DEVELOPMENT, CHARACTERIZATION AND *IN VIVO* ANTIBACTERIAL AND ANTIOXIDANT STUDY OF A COCONUT MILK-BASED KEFIR BEVERAGE

By

MUNA MAHMOOD TALEB ABADL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

To my mother who has been so supportive throughout my journey. To my grandmother, may she rest in peace, who was one of my greatest allies and supporters. To my friends who made Malaysia a second home.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Kefir, a fermented beverage traditionally produced from cow's milk using kefir grains, faces challenges due to the global shift away from dairy products driven by concerns such as lactose intolerance, milk allergies, and environmental impacts. To address this, the present study was undertaken to develop a plant-based alternative – coconut milk kefir. This research aims to explore the effect of fat content on the physicochemical, nutritional, and biochemical characteristics of Reduced Fat Coconut Milk Kefir (RFCMK) and High Fat Coconut Milk Kefir (HFCMK), optimize the pH, antibacterial, and antioxidant properties of coconut milk kefir, evaluate its biochemical, metabolomic, sensory attributes, and shelf life, and assess its *in vivo* antibacterial and antioxidant effects. In the investigation of fat content's influence on beverage properties, RFCMK exhibited higher lactic acid bacteria (LAB) growth and superior antibacterial activity against *Bacillus subtilis* (94.62±2.46%), *Staphylococcus aureus* (96.82±0.8%), *Escherichia coli* (100±1.63%), and *Salmonella* Typhimurium (98.67±1.37%) through antibacterial inhibitory tests. Furthermore, RFCMK demonstrated significantly elevated levels of DPPH, ABTS, and FRAP compared to HFCMK. Remarkably, through Response Surface Methodology (RSM), an optimal formulation was achieved solely by adjusting fermentation parameters, resulting in high antimicrobial activity (93.1 – 100%) against various pathogens and robust antioxidant properties (DPPH: 72.6%, ABTS: 47.06%, FRAP: 437.045 µM TE/100 mL). Metabolomic analysis using ^{1}H–NMR revealed significant changes in metabolites like lactic acid, GABA, Biotin, Riboflavin, Butyrate, and Caprylate during fermentation, known to enhance antioxidant status and reduce lipid peroxidation. LC-MS/MS (Q-TOF) also identified 11 bioactive peptides with antibacterial properties. Consumer acceptability comparisons between cow's milk and coconut milk kefir showed no significant difference between the two products based on a 9-point hedonic scale. During a 30-day storage period at 4°C, syneresis significantly increased after day 15, accompanied by a gradual rise in acidity, reaching pH 3.5 by day 17. The microbial count of LAB and yeast dropped below the Codex Alimentarius Commission recommended values of 7.00 and 4.00 Log CFU/mL after days 17 and 14, respectively. *In vivo* assessment of the optimized coconut milk kefir in Wistar rats revealed increased LAB levels in the cecum, reduced Alkaline Phosphatase (ALP) and Aspartate Transferase (AST) levels, elevated High-Density Lipoprotein (HDL) levels in the blood, and heightened Total Superoxide Dismutase (T-SOD) levels in the liver. In conclusion, this study highlights the potential of coconut milk kefir as a healthy, plant-based alternative to traditional dairy kefir. Its diverse benefits include improved antibacterial and antioxidant properties, appealing sensory characteristics, and positive *in vivo* health effects, offering a promising option for those seeking dairy-free fermented beverages.

Keywords: kefir; coconut milk; antioxidant; antimicrobial; lactic acid bacteria; dairy alternatives; response surface methodology; metabolites; bioactive peptides; *in vivo*

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN, PENCIRIAN DAN KAJIAN ANTIBAKTERIA DAN ANTIOKSIDAN *IN VIVO* MINUMAN KEFIR SANTAN KELAPA

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Kefir, sejenis minuman yang difermentasi secara tradisional daripada susu lembu dengan menggunakan bijirin kefir, kini menghadapi cabaran akibat daripada peralihan global yang mendukung pengurangan penggunaan produk tenusu disebabkan oleh masalah seperti intoleransi laktosa, alahan susu, dan kesan alam sekitar. Dalam usaha menangani isu ini, kajian ini telah dijalankan untuk menghasilkan alternatif berasaskan tumbuhan iaitu kefir santan kelapa. Kajian ini bertujuan untuk mengkaji kesan tahap lemak terhadap sifat fizikokimia, pemakanan, dan biokimia Kefir Santan Lemak Berkurangan (RFCMK) dan Kefir Santan Berlemak Tinggi (HFCMK), mengoptimumkan pH, sifat antibakteria, dan antioksidan kefir santan, menilai sifat biokimia, metabolomik, deria, serta jangka hayatnya, dan menilai kesan antibakteria dan antioksidan secara *in vivo*.

