (Jayabalan et al., 2008). Among the promising health effect of kombucha tea consumptions are anti-carcinogenic (Deghrigue et al., 2013; Jayabalan et al., 2011), anti-diabetic (Aloulou et al., 2012), antimicrobes (Bhattacharya et al., 2016; Sreeramulu, Zhu & Knol 2000), anti-ulceration (Marzban et al., 2015) and hepatoprotective (Murugesan et al., 2009; Wang et al., 2014). Recent research has shown the development of plant-based beverage using kombucha cultures with herbal extracts and fruit juices such as grape juice (Ayed, Ben Abid & Hamdi, 2016), lemon balm (Velicanski et al., 2014), *Eucalyptus camaldulensis* and *Litsea glaucescens* (Gamboa-Gomez et al., 2016). Nowadays, kombucha tea is more of a health drink than a typical beverage, containing bioactive molecules with important antioxidant and as potential functional food with beneficial health properties.

2.8 Colorectal Cancer

Globally, the prevalence of colorectal cancer (CRC) has risen enormously due to population expansion, demographic changes, and lifestyle modernisation. In 2018, 18.1 million new CRC cases were documented worldwide (Wong et al., 2019). On the other hand, 9.6 million CRC-linked mortalities were reported in the same year. CRC ranks the second most frequently diagnosed cancer (9.2%) and third in terms of recognition (6.1%). By 2035, mortality related to rectal and colon malignancies is predicted to increase by 60% and 71.5%, respectively (Sawicki et al., 2021). Generally, the CRC incidence is higher in high-income nations such as Western nations. Conversely, developing nations, including Asian, African, and South American nations recorded lower CRC diagnosed cases. However, in the past decade, CRC-reported cases and mortalities showed the most significant increase in nations with a medium or high

human development index (HDI) in which the citizens are adopting a "Western" style of life (Sawicki et al., 2021; X. Wang et al., 2019).

The association between trends in colorectal cancer (CRC) incidence and death rate can be divided into three different global groups. The initial category comprises countries with medium HDI, including Brazil, Russia, China, Latin America, the Philippines, and the Baltics. These nations recorded a rise in both the occurrence and fatality rates during the last ten years. The observed rise in CRC incidence can likely be attributed to the economic change currently taking place in these nations. CRC is frequently reported in the developing countries as obesity, sedentary lifestyle, red meat consumption, alcohol, and tobacco usage are common in these countries. The second category consists primarily of high-HDI nations, including Canada, the United Kingdom (UK), Denmark, and Singapore. This category has had a rise in incidence but a decrease in mortality, attributed to advancements in treatment options. Finally, the third category refers to nations with the highest HDI that include the United States, Iceland, and Japan. Specifically, France has shown a decline in both mortality and morbidity rates. This phenomenon is likely contributed by the advancement in cancer prevention and treatment (Goodarzi et al., 2019).

Among all the cancers diagnosed, the percentage of CRC among the general population in Malaysia is 12.7%. The incidence of CRC is highest amongst the Chinese (27.35%) followed by the Malay (18.95%) and Indian (17.55%) (Muhammad Radzi et al., 2016; Schliemann et al., 2020). Sedentary lifestyles, body fatness, and dietary patterns influence the disease morbidities. There is compelling evidence that physical activity is protective (Shaw et al., 2018), but frequent consumption of red and processed meat, as

well as alcohol, enhances the chance of developing the disease (Chen, Hoffmeister, et al., 2022; Chen, Li, et al., 2022).

2.9 Risk Factors

Cancer is a disease linked to genetic alterations, which can either involve the activation of oncogenes or repression of tumor suppressor genes. This phenomenon results in uncontrolled cellular proliferation. Colorectal cancer often requires alterations in many genes (Klos & Dharmarajan, 2016). The uncontrolled growth of colon epithelial cells can cause the formation of non-malignant adenomatous polyps (Klos & Dharmarajan, 2016). Subsequently, the polyp can progress to become an advanced adenoma before transforming to become an invasive cancer (Klos & Dharmarajan, 2016). Few nonmodifiable and modifiable risk factors are known to promote the tumourigenesis process of colorectal cancer. Non-modifiable components comprise genetic characteristics, race, age, gender, body height, and a family history of CRC. On the contrary, modifiable elements include environmental and lifestyle factors (Sawicki et al., 2021; M. C. S. Wong et al., 2019).

2.9.1 Non-modifiable factors

A family history of colorectal cancer increases the risk of developing the cancer considerably. This phenomenon is contributed by inherited genetic predisposition and environmental variables. Inherited syndromes contribute to about 2 and 8 percent of colorectal cancer cases. Lynch syndrome, and familial adenomatous polyposis coli (FAP) are the two most frequent hereditary syndromes that promote colorectum carcinogenesis (Rawla et al., 2019). Presumably, almost all patients with undiagnosed and untreated FAP syndrome before the age of 35–40 will eventually develop colorectal

cancer (Yang et al., 2020). Colon polyps (precancerous neoplastic lesions) are an abnormal development of tissue protruding from the colon's mucous layer. Histologically, these polyps can be categorised into two primary groups. Non-neoplastic polyps are hamartomatous, hyperplastic, and inflammatory polyps. On the contrary, neoplastic polyps are specifically adenomatous and serrated polyps. If untreated, neoplastic adenomas can develop malignant changes. Depending on their size and location, serrated polyps may undergo tumourigenesis changes as well. In general, the size of the neoplastic polyp is positively correlated to the risk of cancer occurrence.

Inflammatory bowel disease (IBD) is a group of intractable, chronic diseases that affect the immune system of the digestive tract. This induces uncontrolled inflammation. Crohn's disease and ulcerative colitis (UC) are examples of IBD. The pathogenesis of IBD can be associated with genetic, immunological, and environmental factors or a combination of these elements. UC is the third highest risk condition behind Lynch syndrome and FAP for the development of CRC. The tumor growth and progression are aided by chronic inflammation. The risk of CRC increases with the duration of IBD and the severity of the disease, resulting in a 2 to 6-fold increased risk of having CRC in comparison to healthy individuals (Hnatyszyn et al., 2019; Windsor & Chand, 2019).

The number of diagnosed colorectal cancer cases is linked positively to increased lifespan. CRC has the potential to manifest in individuals among youth and adolescents; nevertheless, most cases are observed in individuals aged 50 years and above. The average age at the time of diagnosis for males with colon cancer is 68, while for women, it is 72 (Ahmed, 2020; Martin et al., 2014). Race and ethnicity can influence the

incidence and survival disparities within a nation. In the United States, individuals of African American and Native American descent show a higher prevalence rate of CRC and worse survival rates across different stages of the disease (Rawla et al., 2019). In the Asian region, there is a tight association between the HDI and CRC incidence and mortality rates. The Republic of Korea, Israel, Singapore, Japan, and Jordan are the nations that documented the highest standardised incidence rates of cancer. On the other hand, Nepal, Bhutan, Bangladesh, Sri Lanka, and Pakistan, which have medium and low HDI levels, exhibited a low prevalence (Ghoncheh et al., 2016).

2.9.2 Modifiable factors

According to research, regular physical exercise reduces the incidence of colorectal cancer by up to 30% with approximately 13–14% of CRC cases attributable to physical inactivity (Conti et al., 2020; Kaplan, 2019). Obesity can be contributed by physical inactivity. This health issue can affect the intestinal microflora growth and promote gut irritation and inflammation. Dysbiosis of the gut microflora can trigger tumourigenesis changes. Therefore, excessive fat buildup secondary to obesity is a prominent risk factor that promotes colorectal cancer development. Obese male and female are 50% and 20% more likely to acquire colorectal cancer than non-overweight individuals. The CRC risk is predicted to be risen by 3% for every five kilogrammes of weight increment (Sawicki et al., 2021).

Besides obesity, diet can play a detrimental or protective role in the development of CRC. Diet has a significant influence on the colon microbiome. Different foods can have varying effects on the microflora population and influence intestinal inflammation (Appunni et al., 2021; Wesselink et al., 2021). Through molecular processes that trigger

chronic inflammation, a diet rich in red and processed meat and a lack of fruits and vegetables have been linked to carcinogenesis progression. Moreover, excessive food consumption can increase body mass index (BMI), and this could initiate chronic inflammation. There is a significant relationship between excess weight and obesity, and cancer development. Several mechanisms have been outlined to explain the possible tumourigenicity of red meat. Heterocyclic amines (HAA) and polycyclic aromatic hydrocarbons (PAH) are chemicals produced during the thermal processing of red meat. These compounds are known to initiate tumour formation in humans. Furthermore, N-nitroso compounds with mutagenic and malignant potential can also be detected in processed meat rich in salts, nitrates, and nitrites. Also, these compounds can be generated endogenously during meat digestion (Genes et al., 2019). A high heme iron content in red meat is catalytically crucial for the formation of harmful nitroso compounds during digestion. The heme iron increases the development of reactive oxygen species (ROS). ROS is highly mutagenic and cytotoxic. It can induce cellular inflammation and promote epithelial cell hyperproliferation (Tuan & Chen, 2016).

Fruits and vegetables are inherently abundant fibre sources. Both are enriched with various bioactive substances including vitamins, minerals, folate, plant sterols, and protease inhibitors. These biochemical compounds have antioxidation and anti-inflammatory properties, which protect DNA from cellular injury. A high-fibre diet has been shown to reduce the risk of colorectal cancer by half. However, current epidemiologic studies do not collectively support the protective effects of fibre against CRC. Instead, the actual mechanism of how fibre exerts anticancer effects remains unclear (Peters et al., 2021; Sawicki et al., 2021; Veettil et al., 2021). Several possible mechanisms by which dietary fibre intake reduces the chances of acquiring colon

cancer have been proposed. Firstly, fibre can decrease stool transit time through the colon. This minimizes the contact between possible carcinogenic substances and the colonic epithelium. Secondly, fibre can increase the quantity of water in stools, which helps dilute carcinogens in the faeces. Additionally, fibre can bind sterols and bile acid metabolites, which are believed to play a part in CRC development. Dietary fibre also can stimulate beneficial intestinal microbiota to perform fermentation to produce short-chain fatty acids. It has been postulated that these fatty acids have tumor-suppressing functions (Murphy et al., 2019; Sawicki et al., 2021).

Alcohol and smoking have long been proven as established risk factors for CRC. Multiple cohort and bioinformatics studies have demonstrated substantial clues linking colorectal cancer to tobacco intake and binge drinking. This suggests that both chemicals are eminent tumourigenic agents in humans (Amitay et al., 2020; Lee et al., 2018; Rossi et al., 2018). Ingesting alcohol in moderate to high quantities is correlated tightly to an increased risk of CRC in a linear dose-response manner. The meta-analysis of cohort research data suggests that there is a positive relationship between ethanol consumption habits and CRC risk. Alcohol users have an estimated 7% higher risk for each daily intake of 10 g of ethanol. Additionally, findings from Amitay et al. (2020) indicate that the risk of developing CRC increases to 30% if one drinks more than 24.6 g of ethanol every day.

There is data suggesting that prolonged cigarette smoking is linked to elevated risks of both overall mortality and morbidity specifically related to CRC in both males and females. The study findings indicate that male individuals who engage in smoking exhibit a 39% higher likelihood of developing cancer in the left (distal or descending)

colon. No significant relationship was observed between smoking and cancer in the right (proximal or ascending) colon in men. Conversely, female smokers showed a 20% elevated risk of acquiring cancer in the right colon, with no notable correlation found between smoking and cancer in the left colon. Female smokers have a heightened susceptibility to developing rectal cancer as compared to their male counterparts (Gram et al., 2020). There exists a correlation between smoking and increased difficulties in diagnosing CRC, as well as decreased survival rates among CRC patients. Furthermore, smoking may impede the immune response, making cigarette users more vulnerable to developing tumourigenesis (Haruki et al., 2020; Huang et al., 2022).

2.10 Development of CRC

CRC is formed through the stages of initiation, promotion, and advancement (Figure 2.2). CRC develops when epithelial cells undergo a sequence of genetic or epigenetic alterations that allow them to proliferate excessively. Initiation involves irreversible genetic damage that predisposes the affected epithelial cells of the intestinal mucosa to neoplastic transformation. During the promotion phase, the initiated cells proliferate, resulting in the development of abnormal growth. Consequently, the transformation of benign cancer cells into malignant ones occurs during the progression phase, whereby they acquire aggressive traits and the capacity for metastasis. The benign precursor lesion, known as a polyp, which is an abnormal growth on the inner lining of the colon, is a significant aspect in the majority of colorectal cancer development processes. Adenomatous polyps (adenomas) and serrated polyps, which are the primary origins of the majority of malignancies, are additional types of lesions observed inside the lumen of the large intestine. The process of colorectal cancer carcinogenesis is characterised by a gradual progression, commencing with a state of mild inflammation. Afterward,

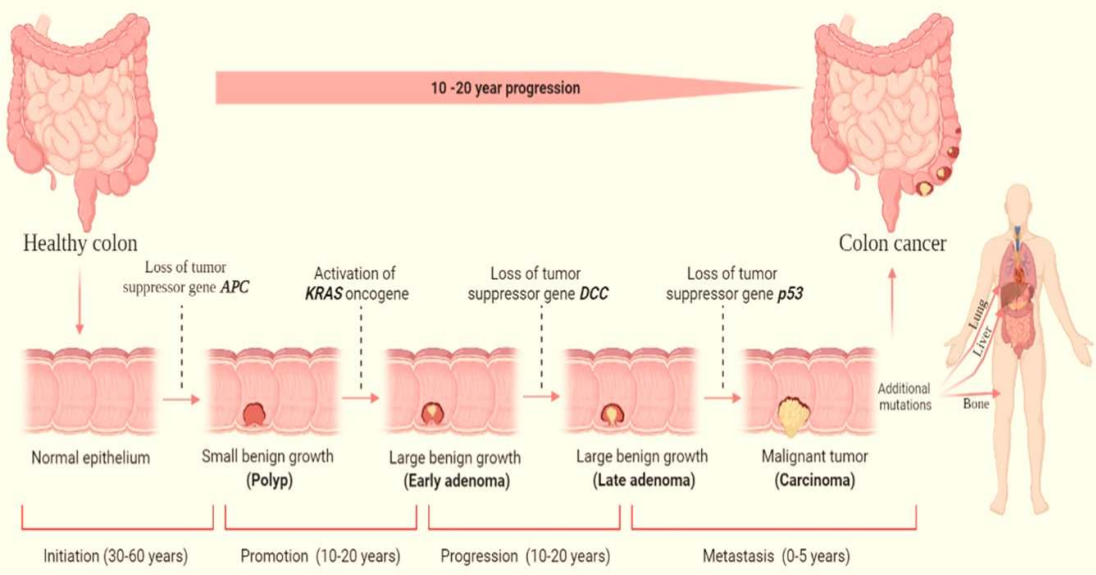


Figure 2.2: Stages and growth of colorectal cancer (CRC). In the process of CRC carcinogenesis, there are four steps: start, promotion, progression, and metastasis. Source: Hossain et al., (2022).

adenomatous polyps will be developed within the epithelial tissue. This eventually forms the adenocarcinoma (Romano et al., 2016; Sabit, 2019). Moreover, the process continues through the consolidation of mutations and genetic modifications and extends a duration of 10-15 years, but it may exhibit rapid progression in certain instances, such as among individuals diagnosed with IBD (Porter et al., 2021; Z. Wang, 2014). A benign adenoma can turn into cancer and spread to other parts of the body through a number of distinct pathways, such as microsatellite instability (MSI), chromosomal instability (CIN), and serrated neoplasia (Sawicki et al., 2021). Approximately 20% of CRC cases may be attributed to hereditary syndromes, namely FAP and Lynch syndrome. These disorders include the inactivation of one allele of the adenomatous polyposis tumour suppressor (APC) gene and one allele of the DNA repair gene in the germline (Valle, 2014). Colorectal cancer may also arise in individuals diagnosed with IBD, including ulcerative colitis, through the inflammatory route. Chronic inflammation is a characteristic of UC. The accumulation of pro-inflammatory

cytokines promotes colonic dysplastic changes. Subsequently, dysplasia advances through a sequence starting from the lack of dysplasia, progressing to indeterminate dysplasia, followed by low-grade dysplasia, high-grade changes leading to neoplastic transformation, and ultimately resulting in CRC (Rivera et al., 2022).

The different pathways involved in CRC development are characterized by distinctive models of genetic instability (Colussi et al., 2013; Sawicki et al., 2021). Most CRC follows the CIN route (chromosomal instability pathway) is characterised by the involvement of APC, p53, and K-ras genes, which are commonly affected. This pathway is responsible for accelerating adenocarcinoma development. Mutations occurring in these genes lead to the activation of oncogenes or the inactivation of tumour suppressors. This eventually leads to the development of malignancy (Rivera et al., 2022). Secondly, MSI is caused by a malfunction of the DNA Mismatch Repair (MMR) mechanism in approximately 15% of CRC cases. The MMR system is responsible for producing proteins that recognise and direct the repair of single nucleotide mismatches that evade DNA polymerase's proofreading (Carethers, 2016). Another aspect that should be considered is the methylator phenotype, specifically the CpG island methylator phenotype (CIMP). The phenomenon under consideration is associated with the hypermethylation of multiple gene promoters (eg. MLH1), the V300E mutation in the BRAF gene, as well as the impairment of TP53 and p16 functionalities (Mojarad et al., 2013). These disorders are responsible for the inactivation of suppressor genes, resulting in disruptions to the functioning of the MMR system. The development of adenomas and cancer can be attributed to the interaction between inherited genetic predisposition and environmental factors (Parmar & Easwaran, 2022). In contrast, most CRC cases are sporadic in nature. In other words,

CRC patients do not possess an inherited predisposition. Instead, the development of this cancer is influenced by lifestyle choices and environmental elements. Additionally, chronic exposure to carcinogens can induce oxidative stress. This triggers DNA damage through a cascade of somatic mutations, ultimately leading to genetic instability and tumorigenesis.

2.11 Apoptosis

The term apoptosis originates from the Greek word that signifies the act of dropping or falling off. The initial introduction of this concept was made by Kerr, Wyllie, and Currie in the early 1970's (Elmore, 2007). Programmed cell death which occurs for specific cells inside human body is part of the mechanism to sustain life. To maintain proper physiology and tissue functionality, the continual process of controlled cell death is used to remove damaged, diseased, or superfluous cells, with the goal of ideally replacing them with new, viable cells (Singh et al., 2019). Apoptosis is a homeostatic process that occurs naturally during development and senescence to maintain tissue cell populations. Apoptosis can also occur as a protective mechanism, such as during immune responses or when cells are damaged by disease or toxins. Although apoptosis can be induced by numerous physiological and pathological stimuli and circumstances but not all cells can die in response to the identical stimulus. DNA damage which occurs in certain cells can be induced by irradiation or chemotherapeutic drugs might result in apoptotic cell death through p53-dependent mechanism (Hientz et al., 2017). In other circumstances, certain hormones, such as corticosteroids, have the ability to induce apoptotic cell death in specific cell types, such as thymocytes, while having little effect or perhaps even promote stimulation in other cell populations (Kalfeist et al., 2022).

2.12 Morphological Changes

The numerous morphological changes that occur during apoptosis have been discovered using light microscopy. Cell shrinkage and reduction of cellular volume (pyknosis) which occurs at the initial phases of apoptosis can be identified using light microscopy. Cell shrinkage can cause reduction in cell size, increment in cytoplasmic density and having a more compact arrangement of organelles. Chromatin condensation is the primary cause of pyknosis, which serves as the most prominent characteristic of apoptosis. Chromatin continues to condense until it undergoes a process known as karyorrhexis in which it ruptures inside a cell with an intact membrane. While at the later stage of apoptosis, morphological features such as membrane blebbing and loss of membrane integrity are observed before these apoptotic cells are engulfed by phagocytic cells such as macrophages, parenchymal cells, or neoplastic cells (Brauchle et al., 2014; Kaczanowski, 2016; Wong, 2011)

2.13 Mechanism of Apoptosis

The mechanisms of apoptosis are extremely complex and sophisticated. It encompassing a series of chemical reactions that are dependent on energy. Recent research findings showed that two primary apoptotic routes are reported, namely the extrinsic pathway, also known as the death receptor route, and the intrinsic pathway, also referred to as the mitochondrial system (Elmore, 2007; Kashyap et al., 2021; Wong, 2011).

2.13.1 Extrinsic pathway

Extrinsic pathway of apoptosis (Figure 2.4) involved the initiation of external signals that lead to the activation of death receptors such as Fas receptors (Fas receptor), DR4/DR5, tumour necrosis factor receptors (TNF-R), and TNF-related apoptosis-inducing ligand receptors (TRAIL-R). These receptors are found on the cell surface of many cell types (Green & Llambi, 2015). Currently, the most extensively studied ligands and their related death receptors include CD95/FasR, TNF-α/TNF-R1, Apo2L/DR4, and Apo2L/DR5. The TNF receptor family is distinguished by the inclusion of extracellular regions that are rich in cysteine, as well as a cytoplasmic domain known as the death domain (Id & Chaudhry, 2019). Death receptors interact with certain ligands on the surface of cells, leading to the recruitment of adaptor proteins such as Fas-associated protein with death domain (FADD) and tumour necrosis factor receptor type 1 associated death domain protein (TRADD). The binding between Fas ligand and Fas receptor leads to the binding of the adapter protein FADD, while the binding between TNF ligand and TNF receptor results in the association of the adapter protein TRADD, which then recruits FADD and RIP. The formation of the FADD-procaspase-8 complex occurs through the process of dimerization of the death effector domain. At this juncture, a death-inducing signalling complex (DISC) is formed. This will lead to the self-catalytic activation of procaspase-8. Hence, it will ultimately initiates the cleavage and activation of other executioner caspases, including caspase-3, caspase-6, and caspase-7 (Brentnall et al., 2013).

2.13.2 Intrinsic pathway

The initiation of the intrinsic pathway (Figure 2.4) takes place intracellularly. Internal triggers that were reported such as irreparable genetic damage, hypoxia, significantly elevation of cytosolic Ca^{2+} concentrations, and severe oxidative stress can initiate the intrinsic pathway. This pathway often causes the increased in mitochondrial permeability and lead to the release of pro-apoptotic chemicals (cytochrome-c) into the cytoplasm. This pathway is closely regulated by Bcl-2 proteins. This group of protein can be divided into two groups: pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk) and anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1) (Singh et al., 2019). Upon activation of pro-apoptotic proteins at the mitochondrial surface, they undergo an allosteric shift, facilitating their oligomerization and subsequent formation of macropores in the membrane. This process leads to mitochondrial outer membrane permeabilization (MOMP) and subsequent decline in the mitochondrial transmembrane potential. This process contributes to the discharge of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Green & Llambi, 2015). The activation of caspase 3 is initiated by the release of cytochrome c from the cytoplasm, which triggers the formation of an apoptosome. This apoptosome is comprised of cytochrome c, Apaf-1, and caspase 9, and it subsequently induces biochemical and morphological alterations that ultimately lead to cellular mortality (Brentnall et al., 2013).

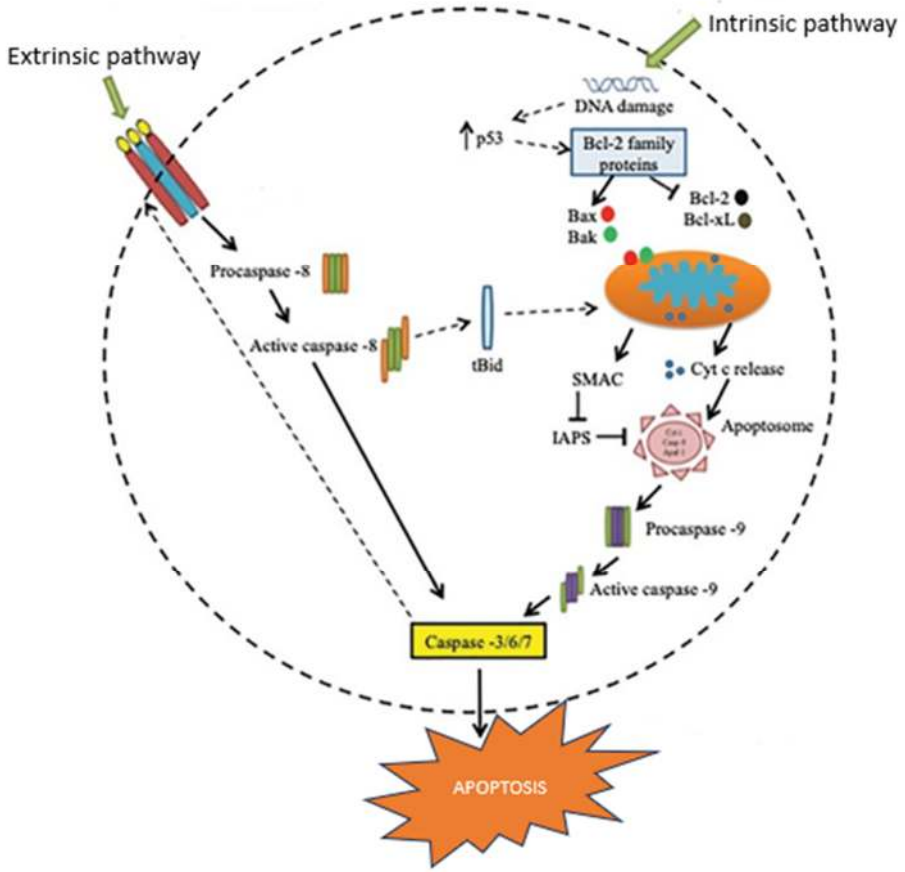


Figure 2.3: Apoptosis is mostly made up of two main pathways, extrinsic pathways are started by outside signals or ligand molecules and involve death receptors (DRs). The intrinsic pathway is controlled by the insertion of Bax/Bak into the mitochondrial membrane. This causes cytochrome c to be released, which then joins with Apaf–1 and procaspase-9 to make an apoptosome. This triggers the caspase 3 cascade of apoptosis, which kills the cell. TNF related apoptosis inducing ligand (TRAIL), cellular FLICE inhibitory proteins (cFLIP), Truncated bid (tBid), B-cell lymphoma protein 2 (Bcl-2), Bcl-2 homologue splice variants (Bcl-xL), Cytochrome (Cyt C), Second mitochondrial activator of caspases (SMAC), Inhibitor of apoptosis proteins (IAPs). Modified from (Id & Chaudhry, 2019).

2.14 Gut Microbiota and Colorectal Cancer

The human gastrointestinal (GI) tract comprises at least 100,000 billion microorganisms. The total human body cell number is 10 to 100 times lower than the intestinal microbiota (Dieterich et al., 2018). Bacteria are categorised based on their taxonomic hierarchy like phyla, classes, orders, families, genera, and species. The composition and relative abundance of the microbiota are dependent on various conditions. These circumstances include oxygen levels, nutrients, pH levels, and dietary patterns. Furthermore, the composition of the intestinal microorganisms can vary in the lumen, mucosa, and crypt-villus axis. 30-50% of the human gut bacteria is *Firmicutes*. *Bacteroides* make up the remaining one-third of the gut microorganisms. Other intestinal microflora include *Actinobacterium*, *Enterobacteriaceae*viruses, archaea, and fungi (Dieterich et al., 2018; Gagnière et al., 2018; Tabowei et al., 2022).

A symbiotic relationship exists between humans and their microbiota. The human intestine is a nourishing environment for the colonisation of gut microflora. The bacteria perform many functions such as enhancing the immune system, regulating the physiological environment, providing nutrition, inhibiting the colonisation of harmful bacteria, and metabolising procarcinogens and carcinogens. The symbiotic interactions between resident micro-organisms and the digestive tract highly contribute to maintaining gut homeostasis. On the contrary, dysbiosis of the intestinal microbiota disrupts the normal functioning of the host's physiological processes. This can lead to the progression of a range of illnesses (Artemev et al., 2022; Rinninella et al., 2019). Factors like genetic composition, lifestyle choices, and environmental factors can affect the population of specific gut bacteria in different individuals. The imbalance in the gut microorganisms can enhance the likelihood of acquiring obesity, autoimmune

disorders, inflammatory diseases, and even some types of cancer. The mechanisms via which the microbiota facilitates the development of CRC remain incompletely understood. The hypothesised mechanisms encompass many factors including DNA damage, the generation of carcinogenic metabolites, and the occurrence of chronic inflammation. Dysbiosis happens when the gut beneficial decrease drastically while the number of harmful bacteria rises exponentially. This promotes the development of cancer-related processes such as angiogenesis, impaired apoptosis, and enhanced cell proliferation. Hence, the composition of the microbiome readily influences the colon carcinogenesis process (Artemev et al., 2022; Rinninella et al., 2019).

Numerous research studies have demonstrated the observable variations in the compositions of intestinal microbiota between individuals suffering from CRC and those who are in good health. Additionally, these investigations have identified certain microbial species that exhibit heightened abundance or diminished presence in patients diagnosed with CRC. Microbiome modifications are also observed in cases of colorectal adenoma, which represents the initial phase of CRC. In a study conducted by Tabowei et al., (2022), it was observed that *Firmicutes* accounted for around 63.46% of the microbial composition in CRC patients. On the contrary, it comprised 43.46% of healthy participants. The phyla of organisms termed *Bacteroidetes* was observed as the second most prevalent in the CRC group (12.7%) and the group of healthy control (13%). Following *Bacteroidetes*, *Fusobacteria* ranks the third most frequent organism present in the gut of both CRC patients (10.58%), in contrast to its occurrence in healthy subjects at a significantly lower percentage of 0.03%. Although there are differences in the composition of gut microbiota, certain microbiota species have been associated with colorectum tumourigenesis progression. *Streptococcus bovis* is a gram-positive

rounded bacterium involved in precipitating the colon carcinogenesis. Enterotoxigenic *Bacteroides fragilis* (ETBF) synthesizes *Bacteroides fragilis* toxin (BFT) that can trigger diarrhoea and inflammatory bowel disease (IBD) (Zamani et al., 2020). Human intestinal adenomas and malignancy are demonstrated to contain a higher abundance *Fusobacterium nucleatum* (Ou et al., 2022). A significantly elevated number of *Enterococcus faecalis* was detected in colorectal cancer (CRC) patients than in the healthy subjects. *E. faecalis* has the ability to generate superoxide. This compound induces DNA damage within epithelial cells. To sum up, a single microorganism does not initiate colorectal cancer (CRC) tumourigenesis. Instead, a group of bacteria overweighs the benefits generated by commensal microbiota to promote CRC development (Cheng et al., 2020; Grenda et al., 2022).

