THE QUANTITATIVE PCR DETERMINATION FOR PROBIOTIC PROMOTION OF BETA-GLUCAN FROM *Cordyceps militaris* AND ANALYSIS ON ANTIOXIDANT PROPERTY

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Lin YunYi

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ABSTRACT

Probiotics are regarded for human health advantages; however, these benefits rely on the specific bacterial strains. *Streptococcus thermophilus* has been extensively used as a starting culture in the dairy products. However, bacterial growth and idenfication of probiotic is commonly used culture technique with labourious and time-consuming. The objective of this study was (1) to develop qPCR technique to determine the *S. thermophilus* growth and (2) evaluate growth promoting using the beta-glucan extract from fungal mycelia of *Cordyceps militaris* by using qPCR technique.

In this study, the genome-based method has been developed to use a specific gene of *S. thermophilus*, the Glucose kinase gene (*GlcK*), by using quantitative PCR (qPCR). The 139-bp PCR product was successfully cloned and used to generate a DNA standard curve by plotting the threshold cycle (Cq) versus log DNA concentration of plasmid DNA, with an amplification efficiency of 97.2%. In addition, coefficient of variation was calculated considering both threshold cycle (Ct) and bacterial cell enumerated by plate counts, which indicated the log CFU/mL (1.69–6.56) and log DNA copies (2.07–6.03). This linear relationship revealed a quantitative curve (R^2 =0.989) with a detection in range from 1.69 to 6.56 log CFU/mL. Next, to determine the prebiotic index and activity, here, the beta-galactosidase, *Lac*Z gene of *E. coli* was cloned using 122-bp PCR product and generated standard curved from Ct and log DNA concentration which revealed indicated the log CFU/mL (2.35–7.35) and log DNA copies (2.61–7.08). This linear relationship revealed a quantitative curve (R^2 =0.9912) with a detection range from 2.35 to 7.35 log CFU/mL. Subsequently, correlations

between bacterial growth obtained from qPCR and plate count method were conducted. Hence, qPCR-based methods facilitated reliable quantification *S. thermophilus* and *E. coli* in growth determination during culture.

Next, we extracted the beta-glucan from *C. militaris*, achieving a yield of about 2.5% and demonstrating an antioxidant activity of 85%. The prebiotic testing was conducted by supplementing with beta-glucan, lactose and inulin in culture media of *S. thermophilus* and *E. coli* (as a negative control) for 48 hrs. The qPCR method was used to monitor the culture every 4 hrs. We calculated the growth and prebiotic properties based on the copy numbers. The results revealed that beta-glucan had potential as a prebiotic, exhibiting a prebiotic index at 3.21, which is comparable to inulin at 0.37. Here the use of qPCR is an alternative method to determine the number of cells and cell growth during culture in the two bacterial strains.

Keywords: Beta-glucan, Glucose Kinase Gene, Probiotic, Quantitative PCR, *Streptococcus thermophilus*

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Probiotics are useful microorganisms that, when consumed in the right amounts, improve human health by balancing the gut microbiome. So far, there are many products have been used the different probiotic strains depend on the fermented products and applications. Particularly, *Streptococcus thermophilus* is a thermophilic bacteria considered as generally regarded as safe (GRAS). which has been used as a starter culture in diary products, including yoghurt along with *Lactobacillus delbrueckii* subsp. *Bulgaricus*.

Prebiotic is described as a substrate that selectively utilized by host microorganisms that provides a health benefit. It is considered as the functional foods that cause specific changes in the composition and activity of the host's gastrointestinal microbiota, resulting in health and wellness benefits. An ingredient must meet three basic criteria to be considered a prebiotic: (i) antidigestion; (ii) fermentation of the colonic microbiota; and (iii) selective action on microbiota with relevant health-promoting effects (Figueroa-González et al., 2011).

Beta-glucan is an active polysaccharide regarded as a prebiotic, found in the cell walls of plants, fungi (including mushrooms), bacteria, algae, and grains. They can generate form in various sources with distinct structures, conformations, physical properties, receptor-binding affinities, and consequently biological roles. They have a common structure consisting of a main chain of non-repetitive but non-randomly arranged beta-(1,3)- and/or beta-glucans (1,4)-D-glucopyranose units, with side chains of varying lengths (Wang et al., 2017). Due to their varying chemical composition, they are also recognized as functional foods due to the offer considerable beneficial impacts on human health. They can play a variety of biological roles, such as anti-cancer, anti-diabetes, anti-inflammatory, antioxidant, immune regulation, blood sugar regulation, etc (Barsanti et al., 2011). In edible fungi, especially Lentinus, Cordyceps, and Ganoderma , comprise