Dalam penyelidikan yang mengkaji pengaruh kandungan lemak terhadap sifat minuman, RFCMK telah menunjukkan pertumbuhan bakteria asid laktik (LAB) yang lebih tinggi dan aktiviti antibakteria yang lebih baik terhadap *Bacillus subtilis* (94.62±2.46%), *Staphylococcus aureus* (96.82±0.8%), *Escherichia coli* (100±1.63%), dan *Salmonella* Typhimurium (98.67±1.37%) melalui ujian perencatan antibakteria. Selain itu, RFCMK juga telah menunjukkan peningkatan yang ketara melalui kaedah DPPH, ABTS, dan FRAP berbanding HFCMK. Melalui Metodologi Permukaan Tindak Balas (RSM), formulasi optimum telah berjaya diperolehi dengan melaraskan parameter penapaian, menghasilkan aktiviti antimikrob yang tinggi (93.1 – 100%) terhadap pelbagai jenis patogen dan sifat antioksidan yang kukuh (DPPH: 72.6%, ABTS: 47.06%, FRAP: 437.045 µM TE/100 mL). Analisis metabolomik menggunakan ^{1}H-NMR telah mendedahkan perubahan metabolit yang signifikan seperti asid laktik, GABA, Biotin, Riboflavin, Butyrate, dan Caprylate semasa proses penapaian, yang telah diketahui dapat meningkatkan status antioksidan dan mengurangkan peroksidasi lipid. Selain itu, melalui LC-MS/MS (Q-TOF), sebanyak 11 peptida bioaktif dengan sifat antibakteria telah berjaya dikenal pasti. Perbandingan penerimaan pengguna antara kefir susu lembu dan kefir santan kelapa tidak menunjukkan perbezaan yang signifikan antara kedua-dua

produk berdasarkan skala hedonik 9 mata. Semasa tempoh penyimpanan selama 30 hari pada suhu 4°C, sineresis meningkat secara ketara selepas hari ke-15, disertai dengan peningkatan keasidan secara beransur-ansur, mencapai pH 3.5 pada hari ke-17. Kiraan mikrob LAB dan yis pula telah menurun di bawah nilai yang disyorkan oleh Kodex Komisi Alimentarius iaitu 7.00 dan 4.00 Log CFU/mL selepas hari ke-17 dan 14, masing-masing. Penilaian secara i*n vivo* terhadap kefir santan yang telah dioptimumkan dalam tikus Wistar telah menunjukkan peningkatan tahap LAB dalam cecum, pengurangan tahap Alkali Fosfatase (ALP) dan Aspartate Transferase (AST), peningkatan tahap Lipoprotein Ketumpatan Tinggi (HDL) dalam darah, serta peningkatan jumlah Superoxide Dismutase (T-SOD) dalam hati. Sebagai kesimpulan, kajian ini menyoroti potensi kefir santan kelapa sebagai alternatif yang sihat, berasaskan tumbuhan kepada kefir susu tradisional. Kelebihan yang merangkumi peningkatan sifat antibakteria dan antioksidan, ciri deria yang menarik, serta kesan positif terhadap kesihatan *in vivo* menjadikannya sebagai pilihan yang meyakinkan untuk mereka yang mencari minuman yang bebas daripada produk tenusu.

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LIST OF ABBERVIATIONS

CM	Coconut milk
CMK	Coconut milk kefir
RFCM	Reduced fat coconut milk
RFCMK	Reduced fat coconut milk kefir
FFCM	Full fat coconut milk
FFCMK	Full fat coconut milk
ROS	Reactive oxygen species
LAB	Lactic Acid Bacteria
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
^{1}HNMR	Proton nuclear magnetic resonance
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
FRAP	Ferric Reducing Antioxidant Power
RSM	Response Surface Methodology
FFD	Full Factorial Design
BBD	Box Behnken Design
CCD	Central Composite Design
SOD	Superoxide Dismutase
CAT	Catalase
GSH-Px	Glutathione peroxidase
MDA	Malondialdehyde
G0	Control group of Wistar rats administrated with water
G1	Treatment group f Wistar rats administrated with coconut milk
G2	Treatment group f Wistar rats administrated with coconut milk kefir