Over the course of recent years, there has been a substantial increase in our understanding of the significance of gut microbiota in colorectal cancer (CRC). The utilisation of the gut microbiota as a biomarker is a newly emerging translational application. A biomarker serves as a detectable sign or measure that signifies the existence or extent of a pathological condition (Veziant et al., 2021). The presence of extensive data supports the idea that screening individuals with average risk can effectively decrease the incidence and mortality rates of CRC. Consequently, the development of a precise and non-invasive screening test has the potential to significantly reduce the global health burden associated with CRC. The growing abundance of metagenomic datasets in CRC presents a valuable resource for the development of faecal microbial indicators for the purpose of disease diagnosis. abundance of metagenomic datasets in CRC presents a valuable resource for the development of faecal microbial indicators for the purpose of disease diagnosis.

Fusobacterium nucleatum has been identified as a significant indicator, either in isolation or in conjunction with other bacterial species, among a range of potential options. The discoveries regarding the microbiota will be a crucial part of future CRC prevention, diagnosis, and treatment as more advances are made in the fields of CRC genetics, metabolomics, and immunology (Wong and Ju, 2019).

CHAPTER 3

METHODOLOGY

3.1 Determination of Kombucha Culture Isolates

Prior to preparation of kombucha beverages from papaya pulp and leaves, the kombucha culture isolates were determined. Firstly, the cultures were isolated and characterized via 16s and 18s DNA, followed by maintenance of kombucha and reference cultures. Next, the kombucha cultures were prepared and lastly, phylogenetic analysis was conducted.

3.1.1 Isolation and Characterization via 16s and 18s rRNA

The kombucha starting culture was acquired from vendors in Selangor, Malaysia and kept at MARDI's food cultures collecting facility. In order to isolate acetic acid bacteria and yeast the sample was prepared by serial dilution in 0.85% saline. Then, a 100 µL aliquot of the material was diluted and spread out on Yeast Peptone Dextrose Agar (YPDA) (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, bacteriological agar 15 g/L, and supplemented with 1.5% calcium carbonate). To distinguish between acid producers of eukaryotes and prokaryotes, the single colonies that created halo-zone and those that did not with various morphologies were chosen and Gram-stained before being viewed under a microscope. All isolates were identified by polymerase chain reaction (PCR) of the 16s rRNA gene with universal primers 27 F (5-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5 ′ -TACGGYTACCTTGTTACGACTT-3′) and 18s rRNA gene primers 18 S F (5′-GCTTGTCTCAAAGATTAAGCC-3 ′) and 18 S R (5 ′ -

CCTTGTTACGACGACTTTTACT-3') The BLASTN platform was used to analyse the acquired sequences.

3.1.2 Maintenance of Kombucha and Reference Cultures

The isolated samples were maintained in acetic acid-ethanol (AE) media with 20% glycerol and YPD with 20% glycerol (*Dekkera bruxellensis* MFS1) (Gullo et al. 2006). (*Komagataeibacter rhaeticus* MFS1). While *Lactobacillus plantarum* was received from UAS Laboratories (Edina, USA) and was maintained in De Man, Rogosa and Sharpe (MRS) media with 20% glycerol, reference cultures (*Acetobactor xylinum* 433, *Acetobacter xylinum* 416) were obtained from MARDI's food cultures collection centre. Prior to usage, all cultures were kept at -80 °C.

3.1.3 Kombucha Culture Preparation

D. bruxellensi MFS1 and *K. rhaeticus* MFS1 glycerol stocks were streaked over PDA and AE agar, respectively, and incubated at 30°C for 2 days and 37°C for 4 days, respectively, before being used as starter cultures.

3.1.4 Phylogenetic Analysis

Using ClustlW multiple alignment, the 16s and 18s rRNA gene sequences of various acetic acid bacteria and yeast were visually aligned. The phylogenetic relationships were inferred using Mega version 5.0, and the distances and neighbour-joining method were computed using bootstrap values based on 2000 replications. The accession codes for each of the nucleotide sequences studied were taken from GenBank.

3.2 Culture Stability Towards Gastrointestinal Environment Conditions

Based on the culture's tolerance for low pH and resistance to bile salts, the stability of the culture toward GI environmental conditions was evaluated.

3.2.1 Tolerance towards Low pH

The isolated bacteria' endurance to low pH conditions was evaluated, with some modifications from Argyri et al. (2013). Yeast and bacterial cultures (OD 600nm 0.5) were taken out by centrifugation at 10,000 rpm for 5 min. They were then twice rinsed in phosphate buffer saline (PBS) solution, which has a pH of 7.2, before being re-dissolved. Following that, the cultures were incubated at 37°C for varying holding durations (0, 0.5, 1, 2, and 3 h), which corresponded to the amount of time food stayed in the stomach. The cells were promptly washed and re-suspended in an equal volume of PBS after each interval time measurement. Serial PBS dilutions of the solution were used to seed it on YPDA (for yeast and acetic acid bacteria) and MRS agar (for *L. plantarum*). For 48 to 72 h, yeast and acetic acid bacteria were cultured on plates at 30 °C. Colony forming units (CFU/mL) were used to express the results.

3.2.2 Bile Salt Resistance

With slight adjustments, Vinderola and Reinheimer's (2003) method was used to examine the isolated microorganisms' bile salt tolerance. Yeast and bacterial cultures (OD 600nm 0.5) were separated by centrifugation at 10,000 rpm for 5 min, and then they were re-suspended in PBS solution containing 0.3, 0.5, and 1.0 percent (w/v) bile salt. The cultures were then incubated at 37 °C for various holding periods 1, 2, and 4 h to mimic how long food stays in the small intestine. The cells were promptly washed and re-suspended in an equal volume of PBS after each interval time measurement.

Serial dilutions of the solution in PBS were used to seed it on YPDA (yeast and acetic acid bacteria) and MRS agar (*L. plantarum*). For 48 h at 30°C for *L. plantarum* and 48 h at 30°C for yeast and acetic acid bacteria, the plates were incubated, respectively. Colony forming units (CFU/mL) were used to express the results.

3.3 Production Of Papaya-Based Kombucha Beverage

Next, the papaya-based kombucha beverages were prepared by setting up the plant materials, juice preparations, and kombucha fermentation of papaya pulp and leaves. This was followed by proximate analysis and analysis of ethanol and acetic acid.

3.3.1 Plant Materials, Juice Preparations, and Kombucha Fermentation of Papaya Pulp and Leaves

The "Sekaki" variety of papaya (*Carica papaya* Linn.) was purchased at the neighbourhood market, and the leaves came from a nearby farm (Exotic star, Pahang, Malaysia). The papaya fruit's developmental stages could be visibly distinguished as follows, depending on the colour of the skin: Green with a hint of yellow is stage 1, totally green is stage 2, more yellow than green is stage 4, more yellow than green is stage 5, yellow with a hint of green is stage 5, and fully yellow is stage 6, respectively (Lam and Zaipun, 1987). Papaya fruits in stage 5 were used for the tests. Both the foliage and the fruits were carefully cleaned. The fruits were carefully chopped into small pieces and skinned. After being quickly dried at 50 °C in a drying oven, the fruits and leaves were ground in a blender to create fruit granules and leave powders, respectively. The powders and granules were vacuum sealed in an aluminium bag and stored at 4°C before use.

Papaya leaves powder and pulp granules containing 10% (w/v) of papaya leaves and 10% (w/v) of sugar were created to produce papaya leaves and pulp juices for kombucha fermentation. Following this, 20% (v/v) of the designated kombucha cultures (*D. bruxellensis* MFS1 and *K. rhaeticus* MFS1) were added to the juices and cultured for 24 hours at 37°C in a shaking incubator. The kombucha fermentation started after the various inoculation fluids containing *D. bruxellensis* and *K. rhaeticus* were blended in an 80:20 ratio and incubated at 37°C for 4 days with 200 rpm shaking.

3.3.2 Proximate Analysis

According to the Association of Official Agricultural Chemists (AOAC) method, the following parameters were measured: moisture, ash, crude fat, crude fibres, and crude protein (Kjeldahl method) (2003). The following equation was used to compute the non-fibre carbohydrates (NFC) by difference:

NFC (%) = 100% - % moisture - % protein - % ash - % fat - % crude fibres

3.3.3 pH, Titratable Acidity (TA) and Total Soluble Solids (°Brix)

A pH metre was used to measure the pH. (Mettler Toledo, Model: Seven Easy GMBH 8603, Switzerland). Acid-based titration was used to quantify the TA in accordance with the method (Chen and Liu, 2000). To determine the endpoint, the pH of the fermented broth (10 mL) was titrated with 0.1 M NaOH and measured using an electronic pH metre (Orion model 290A), where pH = 7.0 was used as the endpoint. In percent v/v, the final TA of the fermented broths was given. Next, a refractometer (Atago, PAL-3, Japan) was used to quantify the total soluble solids in each sample. The results were represented as an °Brix value.

3.3.4 Ethanol and Acetic Acid Analysis

Both the papaya and leaves kinds of kombucha produced cell-free supernatants after being centrifuged at 10,000 rpm for 5 min. Before analysis, these underwent syringe filtration at 0.22 m. Then, gas chromatography was used to identify the presence of ethanol and acetic acid in the clear cell free supernatants.

A flame ionisation detector-equipped Agilent 6890N GC system was used for the chromatographic analysis (FID). A Zebron ZB-Waxplus capillary column with dimensions of 30 m, 0.25 mm internal diameter, and 0.25 m film thickness was used to separate ethanol and acetic acid using a gradient heating profile. The beginning temperature of the oven was 50 °C for 1 min. The column was then heated to 200 °C at a rate of 60 °C/min and maintained there for 1 min before being elevated to 250 °C at a rate of 20 °C/min and maintained there for 3 min. Nitrogen was employed as the carrier gas, with a split flow of 99.4 mL/min and a split ratio of 100:1. The injection port and FID were both set to a fixed temperature of 250 °C. With a total run period of 10 min, the flow rates of hydrogen, air, and make-up gas were kept at 40, 350, and 30 mL/min, respectively. Using HP Chemstation Plus software, the quantities of ethanol and acetic acid were determined from external calibration standards curves (acetic acid and ethanol) (Agilent, USA). Alcohol and acetic acid and ethanol had specific retention durations of 4.299 and 2.749, respectively.

3.4 Metabolite Profiling of Papaya-Based Kombucha Beverages

The metabolite profiling of papaya-based kombucha beverages included the determination of organic acids content and flavonoid compounds.

3.4.1 Determination of Organic Acids Content

Analyses of the organic acids profile of fermented and unfermented papaya pulp and leaves were carried out using high performance liquid chromatography (HPLC) (Alliance Separation Module, Waters, 2695), equipped with a diode array detector (Waters, 2996). With the oven temperature maintained at 30 °C, a 10 L aliquot of the sample solution was separated using a Synergi 4 m, Hydro-RP80A (250 x 4.6 mm) column. A 20 mM KH_2PO_4 solution with an adjusted pH of 2.9 made up the mobile phase, and water with a flow rate of 0.6 mL/min made up the mobile phase B. The following linear gradients from 100 to 0% A were used in the gradient elution mode: from 0 to 30 min, 100% A; from 30 to 45 min; and from 45 to 55 min. Retention durations and UV spectra at 190, 210, and 254 nm were compared with those of common organic acids to identify the peak. Utilizing calibration curves produced by injecting standardised organic acids at known concentrations as an external standard, quantification was performed. Each analysis was carried out three times.

3.4.2 Determination of Flavonoid Compounds

The AcquityTM Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, USA) was used to separate the phenol content of the filtered extracts. The UV spectrum of 280, 330, and 360 nm was used, and the Kinetex C18 100 A column (100 mm 2.1 mm; 1.7 m particle size) was employed. The flow rate was set at 0.4 ml/min, and the oven temperature was maintained at 40 °C. The gradient elution was composed of mobile phase A (water: acetic acid = 97:3) and mobile phase B. (methanol). The gradient elution mode was performed as follows: 0–1 min at 100% A, 1–10 min at 100% to 40% A, 10–12 min at 40% to 100% A, and finally, maintained at 100% A for an additional 2 min. Epigallocatechin, vitexin, rutin, quercetin, luteolin, apigenin,

tannic acid, and ellagic acid were used as external standards with known retention times to generate calibration curves for the quantification of flavonoids (gallic acid, vanillic acid, protocatechuic acid, syringic acid, 4-hydroxybenzoic acid, caffeic acid, o-coumaric acid, ferulic acid, sinapic acid and p-coumaric acid).

3.5 Antioxidant Activities f Papaya-Based Kombucha Beverages

The antioxidant qualities of papaya-based kombucha were assessed based on the total amount of phenolic components, flavonoids, and tannin, as well as the free radical scavenging assay and ferric reducing antioxidant power.

3.5.1 Total Phenolic Content (TPC)

TPC was computed using the Folin-Ciocalteu technique, according to Koh et al (2017). In a nutshell, 1 mL of sample was mixed with 5 mL of diluted Folin-Ciocalteu reagent, and the mixture was left to react for 5 min. The mixture was then combined with 4 mL of a 7.5% sodium carbonate solution and left in the dark at 29 °C for 2 h. A UV-Vis spectrophotometer was employed to measure the absorption at 765 nm. Gallic acid served as the reference, and the results were given in milligrammes of gallic acid equivalent per millilitre (mg GAE/mL).

3.5.2 Total Flavonoid Content Analysis (TFC)

The total flavonoid concentration was assessed using the aluminium chloride method, which was developed from Aziz et al (2018). The quercetin standard curve was used to build the standard. After adding 0.3 mL of a 5% $NaNO_3$ solution, 1 mL of sample was mixed with 4 mL of distilled water. The $AlCl_3$ (0.3 mL) solution was added to the mixture after 5 min. The mixture was properly mixed and allowed to stand for 6 min

before being injected with 2 mL of a 1M NaOH solution. The total volume was then increased to 6 mL by adding the distilled water. The absorbance at 510 nm was measured using a UV-Vis spectrophotometer, and the results were expressed as mg quercetin equivalent per mL of sample (mg QUE/mL).

3.5.3 Total Tannin Determination

To calculate the total tannin concentration, a spectrophotometric technique called Folin-Ciocalteu was utilised. A volumetric flask containing distilled water (7.5 mL), the Folin-Ciocalteu-phenol reagent (0.5 mL), and 35% Na_2CO_3 solution was added with the designated papaya Kombucha samples (0.1 mL) (1 mL). The mixture was then shaken, 10 mL of distilled water was added, and it was allowed to sit at room temperature (29 °C) for 30 min. Using a UV/Visible spectrophotometer, all samples were measured at 725 nm against a blank according to a standard curve made using gallic acid. The amount of tannin was specified as mg of GAE/mL of extract (Durai et al. 2016).

3.5.4 DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Free Radical Scavenging Assay

Based on the modified methodology suggested by Thaipong et al. (2006), DPPH radical scavenging activity was conducted. The measurement was based on the DPPH-infused purple methanol solution's bleaching impact. In a nutshell, 2.85 mL of DPPH methanolic solution was added to 0.15 mL of sample extract, and the mixture was allowed to react for 30 min in the dark. Next, the absorbance at 515 nm was measured. Using ascorbic acid as the reference, results were represented as mg ascorbic acid equivalents per mL of material (mg AAE mL-1). The following formula was used to determine the radical scavenging activity percentage (% RSA):

$$\%RSA = \frac{(Ab_{control} - Ab_{sample})}{Ab_{control}} \times 100\%$$

3.5.5 Ferric Reducing Antioxidant Power (FRAP) Assay Free Radical Scavenging Assay

With some adjustments, the FRAP assay was conducted using the method suggested by Benzie and Strain (1996). The 25 mL acetate buffer, 2.5 mL 10 mM TPTZ solution, and 2.5 mL 20 mM $FeCl_3 6H_2O$ solution were combined to create the FRAP working reagent, which was then heated for 10 min at 37 °C before use. The working reagent (2, 850 mL) and sample (150 mL) were allowed to react for 30 min in the dark. A UV-Vis spectrophotometer set to 593 nm was then used to measure the reaction mixture absorbance. The ascorbic acid equivalents per millilitre of sample (mg AAE/mL) were used to express the results.

3.6 Antimicrobial Effects of Papaya-Based Kombucha Beverages Against Food Borne Pathogens

The fermented samples were fixed to 1.5% acidity using TA method. Then, the fermented samples were centrifuged (10, 000 rpm for 5 min, 4°C) and filtered using 0.20 µm sterile filter to obtain cell-free supernatant (CFCS). The CFCS of fermented papaya pulp, fermented papaya, their neutralised fermented CFCS (prior to filtration, pH of fermented samples were adjusted to pH 7 with I M NaOH, to eliminate the influence of organic acids and low pH) and their non-fermented juices were tested for their antimicrobial activities via agar well diffusion assay. The antimicrobial activity of all the samples were assayed against pathogenic bacteria such as the Gram-negative bacteria (*Escherichia coli, Salmonella thypimurium*, and *Salmonella enteritidis*) and

Gram-positive bacteria (*Listeria monocytogenes* and *Streptococcus gallolyticus*). These pathogens were incubated in Tripticase soy broth at 37 °C temperature for 18 h.

The bacterial solutions were diluted to obtain 0.5 Mac Farland turbidity standard index. The bacterial solutions were then uniformly spread onto a petri dish containing 30 mL of Muller Hinton agar. Five wells were made on the surface of the agar plate and filled with (1) 100 µL of the unneutralised fermented CFCS, (2) neutralised fermented CFCS, (3) non-fermented supernatant, (4) 0.7% acetic acid and (5) 1% Pen-Strep (penicillin G and streptomycin). The petri dish was incubated at 37°C for 18 h. The antimicrobial activity was determined by measuring the clear zone surrounding the wells using a calliper (measurement in mm).

3.7 *In Vitro* Anti-Proliferative and Pro-Apoptosis Activities of Papaya-Based Kombucha Beverages

In vitro anti-proliferative and pro-apoptosis activities of papaya-based kombucha beverages involved a series of procedures, namely cell culture, MTT assay, annexin V assay, and cell cycle analysis.

3.7.1 Cell Culture

HT 29 and SW 480 human colon cancer cell lines were bought from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Both cells were grown in DMEM media with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin supplements (Gibco, USA). The cell lines were sub-cultured when they had reached 70% confluency during their 37 °C incubation in a humidified incubator with 5% CO_2.

3.7.2 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] Assay

MTT assay is a spectroscopic method measuring cell viability. Theoretically, the reduction of yellowish tetrazolium salt to blue purplish color of formazan is directly proportional to the number of living cells. In this study, the half maximal inhibitory concentration ($TCID_{50}/IC_{50}$) of papaya-based kombucha beverages on cancer cell viability was performed using the MTT assay. In summary, a volume of 100 µL of cells with a concentration of 8,000 cells/well was introduced into a 96-well plate and allowed to incubate overnight. Subsequently, the cells were subjected to papaya-based kombucha beverages that were diluted with DMEM media to achieve final concentrations of 30%, 15%, 7.5%, 3.75%, 1.87%, 0.94%, and 0.47% (v/v) of papaya kombucha.

The plate was then incubated for 72 h (72-h treatment time point). After reaching the time-point, each well was filled with 20 µL of MTT solutions (5 mg/mL in PBS) and incubated for another 2-3 h. The solutions were then removed. The formazan crystals produced as a result of the MTT reaction with cells, were dissolved in 100 µL of DMSO. Finally, the plate was read at 570 nm using a QuantTM enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-tek Instruments, USA). The following formula was used to measure the percentage of cell viability:

$$\% \text{ Cell viability} = (Abx/ Ab0) \times 100\%$$

3.7.3 Annexin V Analysis

This assay was carried out using Annexin V FITC Kit (BD Pharmigen, USA) according to the manufacturer's protocol to test the apoptosis induction of papaya-based kombucha beverages towards cancer cell lines. In brief, the seeded cell (2 x 10^5 cells/well) was incubated overnight prior to treatment. The treated cells were then incubated for 72 h and the cells were harvested once they reached the time point. The cell pellets were re-suspended into the 1x binding buffer and were then stained with 5 µL of Annexin V-FITC and 5 µL propidium iodide (PI). Cell suspension was then allowed to stand in the dark at room temperature (29 °C) for 15 min before being analysed using NovoCyte Flow Cytometer (ACEA Biosciences Inc., USA), NovoExpress version 1.2.4 software (ACEA Biosciences Inc., USA). Apoptotic cells undergoing phosphatidylserine externalisation were detected using Annexin V-FITC binding via FITC signal detector while dead cells were detected using PI binding via phycoerythrin emission (PE) signal detector.

3.7.4 Cell Cycle Analysis

Cell cycle analysis was performed to assess the step of the cell cycle in which the cancer cells HT 29 and SW 480 were attributable to the apoptotic effect induced by papaya-based kombucha beverages. The HT 29 and SW 480 cancer cells were seeded into 6-well plates at concentrations of 2 x 10^5 cells per well. The next day, the media was discarded and replaced with fresh media containing papaya-based kombucha beverages (diluted to IC_{50} concentration). After 72 h of treatment, the cells and media were harvested by tripsinisation followed by 5 min centrifugation process at 2000 rpm to acquire a cell pellet. The cell pellet was then subjected to cell cycle analysis using Cycletest^{TM} Plus DNA Reagent Kit (BD Biosciences US A) following the

manufacturer's manual. After that, the cell pellet was re-suspended into solution A (trypsin) and incubated for 10 min prior to the addition of solution B (RNAse A). Further 10 min of reaction time were required at this stage. Lastly, solution C (PI stain) was added to the mixture and incubated for another 10 min. The mixture was then analysed using NovoCyte Flow Cytometer (ACEA Biosciences Inc., USA), NovoExpress version 1.2.4 software (ACEA Biosciences Inc., USA).

3.8 The Biological Effects of Papaya-Based Kombucha Beverages on Azoxymethane-Mediated Colon Carcinogenesis in The Institute of Cancer Research (ICR) Mice

3.8.1 Pilot Study for Carcinogenesis Induction with Azoxymethane (AOM)

This study was carried out at Malaysian Agricultural Research Development Institute (MARDI) facilities located in Serdang, Selangor, Malaysia. An application was made, and the animal use protocol was approved by MARDI Animal Ethics Committee (20190215/R/MAEC00046) and Unversity of Nottingham Animal Welfare and Ethical Review Body (AWERB) (20190220/UNMC 22). All procedures were conducted according to the animal ethics guidelines. A total of four, 4-week-old, male ICR mice were housed in a plastic cage in a well-ventilated animal facility at controlled temperature of 22-24 °C with relative humidity of 50% and 12/12 h light/dark cycles. These mice were acclimatised for seven days and fed with fresh normal diet, water *ad libitum* and continued for another 4 weeks. At week 5, two mice were given a single AOM through intraperitoneal injection (10 mg/kg body weight). One week after the injection, the mice received 2.5% Dextran sulfate sodium (DSS) in their drinking water for seven days continuously. All animals were sacrificed five weeks after receiving the

AOM injection, based on the findings by Kawabata et al. (2012) and Tanaka (2012) where adenomas were observed within 3-4 weeks after administration of AOM on ICR mice. Other researchers had reported the development of neoplasm and tumour within 3-6 weeks after AOM exposures on AJ or SWR/J and ICR mice strains (Robertis et al. 2016). The large intestines were cut open longitudinally along the main axis, and gently washed with phosphate buffer saline (PBS) to remove faeces and fixed in 10% buffered formalin for 24 h. Histopathological examinations were performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining.

3.8.2 Studies of Papaya-based Kombucha Beverages on AOM-induced ICR Mice *In Vivo*

3.8.2.1 Animal subject

The ICR mice were housed in a ventilated animal housing at temperature of 22-24°C with a relative humidity of 50% and 12/12 h light/dark cycles. The animals were acclimatised for 7 days and fed with fresh normal diet and water *ad libitum*. This study was carried out at MARDI facilities. An application was made, and the animal use protocol was approved by MARDI Animal Ethics Committee (20190215/R/MAEC00046). All procedures were conducted according to the animal ethics guidelines.

3.8.2.2 Experimental Design

The ICR mice were randomly separated into 6 groups with 8 mice in each group. The dosing of papaya-based kombucha used in this study was chosen based on the product formulation of 500ml per-serving, calculated against an average man's weight of 60kg. Two concentrations of papaya-based kombucha beverages (0.7ml/kg body weight and

1.8ml/kg body weight) were given to the subjects based on the safe dosing range which showed functionalities. The grouping of this study is shown in Table 3.1.

Table 3.1: Summary of the treatment given to the ICR mice

Group name	Treatment
Normal	Normal saline (vehicle control)
AOM (-ve control)	AOM + DSS + saline
PPL	AOM + DSS + Papaya pulp kombucha (0.7mL/kg of 100% of papaya pulp kombucha)
PPH	AOM + DSS + Papaya pulp kombucha (1.8mL/kg of 100% of papaya pulp kombucha)
PLL	AOM + DSS + Papaya leaves kombucha (0.7mL/kg of 100% of papaya leaves kombucha)
PLH	AOM + DSS + Papaya leaves kombucha (1.8mL/kg of 100% of papaya leaves kombucha)

After a week of acclimatisation, the experiments were initiated by giving normal diets alongside defined kombucha or saline (oral dosing procedure) for another 4 weeks. The mice were given fresh normal diets and water *ad libitum* daily. For control group (G1), ICR mice were given normal saline (vehicle control). For group G2-G6, at week 5, the mice were given AOM through intraperitoneal injection at dose of 10mg/kg body weight once. One week after the injection, all mice in group G2-G6 were given 2.5% DSS in drinking water for continuous 7 days followed by defined kombucha or saline. Figure 3.1 displays the overview of this process. After 18-20 weeks of overall experimentations, the animals were euthanized.

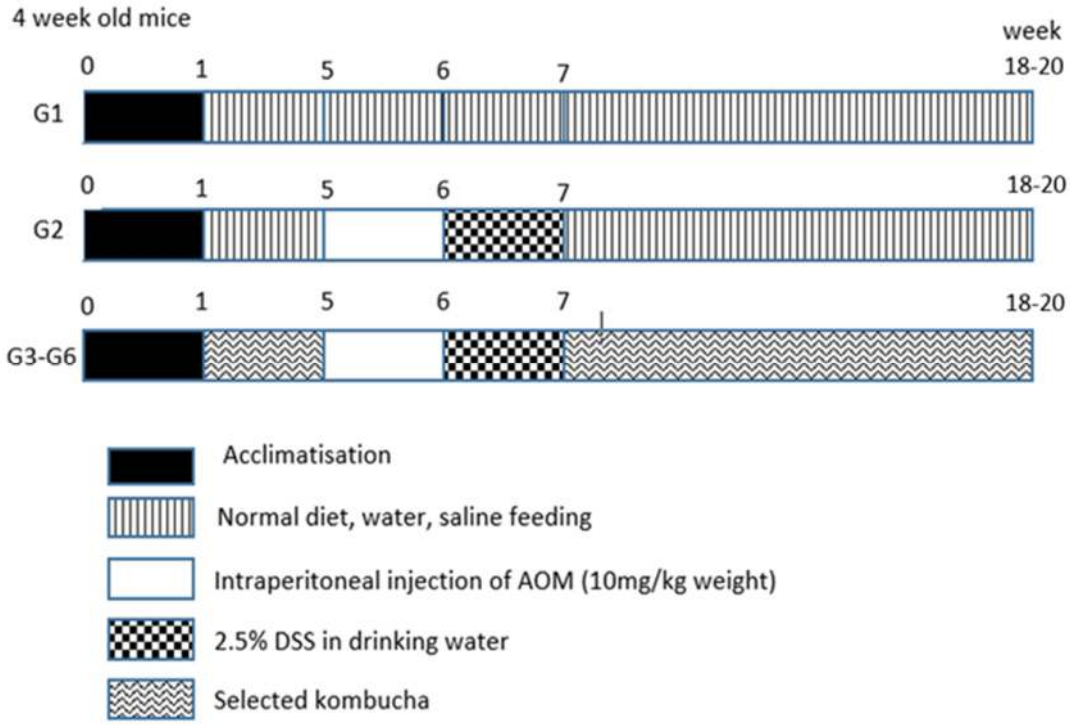


Figure 3.1: Overview diet meal plan for the mice

3.8.2.3 Tissue Collection

All IRC mice were sacrificed by cervical dislocation at the endpoint of the experiment according to the Institutional Animal Care and Use Committee (IACUC) guidelines. Cardiac puncture was performed and the whole blood and serum were pooled for further analysis. The vital organs such as liver, kidney, spleen, lung, and colon were harvested. The harvested organs were further cut into two pieces whereby half of the tissues were placed in tube containing 10% buffered formalin for fixation and histopathological analysis and the remaining half were placed in RNAlater solution (ThermoFisher Scientific, USA) and stored at -80 °C for further analysis.

3.8.2.4 Hematoxylin and Eosin (H&E) Histopathology Staining

In summary, following an overnight immersion in 10% buffered formalin, the tissues were subjected to a dehydration protocol involving a series of ethanol solutions with varying percentages (ranging from 70% to 100%). Subsequently, the tissues were cleaned using xylene with the use of the TP1020 automated tissue processor manufactured by Leica, Germany. The tissues then underwent two changes of 100% ethanol, with each change lasting 30 minutes. Subsequently, they were subjected to two changes of xylol, each lasting 30 minutes. Finally, the tissues were immersed in paraplast for two hours, with two changes during this period. Following that, the tissues were placed in paraffin wax blocks utilising the EG 1140 H embedding station manufactured by Leica in Germany. The blocks were further divided into sections of 4µm in thickness using the Jung Multicut 2045 microtome (Leica, Germany) and afterwards affixed onto glass slides. Subsequently, the deparaffination procedure was performed by immersing the slides in Xylol for a period of 3 minutes, followed by sequential immersion in 100% ethanol for 1 minute, 96% ethanol for 1 minute, 80% ethanol for 1 minute, and 60% ethanol for 1 minute. Finally, the slides underwent stained with haematoxylin (Sigma, USA) and eosin (Sigma, USA), followed by placement under coverslips using DPX mounting media (BDH Laboratory, England). The histological alterations in the tissue slices were examined and captured using the BX51 light microscope, manufactured by Olympus in Japan, at magnifications of x 100 and x 400.