α-glucans, predominantly highly branched glucan containing mainly 1,3- and 1,6-linkages which revealed antibacterial activity in vitro against gram-positive and gram-negative bacteria. They avoid catabolism in the upper gastrointestinal tract and reach the large intestine undigested, as do all dietary fibers. In addition, rea-ctive oxygen species (ROS) play a critical role in the process of ageing and carcinogenesis. ROS attacks the unsaturated fatty acids in the cell membranes, resulting in the membrane lipid peroxidation, the loss in membrane fluidity, the decrease of antioxidant enzymes and the cell inactivation (Barsanti et al., 2011). Recent studies have reported that *C. militaris* beta-glucan exhibits significant antioxidant capacity, immunomodulatory effect, and high anti-inflammatory effect. It also possesses potential lipid-lowering activity, anti-atherosclerosis activity, anti-tumour activity, and anti-diabetes activity (Miao et al., 2022). The beta (1,6) dextran extracted from *C. militaris* exhibits considerable antioxidant capacity, potentially linked to its beta-(1,6)-dextran content or monosaccharide composition (Liu et al., 2020). Furthermore, linear beta-(1,3) glucan derived from *C. militaris* exhibits a pronounced anti-inflammatory impact and is the most potent anti-inflammatory molecule within the polysaccharide extract of *C. militaris* (Smiderle et al., 2014).

Since the prebiotic has been studied the method is commonly used culture method to determine pretiotic property which labouious and time-comsuming. The genome-based method has been recognized as an alternative rapid method. So far, PCR-based methods, especially quantitative PCR (qPCR), are mainly used for the detection, identification and quantification of pathogens or beneficial flora, such as fermenting microbes or probiotics. This technique combines the amplification of a target DNA sequence with the determination of the concentration of that DNA species in the reaction. The approach calculates initial template concentrations, making it a common analytical tool for determining DNA copy number is faster, more sensitive, and more specific than culture-based approaches. In this study, the specific gene *glcK* gene and *lacZ* gene were used to evaluate the prebiotic and *S. thermophillus* and *E. coli.*

Using qPCR assay, an efficient and quick method for detecting and quantify the *S. thermophillus* was developed and its specificity, repeatability and reproducibility were

validated. In addition, beta-glucan has been identified as a primary active component of fungi and mushrooms but information from *C. miliatris* is still little-known to understand antioxidant activity and probiotic properties. This information could be revealed for potential medical or functional food applications. In addition, this work presents a new genomic approach to quantify the cell density of a specific strain and to describe the microbial growth during prebiotic test.

1.1 Objectives

This study aimed to develop a qPCR technique for biomass quantification to address prebiotic tests on microbial growth and determine the prebiotic activity of beta-glucan from the fungal mycelia of *C. militaris*. In addition, it also determined the beta-glucan content in fungal mycelia and antioxidant activity.

1.2 Scope of Research

This study was (1) to determine beta-glucan content from the fungal mycelia of *C. militaris* and biological activity including antiantioxant activity by using ABTS and DPPH assay. and prebiotic property and (2) to develop qPCR technique to describe the prebiotic property on *S. thermophilus* and *E. coli* growth by using the Glucose kinase gene (*GlcK*) and *E. coli*, the beta-galactosidase (*LacZ*) by quantitative PCR. PCR products were successfully cloned into TOPO plasmid and a DNA standard curve was generated by plotting the threshold cycle (Ct) versus the log DNA concentration of the plasmid DNA (from $3x10^3$ to $3x10^8$ copies). Next, the gene amplicons are quantified by qPCR. Then, a calibration curve was established between the obtained Ct values and log CFU, and a correlation analysis of bacterial counts between plate counts (CFU) and qPCR (gene amplicons) was performed. Finally, the bacterial strains; *S. thermophilus* and *E. coli* were

grown in medium supplemented with beta-glucan extracted from *C. militaris* as the sole carbon source, and the bacterial counts were quantified in qPCR every 4 hours for 48 hours.

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

Probiotics are live microorganisms that, when consumed in sufficient quantities, benefit the host health. Generally, these microbes are used in fermented products that could improve human health such as yogurt, cheese, Japanese miso, sauerkraut, and beer are the most common food sources. Non-dairy fermented substrates such soy products, cereals, legumes, cabbage, corn, and sorghum also contain probiotic bacteria. Recently, probiotics has become popular and high demans in the healthcare industry. The field of probiotic research have been investigating a variety of probiotics strains within the genera such as *Streptococcus, Enterococcus, Alloiococcus, Aerococcus, Lactococcus, Oenococcus, Vagococcus, Lactobacillus, Carnobacterium, Pediococcus, Leuconostoc, Tetragenococcus, Weissella, Bifidobacterium, Symbiobacterium,* and *Atopobium* (Venkatesh et al., 2024).