LDL	low-density lipoprotein
HDL	High-density lipoprotein
Amino Acids	
A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
K	Lysine
L	Leucine
M	Methionine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine

CHAPTER 1

INTRODUCTION

1.1 Background of study

Kefir, a functional fermented beverage with its origins in the Caucasus Mountains, is traditionally prepared by introducing kefir grains into predominantly animal milk. These kefir grains, which vary in dimension from 0.3 to 3.5 cm in diameter (Silva et al., 2009), comprise a heterogeneous amalgamation of lactic acid bacteria, yeast, and acetic acid bacteria. Encased within a water-soluble branched glucogalactan matrix known as kefiran, these microorganisms produce this polysaccharide matrix through the enzymatic digestion of lactose in milk (Moradi & Kalanpour, 2019). Numerous studies have underscored the probiotic nature of kefir, replete with substantial nutritional and health advantages (Leite et al., 2013). The antibacterial properties of kefir can be attributed to a repertoire of bioactive compounds, including bioactive peptides, H_2O_2, organic acids, CO_2, and acetaldehyde, which emerge during the fermentation process (Rosa et al., 2017). These bioactive constituents are reported to alleviate gastrointestinal disturbances and promote the development of a healthy and balanced microbial ecosystem within the intestinal milieu (Ahmed et al., 2013). Given the mounting concern surrounding antibiotic-resistant pathogenic bacteria, recent research has underscored the urgent necessity for natural alternatives to mitigate antibiotic usage. Kefir, through its metabolic, immunomodulatory, and defensive attributes against deleterious processes, can significantly enhance consumers' well-being (Rosa et al., 2017). Kefir beverages have also demonstrated the capacity to reduce reactive oxygen species (ROS), consequently reducing the risk of developing diseases such as cancer, atherosclerosis, hypertension, and arthritis (Yang & Lian, 2019), thus minimizing cellular damage through natural means (Rosa et al., 2017).

Coconut (*Cocos nucifera* L.), often hailed as the "tree of life," stands as a vital nutrient source in tropical and subtropical regions (Yang et al., 2018). Derived from the mature coconut's kernel flesh (endosperm), coconut milk is prepared, either as is or with the addition of water (Patil & Benjakul, 2018). Coconut milk typically consists of approximately 54% water, 37-35% fat, and 11-9% non-fat solids (Lad & Murthy, 2016), alongside a rich array of micronutrients including proteins and various essential vitamins and minerals. These vitamins encompass essential nutrients such as ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), thiamine (vitamin B1), niacin (vitamin B3), pantothenic acid (vitamin B5), and pyridoxine (vitamin B6), enhancing its nutritional value (DebMandal & Mandal, 2011). Coconut milk is an integral component of numerous traditional dishes across various Asian countries (Patil & Benjakul, 2018). The surge in plant-based diets and the heightened demand among lactose-intolerant consumers have contributed to a substantial increase in the popularity of coconut products in developed nations (Mauro & Garcia, 2019). In Southeast Asian regions, coconut milk is enjoyed either in its fresh or fermented form. For instance, cendol, a beloved dessert/drink, is prepared with coconut milk and green rice flour, and it enjoys widespread popularity throughout Southeast Asia (Nurul et al., 2017). In Indonesia, Bandrek, a spicy and invigorating beverage, incorporates coconut milk into its preparation (Wahyudi et al., 2017). Furthermore, in locales such as Southern China and Taiwan, coconut milk is relished as a refreshing beverage, while South American countries like Brazil employ it to craft alcoholic beverages like piña colada and Batida de Côco cocktails (D'Amato et al., 2012).

Numerous studies have documented the intrinsic antioxidant, antibacterial, and anti-inflammatory attributes of coconut milk (Aggarwal et al., 2017; Roopan, 2016). Öner et al. (2010) have proposed that kefir beverages can be crafted from diverse milk sources, whether they originate from animals or plants. While substantial research has been dedicated to animal-based kefir (Gul et al., 2015; Satir & Guzel-Seydim, 2015; Yilmaz-Ersan et al., 2018), soy milk has emerged as the primary plant-based milk explored in kefir production (Kesenkaş, 2011; Liu, Chen, et al., 2005; McCue & Shetty, 2005).