3.8.2.5 Immunophenotyping of Spleenocytes

In brief, the harvested spleens were washed and mashed using 70mm cell strainer (SPL, Korea) in PBS. The supernatants were centrifuged at 2000 rpm for 5 min before the

pellets were lysed using lysis buffer (8g NH_4Cl, 1 g Na_2HCO_3, EDTA, 0.1g KH_2PO_4 at pH 7.4) for 10 min at 4 °C. Then, the splenocytes were stained with flurochroe-conjugated antibodies (CD3, CD4 and CD8) (Abcam, USA) for 2 h before subjected to flow cytometry analysis using NovoCyte flow cyotmeter (ACEA Biosciences Inc., USA). The analysis was done using NovoExpress® version 1.2.4 software (ACEA Biosciences Inc. USA).

3.8.2.6 Metagenomic Study

The final treatment week's faeces were collected and stored at -80 °C for later use. Each group's faeces were pulverised in a pestle with liquid nitrogen. The DNA was extracted from the faeces using the QIAamp DNA stool micro kit (QIAGEN, Germany) in accordance with the manufacturer's instructions. Agencourt® AMPure® XP beads from Beckman Coulter (USA) were used to purify the DNA, and a Qubitfluorometer was used to determine the concentration (Life Technologies, USA). In the first PCR, the 16S amplicon of the V3 and V4 region was amplified (Forward primer: 5'-CGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWG CAG-3'; Reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT AATCC-3') (Klindworth et al., 2012).

Then, 1 M of the forward and reverse primers for the 16S amplicon PCR were mixed with 25 L of DNA samples with a concentration of 12.5 ng of DNA, 12.5 L of 2x KAPA Hifi Hot Start Ready Mix (Kapa Biosystems), and 25 L of the mixture. The PCR was run for 3 min at 95 °C, then 35 cycles of 30 s each at 95 °C, 55 °C, and 72 °C were completed. Prior to starting the second PCR, the first PCR product underwent a quality

check by being purified using Agencourt® AMPure® XP beads from Beckman Coulter in the United States. This confirmed the beads were the right size. Using the Nextera XT index kit, the first PCR product was combined with the distinctive barcode to create the second PCR amplification (Illumina, USA). 5 L of the first PCR products, 25 L of 2x KAPA Hifi Hot Start Ready Mix, and Nextera XT index Primer 1 and 2 were combined for the second PCR amplification.

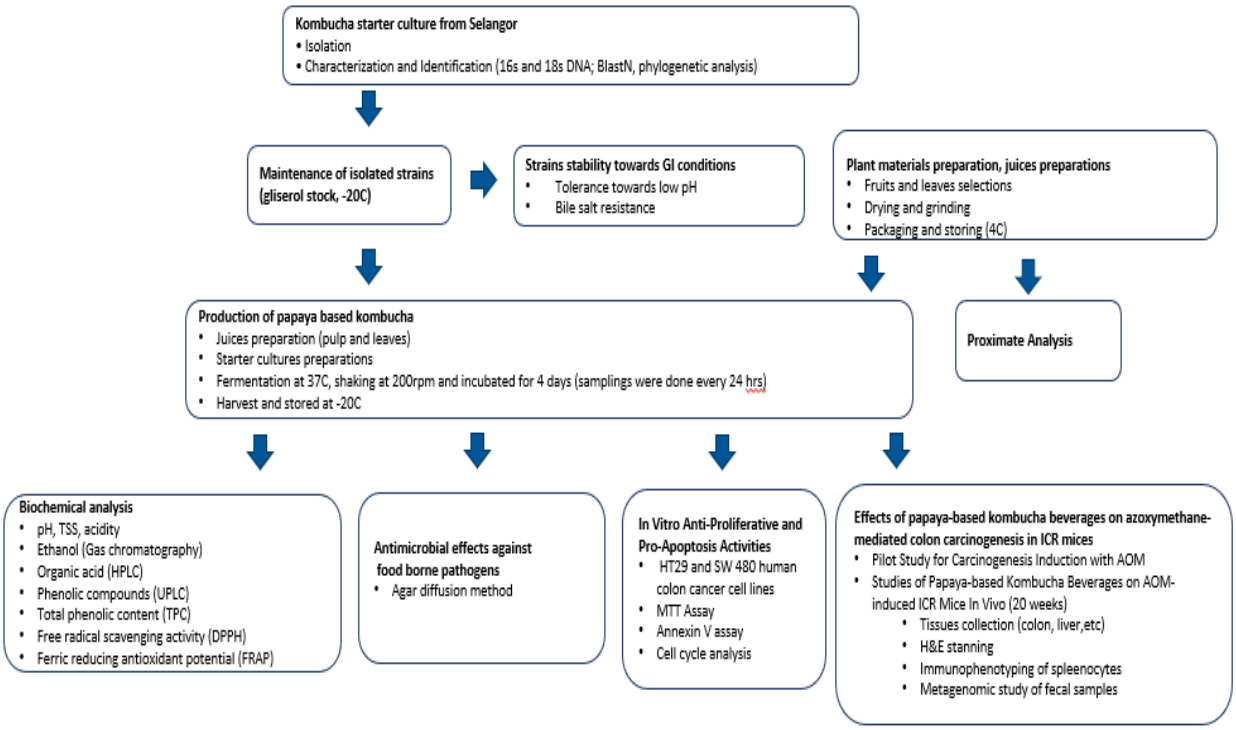
After starting the PCR at 95 °C for 3 min, it went through 8 cycles of 30 s at 95 °C, 55 °C, and 72 °C, followed by 5 min at 72 °C. With the Agilent Bioanalyzer 2100 and Agencourt® AMPure® XP beads, the second PCR product's quality was checked (Agilent Technologies, USA). The concentration of the products was adjusted to 4 nM and pooled together based on the bioanalyzer's findings. Then, pooled samples were processed according to the established library methods to a final concentration of 9 pM and run on an Illumina MiSeq Sequencer utilising 600 cycles of MiSeq V3 reagents (Illumina). Based on a BLASTX comparison against the NCBI database, the 16S metagenomics analysis was carried out using MEGAN (MEtaGenomeANalyzer).

3.9 Statistical Analysis

Statistical Analysis Software (SAS) was used in all statistical analysis. Means and standard deviations were used to communicate all quantitative measurements (SD). One-way analysis of variance (ANOVA) was used for the analysis, and Duncan's multiple range test was used to compare group averages. P-values lower than 0.05 were regarded as statistically significant.

3.10 Flow Chart of Experimental Design

Figure 3.2: Flowchart of the experimental design and analysis method



CHAPTER 4

RESULTS AND DISCUSSIONS

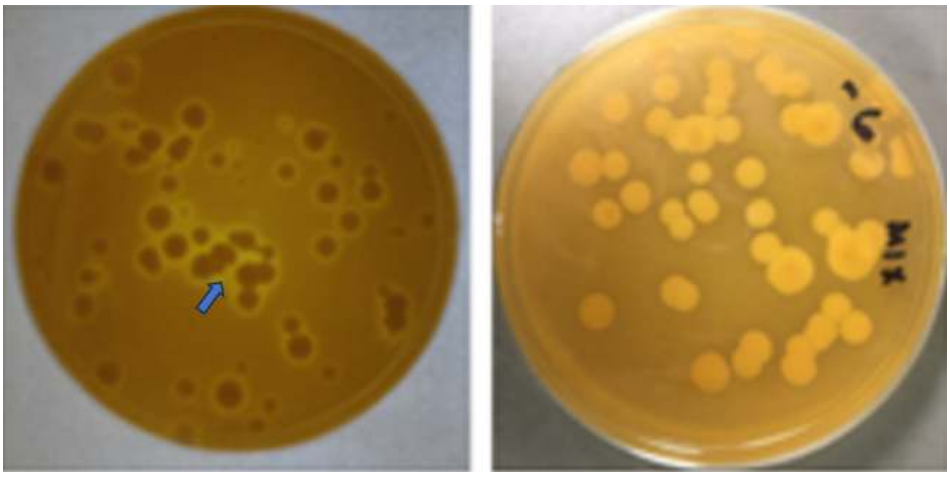
4.1 Isolation and Identification of Kombucha Culture

Symbiotic cultures of yeast and bacteria that produce acetic acid make up the kombucha culture. In this work, simple modified media including glucose, yeast extract, peptone, and calcium carbonate supplement were used to screen the microorganisms found to be present in the starter culture. Acetic acid bacteria had previously been isolated using a variety of media formulations, all of which contained glucose, yeast extract, and peptone (Gomes et al., 2018; Kim et al., 2019). Different yeast strains can grow in substrates with comparable ingredients (Zonneveld, 1986). Kim et al. (2013) cultivated acetic acid bacteria and yeast using a similar medium composition.

Through visual examinations of halo zone and non-halo zone generating microorganisms, potential isolates were chosen at random. Due to their synthesis of organic acids, acid producing bacteria (e.g acetic acid bacteria) may typically be identified from other strains based on the halo zone that surrounds their colonies (Figure 4.1). In this work, kombucha starting culture was acquired from vendors in Selangor, Malaysia was effectively used to extract two different types of microbes. The isolated cultures contained yeast and the acetic acid bacteria *Komagataeibacter rhaeticus* MFS1 and *Dekkera bruxellensis* MFS1, respectively. According to a study conducted by Rasu Jayabalan et al. (2014), the predominant acetic acid bacteria identified in kombucha tea are *Acetobacter*, *Gluconoacetobacter,* and *Gluconobacter*. While *Zygosaccharomyces*, *Brettanomyces* (*Dekkera*), and *Saccharomyces* are widely recognised as the most frequent yeast strains (Jarrell et al., 2000; Marsh et al., 2014). In order to make

kombucha tea, at least one strain each of acetic acid-producing bacteria and yeast is needed. The yeast will turn the medium's sugar into ethanol, which the acetic acid bacteria will then oxidise into acetate (Jayabalan et al., 2008). Because of the low pH and organic acids present as antimicrobial metabolites produced by this symbiotic fermentation, there is less risk that the final product will be contaminated by other bacteria, fungi, or yeast (Teoh et al., 2004). Similar to this, a study by Mukadam et al. (2016) revealed that two isolates from traditional kombucha culture that originated in India were found to contain acetic acid bacteria from the *Komagataeibacter* genus (*Komagataeibacter saccharivorans*) and yeast (*Zygosaccharomyces bailli*).

A combination of yeast and acetic acid bacteria make up the composition and quantity of microorganisms present in kombucha tea, according to previous studies. These bacteria' makeup frequently varies from one culture to another (Villarreal-soto et al., 2018). This kombuha tea diversity is frequently brought by elements like climate, source, or location (Rasu Jayabalan et al., 2014).



(a) (b)

Figure 4.1: Isolation of kombucha culture using YPDA supplemented with $CaCO_3$, blue arrow showing colonies producing halo zone. Growth of microbes from kombucha culture on YPDA supplemented with $CaCO_3$. Colonies producing (a) halo zone produced by potential acid producing microorganism (indicated by blue arrow) (b) and non-halo zone producing were isolated for further characterisation.

Figure 4.2 shows the results of PCR amplification from random samples using the 18s rRNA and 16s rRNA primers, which produced two amplified products with approximate sizes of 1.5 kb (band A) and 1.8 kb (band B) (band B). BLASTN analysis of the retrieved sequence revealed that PCR band A was correctly recognised as *Dekkera bruxellensis* at 99% identity with a query cover of 99% and an E value of 0.0 (Appendix A). Band B, on the other hand, displayed a 99% identification to *Komagataeibacter rhaeticus* with a 98% query cover and an E value of 0.0 (Appendix B). *K. rhaeticus* and *D. bruxellensis*, commonly known as *Brettanomyces bruxellensis*, were both identified in several studies employing sequence-based analysis on numerous kombucha samples to be among the major genus in the populations of examined samples (Marsh et al., 2014; Reva et al., 2015; Villarreal-soto et al., 2018).

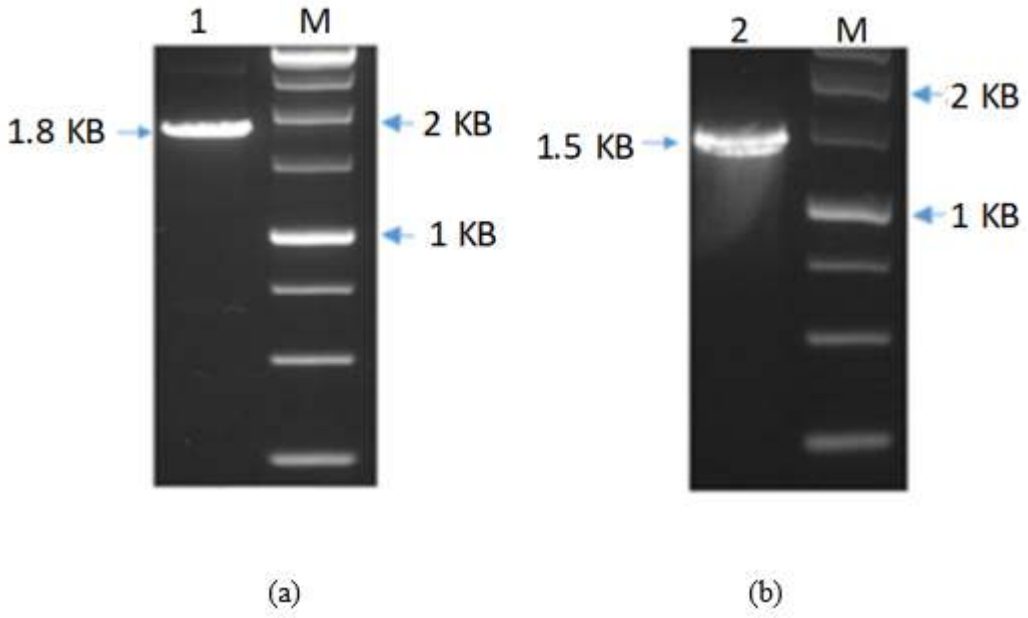


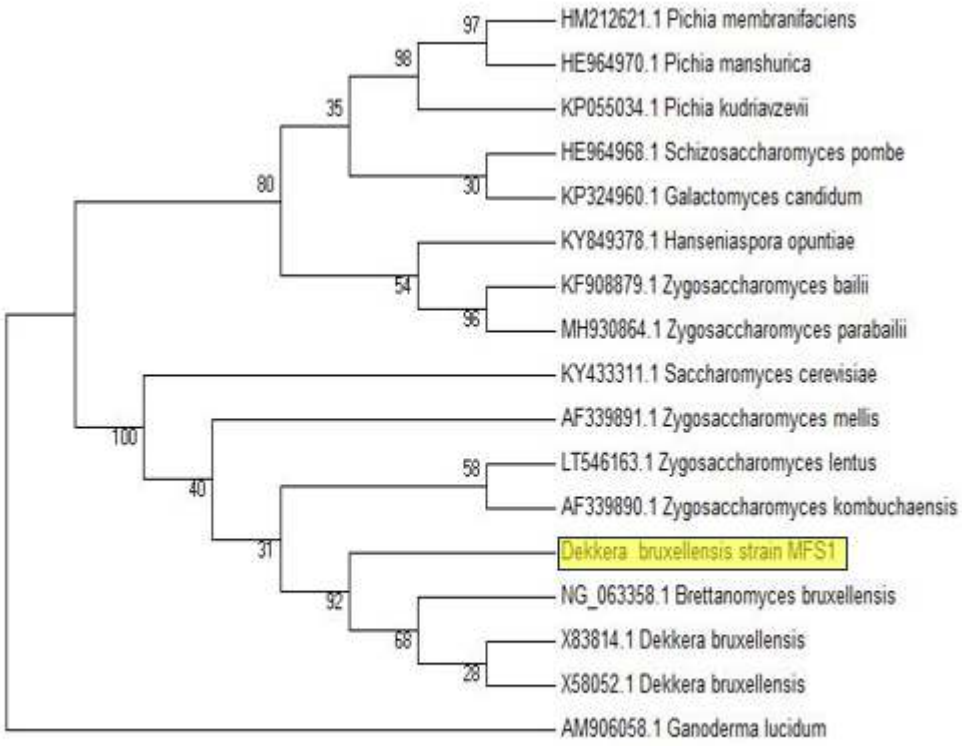
Figure 4.2: Analysis of PCR product amplified from the DNA of two random samples using the 18s rRNA and 16s rRNA primers on 1% agarose gel in 1× TAE buffers. PCR Lane 1 (a): 1.8 kb; Lane 2 (b): 1.5 kb, lane M: 1 kb ladder molecular weight marker (Invitrogen).

Isolated yeast *D. bruxellenxis* is thought to be the contaminating species in charge of the off flavours found in wine, cider, or dairy products (Blomqvist & Passoth, 2015; Steensels et al., 2015). According to recent reports, fermented beverages can taste better, have more complex flavours and specific aromas (Joseph, Albino, Ebeler and Bisson, 2015). Additionally, it shows promise as a new yeast strain for the ethanol manufacturing sector (Blomqvist, 2011). This is due to its great tolerance for ethanol and osmotic stress (Steensels et al., 2015). On the other hand, *D. bruxellensis* was shown to be the dominant culture isolated from commercial kombucha beverages in a study on yeast ecology conducted in New South Wales, Australia (Teoh et al., 2004).

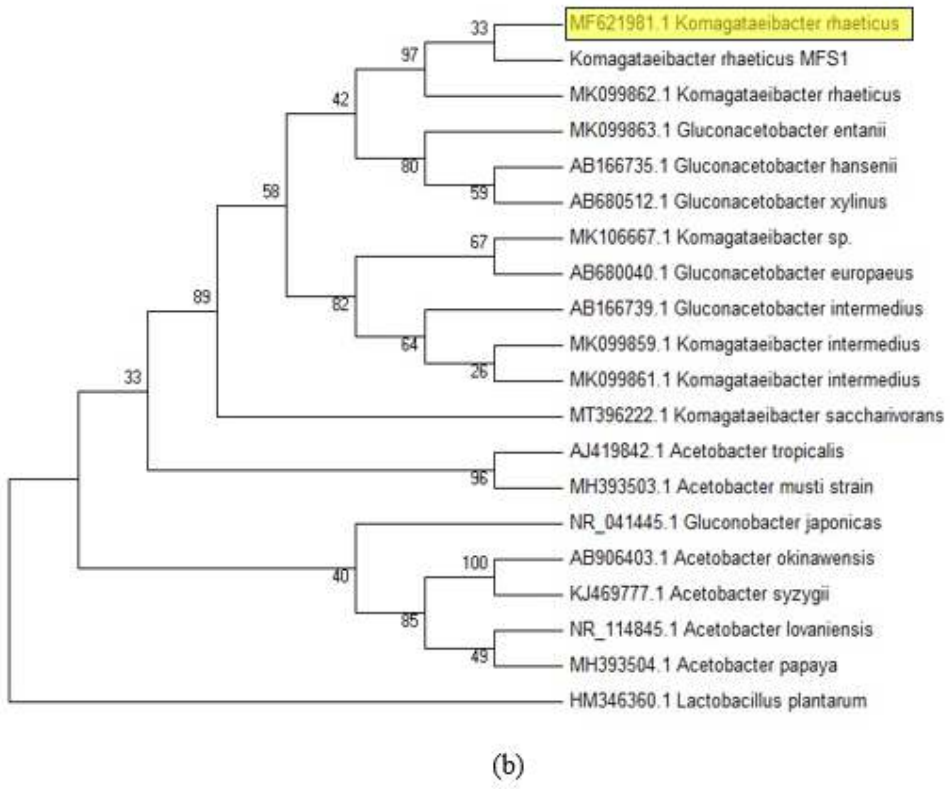
The acetic acid bacteria *K. rhaeticus*, on the other hand, is strictly aerobic and can oxidise alcohol, aldehydes, and sugar alcohols in the presence of oxygen (Gomes et al., 2018). Additionally, it has been shown to produce bacterial cellulose (Machado et al., 2016; Prudnikova et al., 2017) and saccharic acid-1,4-lactone (Wang et al. ,2010), both of which are present in kombucha tea and are known to inhibit the activity of glucuronidase, an enzyme that is tangentially linked to cancer (Bhattacharya et al., 2011; K. Wang et al., 2010). *K. rhaeticus,* formerly known as *Gluconacetobacter rhaeticus*, was isolated from kombucha tea, according to another investigation from Brazil (Dos Santos et al., 2014). Additionally, *K. rhaeticus* was discovered by Semjonovs et al. (2017) in commercial Latvian kombucha tea beverages that exhibited the ability to create bacterial cellulose with high tensile strength and polymerization levels.

The employment of various beginning materials and ambiguous starter cultures during spontaneous food fermentations might result in the creation of several variable metabolites, each of which may have a different bioactivity. In addition, there is a

chance that food pathogen contamination could put human health at risk (Capozzi et al., 2017). Determining the type of microorganism present in kombucha cultures, as well as any potential traits, metabolites they may create, and the safety of the microbial cultures, has therefore become of greatest importance. The evolutionary relationship between the SCOBY isolated from several kombucha teas (Coton et al., 2017; Gomes et al., 2018) and *K. rhaeticus* MFS1 and *D. bruxellensis* MFS1 is then shown in Figure 4.3. It is clear that *Komagataeibacter sp.* and *Gluconoacetobacter sp.* are grouped together with *K. rhaeticus* MFS1. *D. bruxellensis* MFS1 is clustered with *Dekkera sp., Zygosaccharomyces sp.,* and *Saccharomyces cerevisiae* whereas the eukaryotes are divided into two sub-groups.



(a)



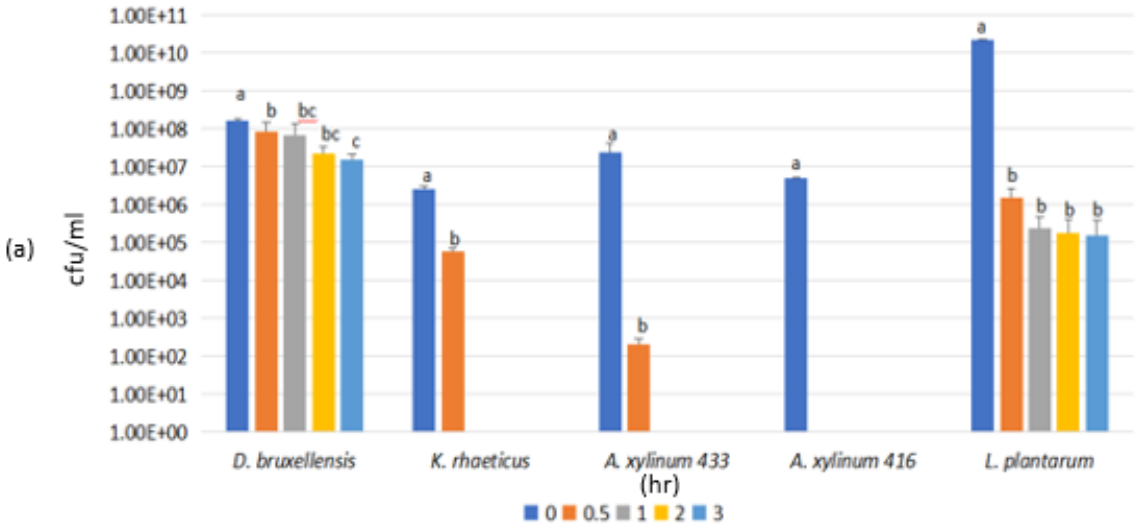
(b)

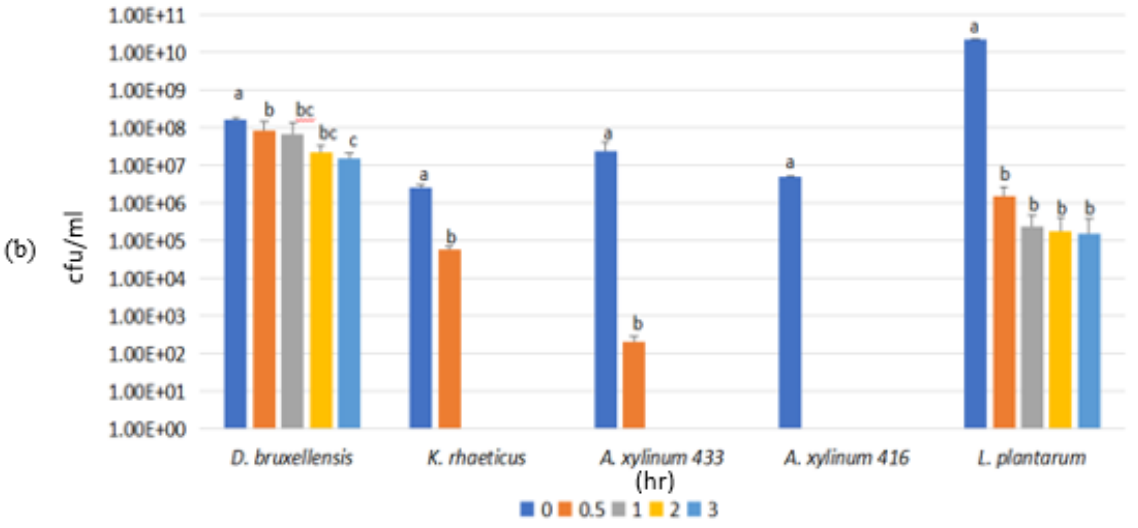
Figure 4.3: Phylogenetic relationship of acetic acid bacteria based on 16s rRNA (a) and (b) eukaryotes 18s rRNA gene sequence of SCOBY isolated from kombucha tea cultures obtained from genebank. Strains of interest are highlighted with yellow box.

4.2 Low ph and bile salt tolerance assays

The bacterium must be extremely tolerant of the low pH and bile salt environment in the gastrointestinal system and able to grow and colonise there in order to be used as a possible probiotic in food applications (Bezkorovainy, 2001; Kechagia et al., 2013; Saad et al., 2013). Overall, testing for low pH and bile salt tolerance were conducted on two isolated kombucha strains (*K. rhaeticus* MFS1 and *D. bruxellensis* MFS1) as well as three other reference strains (*A. xylinum* 433, *A. xylinum* 416, and *L. plantarum*). The five strains that were examined for cell viability under low pH conditions are shown in Figure 4.4. Over the course of the 3 h incubation period, all bacteria shown, on average, better tolerance at pH 3 and pH 4 than pH 2. After 3 h of incubation at pH

3, *A. xylinum* 416 had the lowest viability (10^4 CFU/mL). Only the *D. bruxellensis* MFS1 and *L. plantarum* cultures of all the investigated strains were still alive at pH 2 after 3 h of incubation. While *D. bruxellensis* MFS1 maintained its viability levels up to 10^7 CFU/mL, one-fold lower than the initial count, it demonstrated greater levels of pH tolerance.





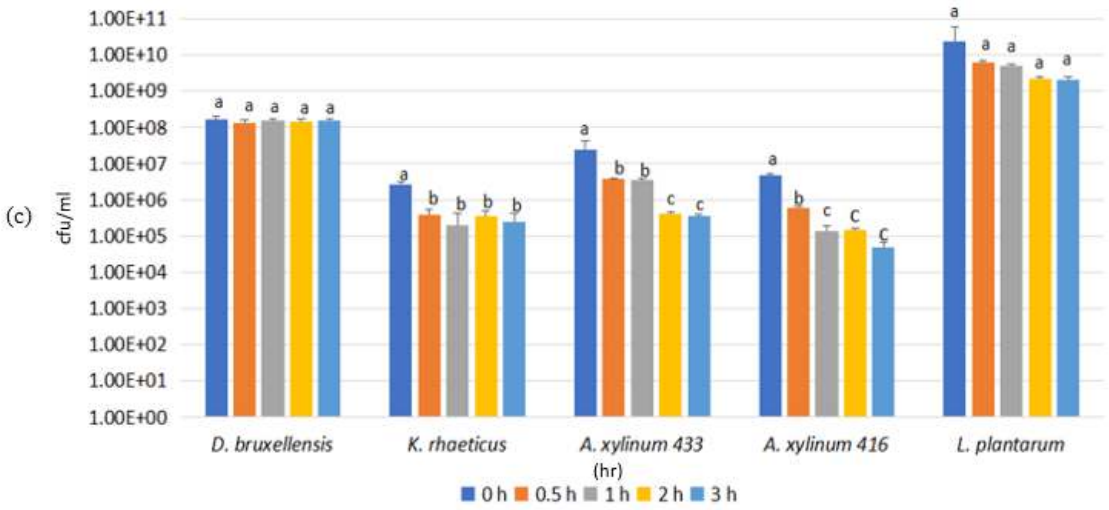


Figure 4.4: Low-pH tolerance of tested strains (cfu/mL) at (a) pH 2; (b) pH 3 (c) pH 4. Values were expressed as mean ± standard deviations of 3 replicates of [a-c]Means with different letters were significantly different ($P < 0.05$) from initial count (0 h).

The findings of the bile salt tolerance assay, which assessed the viability of these five strains under various bile salt concentrations using oxgall, are displayed in Figure 4.5. Only *D. bruxellensis* MFS1 and *L. plantarum*, were able to demonstrate viable growth following incubation in various bile salt concentrations. Only after exposure to a bile salt environment could the remaining three acetic acid bacteria, including *K. rhaeticus*, maintain their viability for longer than 2 h. *D. bruxellensis* MFS1 and *L. plantarum*, on the other hand, shown comparative better tolerance to various bile salt concentrations. Even after being exposed for up to 4 h, both strains were still able to produce a viable count of more than 10^6 CFU/mL. At a bile salt concentration of 1%, *D. bruxellensis* MFS1 was shown to have a 1-fold drop in viability from the initial 10^8 to 10^7 CFU/mL.

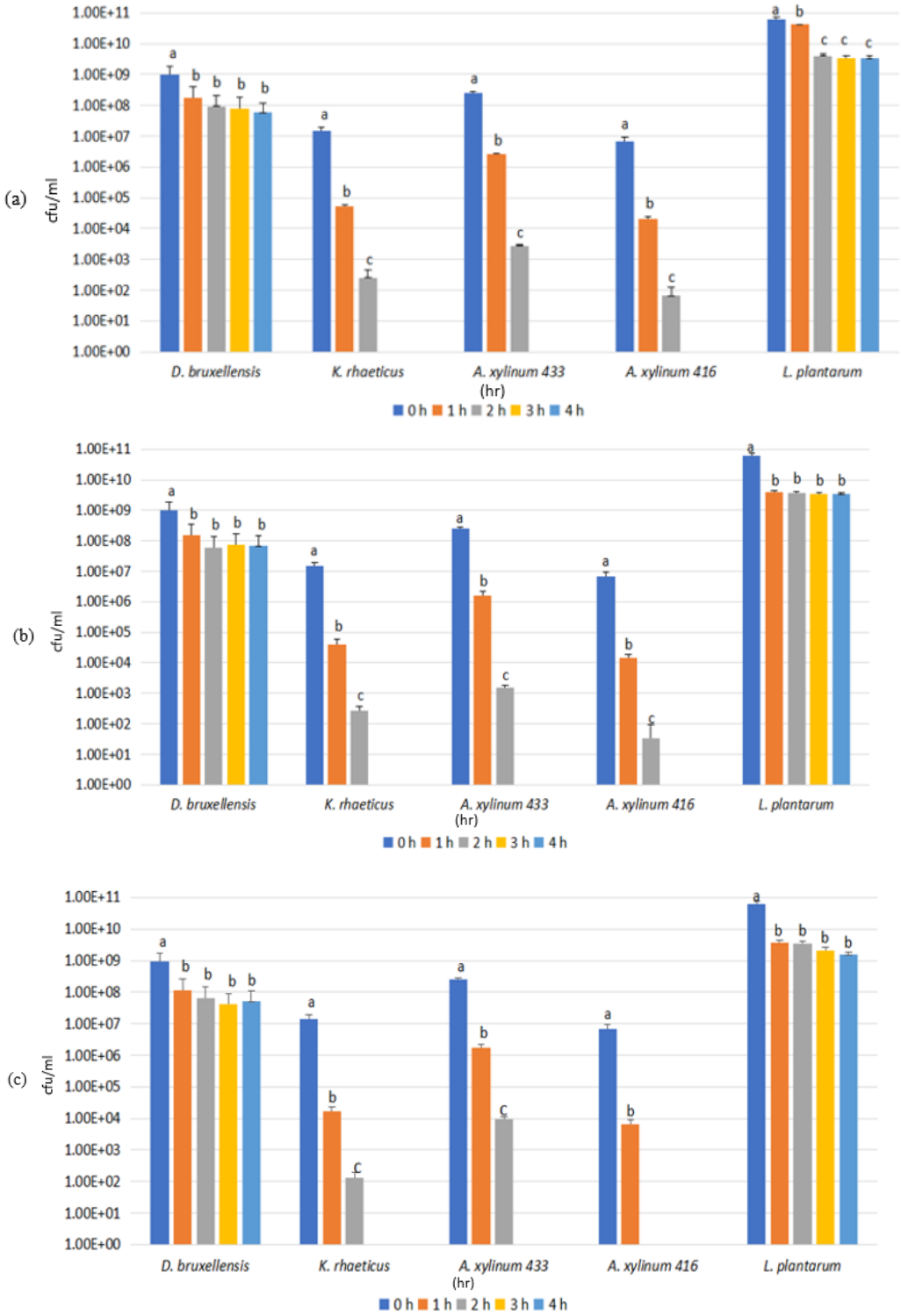


Figure 4.5: Bile salt tolerance of tested strains (log10 CFU/ml); (a) bile salt 0.3%; (b) bile salt 0.5% (c) bile salt 1.0%. Values were expressed as mean ± standard deviations of 3 replicates of [a-c]Means with different letters were significantly different ($P < 0.05$) from initial count (0h).

The studied kombucha isolated strains and the reference strain were found to differ in their susceptibility to low pH and bile salt environments (*L. plantarum*). Since the average digestive process can take up to 3 h, the pH of the human stomach can range from 1 (when the stomach is empty) to 4.5 (after food ingestion) (Wang et al., 2009). Most bacteria may be harmed by this low pH environment. Except for *D. bruxellensis* MFS1 and *L. plantarum*, all the acetic acid bacteria tested were found to be pH 3 tolerant, although they did not preserve any viability after 0.5 h of exposure at pH 2. Previous research had demonstrated that exposing lactic acid bacteria to gastric acid with a pH 2 for a 3 h incubation period had significantly reduced their viability count (Sahadeva et al., 2011; Vinderola & Reinheimer, 2003). In another investigation, probiotic-like lactic acid bacteria exposed in vitro for 1 h showed diminishing tolerance at pH 2 and no viability at pH 1 (Angmo et al., 2016). However, as shown by other studies, an effective probiotic strain should be able to resist pH 3 (Fernandez et al., 2003; Song et al., 2015).

Another crucial probiotic trait to be identified is the tolerance to bile salts. Microorganisms need to be able to withstand the harsh conditions in the GI tract, including the presence of bile salt in the small intestine, in order to survive and flourish there. A significant portion of bile is composed of bile salts, which are generated in the liver and conjugated with amino acids (glycine or taurine) before being deposited in the gall bladder and secreted into the duodenum during digestion to aid in the emulsification and solubilization of lipids (Begley et al., 2005). Because of altered cell permeability caused by bile salts in the small intestine, which causes oxidative stress and impairs DNA repair mechanisms, the rate of bacterial survival is decreased (Begley et al., 2005; Ruiz et al., 2013). Bile salts content in the human body is typically in the

range of 0.05 – 2%, which may have a role in the development of the microbial profile in our guts (Islam et al., 2011). Bile salt resistance is regarded as a crucial factor for choosing probiotic strains. It is challenging to pinpoint the exact quantity that the chosen strain should be resistant to, although 0.15 to 0.30% bile salt has been suggested as an acceptable human concentration for choosing probiotic bacteria (Fernandez et al., 2003; Song et al., 2015).

All of the acetic acid bacteria evaluated in this study were found to be able to resist bile salt concentrations between 0.30 and 0.50% for up to 2 h, but only with a significant drop in viability from the starting cell count. However, *D. bruxellensis* MFS1, an acid-producing yeast, has demonstrated the ability to adapt to the environment of bile salts and pH stress (1%) (pH 2). The observation made on the yeast probiotic strain *Saccharomyces ceveresiae* var. Boulardii (Czerucka et al., 2007; Brando et al., 2014) was similar to the finding made on *D. bruxellensis* MFS1. However, it's possible that the bile salts in both systems do not accurately represent their capacity for bile in vivo. This is due to the fluctuating and relatively low amounts of bile salts that exist before the digestion of fatty foods. The activity of the bile salts may also be impacted by the presence of food because they may attach to the food matrix and prevent germs from being exposed to their toxic effects (Begley et al., 2005). However, in vitro testing provides a simple method to assess a microorganism's tolerance to bile salts and determine its possible resistance to their effects.

4.3 Papaya pulp and leaves characterisation

For fresh consumption and the processing business, Malaysia has various papaya cultivars grown, including Sekaki, Eksotika 1, Eksotika II, Batu Arang, Sitiawan, and Subang 6. Different papaya cultivars, farming methods, planting locations, sunlight exposure, ripening stages, and postharvest processing are said to have a substantial impact on the physiological and nutritional makeup of papaya fruits (Ikram, 2019; Sancho et al., 2011). Sekaki was chosen for this study because it is the most widely used variety for domestic consumption and a crucial cultivar for export (Choo et al., 2020). As a result, the study relied on the Sekaki cultivar, which was acquired from a plantation in Serdang, Selangor.

Table 4.1 displays the physical characteristics of processed papaya pulp and leaves. Papaya pulp granules had a lower moisture content (4.25 ± 0.08%) than papaya leaves (5.47 ± 0.05%) after drying processes. The low moisture level of the samples makes it simple to store them and may prevent the formation of mould and fungus. According to a previous study by Martial-didier et al. (2017), moisture levels between 5 and 10% can prevent the growth of mould, whereas levels between 13 and 18% can lead to mould growth and a significant risk of mycotoxin generation (Drusch & Ragab, 2003).

Table 4.1: Physical appearances and moisture content of processed papaya pulp and leaves

Sample	Colour	Appearance	Moisture content (%)
Papaya pulp	Orange-red	Granule	4.25 ± 0.08^{a}
Papaya leaves	Green	Powder	5.47 ± 0.05^{b}

Values were expressed as mean±standard deviations of 3 replicates. $^{a\text{-}b}$Means within a column with different letters were significantly different ($P<0.05$)

Meanwhile, the proximate analysis data as shown in Table 4.2 had provided the nutritional composition of Sekaki cultivar. Papaya pulp was found to have a 2-fold higher carbohydrate content ((78.18%) by difference) than papaya leaves. The carbohydrate content of papaya pulp and leaves was 70.70% and 38.4%, respectively, in the study published by Am et al. (2014). For the fermentation of kombucha, carbohydrates are a crucial supply of carbon. Before beginning the fermentation operations, the sugar concentration of the substrates is often standardised to be in the range of 5 to 15% when manufacturing kombucha (Amarasinghe, Weerakkody & Waisundara, 2018; Greenwalt et al., 2000).

As opposed to papaya pulp sample, papaya leaves had higher levels of ash, crude fibre, fat, and protein. The availability of all minerals contained in these samples was shown by the ash content of the papaya leaves (13.61%) and pulp (4.62%). The crude fibre in leaves was discovered to be two times more abundant (14.16%) than in papaya pulp, which is helpful for regulating bowel movements and enhancing nutritional absorption. Papaya leaves had a greater concentration of ash and crude fibre than was reported by Nwofia et al. (2014) in their study of numerous *Carica papaya morphotypes.*

Compared to high protein legumes, which can contain up to 30% protein, papaya fruits and leaves in this study were assessed to have a moderate concentration of total protein (Maphosa, 2017). When compared to papaya pulp, the total protein contained in leaf samples was substantially higher (P 0.05) at 23.35%. These findings concurred with those of Am et al. (2014), who reported papaya pulp and leaf values of 6.1% and 33.4%, respectively. The amino acids and other nitrogenous-related substances that make up the total protein found in fruits and vegetables are also present. On the other hand,

papaya pulp had a crude fat content of 0.19% and leaves had 5.47% crude fat. Fruits typically contain less than 1% fat, though this can change depending on the product. Papaya leaves and pulp were found to have low levels of fat, according to Am et al. (2014), despite the fact that evidence from a number of other research suggested that the fat content of papaya leaves might range from 2.8 to 5.5% (Joseph et al., 2015; Verma & Kaushal, 2014).

Table 4.2: Proximate analyses of papaya pulp and leaves

Sample	Carbohydrate (g/100g)	Protein (g/100g)	Fats (g/100g)	Ash (g/100g)	Crude fibre (g/100g)
Pulp	78.18 ± 0.57^{a}	6.82 ± 0.14^{a}	0.19 ± 0.04^{a}	4.62 ± 0.02^{a}	5.93 ± 0.06^{a}
Leaves	37.94 ± 0.32^{b}	23.35 ± 0.15^{b}	5.47 ± 0.03^{b}	13.61 ± 0.05^{b}	14.16 ± 0.15^{b}

Values were expressed as mean±standard deviations of 3 replicates. $^{a-b}$Means within a column with different letters were significantly different ($P<0.05$)

4.4 Kombucha fermentation using papaya pulp and leaves juices

An essential indicator in fermentation processes is pH value. Not only can it affect the final flavour or taste, but it can also encourage or discourage the growth of the microorganisms used in fermentation. On the other hand, low pH of kombucha juice at the lower extreme can also reduce the sensory quality and drinking level of the beverage (Chu & Chen, 2006; Neffe-skociska et al., 2017). As a result, pH value serves as an indicator for when kombucha processing has finished. According to the species and strain, both AAB and yeast can endure a pH range between 3.6 and 6.3, while yeast can often withstand a pH range between 4.5 and 6.5 (Neffe-skociska et al., 2017). The values of pH and TSS were reduced significantly for both papaya pulp kombucha (PPK) and papaya leaves kombucha (PLK) during fermentation process (Figure 4.6). The pH

of PPK dropped from 5.14 to 2.75 (a reduction of 53.5%), while PLK had a reduction of 50.4% where the pH dropped from 6.13 to 3.09. The pH value had changed rapidly within the 48 h of fermentation, where both papaya kombucha reached the end point pH value in the range of 3.12 and 2.95 for PLK and PPK, respectively. Other research with similar results (Greenwalt et al., 1998; Neffe-skociska et al., 2017) found that the pH of their kombucha juice had fallen from 5.0 to 2.5 between 6 and 10 days of fermentation. Due to yeast and bacterial synthesis of organic acids, the pH value fell (Amarasinghe, Weerakkody & Waisundara, 2018).

Total soluble solids are defined as sugars, organic acids, trace amounts of dissolved metabolites, and minerals (TSS). The most crucial quality parameter for determining how sweet fresh fruits and juices is TSS. Additionally, it indicates the amount of sucrose in the solutions, with 1 degree of Brix corresponding to 1 g of sugar for every 100 mL of solution (Samukelo and Linus, 2015). After four days of fermentation, the TSS content indicated a drop of 32.6% (PPK) and 31.03% (PLK) on their °Brix values. The hydrolysis of sucrose to glucose and fructose, which was subsequently metabolised for microbial development and the generation of organic acids and other metabolites, may be the cause of the variations in TSS levels. According to studies, the amount of sucrose in all kombucha decreases over the course of the fermentation process, while the amount of glucose and fructose increases (Gagg et al., 2018; Neffe-skociska et al., 2017). In the meantime, Jayabalan et al. (2014) evaluated how fermentation raised the quantities of organic acids and monosaccharides. It has been found that chemical changes are closely tied to a variety of factors, including starter cultures, time, and temperature.

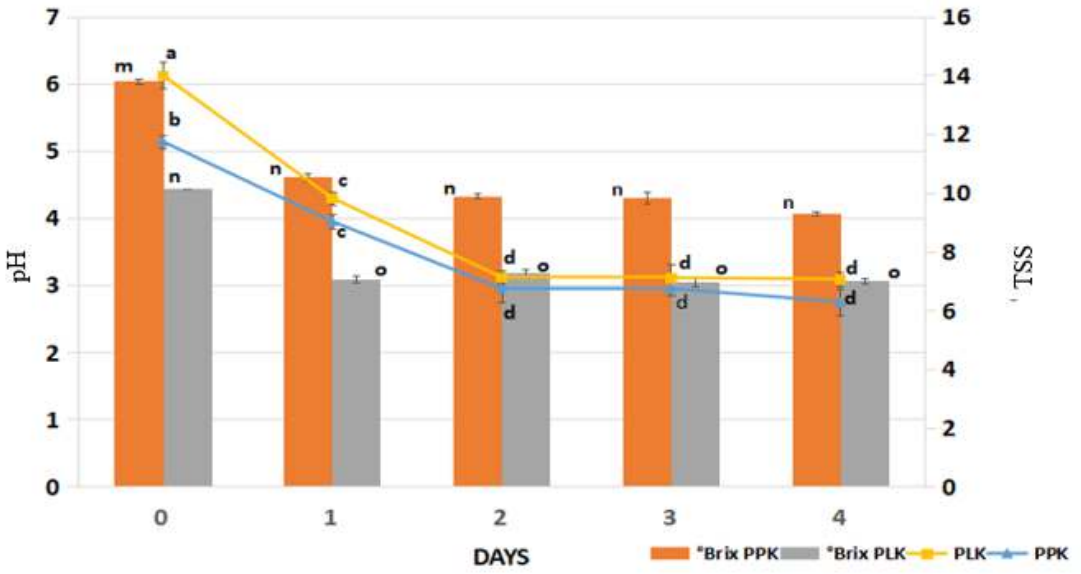


Figure 4.6: Changes of pH and Total soluble solids (TSS) content during 4-days fermentation of PPK and PLK.

Bar graphs represent mean ± standard deviations of 3 replicates.[a-d]Means and [m-o]means with different letters were significantly different ($P < 0.05$) from initial count (0 h) for pH and Brix, respectively.

The ethanol concentrations in the papaya pulp and leaves kombucha increased quickly after four days of fermentation, reaching their greatest levels on day two (Figure 4.7). On the fourth day of fermentation, the accumulated ethanol concentration for PPK was 0.04% after being initially discovered at 0.95%. Like PLK, the ethanol concentration there gradually rose to 1.18% before falling to 0.10% on the fourth day of fermentation. On the other hand, it was found that the levels of acetic acid in both papaya kombucha juices steadily grew, reaching 1.62% (PLK) and 1.45% (PPK) at the end of day four.

The acetic acid bacteria (*K. rhaeticus* MFS1) will grow concurrently and oxidise the ethanol into acetic acid to become the principal organic acid produced in kombucha manufacturing while the yeast (*D. bruxellensis* MFS1) converts carbohydrates into ethanol and carbon dioxide. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), two successive catalysts produced by acetic acid bacteria,

were responsible for both kombucha drinks' continuous decline in ethanol content (Gomes et al., 2018). Acetic acid, which can build to up to 10 to 20% when

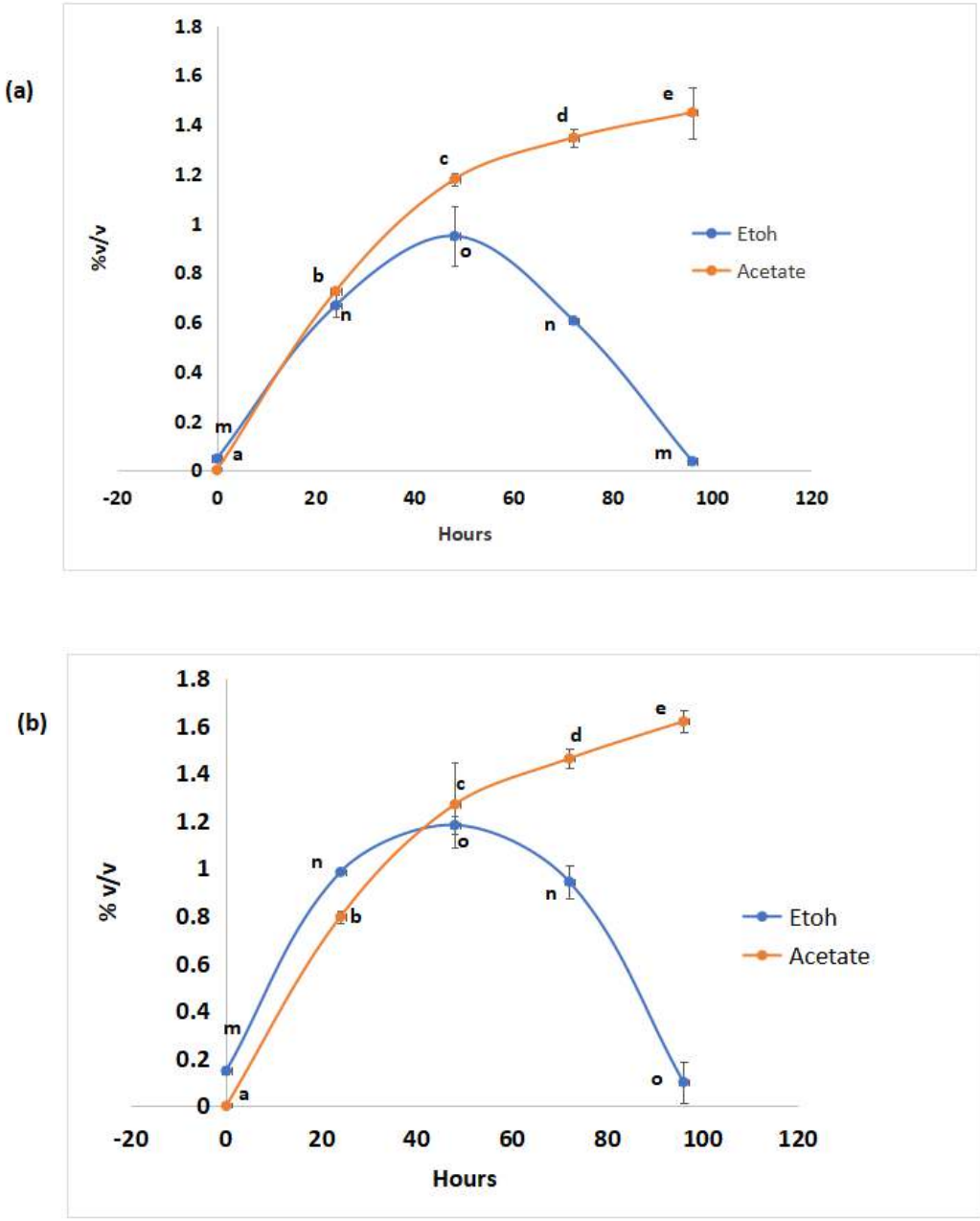


Figure 4.7: Changes of ethanol and acetic acid concentrations during kombucha fermentation of PPK (a) and PLK (b).

Bar graphs represent mean ± standard deviations of 3 replicates [a-e]Means and [m-o]means with different letters were significantly different ($P < 0.05$) from initial count (0 h) for acetate and ethanol, respectively.

Komagataeibacter species is utilised but only up to 5 to 10% in other *Acetobacter* species, is released into the media during acetification. Furthermore, because this strain is more resistant to extremely high acidity, *Komagataeibacter* species has reportedly been found to be preferable in submerged cultures (Gomes et al., 2018). When comparing the shaken culture of PPK and PLK to the conventional static kombucha tea fermentation method, it was found that the oxidation of ethanol to acetic acids occurred more quickly.

The concentrations of 0.60 - 3.00% were recorded using standard static fermentation, which typically takes up to 14 days (Greenwalt et al., 1998; Kochman et al., 2020), while with shaken culture of PPK and PLK the acetic acid concentrations were at 1.62% for PLK and 1.45% (PPK) within four days of fermentation. On the other hand, PPK and PLK both had lower ethanol concentrations than standard kombucha static cultures, which had ethanol concentrations of 2.75% and 0.04%, respectively (Kochman et al. 2020). The use of a shaking culture during the fermentation, which produced a better dissolved oxygen rate than static cultures, may have contributed to the lower concentration of ethanol and faster oxidation to acetic acid. Shaken culture, as demonstrated by Henzler and Schedel (1991), can increase the dissolved oxygen content of substrates and promote better microbial growth.

The following catalytic activities take place during kombucha fermentation: ADH will oxidise ethanol to aldehyde, and ALDH will subsequently oxidise this intermediate product to acetic acid. The respiratory chain, in which oxygen serves as the last electron acceptor, is connected to the membrane bound ADH and ALDH complexes. Since the ALDH is more sensitive to oxygen concentrations, a shortage of oxygen may result in

decreased conversion activity, which would lead to an accumulation of more aldehydes and a reduction in the synthesis of acetic acid (Mamlouk & Gullo, 2013). In order to make kombucha juice with a high level of acetic acid production and a low level of ethanol, adequate levels of oxygen must be present. Therefore, in this experiment, it was demonstrated that adding a shaking culture, which increased the quantity of soluble oxygen, sped up the oxidation process and significantly reduced the amount of ethanol within four days of fermentation. The use of an aerated bioreactor, which may open the door to more effective process control for kombucha fermentation, can help to further develop this innovative method of kombucha beverage production.

Due to its promotion as a non-alcoholic beverage, kombucha must adhere to the following legal requirements: in the United States, a non-alcoholic beverage must have an ethanol content of less than 0.5% alcohol by volume (ABV), in the European Union, an ABV of less than or equal to 1.2%, and in Malaysia, the Malaysian Halal certification board, governed by the Department of Islamic Development Malaysia (JAKIM), which stipulates that kombucha must have an ABV of less than 1.0% (Ebersole et al., 2017; Najiha & Nadiah, 2015). These restrictions and safety precautions are intended for a specific population that is most at danger from low-level alcohol consumption, including children, pregnant women, persons who drive for a living, and people who abstain from alcohol due to personal or religious convictions.

4.5 Organic acids content of papaya pulp, leaves juices and papaya kombucha

Table 4.3 shows the differences of organic acids content in papaya pulp and leaves before (papaya pulp and leaves juices column) and after fermentation with isolated kombucha cultures (papaya pulp and leaves kombucha column). Fermentation using kombucha cultures produced significant changes in organic acids composition for both papaya pulp and leaves samples. Organic acids such as acetic acid, citric acid, oxalic acid, malic acid, kojic acid and quinic acid were detected in both papaya pulp and leave juices and their respective kombucha samples. Similar organic acids compositions were also reported in several kombucha beverages studies (Jayabalan et al., 2008; Vitas et al., 2018). Acetic acid is the primary organic acid present in every kombucha tea produced as it has become the signature component for kombucha beverages (Leal et al., 2018; Villarreal-soto et al., 2018). The kombucha cultures used in this study were the locally isolated *K. rhaeticus* MFS1, a known acetic acid producing bacteria (Machado et al., 2016). Thus, in this study, the acetic acid was the highest organic acids concentration detected in both papaya pulp (13439.60 ± 571.30 ppm) and leaves kombucha (19262.84 ± 347.65 ppm).

Both fermented papaya pulp and leaves kombucha showed a significant increase in quinic acid concentration with 25-fold (2169.84 ± 114.40 ppm) and 11-fold (985.25 ± 7.03 ppm), respectively. The increasing trends of quinic acid observed during kombucha fermentation were similar with the findings conducted by Neffe-skocińska et al. (2017). An increase in kojic acid was also observed in both fermented papaya pulp and leaves samples where the latter showed higher increment of up to 2.5-fold from its initial concentration. *Aspergillus* species is the prominent producer of kojic acid (Rosfarizan et al., 2010). However, several acetic acid bacteria were also reported to

produce kojic acids (Bentley, 2006), which may also contribute to the slight increment of kojic acid after fermentation.

The initial concentration of oxalic acid in non-fermented substrates was significantly reduced after four days of fermentation to 44.09 ± 3.31 ppm for PPK and 70.45 ± 3.63 ppm for PLK. Organic acids could be utilized as an important secondary source of carbohydrates for microorganism during food fermentation (Torija et al., 2003). This could explain the reduction pattern of oxalic acid in both kombucha, due to microbial metabolism or breakdown during fermentation. The reduction of oxalic acid content in food may give a positive effect to consumer's diet as the occurrence of stone formation in urinary tract due to its high consumptions can be avoided (Peck et al., 2015).

Table 4.3: The changes in organic acids content

Organic acid (ppm)	Papaya pulp juices	Papaya pulp kombucha	Papaya leaves juices	Papaya leaves kombucha
Acetic acid	0.00^{a}	13439.60 ± 571.29^{b}	0.00^{a}	19262.84 ± 347.65^{c}
Citric acid	726.36 ± 12.27^{a}	793.54 ± 39.59^{b}	327.00 ± 11.80^{c}	229.91 ± 8.83^{d}
Oxalic	180.56 ± 4.66^{a}	44.09 ± 3.31^{b}	236.89 ± 2.17^{c}	70.45 ± 3.63^{d}
L-malic acid	55.59 ± 3.45^{a}	251.95 ± 8.80^{b}	116.03 ± 3.30^{c}	125.40 ± 4.26^{c}
Kojic acid	2.57 ± 0.38^{a}	4.78 ± 0.40^{b}	8.29 ± 0.56^{c}	20.38 ± 0.84^{d}
Quinic acid	84.37 ± 4.02^{a}	2169.84 ± 114.40^{b}	84.50 ± 3.95^{a}	985.25 ± 7.03^{c}
Total	**1049.45**	**16703.8**	**772.71**	**20694.23**

Values were expressed as mean ± standard deviations of 3 replicates. $^{a-c}$Means within a row with different letters were significantly different ($P<0.05$)

4.6 Total phenolic contents (TPC) and total flavonoid content (TFC) analysis

Polyphenolic compounds are commonly regarded as antioxidant due to their capabilities to scavenge free radicals and reactive oxygen species. In this study, Folin–Cioacalteu method was used to assay the total phenolic contents (TPC) while the total flavonoid content (TFC) assay was performed based on flavonoid-aluminum chloride ($AlCl_3$) complexation. Table 4.4 shows the level of TPC detected in papaya pulp juice (0.226 ± 0.004 mg GAE/ml) that was significantly lower when compared to papaya leaves juice (0.460 ± 0.006 mg GAE/ml). Similarly, the TFC component of papaya leaves juice was also significantly higher (0.577 ± 0.028 mg QUE/mL) than the papaya pulp juice (0.052 ± 0.005 mg QUE/mL). This indicated that papaya leaves contained higher amount of polyphenolic content than stem, which was similar with previous reports (Hadadi et al., 2018; Maisarah et al., 2014; Runnie et al., 2004), and thus can be a good source of dietary antioxidants.

Table 4.4: Total phenolic content and total flavonoid content of fermented and non-fermented papaya pulp and leaves juices.

Samples	TPC	TFC
Non fermented papaya pulp	0.226 ± 0.004^{a}	0.052 ± 0.005^{a}
Fermented papaya pulp/PPK	0.236 ± 0.008^{b}	0.004 ± 0.002^{b}
Non fermented papaya leaf	0.460 ± 0.006^{c}	0.577 ± 0.028^{c}
Fermented papaya leaf/PLK	0.390 ± 0.004^{d}	0.433 ± 0.039^{d}

Values were expressed as mean±standard deviations of three replicates. $^{a-d}$Means within a row with different letters were significantly different ($P<0.05$)

After four days of fermentation, TPC value for PPK showed a slight increment [increased from 0.226 ± 0.004 mg GAE/ml to 0.236 ± 0.008 mg GAE/ml (increment of 4.42 %)]. Meanwhile, TPC value for PLK was decreased from 0.460 ± 0.006 mg GAE/ml to 0.390 ± 0.004 mg GAE/ml. Decrease in TFC was also observed in both PLK (24.95%) and PPK (92.3%).