1.2 Problem statement

A quarter of greenhouse gas (GHG) emissions are attributed to current food systems, which are also major contributor to freshwater depletion, deforestation, biodiversity loss, and water pollution (Clune et al., 2017). 14% of the GHG emissions are caused by animal farming alone (Myers et al., 2017). The treatment of farm animals raised for food and feed creates significant ethical concerns around the globe. There is an increased interest in and demand for plant-based dairy substitutes made from soy, legumes, seeds, nuts, or cereals worldwide. These plant-based alternatives hold the potential to complement and even replace dairy products in individuals' diets, potentially reducing the environmental impact of food consumption. There is a growing body of evidence supporting the need for transitioning to more sustainable diets and food systems, necessitating substantial changes in food production and consumption practices (Röös et al., 2018). According to the American National Institute of Health, approximately 68% of the global population experiences lactose malabsorption (Storhaug et al., 2017). This prevalence varies widely, from under 5% in northern European nations such as Denmark, Britain, and the Netherlands to over 80% in countries like China, Korea, Thailand, as well as among the Yoruba population in Nigeria and Native Americans in North America (Itan et al., 2010; Storhaug et al., 2017; Wilt et al., 2010). Nevertheless, it's important to note that this estimate is likely a rough approximation due to variations in diagnostic methods and a limited sample size, with many individuals being hospitalized for evaluation (Granato et al., 2010). Fermented dairy products like yogurt and cheese are supposed to be lactose-free; however, many of the products available on the international market undergo only partial fermentation (Bianchini et al., 2020). When analyzing the lactose content of different yogurt products in Brazil, Batista et al. (2008) concluded that these products cannot be consumed by lactose-intolerant people due to insufficient reduction in the lactose levels. The same conclusion was also drawn by a study conducted to survey the bio- yogurt products in the market in Israel as they had significant amount of lactose that could cause digestive issues for people who are lactose-intolerant (Shapiro & Silanikove, 2010). The best solution is therefore to create plant-based alternatives to avoid any gastrointestinal problems for people who can’t digest lactose.

The market of plant- based dairy alternatives is growing rapidly and is expected to reach a CAGR of 9% to reach $ 30 billion share by 2030 (John, 2023). Even though soymilk has the largest market share of the food and beverage segment (Singh, 2022), it still does not cater to all consumers. Many consumers don't enjoy the beany flavor of soymilk, which adds extra processing steps in the production to remove. Moreover, the claims that soy disrupts the hormonal balance (Paul et al., 2020). Therefore, it is important to provide various alternatives to consumers to meet their needs.

1.3 Objectives of study

In the present study, coconut milk kefir was created by using optimized conditions to produce a product with maximum antibacterial and antioxidant activities, subsequently evaluating their bioactivities using an in vivo animal model. Coconut milk kefir can be used as a standalone product or for producing other dairy-based products such as ice cream. Therefore, a series of investigations were conducted to achieve the following four objectives:

1) to compare the effect of fat level on the physicochemical, nutritional, and biochemical properties of Reduced Fat Coconut Milk Kefir (RFCMK) and High Fat Coconut Milk Kefir (HFCMK)
2) to optimize the pH and antibacterial and antioxidant properties of kefir coconut milk
3) to characterize the biochemical, metabolomic, sensory properties and shelf life of the optimized coconut milk kefir
4) to examine the antibacterial and antioxidant effects of this drink *in-vivo*.

1.4 Study hypotheses

1) By optimizing the fermentation parameters, a coconut-based kefir beverage can have high antioxidant and antimicrobial properties.
2) A significant amount of bioactive peptides and molecules result from the fermentation process of coconut milk kefir.
3) Consumers find coconut milk kefir as favorable as cow milk kefir drink.
4) The administration of coconut-based kefir beverage to rats increases the lactic acid bacteria count in the cecum of the animals, antioxidant enzymes in the liver, decreases the pathogenic bacterial count in the digesta and improves overall health of the animals.

1.5 Novelty of study

This study is novel in that it focuses on creating an optimal coconut milk kefir by optimizing the process parameters. The optimized coconut milk kefir has optimal levels of antioxidant properties, which according to the author’s knowledge has not been reported previously, and antimicrobial properties. An *in vivo* study has also been conducted for this product, which has not been conducted before to the best of the author’s knowledge.

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