During fermentation, metabolic activities of kombucha cultures consisting of yeast and bacteria may change the physical (texture, colour) and biochemical compositions (polyphenols, proteins, organic acids) of both papaya pulp and leaves juices (Jayabalan et al*.,* 2008). Fermentation can cause sugars and other molecules to be transformed or decomposed. Decrease in the phenolic content could be contributed by a number of factors, namely (1) oxidation via the activity of phenoloxidase (PPO) (Pérez-Gregorio et al., 2011; Álvarez et al., 2017), (2) condensation reactions of single polyphenols to insoluble complex tannins that interact with proteins (Suazo et al., 2014), and (3) precipitation or adsorption to the solids or microbes and polymerisation, which results in the losses of these compounds (Chen et al., 2018). In contrast, an increase in TPC can be related to enzymatic hydrolysis or by organic acids produced by the starter cultures, which facilitates the release of simple soluble phenolic during fermentation (Sun et al., 2015). Moreover, the concentration detected for TPC and TFC depends on the methods, solvent system, parts of the plant or species and cultivars adopted (Kuppusamy et al., 2018).

4.7 Polyphenolic Constituents in Papaya Juices and Their Kombucha Beverages

Papaya, like any other fruits and vegetables that are rich in phytochemicals, is often associated with providing health benefits to human body. Several studies in analysing the phytochemical compositions of papaya have found that this fruit is rich in polyphenolic compounds, minerals, and other beneficial substances (Ikram, 2019; Kadiri et al., 2016). The polyphenolic compositions of papaya juices before fermentation are listed in Table 4.5 under the papaya pulp and leaves juices column, whereas the compositions after fermentation are given in the papaya pulp and leaves kombucha column. There were 13 types of polyphenolic compound detected in papaya juices and their respective kombucha beverages. Two classes of phenolic acid in the form of benzoic acid and cinnamic acid derivatives were found in those samples.

Table 4.5: Polyphenolic constituents in papaya pulp and leaves juices and their kombucha counterparts

Phenolic compounds	Papaya pulp juices (ppm)	Papaya pulp kombucha (ppm)	Papaya Leaves Juices (ppm)	Papaya leaves kombucha (ppm)
Gallic acid	7.13 ± 0.1^{a}	0.78 ± 0.08^{b}	0.83 ± 0.08^{b}	0.45 ± 0.05^{c}
Protocatechuic acid	1.31 ± 0.07^{a}	1.22 ± 0.11^{a}	0.72 ± 0.19^{b}	1.51 ± 0.06^{c}
Hydroxybenzoic acid	ND^{a}	0.29 ± 0.03^{b}	0.91 ± 0.04^{c}	0.80 ± 0.06^{c}
Syringic acid	ND^{a}	0.04 ± 0.002^{b}	1.06 ± 0.03^{c}	1.34 ± 0.03^{c}
Ellagic acid	ND^{a}	0.07 ± 0.01^{a}	4.01 ± 0.59^{b}	8.76 ± 0.40^{c}

2,5 Dihydroxybenzoic acid	0.36 ± 0.01[a]	0.45 ± 0.04[b]	0.22 ± 0.02[c]	0.82 ± 0.04[d]
Caffeic acid	0.02 ± 0.003[a]	0.02 ± 0.002[a]	2.48 ± 0.09[b]	0.32 ± 0.05[c]
Chlorogenic acid	ND[a]	0.24 ± 0.02[b]	1.01 ± 0.004[c]	2.34 ± 0.04[d]
Ferulic acid	ND[a]	ND[a]	0.26 ± 0.02[b]	0.25 ± 0.02[c]
Catechin	0.10 ± 0.02[a]	0.15 ± 0.02[a]	1.18 ± 0.10[b]	7.34 ± 0.55[c]
Epigallocatechin	0.36 ± 0.006[a]	0.07 ± 0.01[b]	0.869 ± 0.16[c]	0.6 ± 0.15[a]
Rutin	0.05 ± 0.006[a]	0.28 ± 0.02[a]	10.83 ± 2.01[b]	10.55 ± 0.52[b]
Tannin*	329.25 ± 28.60[a]	298.67 ± 33.78[b]	576.67 ± 25.62[c]	426.20 ± 29.42[d]

Values were expressed as mean ± standard deviations of 3 replicates. [a-d]Means within a row with different letters were significantly different ($P<0.05$).
Abbreviation: ND = not detected
*Tannin was analysed using spectrophotometric methods and were expressed as mg GAE/mL

In this study, the benzoic acid derivatives detected in papaya juices were gallic acid, protocatechuic acid, hydroxybenzoic acid, syringic acid, 2,5-dihydroxybenzoic acid and ellagic acid. Gallic acid (7.13 ± 0.10 ppm) and protocatechuic acid (1.31 ± 0.07 ppm) were the main benzoic acid derivatives found in papaya pulp, while ellagic acid (4.01 ± 0.59 ppm) and syringic acid (1.06 ± 0.03ppm) were the two main components present in papaya leaves. The cinnamic acid derivatives detected were caffeic acid, chlorogenic acid and ferulic acid. Even though papaya pulp only contained caffeic acid (0.02 ± 0.00 ppm), all three types of cinnamic derivative mentioned above were also detected in papaya leaves juice. The order of concentration from the highest to the lowest was as follows: caffeic acid (2.48 ± 0.09 ppm), chlorogenic acid (1.01 ± 0.04 ppm) and ferulic acid (0.26 ± 0.02 ppm).

Flavonoids, which can be usually found in leaves and skin of fruits are the structurally diverse secondary metabolites in the plant kingdom. It often involves in plant development, pigmentation, UV protection, disease protection and resistance (Mathesius, 2018). The flavanols detected in papaya juices (pulp and leaves) were catechin, epigallocatechin and rutin. The flavonoid compounds found in papaya pulp juices were lower when compared to papaya leaves juice. Higher levels of flavonoids were observed in papaya leaves juice with rutin (10.83 ± 2.01 ppm) and catechin (1.18 ± 0.10 ppm) to be reported as the most abundant types of flavonoids. Tannins is another class of polyphenolic compound detected in papaya juices. Tannins may bind to salivary proteins, which produced astringency taste which can render some plant tissues inedible (Ashok & Upadhyaya, 2012). Among all the polyphenolic compounds detected, tannin was observed to have the highest concentration in papaya pulp juices (329.25 ± 28.60 mg GAE/mL) and leaves (576.67 ± 25.62 mg GAE/mL).

The polyphenolic compositions detected in both papaya pulp and leaves were found to be altered after four days of fermentation with kombucha cultures. No uniformity changes in the polyphenolic compositions were observed but significant changes in their concentrations were detected after four days of fermentation. This might be due to the biotransformation processes during the fermentation. A single reaction or combinatory of processes during fermentation may affect the formation of bioactive components in the beverage which includes loss of compounds through oxidation, absorption to solids, protein, microbes, and enzymatic polymerisation. The improvement or enrichment of compounds may also be due to the enzyme and acids produced by microorganisms which may facilitate the release of these compounds (Chen et al., 2018).

Gallic acid was sharply decreased to 0.78 ± 0.08 ppm and 0.45 ± 0.05 ppm in both PPK and PLK, respectively. Decrease of the gallic acid concentration was due to the degradation or metabolism by the starter cultures. Microorganisms, such as bacteria (Alberto et al., 2004; Müller et al., 2007) and yeast (Meier et al., 2017) have been known to metabolise gallic acid. Besides, gallic acid could also be polymerised into ellagic acid, which is a dimeric derivative of gallic acid (Muthukumaran et al., 2017). This phenomenon was observed when ellagic acid (0.07 ± 0.01ppm) was detected in PPK after fermentation, while a significantly increase in ellagic acid (8.76 ± 0.40ppm) content was observed for PLK. Another possibility for the increase amount of ellagic acid in both PPK and PLK was the degradation of tannins. Tannins are composed of gallotannins and ellagitannins, was found to have a reduction of approximately 9.50% in PPK and 26.09 % in PLK after fermentation, respectively.

On the other hand, chlorogenic acid was observed to be increased in concentration for both PPK and PLK. Its content in PPK had increased from non-detected to 0.24 ± 0.02ppm while PLK had doubled its concentration from 1.01 ± 0.004ppm up to 2.34 ± 0.04ppm. Chlorogenic acid is an ester of caffeic acid and quinic acid. Recent study showed that certain bacteria and fungi can biosynthesise chlorogenic acid through the esterification of quinic acid and caffeic acid in their secondary metabolic pathway (Wang et al., 2018). Catechin concentration was increased significantly for PLK from 1.18 ± 0.10ppm to 7.34 ± 0.55ppm. For epigallocatechin, both PPK and PLK were found to reduce significantly into 0.07 ± 0.01ppm and 0.6 ± 0.15ppm, respectively.

4.8 Antioxidant activities

Reactive oxygen species (ROS) are reactive groups of atoms with free radicals, which may cause damages to the components of living cells when produce in excessive quantities (Valko, 2007). Phenolic compounds found in fruits and vegetables are parts of diet where their consumptions can render possible health benefits from its antioxidant properties (Kumar et al., 2014). Antioxidant capacity assays can be categorised into two types of mechanism, namely: hydrogen atom transfer (HAT) and single electron transfer (SET). The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) Ferric reducing antioxidant power (FRAP) assays is operated using the SET mechanism, while 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay determined antioxidant capacity through HAT and SET mechanism (Huang et al., 2005; Liew et al., 2018). The results of DPPH and FRAP assays are presented in Table 4.6. All samples regardless of papaya pulp and leaves or kombucha beverages had exerted antioxidant activities against free radical DPPH. The PLK had the highest antioxidant activity against DPPH with 89.60% (an increase of 20% from its non-fermented juice), while PPK showed a slight increase of 3.7%, from 52.90% in the non-fermented form to 54.95%.

From Table 4.6, the results for FRAP assay showed that antioxidant activity had increased from 0.98 ± 0.02 mg AAE/ml to 1.10 ± 0.006 mg AAE/ml for PPK. In contrast, a decrease in FRAP value was observed in PLK where its antioxidant activity had dropped from the initial 2.80 ± 0.04 mg AAE/ml to 2.13 ± 0.02 mg AAE/ml. Meanwhile, similar trends with FRAP assay results were also observed for TPC results in papaya kombucha samples. The TPC value obtained for PPK had increased slightly from the initial concentration of 0.23 ± 0.004 mg GAE/ mL to 0.24 ± 0.01 mg GAE/

mL whereas the PLK had dropped from 0.50 ± 0.01 mg GAE/ mL to 0.40± 0.004 mg GAE/ mL, respectively.

The observed differences in the antioxidant capacities were mostly depending on the specific assays and metabolites involved. Generally, the polyphenolic compounds present in fruits and vegetables are reported to be associated with the antioxidant activities of their samples. However, the quantity of phenolic compound does not necessarily determine the antioxidative capacities of kombucha or fermented products, but the types of metabolites produced, or types of phenolic compound might have played the key roles (Chu & Chen, 2006; Fernandez-Panchon et al., 2008; Xu et al., 2019). As for phenolic compounds, it is reported that monophenols are found to be less efficient to react and quench free radicals than polyphenols. Besides, the radical scavenging potentials of phenolic compounds also depend on the number and position of hydroxyl and methoxy groups in the phenolic rings (Fernandez-Panchon et al., 2008; Mathew et al., 2015).

Different antioxidant scavenging capacities of the fermented papaya kombucha samples than their non-fermented juices (pulp and papaya) might be attributed to the bioactive components that would determine their abilities to scavenge radicals through either HAT or SET mechanisms. However, the inhibition remained within the range of 50.0% - 89.6%. From past studies, traditional kombucha tea produced using black tea showed an inhibition towards DPPH radicals in the range of 37% to 70%, while around 63.0% to 95.3% inhibition was observed for green tea (Fu et al., 2014; Jayabalan et al., 2008; Malbaša et al., 2011). On the other hand, fermented papaya juice produced using several *Lactobacillus* species strains had shown DPPH radical scavenging activities in

the range of 55.60% to 55.60% and an ABTS scavenging activities of more than 80% (Chen et al., 2018). In addition, the antioxidant protective effects of kombucha tea or other fermented products are mainly due to (1) the polyphenolic activities and (2) the compounds produced during fermentation using different types of starter culture, which subsequently produce synergistic effects (Jayabalan et al., 2008; Leal et al., 2018).

Table 4.6: The changes in antioxidant capacities of samples

Samples	TPC (mg GAE mL-1)	FRAP (mg AAE mL-1)	(%) Inhibition of DPPH
Papaya pulp juices	0.23 ± 0.004^{a}	0.98 ± 0.02^{a}	53.00 ± 1.81^{a}
PPK	0.24 ± 0.01^{b}	1.10 ± 0.01^{b}	55.00 ± 0.22^{b}
Papaya leaf juices	0.50 ± 0.01^{c}	2.80 ± 0.04^{c}	74.25 ± 0.16^{c}
PLK	0.40 ± 0.004^{d}	2.13 ± 0.02^{d}	89.60 ± 0.85^{d}

Values were expressed as mean ± standard deviations of 3 replicates. $^{a-d}$Means within a column with different letters were significantly different ($P<0.05$)

4.9 Antibacterial activities

The antibacterial activities of PPK and PLK supernatants were tested against several pathogenic bacteria obtained from The American Type Culture Collection (*ATCC)* (Table 4.7). Both un-neutralised papaya pulp kombucha (un-PPK) and papaya leaves kombucha (un-PLK) supernatants, acetic acid (ACE, 0.7%) and antibiotic (Pen-Strep, 1%) had exerted inhibition activities, but no inhibitions were observed for neutralised (pH 7) papaya pulp kombucha (n-PPK), neutralised papaya leaves kombucha (n-PLK), papaya pulp juices and papaya leaves juices supernatants. Both PPK and PLK supernatants produced clear inhibition zones (Figure 4.8) on all Gram-positive and Gram-negative pathogens at the range of 16 to 21mm, with PLK had larger clearing zone of inhibition on all pathogenic strains tested. Acetic acid solution (0.7%) did not

exhibit any inhibition for *Listeria monocytogenes* and both acetic acid and Pen-Strep (1%) showed no inhibition towards *Streptococcus gallolyticus*. Neutralised cell free supernatants from both PPK and PLK showed no inhibition zones against the pathogenic strains examined.

Table 4.7 Comparison of antibacterial activities

Sample/ Bacteria	**Inhibition zones (mm)±SD**				
	***S. typhimurium* (Gram -)**	***E. coli* (Gram -)**	***S. entiritidis* (Gram -)**	***S. gallolyticus* (Gram +)**	***L. monocytogens* (Gram +)**
PPK	18.63 ± 1.46^{b}	17.67 ± 1.65^{e}	16.28 ± 0.07^{e}	19.02 ± 2.19^{b}	18.03 ± 0.85^{c}
PPJ	ND^{d}	ND^{d}	ND^{d}	ND^{c}	ND^{d}
NPPK	ND^{d}	ND^{d}	ND^{d}	ND^{c}	ND^{d}
PLK	20.27 ± 0.85^{b}	21.65 ± 1.30^{b}	20.27 ± 1.60^{b}	21.65 ± 1.01^{a}	21.55 ± 0.82^{b}
PLJ	ND^{d}	ND^{d}	ND^{d}	ND^{c}	ND^{d}
NPLK	ND^{d}	ND^{d}	ND^{d}	ND^{c}	ND^{d}
ACE	10.42 ± 0.87^{c}	11.62 ± 1.77^{c}	13.02 ± 1.30^{c}	ND^{c}	ND^{d}
Pen-Strep	26.55 ± 0.92^{a}	25.70 ± 2.55^{a}	25.78 ± 3.01	ND^{c}	26.52 ± 1.58^{a}

Abbreviation: PPK = un-neutralised papaya pulp kombucha; PPJ = papaya pulp juices; NPPK = neutralised (pH 7) papaya pulp kombucha; PLK = papaya leaves kombucha; PLJ = papaya leaves juices; NPLK = neutralised papaya leaves kombucha; ACE = acetic acid (0.7%); ND, not detected. All kombucha samples were fixed at acidity of 1.5% v/v in 100µl for this assay. Values are expressed as mean ± standard deviations of 3 replicates. $^{a-e}$Means within a column with different letters were significantly different ($P<0.05$)

Traditional kombucha tea is reported to inhibit the *in vitro* growth of various food-borne pathogens that can cause food poisoning (Sreeramulu et al., 2000). Previous studies have shown that kombucha tea contains several organic acids such as acetic, glucoronic, citirc, malic, oxalic, succinic and malonic (Guida et al., 2018; Villarreal-soto et al., 2018). During kombucha tea fermentation using sucrose as the carbon source, acetic acid becomes the major metabolites produced by acetic acid bacteria (Jayabalan et al.,

2014). Acetic acid as low as 0.5% v/v in kombucha tea have been shown to inhibit the growth of food-borne pathogens (Greenwalt et al., 1998; Trček et al., 2015). Therefore, the higher concentration of acetic acid in papaya kombucha (PLK and PPK) are responsible to the superior microbial inhibition observed compared to ACE (0.7% v/v). However, in this study, the growth of *Streptococcus gallolyticus* was unsuccesfully inhibited by ACE and Pen-Strep. Most probably, the concentration of ACE at 0.7% v/v is not adequate enough to inhibit the growth of *S. gallolyticus* as compared to PLK and PPK. Pen-Strep is classified as a beta-lactam and aminoglycoside antibiotic. Various *Streptococcus* species are known to adapt resistant towards beta lactam antibiotics (Matsui et al., 2011; Specht et al., 2021), while streptomycin concentration might not have an effective dose for *S.gallolyticus* to be inhibited.

The concentrations of acetic acid may vary during kombucha fermentation. Some studies showed that prolong fermentation duration for up to 30 days can produce higher acetic acid concentration such as 11-12 g/L acetic acid (Jayabalan et al., 2014) and 24 g/L acetic acid (Greenwalt et al., 1998) respectively. In this study, PPK and PLK samples had exhibited anti-bacterial activity against both G*ram-positive* and *Gram-negative* bacteria. Sreeramulu et al. (2000) and Greenwalt et.al (1998) had reported that kombucha' antimicrobial inhibition activity was correlated to the concentrations of acetic acid formed. Studies conducted by Bhattacharya et al. (2016) had reported that several types of G*ram-positive* and *Gram-negative* bacteria strains were inhibited by the polyphenolic fraction found in kombucha tea. In short, the anti-bacterial activity against pathogenic bacteria by PPK and PLK could be the symbiotic effects of organic acids and polyphenolic compounds. However, both neutralised fraction of papaya and leaves kombucha did not show any inhibition on food-borne pathogens tested. This was

due to the inactivation of organic acids which were the main component in repressing the microbial growth.

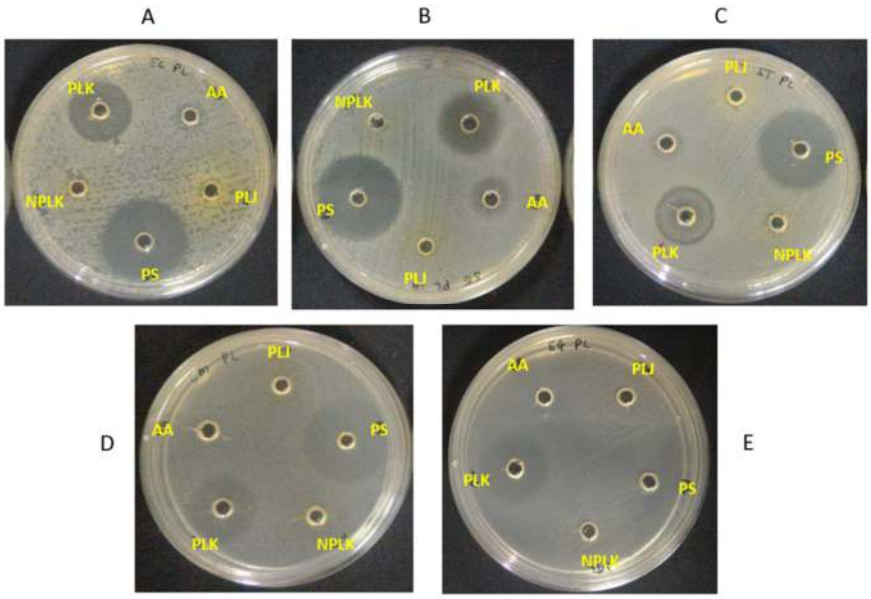


Figure 4.8: Antimicrobial activities of PLK= papaya leaves kombucha; PLJ = papaya leaves juices; NPLK = neutralised papaya leaves kombucha; ACE = acetic acid (0.7%); All kombucha samples were fixed at acidity of 1.5% v/v and 100µl were used in this assay; PS=Penicillin/strepromycin (1%) against Gram-negative *Escherichia coli* (A), *Salmonella enteritidis* (B), *Salmonella thypimurium* (C) and Gram-positive bacteria *Listeria monocytogenes*(D) *Streptococcus gallolyticus* (E)

Simirlarly, kombucha produced from *Satureja montana* and tea (Black and green) when neutralised, no inhibition of pathogenic strains (*Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*) were observed (Cetojevic-Simin et al. 2008; Battikh et al., 2012). In another study, neutralised fermented cell free product using lactic acid bacteria also showed a significant reduction in its inhibitory effects mainly due to the neutralisation of its organic acids (lactic acid, acetic acid) and hydrogen peroxide produced by the bacteria (Hor & Liong, 2014).

Thus, organic acids as the main metabolites had contributed to the *in vitro* antimicrobial effects. Acetic acid classified as generally regarded as safe (GRAS) in United State is an organic acid than may exert growth inhibition effect as low as 0.5% v/v and is widely used as food preservative or in food preparations. Therefore, it will be advantageous to have adequate amount of acetic acid in kombucha fermentation or formulation to act as a natural preservative and flavour enhancer. However, the higher concentration of acetic acid in kombucha beverages can render the beverage sensory and quality to an undesirable drinking level (Chu & Chen, 2006).

4.10 Papaya Kombucha-Like Beverages Induced Cytotoxicity in HT29 and SW480 Colon Cancer Cells

Apart from a series of analysis conducted e.g., antioxidants and antibacterial activities to highlight the nutraceutical elements of papaya based-kombucha beverages prepared in this study, anticancer properties were also evaluated based on their cytotoxicity effects and oncolytic properties. Two colon cancer cell lines; HT29 and SW 480 were selected as both are commonly used in anticancer studies for colon carcinoma. Furthermore, their molecular set-up is relevant to determine the sensitivity of anticancer element of kombucha beverages prepared in this study that was based on organic acids content.

4.10.1 Cytotoxicity of papaya kombucha-beverages

Cytotoxic effects of both papaya kombucha beverages on human carcinoma cells, HT29 and SW 480 were assessed using MTT assay (Figures 4.9a and 4.9b). This assay helps researchers in revealing the metabolic activity of the cells that are being studied. Therefore, this would enable the estimation of specific cell death after treatment with

kombucha supernatants. Overall, the responses of HT29 and SW 480 colon cancer cells towards the increased concentrations of both PPK and PLK were exponential. Both HT29 and SW 480 colon cancer cells experienced gradual decrease in viability at low concentrations and eventually declined at the highest concentration tested as can be seen in Figure 4.9a and 4.9b. The estimated IC_{50} values (concentrations causing death of 50% of cancer cells) of HT-29 for papaya kombucha-like beverages were 6.0±0.21 %v/v and 5.5±0.14 % v/v for PLK and PPK, respectively. Meanwhile, the estimated IC_{50} values of SW 480 due to papaya kombucha-like beverage were 5.5±0.10 %v/v and 5.0 ±0.14 % v/v for PLK and PPK, respectively.

As low IC_{50} value indicates strong cytotoxicity, PPK showed slightly better anticancer activities than PLK. Nevertheless, the IC_{50} values were in the range of 5.0 – 6.0 %v/v for both PLK and PPK to display closely similar effects of cytotoxicity. This could be attributed to their comparable amounts of organic acids, particularly acetic acid to give the anticancer property. Rasouli et al. (2022) mentioned that acetic acid is mainly responsible for the anticancer properties of kombucha beverages.

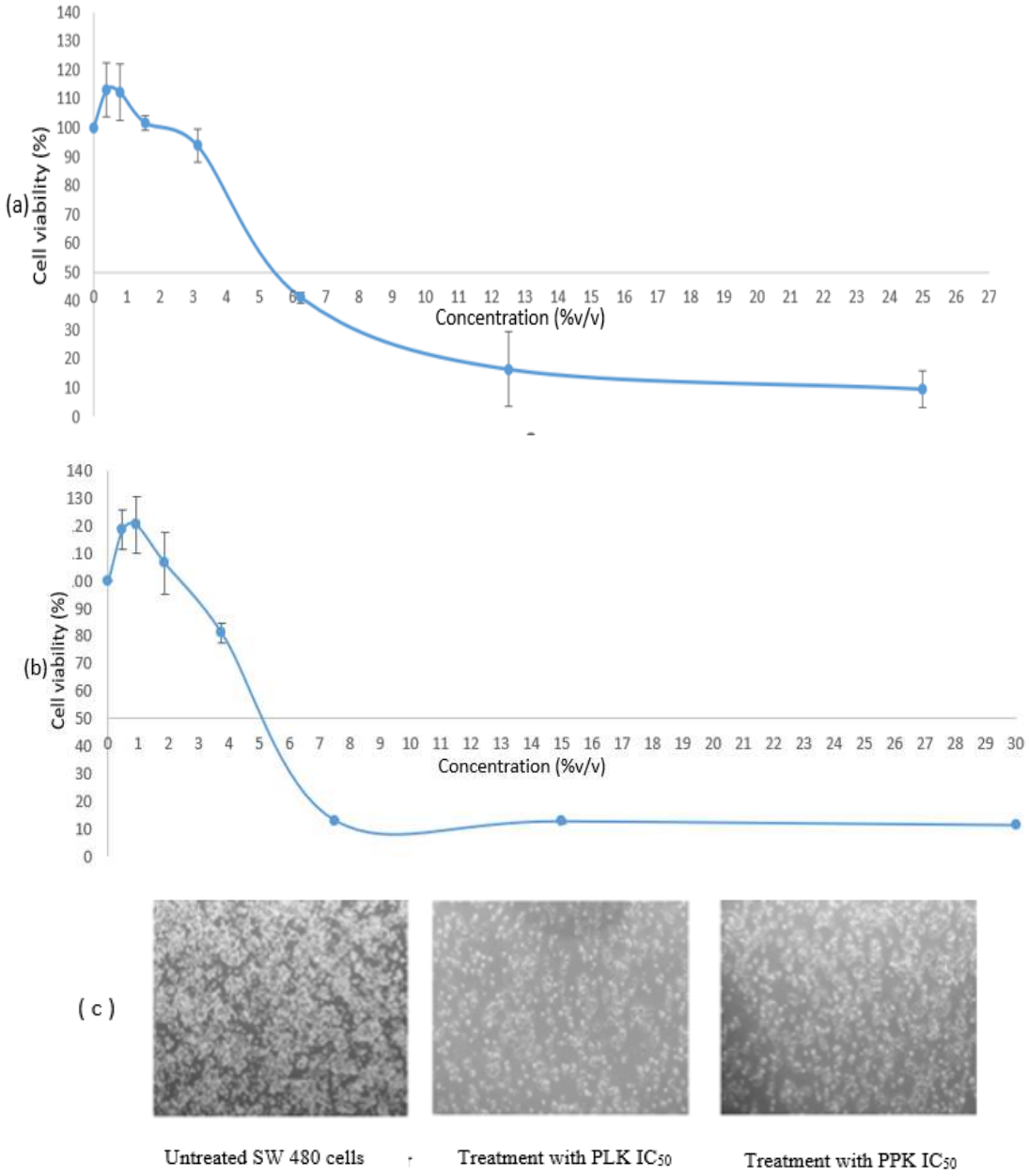


Figure 4.9a: Representative MTT assay showing the cytotoxicity activity of PLK (a) and PPK (b) at 72 h against SW 480 cells after 72-h incubation In vitro. (c) Cells of untreated, and under treatment of papaya kombucha (leaves and pulp) after 72 hours. Note: The IC_{50} value (half-maximal inhibitory concentration, PLK = 5.5±0.10 % v/v: PPK = 5.0 ±0.14 % v/v.

Each data was expressed as mean ± standard deviations of triplicate determinations.

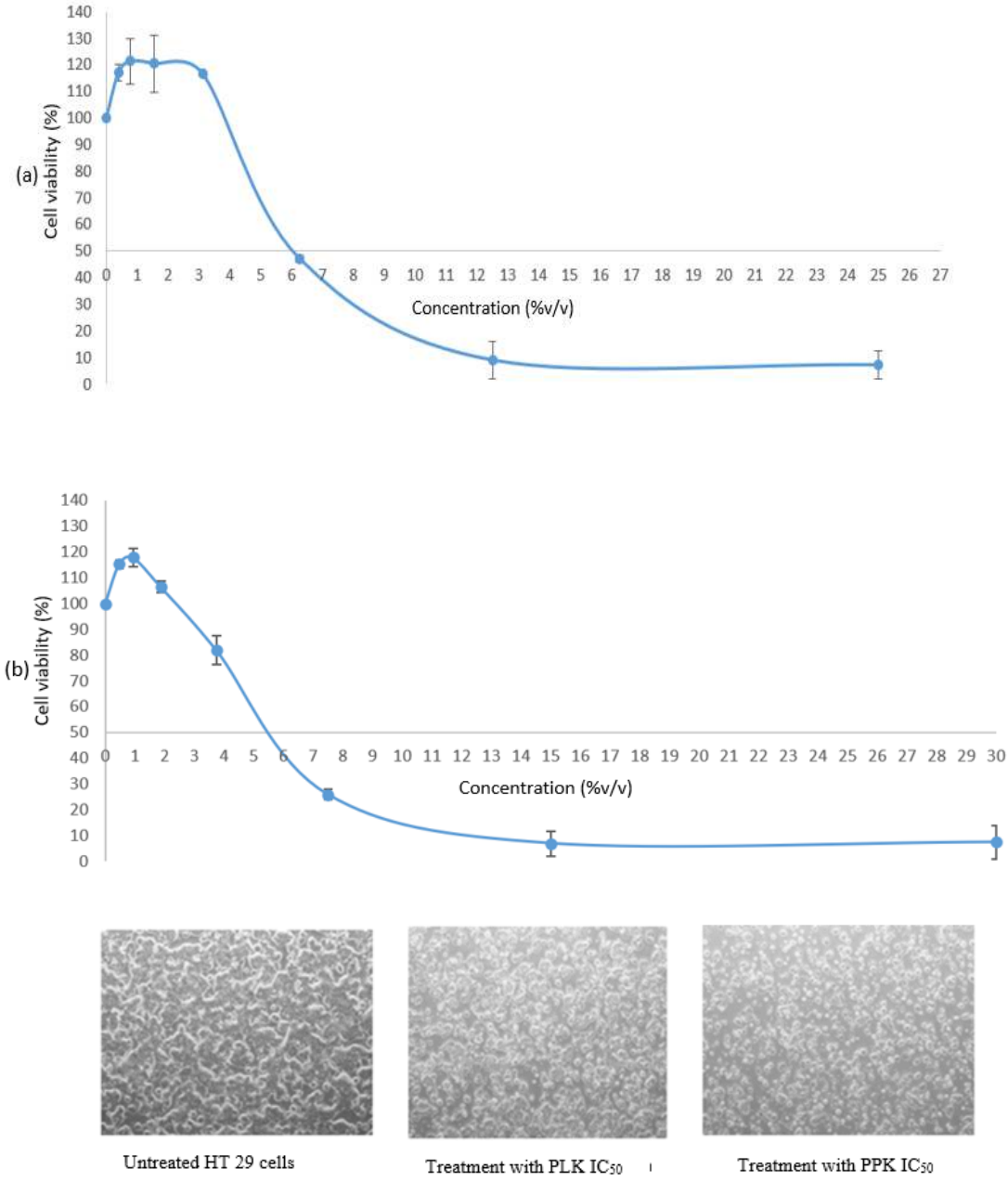


Figure 4.9b: Representative MTT assay showing the cytotoxicity activity of PLK (a) and PPK (b) at 72 h *against* HT29 cells after 72-h incubation *In vitro*. (c) Cells of untreated, and under treatment of papaya kombucha (leaves and pulp) after 72 hours. Note: the IC_{50} value (half-maximal inhibitory concentration, *PLK* = 6.0±0.21 % v/v*: PPK* = 5.5±0.14 % *v/v.*

Each data was expressed as mean ± standard deviations of triplicate determinations.

4.10.2 Apoptosis Study

Further evaluation of papaya kombucha-like beverages in causing colon cancer cell death or apoptosis was conducted using Annexin V FITC assay. Figure 4.10a shows the results of Annexin V FITC assay of HT29 and SW480 (4.10b) after being treated with both PPK and PLK for 72h. It was observed that both treated HT29 and SW480 shared similar cell population percentage pattern shift from viable cells to early apoptotic, then to the late apoptotic cell (Figure 4.11). For HT29 cells, the percentage of early apoptotic cells was increased from 3.14 ± 0.84% in control group to 37.34 ± 8.81% and 43.03 ± 5.26% for PLK and PPK, respectively. The late apoptotic HT29 cells were further decreased to 6.21 ± 1.96% and 6.12 ± 1.04% for PLK and PPK treated cells, respectively. Similarly, for SW480 cells, the percentage of early apoptotic cells was increased from 1.89 ± 0.84% in the control group to 26.83 ± 4.10% (PLK) and 25.15 ± 3.18 (PPK). The late apoptotic SW480 cells were further decreased to 5.78 ± 2.16% and 5.10 ± 0.42% for PLK and PPK treated cells, respectively.

Apoptosis is a synchronized form of cellular suicide that happens in multicellular organisms caused by exogenous or endogenous death stimuli (Saccheri and Travan, 2021). This includes death ligands such as TNF-related apoptosis-inducing ligand, tumor necrosis factor alpha (TNF-α), FasL/CD95/Apo1 and chemotherapeutic agents, for instance, doxorubicin, cisplatin and 5'FU (Han, Kim & Kim, 2008). The existence of p53 proteins, which cause apoptosis, is what enables cellular systems to restrict the growth of tumours and the response to many different types of cancer therapy. Following cellular stressors, the p53 protein is stabilised and forms a tetramer that binds to DNA in a sequence-specific manner, activating a number of apoptosis-associated

proteins such as Bcl-2, Bax, the Bcl-2 antagonist killer, and p53-upregulated modulator of apoptosis. Bcl-2 proteins, which are found on the mitochondrial membrane, control apoptosis by maintaining a balance between pro- and antiapoptotic members (Wei et al., 2021).

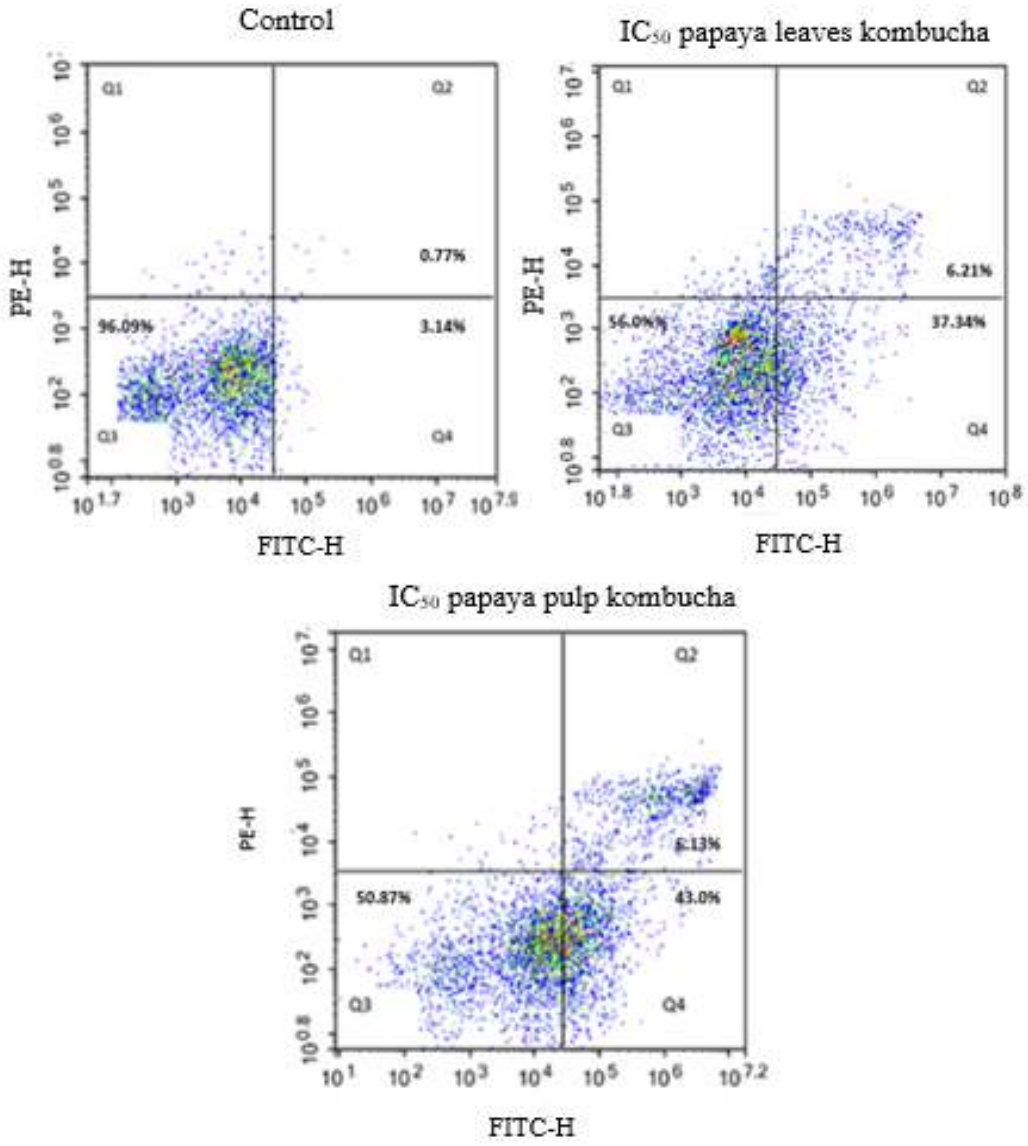


Figure 4.10a: Representative imaging of histogram analysis of Annexin V-FITC assay of HT 29 colon cancer cells after 72 h papaya kombucha-like beverage treatment. Note: the lower left quadrant of each group indicated the viable cells population; the lower right quadrant indicated the early apoptotic cells population; the upper right quadrant indicated the late apoptotic cells population; and the upper left quadrant indicated the necrotic cells population. Two fluorescent dyes were used in this assay; FITC (x-axis) and PI (y-axis).

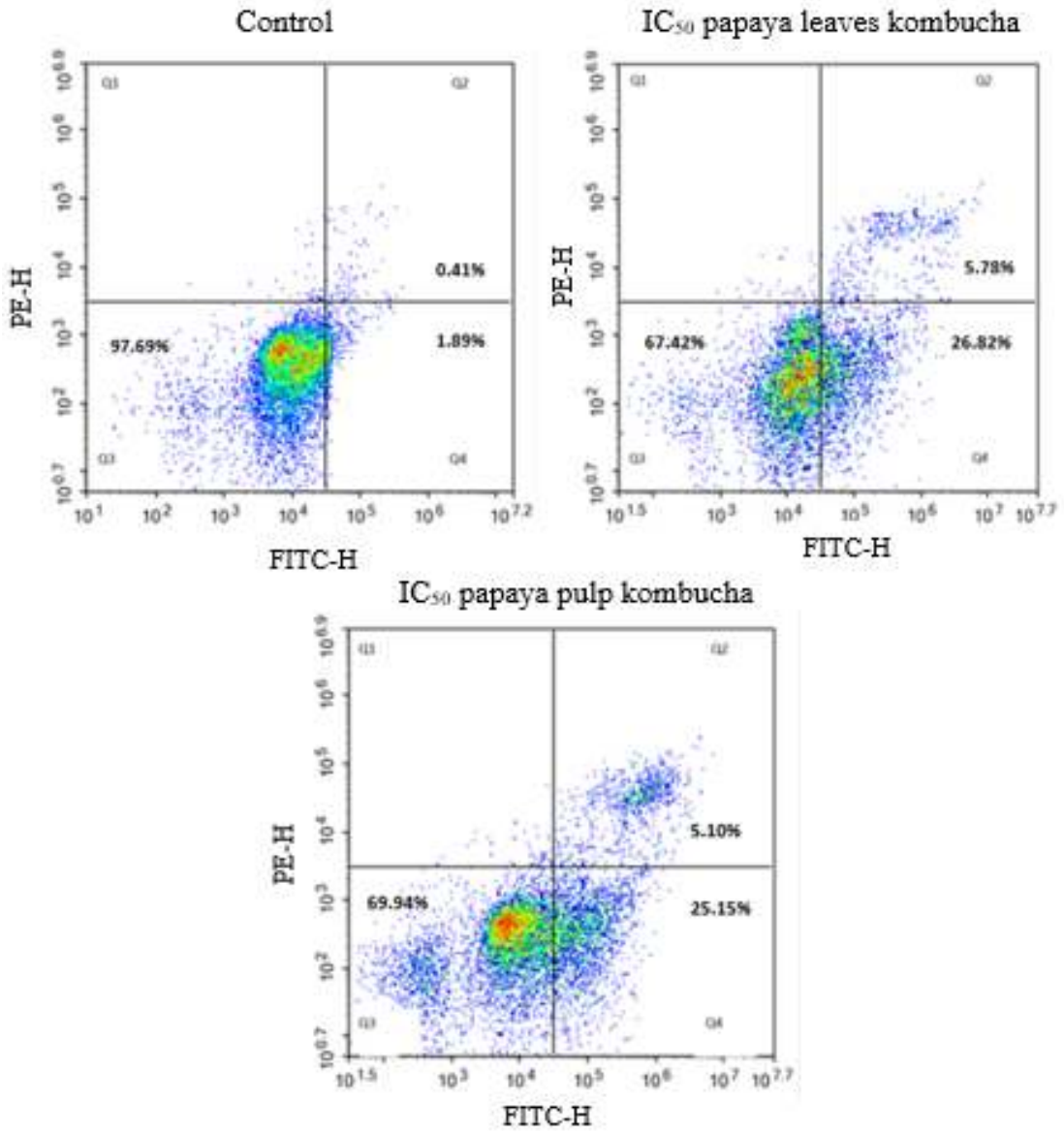
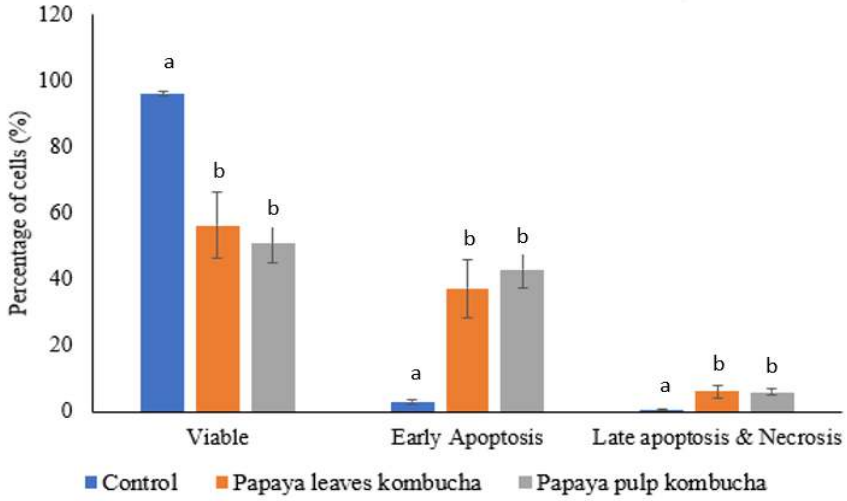


Figure 4.10b: Representative imaging of histogram analysis of Annexin V-FITC assay of SW 480 colon cancer cells after 72 h papaya kombucha-like beverage treatment. Note: the lower left quadrant of each group indicated the viable cells population; the lower right quadrant indicated the early apoptotic cells population; the upper right quadrant indicated the late apoptotic cells population; and the upper left quadrant indicated the necrotic cells population. Two fluorescent dyes were used in this assay; FITC (x-axis) and PI (y-axis).

In this study, the evaluation of apoptosis progress by Annexin V-FITC and propidium iodide staining in colon cancer cells treated with the desired concentrations of the kombucha beverages (IC_{50} concentration for both PLK and PPK) revealed a significant increase in early phase apoptosis rate. The present study has showed that, papaya based

kombucha has managed to demonstrate an early apoptosis activity in human CRC cell lines. Therefore, the findings have corresponded to the work conducted by Rasouli et al. (2022), which examined the effects of green tea kombucha on the HCT-116 colorectal cancer cell line. Rasouli et al. (2022) also reported that the presence of polyphenolic chemicals and organic acid are the component contributed to the anti-cancer activities.

(a)



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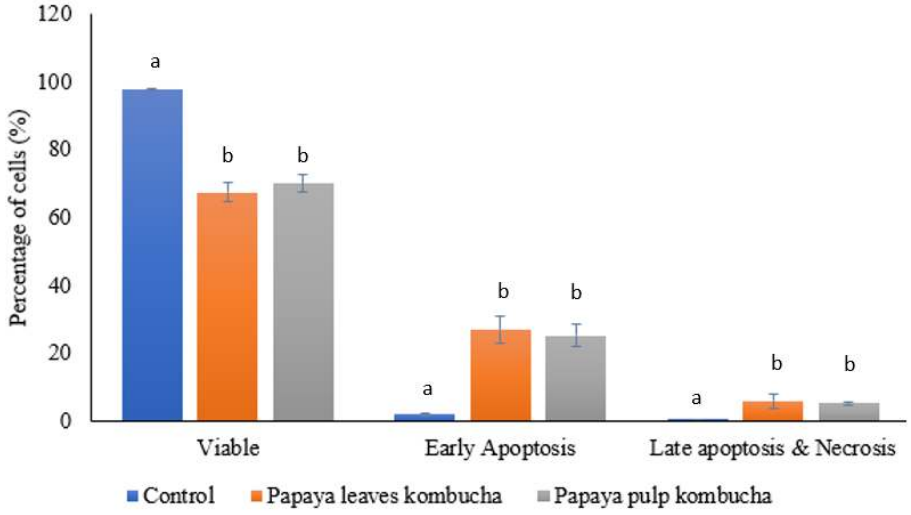


Figure 4.11: Percentage of viable, early apoptotic and late apoptotic cells population analysed using flow cytometer following the treatment with papaya kombucha (pulp and leaves) on (a) HT29 and (b) SW 480 colon cancer cells.

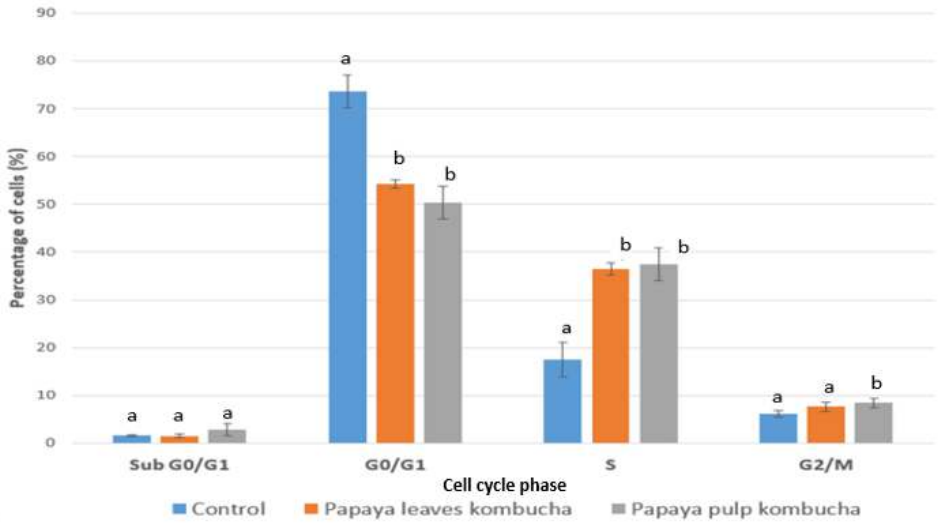
Values were expressed as mean standard deviations of 3 replicates. [a-d]Means with different letters were significantly different ($P<0.05$)

4.10.3 Cell Cycle Analysis

Flow cytometry was used to perform cell cycle analysis in order to investigate the impact of papaya kombucha treatment on the progress of HT29 and SW480 cell cycles, as depicted in Figure 4.12. In the case of both HT29 and SW480 cancer cells, the introduction of papaya kombucha treatment did not result in a significant increase in the G0/G1 phase, as compared to the control samples. In contrast, the proportion of cells during the S phase was shown to be greater in the group treated with kombucha supernatants compared to the control samples. In both HT29 and SW 489 cell lines, there is a significant increase in the proportion of cells during the S phase. The notable increased in the proportion of cells in the S phase indicated the ability to induce cell arrest and initiate processes that ultimately result in apoptosis. Therefore, treatment of kombucha derived from PPK and PLK effectively suppressed the proliferation of HT29and SW480 cells. To ensure that the integrity of cells is retained or survived, each cell needs a strict regulation of the cell cycle that drives them into multiple checkpoints in cell cycle stages and completes them before the cells divide in mitosis (Barnum & O'Connell, 2014). Once the cells are deemed damaged, this cycle may be stopped at any stage to stop the normal cells from developing into cancerous cells (Houtgraaf, Versmissen & van der Giessen, 2006). In this study, the growth and progression of HT29 and SW480 were arrested at S phase, respectively. According to Rasouli et.al (2021), kombucha tea has demonstrated an anti-proliferative impact on HCT-116 cancer cells. The inhibition of cancer cell development has been noted to be influenced by metabolites, specifically phenolic chemicals and organic acids (Lan et al., 2007; Zhang et al., 2022). The kombucha papaya beverage is known to have metabolites that are abundant in phenolics and organic acids. Additionally, a component of SCFA

(acetic acid) significantly influencing the pH and acidity levels in papaya based kombucha. Multiple studies have shown that SCFAs can caused apoptosis in CRC cell lines, these findings can enable new CRC treatments based on the manipulation of SCFA level via pH modulation and colon microbiota composition (Gomes et al., 2022; Matthews et al., 2012; Preto, 2013).

(a)



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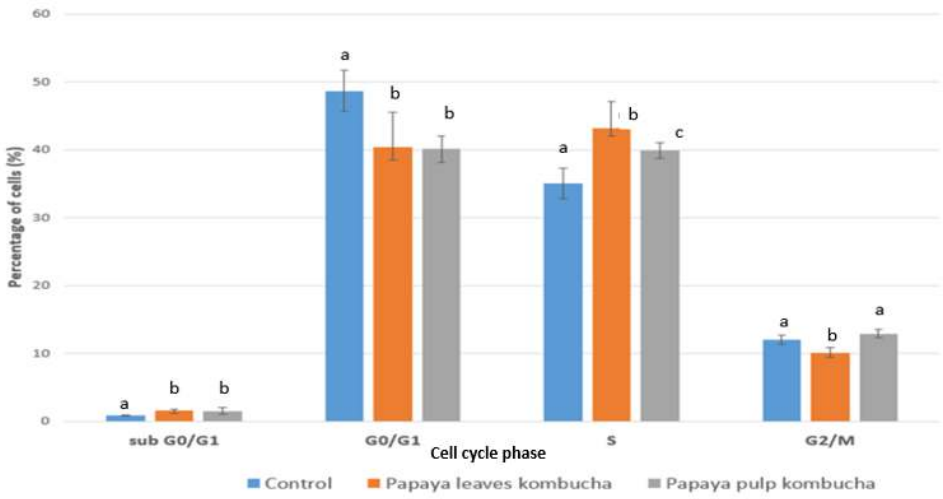


Figure 4.12: Percentage of cells population at different cell cycle phases analysed by flow cytometer for papaya kombucha (PPK and PLK) treated (a) HT29 and (b) SW480 cells. Values were expressed as mean standard deviations of 3 replicates. [a-d]Means with different letters were significantly different ($P<0.05$)

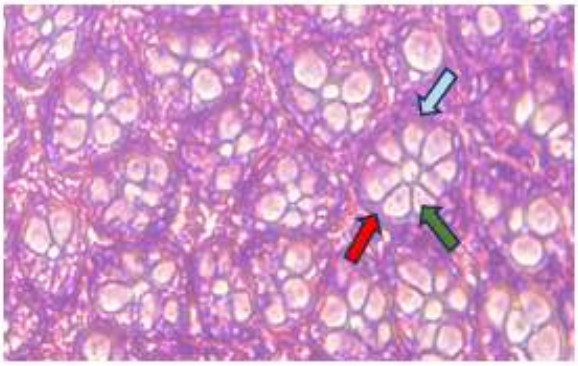
4.11 Pilot study for carcinogenesis induction with AOM/DSS

The tissue section of the colon stained with H&E is shown in Figure 4.14. The normal control group (Figure 4.13) showed a circular shape crypts and basal location of nuclei. A normal and healthy crypt structure is composed of a lumen and goblet cells in cytoplasm, surrounded by a thin layer of nuclei (Rivlin & Shimshoni, 2014). The azoxymethane (AMO) treated mice (Figure 4.13) exhibited elongated crypts and larger than normal cells, reduced in goblet cells and slight loss of cell polarity. These structural differentials have been also reported by several researchers (Almagrami *et al.* 2014; Jorge *et al.* 2014). Meanwhile, Figure 4.14 shows the opened distal region of colon, where several mucosal nodules could be clearly visualized with normal eyes. This observation could be an indication of adenocarcinoma progession as was noted by Metzger et al. (2019).

Colitis is the inflammation symptoms occur at the inner lining parts of colon tissues. This digestive disease is a potential risk factor which can lead to the development of colitis-associated colorectal cancer (CAC). Azoxymethane/dextran sulphate-sodium (AOM/DSS) animal model is commonly used in CAC research (Schepelmann et al., 2022). The use of animal models is good to study the biology of disease development. In addition, animal models allow the testing of hypothesis related to environmental factors to etiology and cancer prevention. AOM is claimed to develop tumours in subject's small intestine, causing liver pathological changes and development of different stages of cystic dilation of the intrahepatic bile ducts. DSS on the other hand is toxic to colonic epithelial cells and trigger epithelial barrier integrity which can lead to inflammatory response. Studies using AOM/DSS mouse model had indicated that colorectal cancer can be induced in a short-term period. In the experiment,

carcinogenesis on male ICR mice was first initiated by using a single intraperitoneal injection (IP) at the concentration of 10 mg/kg body weight. The subject was then being administed with 2% DSS in drinking water (DSS can be varied from 0.5-2.5%) for one week. Observation results showed that colorectal adenoma and adenocarcinoma were developed within three to four weeks time. AOM induced morphological changes associated with aberrant crypt foci (ACF) development and DNA damage in proliferated cells (Waly et al., 2014). ACF that was present in the colon of mice treated with carcinogens could be the possible precursor lesions for colon cancer and acted as a useful biomarker for detecting colon carcinogenesis (Kawabata et al., 2012; Tanaka, Makita & Kawabata, 1997).

(a)



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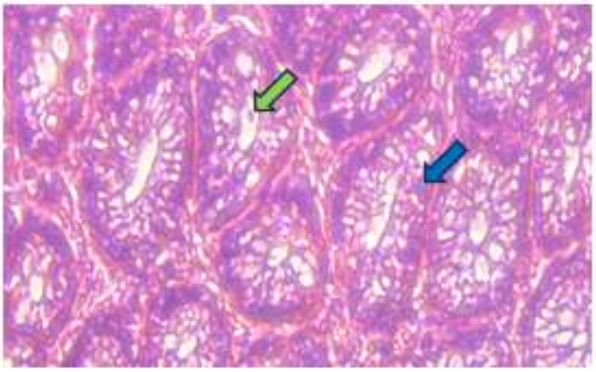


Figure 4.13: Effect of AOM on histological sections of AOM induced IC (a) normal colon mucosa, normal crypt structure (light blue arrow), thin layer of basal nucleus (red arrow), goblet cell (dark green arrow) (b) 10mg/kg AOM + 2% DSS treated colon mucosa, dysplasia aberrant crypt foci showing elongated crypt (Dark blue arrow) and neutrophil infiltration in crypts (green arrows)

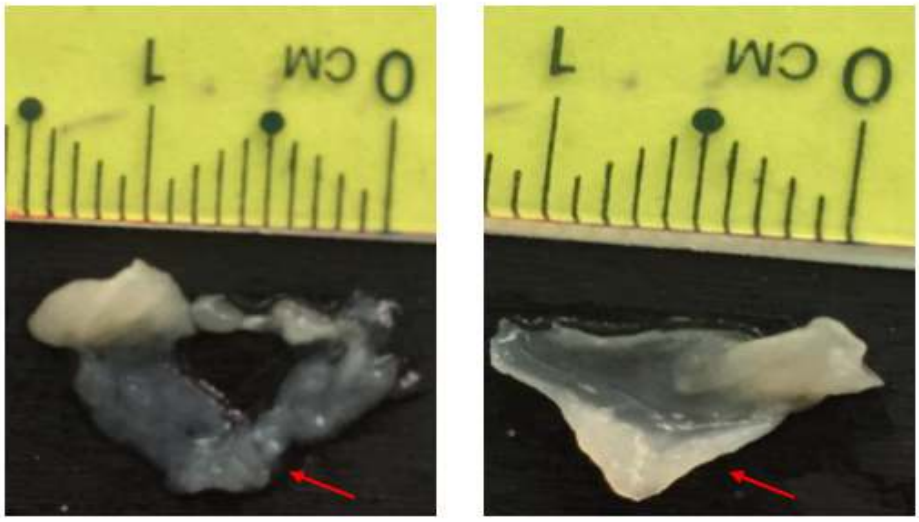


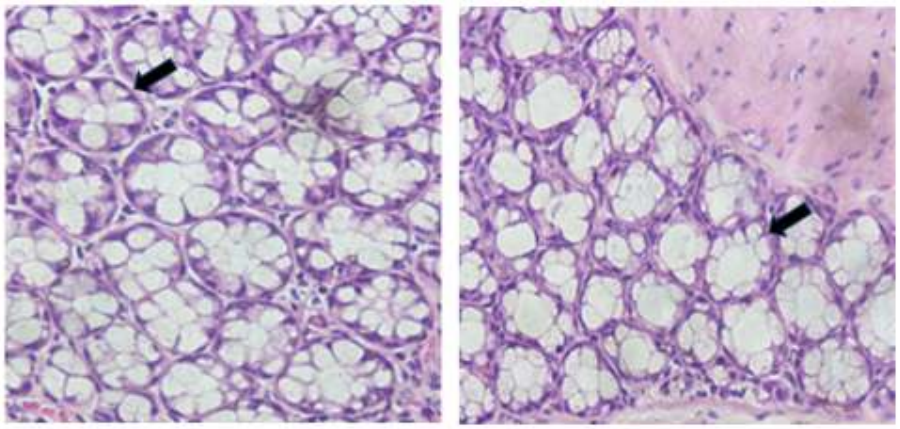
Figure 4.14: Open distal colon areas of mice, visible mucosal nodules (arrows)

4.12 Effects of papaya kombucha-like beverage on AOM/DSS -induced ICR mice *In Vivo*

The epithelial layer of the human colon is composed of a single sheet of columnar epithelial cells that generate finger-like intrusions into the underlying connective tissue of the lamina propria to form the crypt, the basic functional unit of the gut (Humphries & Wright, 2008). The continuous formation of new epithelial tissues is produced by colonic stem cells located in the basal part of the crypt. Metaplasia known as a change that can happen to one specific epithelial cell that is often observed when there is a chronic inflammation of the epithelial cells in the digestive tract. These alterations may lead to the formation of cancer. Ulcerative colitis is one of a chronic digestive illness that can cause severe inflammation throughout the colon. The occurrence of inflammation leads to the development of a specific form of intestinal metaplasia characterised by the predominant presence of goblet cells that produce mucin (Giroux & Rustgi, 2018). The mentioned epithelium possesses inherent genetic instability, resulting in the accumulation of mutational and epigenetic changes. These

modifications have the potential to induce dysplasia, characterised by the distortion of tissue architecture and cytology, ultimately culminating in the development of cancer (Giroux & Rustgi, 2018; Vanoli et al., 2023).

Figure 4.16 displays a series of photomicrographs of colon cells of AOM-induced ICR mice *in vivo* with different treatments. In Figure 4.15 (a), the mice on normal diet had circular shape crypts and basal location of nuclei. Figure 4.15 (b) shows AOM/DSS induced mice on normal diet with severe dysplasia. The histological examination of H&E colon samples reveals distortion of the crypts and neutrophils infiltration within the crypts. Furthermore, an increase in chronic inflammation within the lamina propria were also observed. Moreover, the colon mucosa exhibits the presence of crypt abscesses containing neutrophils. These findings suggest a potentially malignant condition known as adenocarcinoma. Next, same subjects with administration of low dose PPK showed mild to moderate dysplasia (Figure 4.15 (c)). Meanwhile, Figure 4.15 (d) showed same subject with alteration on the administration of high dose PPK with moderate to severe dysplasia. Subjects treated with low dose of PLK (Figure 4.15 (e)) showed moderate dysplasia and subjects treated with high dose of PLK (Figure 4.15 (f)) showed moderate to severe dysplasia respectively. The potential risk factor for the onset of CRC have been identified due to chronic inflammation. One of the assumptions that can be given to the formation of cancer caused by colon cell damage is the repeated destruction followed by the formation of epithelial cells. This repeated process will increase the probability of cell mutations. (Hu et al., 2010; Spit et al., 2018).



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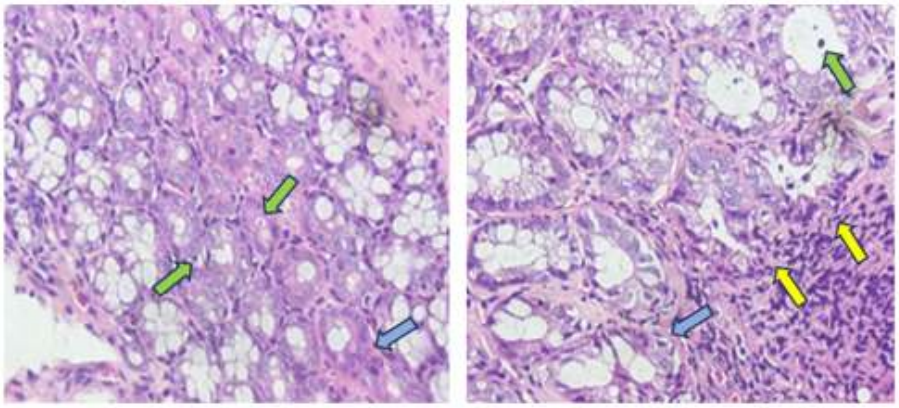

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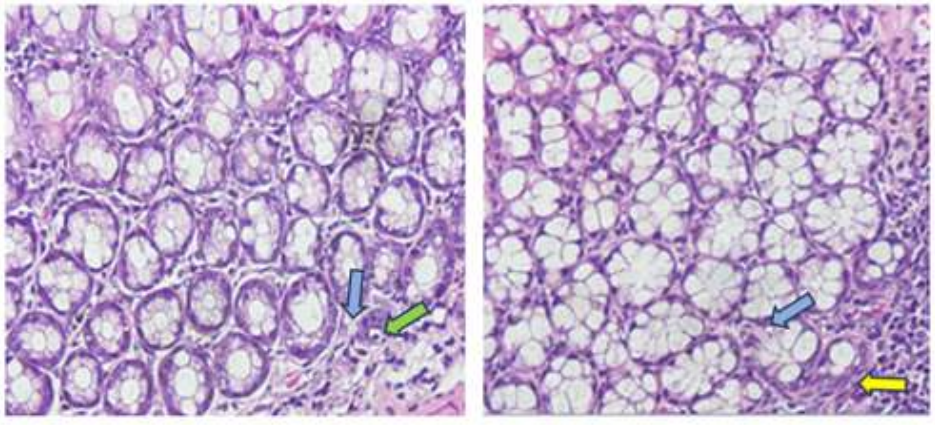

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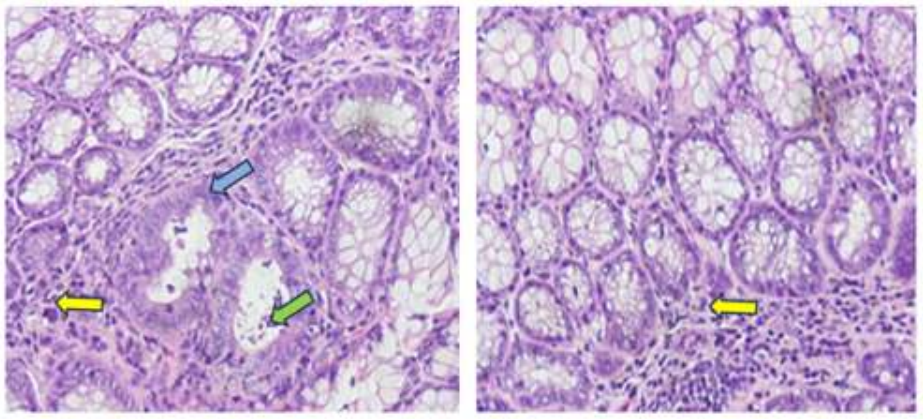

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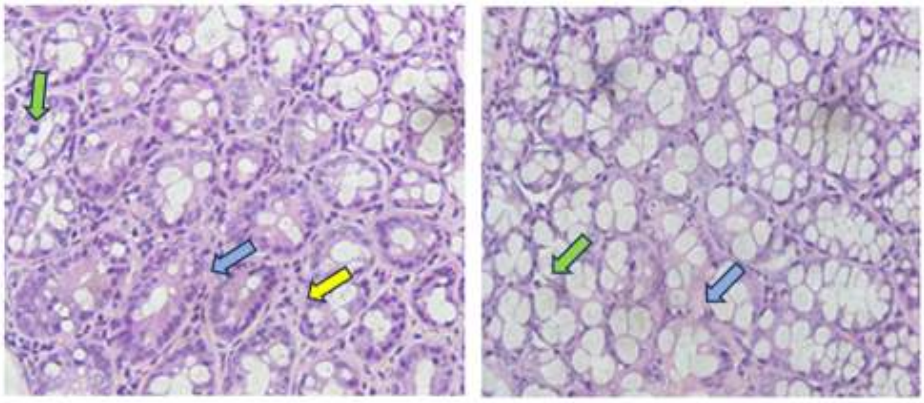

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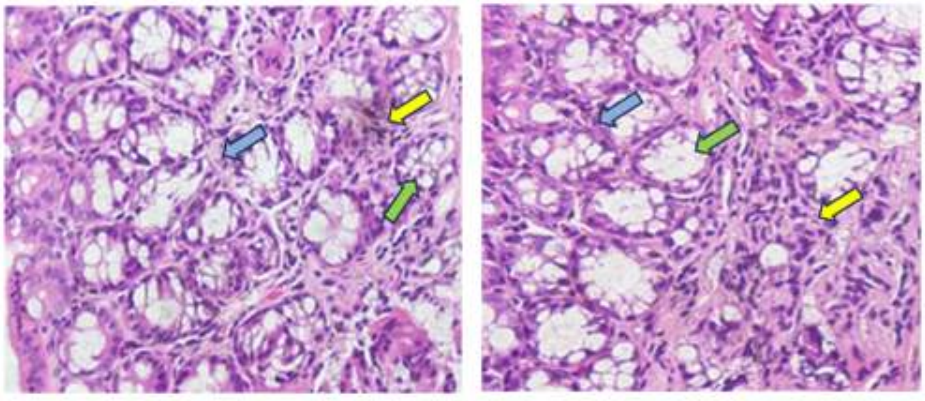


(f)

Figure 4.15: Photomicrographs of different colon cancer cells with different treatments. Colon histology showing normal crypt (black arrow), crypt distortion (blue arrows) and neutrophil infiltration in crypts (green arrows). Increased chronic inflammation within the lamina propria, arrows(yellow) denote crypt abscesses including neutrophils within colon mucosa (a) Normal mice; (b) AOM/DSS carcinogenesis with normal diet (c) AOM/DSS carcinogenesis with normal diet treated with low dose PPK (d) AOM/DSS carcinogenesis with normal diet treated with high dose PPK (e) AOM/DSS carcinogenesis with normal diet treated with low dose PLK and (f) AOM/DSS carcinogenesis with normal diet treated with high dose PLK.

Treatment of AOM/DSS on mice had shown development of potential adenocarcinoma within 20 weeks. When given twice daily consumptions of low dose (0.7mL/kg) papaya based-kombucha, a mitigating progress of adenocarcinoma in colon was observed (Figure 4.16 (c and e)), while the consumptions of high dose papaya kombucha at 1.8mL/kg twice daily showed probable aggravation in the progress of adenocarcinoma in the colon (Figure 4.16 d and f). study have shown that, by increasing the intracellular pH of colon through acute (but short duration) addition of ascorbic acid (vitamin C) to obtain low intracellular pH resulting in the cessation or prevent malignant growth. Most common facts in the development, proliferation and infiltration of cancer cells are due to maintaining elevated intracellular pH (pHi) together with a significantly lower extracellular pH (pHe) (Aldajaniet al. 2017). Notably, when colonic crypts were subjected to varying concentrations of vitamin C, distinct pH modulation patterns were observed, leading to a response that was dependent on the dosage administered (Aldajani et al. 2017; Ward et al., 2020). Pandey et al. (1995) established the risk of all cancers in the body being reduced when >113 mg/d Vitamin C was consumed, while Sahuri-arisoylu et al., (2021) observed that short chain fatty acid acetate reduced the viability of colon cancer *in vitro*. When high concentration of organic acid present, especially acetic acid, it may cause pH modulation within the intestinal environment may be possible due to the consumption of papaya kombucha beverages. Kombucha beverages can be the source of acetic acid which is one of the short-chain fatty acid (SCFA) that is important in maintaining a healthy gut (Roos & Vuyst, 2018). Moreover, SCFA can be produced in the gut through the metabolism of gut microbiota which involves the saccharolytic fermentation of dietary fibers such as starch, polysaccharides, and sugars not fully digested and absorbed (Nogal et al. 2021). SCFA such as acetate, propionate, and butyrate have an influential role in the maintaining and regulating the

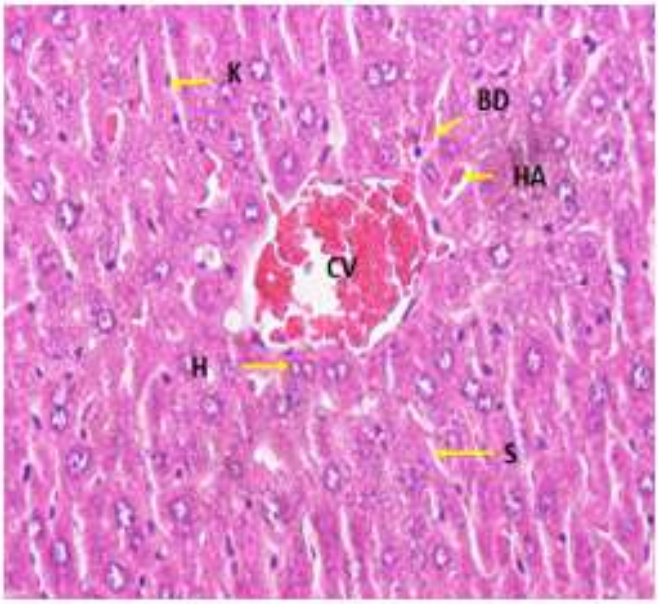
intestinal homeostasis. Their properties exhibit reactions within the intestinal mucosa by exciting intracellular and extracellular reactions that lessen the proliferation of pathogenic microorganisms, exert anti-inflammatory effects, enhancing functionality of epithelial barrier, modulation of gene expression in the epithelial cells to enhance their proliferation, induce apoptosis specifically in colorectal cancer cells while sparing healthy cells, and demonstrate anti-neoplastic effects (Al-qadami et al., 2022; Beena Divya et al., 2012; Venegas et al., 2019).

However, the extracellular environment's lower pH levels may encourage cancer cells' capacity for invasion and metastasis. Tumor cells provide most of the extracellular acidity due to increased synthesis of lactic acid and proton [H+] (Robey, 2012). The systemic acid-base balance may change over time because of dietary habits. There are no extensive studies linking diet induced acidity to cancer, but the effect of acidosis may cause cancer is still debated. Nevertheless, due the reactions from intracellular and extracellular pH triggers certain immunological and inflammatory mechanism, the connection between the two must not be ignored. One of the most typical anomalies in people with serious illnesses is metabolic acidosis. An increase in research has shown that even though various types of organic acid are set at the same pH, they still impact different immune and inflammation responses. (Nadai et al., 2013).

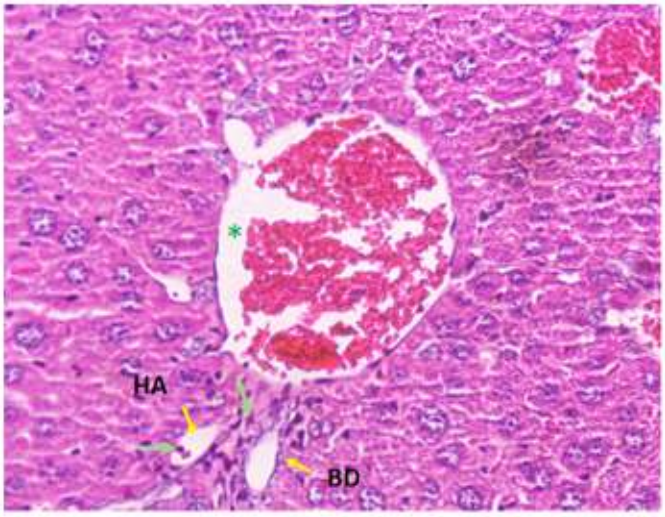
Beside producing anti tumor functions, the immune system also involved in the growth and the spread of tumor (Rumba et al., 2018). Changes in nutrition and food consumptions will have an effect in the increased rates of cancer incidence. Furthermore, meal combination may produce synergistic effects and can either enhance or lower the CRC risk (Pietrzyk, 2017). A high-fiber diet has been linked to lower rates

of CRC, which led to the hypothesis that diet plays a significant role in determining risk of the disease being developed roughly 50 years ago. Changes in food habits and body mass index have been identified as significant risk factors for CRC (Pietrzyk, 2017).

Meanwhile, Figure 4.16 (a-f) shows the photomicrographs of mice liver stained with H&E with different treatments. The photomicrographs of normal mice (Figure 4.16 (a)) display liver cells comprising central vein (CV), bile ducts (BD), hepatic artery (HA), sinusoids (S), hepatocyte (H) and Kupffer cells (K). Liver samples from mice induced with AOM/DSS and fed a normal diet showed possible symptoms of inflammation due to leucocyte infiltration into renal tubules, as well as possible signs of CV dilation (Figure 4.16 (b)). All additional samples (Figure 4.16 (c-f)) that were subjected to PPK and PLK administration displayed comparable morphologies, with observations made about potential indications of inflammation and CV dilation of the liver cells. AOM/DSS-induced colitis and inflammation, a kind of IBD, can lead to the development of crypt dysplasia and consequent liver problems. Inflammation has a pivotal role in the pathogenesis of various chronic liver disorders. The liver is also commonly recognised as the predominant site for distant metastasis arising from solid malignancies (Strathearn et. al 2020). Blood supply to the liver can be a factor in the occurrence of CRC metastasis. This is due to the confluence of blood vessels from the GI tract to the hepatic portal vein. Because of this, the blood vessel system has helped transfer CRC cells to the hepatic parenchyma and subsequently form CRC metastasis in the liver (Kamarajah & Christou, 2021).



(a)



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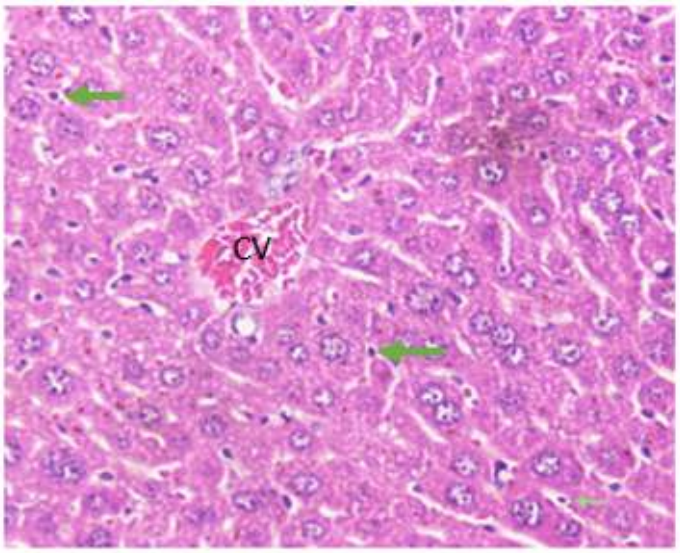

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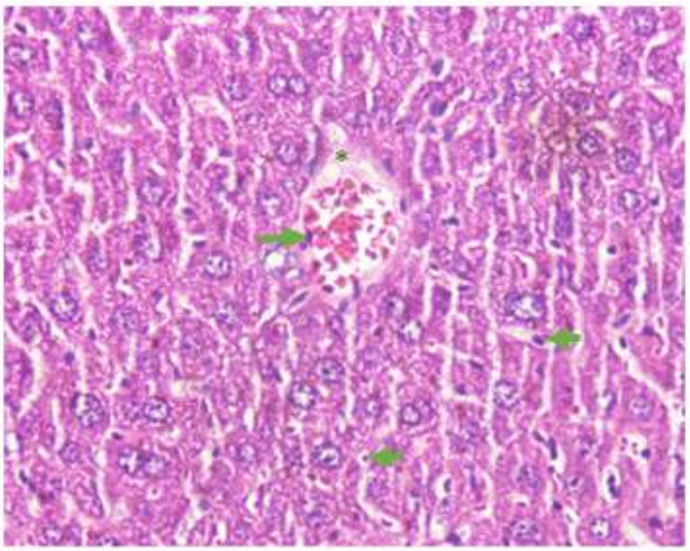

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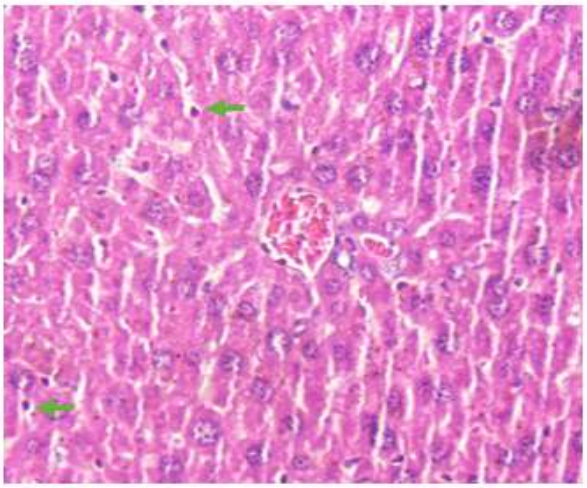

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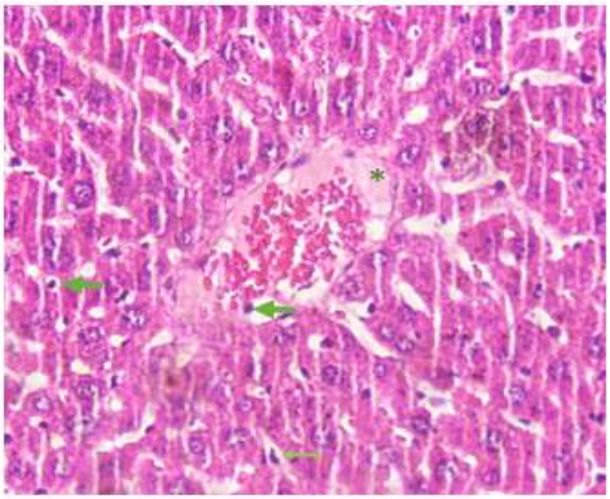


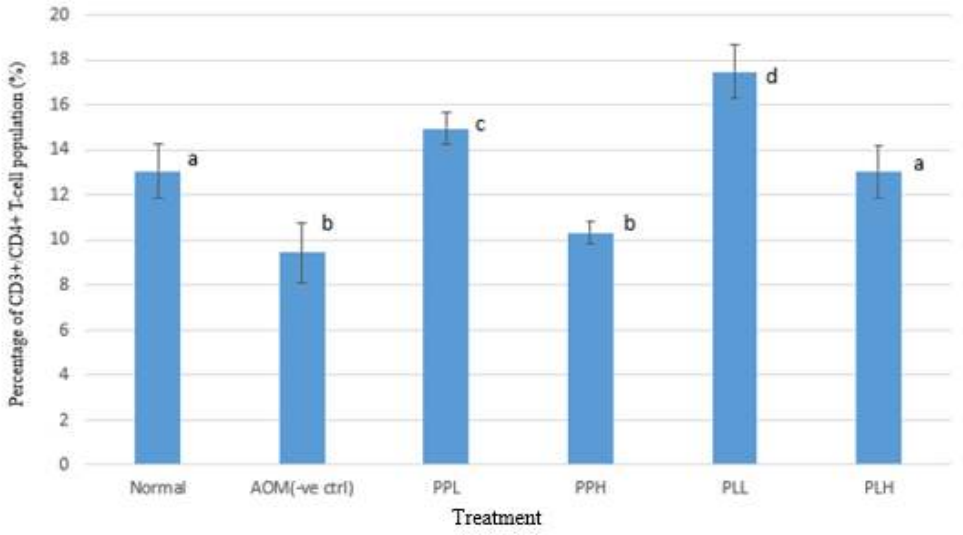
(f)

Figure 4.16: Photomicrographs of liver cells of (a) normal mice, (CV), bile ducts (BD), hepatic artery (HA), sinusoids (S), hepatocyte (H) and Kupffer cells (K) (b) induced mice on normal diet, Possible sign of inflammation (infiltration of leucocytes into renal tubules) , possible sign of central vein dilation * (c) induced mice on low dose PPK, possible sign of inflammation (infiltration of leucocytes)→ (d) induced mice on high dose PPK, possible → sign of inflammation due to the infiltration of leucocytes → , possible sign of vein dilation* (e) induced mice on low dose PLK possible sign of inflammation (infiltration of leucocytes) → (f) induced mice on high dose PLK, possible sign of inflammation (infiltration of leucocytes) → , possible sign of vein dilation *

4.13 Immunophenotyping of Spleenocytes

Immuophenotyping of spleenocytes was performed to investigate the effects of papaya based-kombucha in modulating important immune markers such as CD3+/CD4+ and CD8+/CD3+ T-cells. In Figure 4.17, the spleenocytes population of CD3+/CD4+ T-cell was decreased by 27.7% from normal mice to induced mice (negative control). However, significant increments were observed in both subjects treated with papaya pulp and papaya leaves from normal subjects at 14.4% and 33.7%, respectively. When papaya based-kombucha beverages were introduced, the values were reduced from their unfermented components and normal subjects. Meanwhile, the population of spleenocytes for CD8+/CD3+ T-cell steadily decreased in the following trend: normal subjects > induced-mice > papaya pulp > papaya leaves > PPK > PLK.

Overall, it was found that kombucha produced from papaya can affect the expression of T cell population CD3+/CD4+ and CD8+/CD3+ T. Haematopoietic stem cells from the bone marrow are the producers of the T cell population and undergo maturation process inside the thymus (Thomas, Wang & Su, 2020). Mice with lack of thymus would face deficiency of matured T-cells population. This leads to immunocompromised phenomenon in mice to allow the engraftment and growth of human HT29 and SW480 cancer cells. To explain the increments of T-cells when papaya supernatants were introduced to the mice, the CD3+/C4+ T-cells population had been propagated as a result of lymphocytes generation or lymphoiesis. The work of Hardy et al. (2005) had also reported similar findings.



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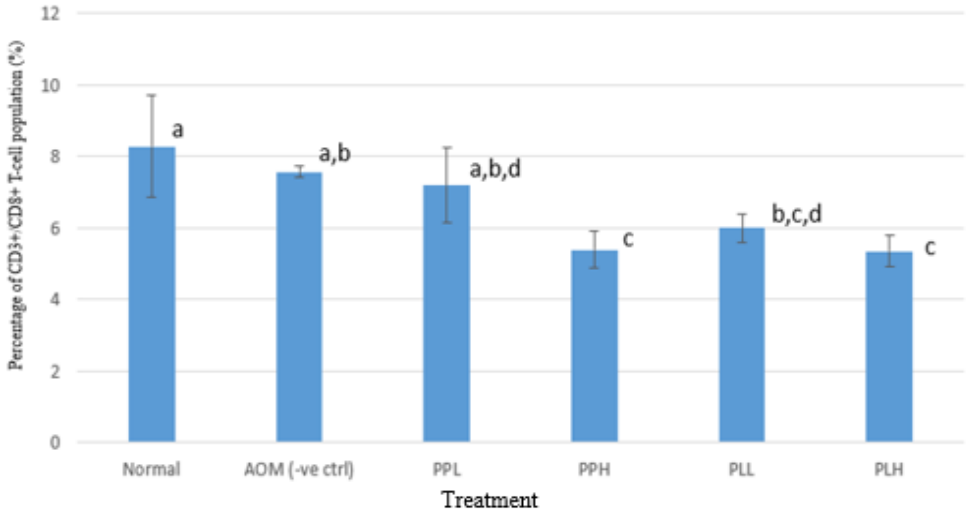

(b)

Figure 4.17: Percentage of CD3+/CD4+ T-cell population (%) from spleenoyctes of normal and treated mice (a); Percentage of CD3+/CD8+ T-cell population (%) from spleenoyctes of normal and treated mice (b) as analysed by flow cytometry.
Values were expressed as mean standard deviations of 3 replicates. [a-d]Means with different letters were significantly different ($P<0.05$)

Tumor-infiltrating lymphoctyes (TILs) can be determined by the ratio of CD4/CD8 T cells as the key parameter in tumor surveillance (Shah et al., 2011). Identification of these TILs contributes to better prognosis in many types of cancer, especially colon cancer. The infiltration of the tumor site with high numbers of CD8+ TILs would be

desirable. However, to have CD8+T cells functioning properly the presence of CD4+T cells is necessary. The ratios of CD4 to CD8 for all samples are showed in Figure 4.19. The increasing trend of CD4:CD8 ratio based on treatment is as follows: AOM-induced < PPK < papaya pulp < normal < PLK < papaya leaves. Overall, the ratios were heavily influenced by the values of CD4+ that were higher than CD8+. Supposedly, high CD8+ values and low ratio of CD4:CD8 could demonstrate higher infiltration of tumor to promote better prognosis and survival of the patients. The trend displayed inconsistent lymphoid infiltration to show possible significance as this was also issued by Diederichsen et al. (2003) due to small data sample. Nevertheless, significant reverse relationships between survival and CD4:CD8 ratio have been reported (Diederichsen et al., 2003; Shah et al., 2011).

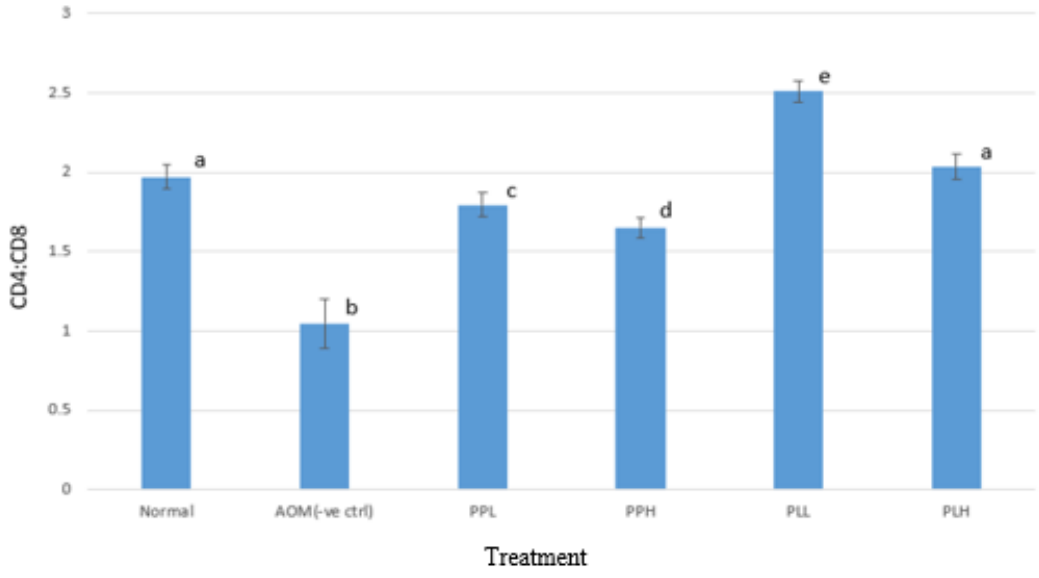

Figure 4.18: CD4:CD8 ratios of spleenoyctes from normal and treated mice as analysed by flow cytometry

Values are expressed as mean standard deviations of 3 replicates. [a-d]Means with different letters were significantly different (P<0.05)

4.14 Microbiota Profiling

Majority constituents of the microbial community in the gastrointestinal (GI) tract would be *Firmicutes* and *Bacteroidetes*. They both primarily reside in the mucosal lining of the digestive tract, predominantly within specific regions of the intestinal system. On the other hand, lowering the abundance of various bacterial phyla, particularly *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*, is crucial for keeping the GI system in a good health. This clarification emphasizes that these two assemblies of microorganisms exist not solely in the intestines but inhabit precise regions within this organ. Dysbiosis, signifying disturbances in the microbial constitution, holds the potential to negatively affect the host's physiological state. Such anomalies are frequently detected during periods of prolonged tension or in particular gastrointestinal ailments like inflammatory bowel disease. Notably, the changes of the microbiota composition might yield interferences on the progression of a colorectal cancer (CRC).

In this study, the effects of papaya kombucha consumption were examined concerning the microbial composition of fecal specimens from mice. This encompassed both control and samples from mice subjected to AOM/DSS treatment. AOM/DSS is a substance recognized to induce gastrointestinal disorders. Through genetic analysis, alterations in the microbial constitution were discerned. Manifestly, in mice exposed to AOM/DSS treatment while adhering to a typical diet, a decrease in Bacteroidetes and an augmentation in Firmicutes were perceived. This is frequently correlated with an effectively functioning and robust gastrointestinal system. Irrespective of papaya consumption in either cohort, the shift in bacterial equilibrium was evident in both the control and AOM/DSS-treated groups. Liu et al. (2022) reported that mice suffered

from adenocarcinomas would have notable changes in their intestinal bacterial profiles. Particularly, fluctuations of Firmicutes and Bacteroidetes can be both found in cancerous and non-cancerous mice, where they proliferated faster and grow slower in cancer cells, respectively (Li et al., 2022; Liu et al., 2022; C. Yu et al., 2022).

Table 4.8: Composition of taxonomy (Phyla) in normal control mice, AOM/DSS carcinogenesis with normal diet treated and with AOM/DSS carcinogenesis with designated papaya kombucha.

Tax Name	Control (%)	AOM/DSS (%)	PLH (%)	PLL (%)	PPL (%)	PPH (%)
Actinobacteria	0.06± 0.04^{f}	0.09± 0.01^{f}	0.18± 0.11^{f}	0.16± 0.06^{f}	0.44± 0.33^{f}	0.08 ±0.04^{f}
Bacteroidetes	70.49± 6.18^{a}	57.11±5.17^{bc}	58.75± 2.99^{bc}	65.52± 3.04^{ab}	69.04± 4.50^{a}	55.03± 5.21c
Candidatus Saccharibacteria	0.50± 0.22^{f}	1.53±0.63^{f}	1.57± 0.59^{f}	1.18± 0.05^{f}	0.64± 0.17^{f}	1.54 ±1.43^{f}
Firmicutes	23.23± 4.01^{e}	36.50±6.17^{d}	31.93± 5.42^{de}	24.49± 2.00^{e}	24.95± 5.23^{e}	38.75± 5.11^{d}
Proteobacteria	5.40± 2.40^{f}	4.65±1.46^{f}	7.34± 1.93^{f}	8.40 ±3.73^{f}	4.76± 0.56^{f}	4.42± 1.08^{f}
Tenericutes	0.23± 0.07^{f}	0.04±0.03^{f}	0.00± 0.00^{f}	0.08± 0.02^{f}	0.12± 0.06^{f}	0.11± 0.06^{f}

Values were expressed as mean standard deviations of 3 replicates. $^{a-f}$Means with different letters were significantly different ($P<0.05$)

Gut bacteria contribute to the development of CRC primarily via two mechanisms: (1) disruption of the bacterial balance, leading to changes that facilitate cancer development by modifying the gut environment, inducing inflammation, and altering cells in a manner conducive to cancer; and (2) aligning with the "driver-passenger" theory, certain gut bacteria act as "bacterial drivers," initiating CRC by inflicting DNA damage and affecting the immune system.

Significant variations in the microbial population were notably observed at the genus taxonomic level. A substantial imbalance was evident in the genera *Alloprevotella, Bacteroides, Barnesiella, Lachnoclostridium*, and *Prevotella* when compared to the microbiota of mice that did not receive DSS/AOM injections (Control), as shown in Table 4.9.

Table 4.9: Composition of taxonomy (Genus) in normal control mice, AOM/DSS carcinogenesis with normal diet treated and with AOM/DSS carcinogenesis with designated papaya kombucha.

	Control	AOM/DSS	PLH	PLL	PPL	PPH
Alloprevotella	13.19± 1.15%^{def}	4.55± 1.96%^{ghijk}	4.02± 0.96%^{ghijk}	2.97± 1.39%^{hijk}	1.90± 0.69%^{ijk}	2.57± 1.66%^{hijk}
Bacteroides	26.45± 2.82%^{ab}	8.15± 2.19%^{fghi}	8.70± 3.42%^{fgh}	7.46± 1.17%^{fghij}	9.84± 1.54%^{ij}	5.62± 1.12%^{ghijk}
Barnesiella	13.99± 1.79%^{ef}	17.91± 0.69%^{cd}	22.59± 4.67%^{bc}	30.29± 1.05%^{a}	21.12± 1.61%^{bc}	18.86± 0.79%^{cd}
Lachnoclostridium	10.29± 1.75%^{efg}	18.53± 4.88%^{cd}	13.37± 2.21%^{def}	9.86± 1.32%^{fg}	10.03± 3.39%^{fg}	16.90± 3.73%^{cde}
Prevotella	0.29± 0.11%	1.15± 0.27%^{j}	0.96± 0.04% ^{j}	1.84 ±0.61%^{ij}	1.06 ±0.02%^{j}	1.28 ±0.39%^{j}

Values were expressed as mean standard deviations of 3 replicates. $^{a-f}$Means with different letters were significantly different ($P<0.05$)

Prevotella distribution is elevated in all AOM/DSS-treated animals compared to the normal controls. Similar discoveries were reported in a study by Gao et al. (2020), revealing that *Prevotella* is one of the genera linked to microbiota imbalances within the epithelial cell layer in patients with CRC. Furthermore, mice subjected to AOM/DSS treatment exhibited a significantly elevated concentration of the *Barnesiella* genus compared to untreated mice. Conversely, the genus *Alloprevotella* exhibited a decrease in abundance. The progression of mucosal intestinal injury in mice has been associated with dysbiosis of both *Barnesiella* and *Alloprevotella* (Wang et al., 2021). The prevalence of the *Lachnoclostridium* genus in the AOM-induced sample aligns

with previous research reporting an increase in its abundance in an animal model exhibiting illness (Cai et al., 2022). *Bacteroides* quantity significantly decreased, nearly by five-fold.

Bacteroides spp., the native bacteria found in the GI system, provide a variety of tasks, including disease protection and nutrition for other microorganisms (Zafar, 2021). The progression of CRC can be triggered by the bacterial engagement with the immune system that produces harmful metabolites (Yu et al., 2022; Yu, 2018). Typically, bacteria within the GI tract aid in the synthesis of short-chain fatty acids (SCFAs). This results in the amplified amounts of SCFAs in the system. As demonstrated by Al-Qadami et al. (2022) and Gomes et al. (2023), the increased levels of SCFAs are correlated with various beneficial health outcomes, including (1) improved differentiation of immune cells, (2) reduced production of inflammatory mediators, (3) inhibition of tumour-induced blood vessel formation, (4) preservation of basement membrane integrity, and (5) pH regulation in the GI tract.

Cultures in fermented foods produce metabolites advantageous to their human host and have thus been associated with human health benefits. Fermented foods can improve health through one or a combination of the following: i) natural nutritional composition, including bioactive chemicals resulting from fermentation; ii) supply of essential nutrients to support gut microbiota proliferation; iii) potential probiotic fermented food bacteria resisting stomach acid, colonizing the intestinal tract, or suppressing or competing with resident gut microbes (Mathur et al., 2020). The potential influence of papaya-based kombucha might be affected by the methods. Regulating pH and acidity levels in the intestine can have diverse beneficial effects, such as maintaining balanced

extracellular and intracellular pH to limit pathogenic microorganism growth (Koh et al., 2017). Additionally, it can affect gene expression in epithelial cells, promoting proliferation, enhancing epithelial barrier functionality, improving nutrient absorption, exhibiting anti-inflammatory properties, and selectively inducing apoptosis in colorectal cancer cells without harming healthy cells (Al-qadami et al., 2022; Beena Divya et al., 2012; Venegas et al., 2019). In the context of gut microbes, enzymatic degradation of phenolic compounds induces structural modifications, leading to antioxidant properties expression. This phenomenon significantly contributes to slowing down cellular aging mechanisms and enhancing the body's ability to resist various illnesses. Moreover, the gut microbiota's breakdown of dietary fiber can lead to the production of SCFAs. This will influence the gut environment, colon functions, provide energy to both host cells and the intestinal microbiota, and participate in various host-specific signaling systems (Ros-Covián et al., 2016). The yeast used in kombucha-making processes has the potential to exhibit a probiotic effect due to its tolerance to pH and bile salts (Sharifudin et al., 2021).

Overall, different ingredients can promptly influence kombucha drinks by affecting microbial growth, metabolite production, and overall functionality (Nyhan et al., 2022). In this research, a significant improvement in gut flora dysbiosis was observed after twenty weeks of consuming papaya-based kombucha, even at a low concentration. This indirectly validates one of the health advantages of consuming papaya kombucha beverages.

CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Summary of Research

Papaya based-kombucha beverages were successfully prepared in this study using papaya pulps and papaya leaves. A series of analyses was conducted to evaluate the proximate analysis, antioxidants properties and antibacterial activities of these novel kombucha beverages from Sekaki papaya. Next, to demonstrate the nutraceutical elements of these beverages, anticancer activities were examined *in vitro* and *in vivo*.

First, from the kombucha starter culture maintained at MARDI's food laboratory, two strains of culturable microorganisms, an AAB (*K. rhaeticus* MFS1) and a yeast (*D. bruxellensis* MFS1) were isolated and identified. The safety of the fermentation processes can be managed to prevent the contamination of harmful microorganisms using this defined starter in conjunction with shaking culture (for greater soluble oxygen content). The findings also showed that, based on its low pH and bile acid tolerance, *D. bruxellensis* MFS1 demonstrated better potential probiotic characteristics than *K. rhaeticus* MFS1.

Both papaya pulp kombucha (PPK) and papaya leaves kombucha (PLK) significantly lower pH and total soluble solids values during fermentation than their unfermented counterparts. The ethanol concentrations in papaya PPK and PLK grew quickly after four days of fermentation, reaching their greatest levels on day two. On the fourth day of fermentation, the accumulated ethanol concentration for PPK was 0.04% after being

initially discovered at 0.95%. Similar to PLK, the ethanol concentration there gradually rose to 1.18% before falling to 0.10% on the fourth day of fermentation. On the other hand, it was found that the levels of acetic acid in both papaya kombucha juices steadily grew, reaching 1.62% (PLK) and 1.45% (PPK) at the end of day four.

Next, fermentation using kombucha cultures produced significant changes in organic acids composition. Organic acids such as acetic acid, citric acid, malic acid, kojic acid and quinic acid were detected higher in both PPK and PLK than their respective kombucha samples, except for oxalic acid. However, total phenolic content and total flavonoid content for both PPK and PLK were decreased as a result of metabolic activities of kombucha cultures. Nevertheless, all samples had exerted antioxidant activities against free radical DPPH. The PLK had the highest antioxidant activity against DPPH with 89.60% (an increase of 20% from its non-fermented juice), while PPK showed a slight increase of 3.7%, from 52.90% in the non-fermented form to 54.95%.

Besides, the antibacterial activities of PPK and PLK supernatants were tested against several pathogenic bacteria. Neutralised cell free supernatants from both PPK and PLK showed no inhibition zones against the pathogenic strains examined. Increased antimicrobial effects by PLK and PPK compared to acetic acid solution (0.7%) were due to their higher concentration of acetic acid composition in the designated papaya kombucha (1.5%). Organic acids as the main metabolites in kombucha beverages had contributed to the *in vitro* antimicrobial effects.

Other than that, anticancer properties were also evaluated based on their cytotoxicity effects. Two colon cancer cell lines; HT29 and SW 480 were selected. The responses of HT29 and SW 480 colon cancer cells towards the increased concentrations of both PPK and PLK were exponential. PPK showed slightly better anticancer activities than PLK. This was in line with lower pH value of PPK and PLK to indicate higher acidity to impose anticancer action. The presence of acetic acid was mainly responsible for the anticancer properties of kombucha beverages. Based on Annexin V FITC assay, both treated HT29 and SW480 shared similar cell population percentage pattern shift from viable cells to early apoptotic, then to the late apoptotic cell. Cell cycle analysis using flow cytometry was conducted to examine the effects of papaya kombucha treatment on HT29 and SW480 cell cycle progression. Conclusively, kombucha treatment from PPK and PLK inhibited the transition of the HT29 and SW480 cancer cells.

When compared to normal control mice, mice with AOM/DSS carcinogenesis displayed significant dysbiosis in their gut microbiota when fed a normal diet and treated with papaya kombucha. Alterations in the intestinal phyla of Bacteroidetes and Firmicutes which make up the majority of gut microbiota were significantly difference. These two phyla often dominate the intestines of healthy and diseased subjects. This study showed more evidence that adenocarcinoma growth inhibited *Bacteroidetes* proliferation while increased *Firmicutes* growth. It was also discovered that at low doses, both PPL and PLL were capable of restoring microbiota levels to those of the control group. This demonstrates that at low doses, both kombuchas are capable of restoring the microbiota phyla to normal levels. This response could be due to pH balancing in the stomach as well as microbial fermentation of kombucha fibre to SCFAs that can regulate the gut environment and hence have a health effect on the host. The

consumption of papaya-based kombucha has the potential to offer many health advantages due to the presence of natural metabolites. Through the process of direct and systematic absorption, the well-being of the host can be acquired. While, microbiota in the gut react symbiotically because it is able to have a significant effect on increasing the bioactivity of phytochemicals and metabolites. The composition and diversity of the microbiota is also affected by the metabolites, therefore the microbiota and metabolites will manifest various affect including antioxidant, anti-inflammatory, anticancer and various metabolic pathways in the intestine.

5.2 Future Recommendations

There are some limitations associated with the study that has been conducted. The experiment did not include the testing of a positive control, such as a normal colon cell line, with the other samples. This may raise concerns regarding the potential toxicity of the substance to non-cancerous cells. However, the study conducted by Koh et al. (2021) demonstrated that the administration of papaya kombucha to ICR mice being treated for obesity did not exhibit any signs of toxicity. The administration of a low dosage papaya kombucha may mitigate the advancement of colon cancer. However, to establish the changes obtained are from the consumption of kombucha or the result of the SCFA reaction from the gut microbiota is difficult and a challenging task. If viewed from the biotic market segment, products such as probiotics, prebiotics, synbiotics and postbiotics are to promote the health of the intestinal digestive system by taking into account the balance of the intestinal environment. This equilibrium is particularly crucial for maintaining pH balance, which significantly influences the composition of microbiota, ion transportation, and immune response. Since papaya kombucha is a drink product rich in polyphenolic compounds and organic acids, a study of its use

together with anticancer medication should be conducted. The study can aim to determine the possibility to increase anticancer drugs activity and at the same time reducing the side effects of the treatment.

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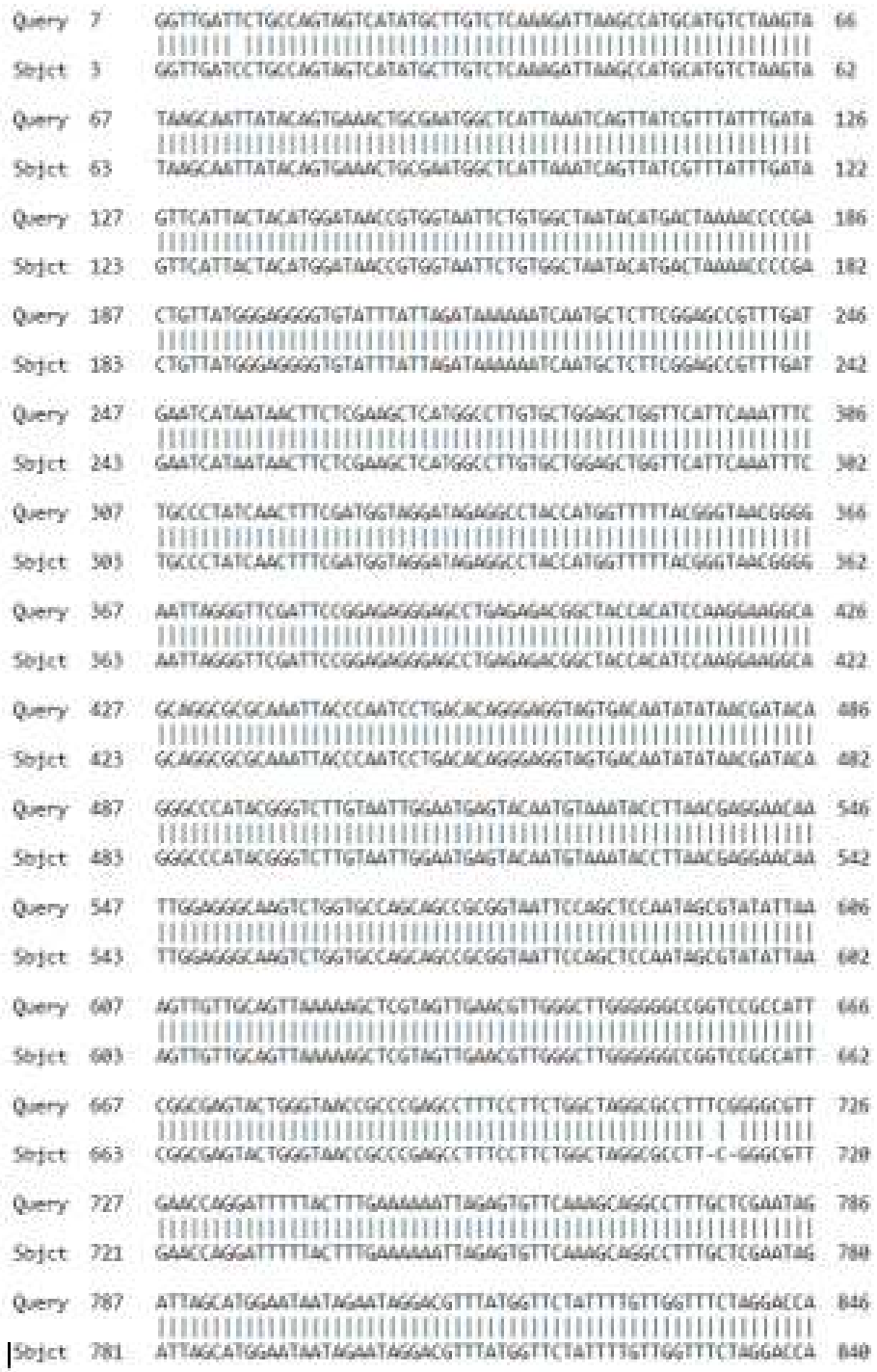
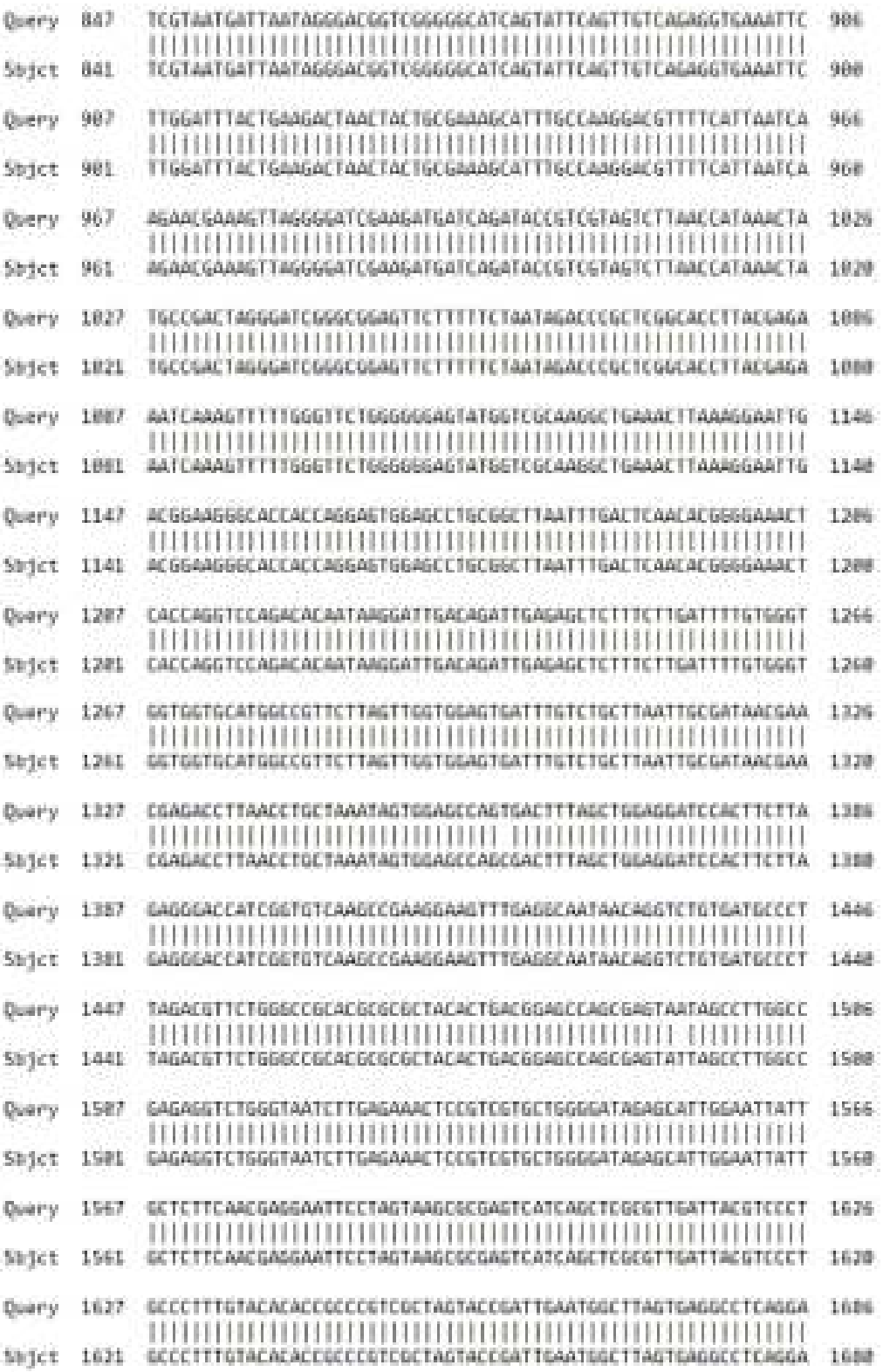
Appendices

Appendix A

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Dekkera bruxellensis 18S rRNA gene, ITS1, 26S rRNA and 5S rRNA genes (rDNA tandem repeat 1), and partial 18S rRNA gene (rDNA tandem repeat 2), strain C	3191	3191	99%	0.0	99%	AM850955.1
Dekkera bruxellensis 18S rRNA gene (NCYC 362)	3177	3177	99%	0.0	99%	X83814.1
D.bruxellensis gene for 18S ribosomal RNA	3149	3149	99%	0.0	99%	X58052.1
Dekkera anomala 18S rRNA gene (NCYC 749)	3144	3144	99%	0.0	99%	X83816.1
Dekkera bruxellensis 18S rRNA gene (NCYC 370)	3137	3137	98%	0.0	99%	X83815.1
Dekkera anomala 18S rRNA gene (CBS 8139)	3131	3131	99%	0.0	99%	X83820.1
Dekkera anomala 18S rRNA gene (NCYC 615)	3126	3126	99%	0.0	99%	X83828.1
Dekkera bruxellensis strain NRRL Y-12961 18S ribosomal RNA gene, partial sequence	3124	3124	97%	0.0	99%	JQ898898.1
Dekkera anomala 18S rRNA gene (NCYC 449)	3117	3117	98%	0.0	99%	X83818.1
Brettanomyces anomalus strain NRRL Y-17522 18S ribosomal RNA gene, partial sequence	3106	3106	97%	0.0	99%	EF550396.1

Figure A1: Multiple sequence alignment (BLASTN) producing significant alignment to *Dekkera bruxellensis*

Appendix B


Query 1 GACTTCACCCCCAGTCGCTGACCCGACCGTGGTCGGCTGCGTCCTTGCGGTTCGCTCACC 60
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Sbjct 2642562 GGCTTAAGGTCAAACCAAC-TCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC 2642620
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Sbjct 2642621 GTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCACCTTCATGCACTCGAGT 2642680
Query 181 TGCAGAGTGCAATCCGAACTGAGACGGCTTTTTGAGATCGGCTCGGTGTCACCACCTGGC 240
Sbjct 2642681 TGCAGAGTGCAATCCGAACTGAGACGGCTTTTTGAGATCGGCTCGGTGTCACCACCTGGC 2642740
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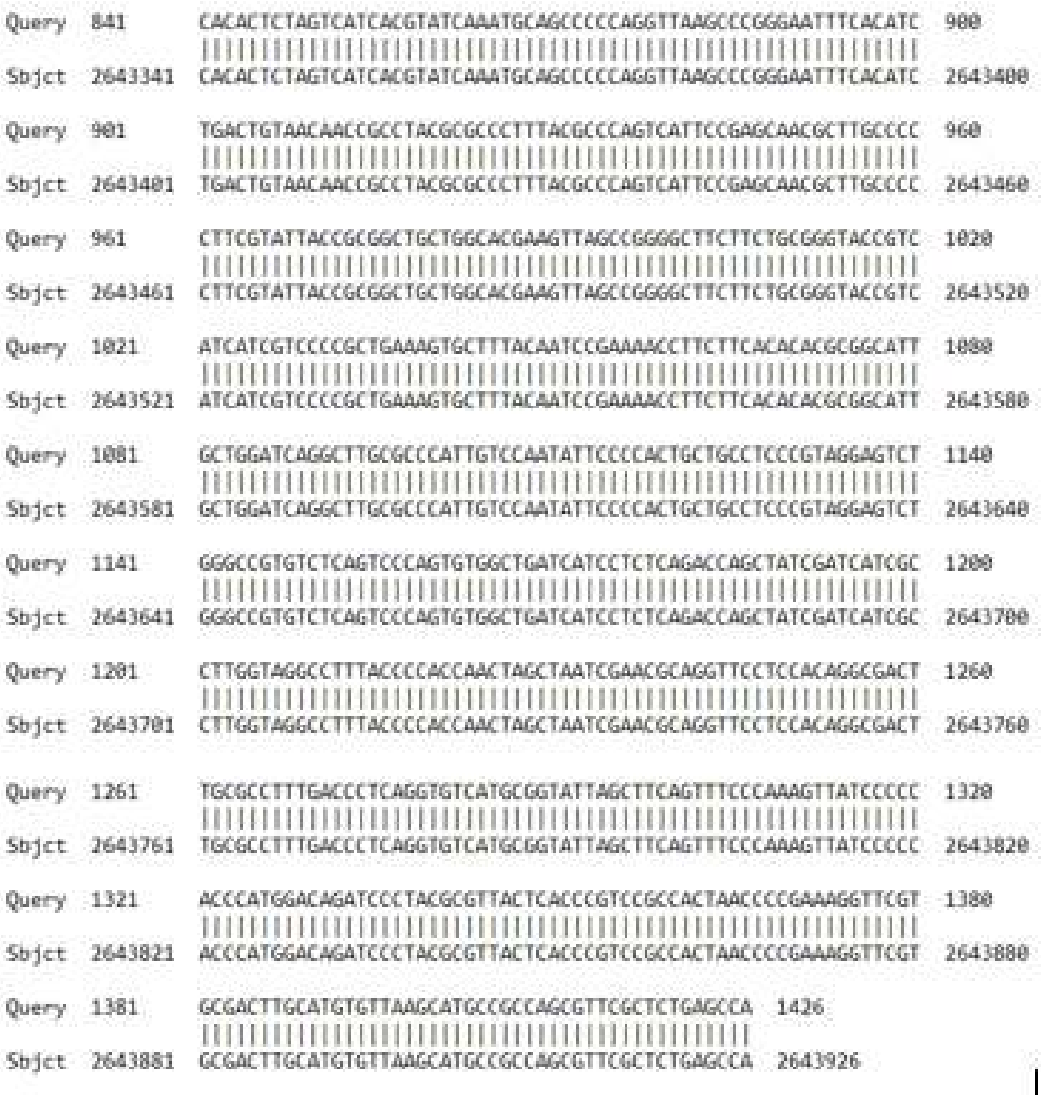

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☑	Komagataeibacter rhaeticus strain JCM 17122 16S ribosomal RNA gene, partial sequence	2535	2535	98%	0.0	99%	NR_113396.1
☑	Komagataeibacter intermedius strain TF2 16S ribosomal RNA gene, complete sequence	2520	2520	99%	0.0	99%	NR_026435.1
☑	Komagataeibacter xylinus 16S ribosomal RNA, complete sequence	2515	2515	99%	0.0	99%	NR_121785.1
☑	Komagataeibacter oboediens strain LTH 2460 16S ribosomal RNA gene, partial sequence	2515	2515	99%	0.0	99%	NR_041295.1
☑	Komagataeibacter oboediens strain LTH2460 16S ribosomal RNA gene, complete sequence	2515	2515	99%	0.0	99%	NR_114683.1
☑	Komagataeibacter europaeus strain DES11 16S ribosomal RNA gene, partial sequence	2511	2511	100%	0.0	99%	NR_026513.1
☑	Gluconacetobacter xylinus NBRC 3288 strain NBRC 3288 16S ribosomal RNA, complete sequence	2507	2507	99%	0.0	99%	NR_074338.1
☑	Komagataeibacter europaeus strain DSM 6160 16S ribosomal RNA gene, partial sequence	2507	2507	99%	0.0	99%	NR_112539.1
☑	Komagataeibacter sucrofermentans strain BPR 2001 16S ribosomal RNA gene, partial sequence	2502	2502	99%	0.0	99%	NR_114695.1
☑	Komagataeibacter oboediens strain JCM 16937 16S ribosomal RNA gene, partial sequence	2498	2498	98%	0.0	99%	NR_113397.1

Figure A1: Multiple sequence alignment (BLASTN) producing significant alignment to *Komagataeibacter rhaeticus*