Streptococcus thermophilus is a *thermophilic* species of considerable interest among the wide variety of probiotic strains. The dairy industry widely uses *S. thermophilus* as a starter culture in many traditional fermented products, including yoghurt along with. *Lactobacillus delbrueckii* subsp. *bulgaricus* (Gram-positive *S. thermophilus* is a member of the Streptococcaceae family and phylum Firmicutes. The Food and Drug Administration (FDA) has designated this strain as generally regarded as safe (GRAS) (Uriot et al., 2017). Numerous studies have explored the technological features of *S. thermophilus*, such as its proteolytic activity, its ability to utilize lactose and galactose and its ability to acidify milk. The probiotic products with *S. thermophilus* are available on the market (Kapse et al., 2024).

Several studies have reported the absence of viable *S. thermophilus* cells in the faeces of healthy individuals who have been administered either pasteurised or fresh

yoghurt. This suggested that *S. thermophilus* is susceptible to gastro-intestinal transit in humans. So far, many studies of genomes of *S. thermophilus* have been reported, significantly enhancing our comprehension of the molecular-level metabolic activities of this bacterial strain. These activities encompass extrapolysaccharide and folate biosynthesis, resistance to bacteriophages, proteolytic systems, and carbohydrate metabolism, among others (Wang et al., 2017).

2.2 Mechanism of Action

2.2.1 Intestinal Microbiota

Probiotics and prebiotics affect the bacteria in the gastrointestinal (GI) tract. 100 trillion microorganisms (bacteria, viruses, fungus, protozoa) of at least 1000 different species reside in the GI tract. The microbial diversity in the gastrointestinal system varies from person to person, and each person has a distinct pattern of microbial composition The initial colonization at birth, food choices, and genetics all have an impact on this. The “gut microbiota” is a collection of bacteria that inhabit the digestive tract. These bacteria, along with their related genes and the environment that influences them, comprise the “gut microbiome” (Anon, 2022). The gut microbiome can be viewed as an organ that produces hundreds of active metabolites that can influence human health and disease both within and beyond the gut (Kaur et al., 2021).

2.2.2 Mechanism of Action Prebiotics

Prebiotics affect the intestinal microbiota by reducing the quantity of potentially harmful microorganisms in the colon and boosting the amount of advantageous anaerobic bacteria, particularly *Bifidobacteria* and *Lactobacilli*. The importance of prebiotics is mostly due to the increased generation of short-chain fatty acids (SCFA), which are the primary by-products of prebiotic fermentation and have several significant functions (Holmes et al., 2020). These effects include maintaining intestinal barrier function, lowering inflammatory markers, and influencing metabolic hormones (including glucagon-like peptide 1 (GLP-1) and peptide Y-Y stimulation), which can reduce appetite, improve

insulin sensitivity, and prevent weight gain and metabolic syndrome (Anon, 2022). Prebiotics may also act locally and systemically in the gut, increasing anti-inflammatory cytokines and decreasing pro-inflammatory cytokines, as well as other immune function markers, modulating the immune system and inflammatory response (Figueroa-González et al., 2011).

2.3 Prebiotic

Prebiotic is described as a substrate that selectively utilized by host microorganisms that provides a health benefit. It is considered as the functional foods that cause specific changes in the composition and activity of the host's gastrointestinal microbiota, resulting in health benefits. An ingredient must meet three basic criteria to be considered a prebiotic: (i) antidigestion; (ii) fermentation of the colonic microbiota; and (iii) selective action on microbiota with relevant health-promoting effects (Obayomi et al., 2024). There are many types of prebiotics, most of which comprise oligosaccharides or short polysaccharides such as inulin, oligofructose, galactofructose, oligogalactose, and others that are not digestible carbohydrates, and each has a unique structure and mode of action (Obayomi et al., 2024). For example, inulin is a fructose polymer held together by beta (2-1) glycosidic linkages. In the colon, it serves as a substrate for fermentation by helpful bacteria. Inulin is disgested by bacteria known to benefit gut health, such as *Lactobacillus* and *Bifidobacterium*. Oligofructose (FOS) is composed of terminal glucose molecules and fructose units joined by β (2-1) glycosidic linkages. FOS consumption has been linked to a variety of health advantages, including increased immunity, improved digestive health, and a potential reduction in the risk of acquiring certain chronic diseases. FOS has been demonstrated to be bifidogenic, which means that they stimulate the growth of intestinal bifidobacteria (Obayomi et al., 2024). The other is oligogalactose (GOS), which is composed of short-chained galactose molecules. GOS supplements modify the composition and activity of the intestinal flora, creating a more diverse and harmonious microbial population. This could

help avoid gastrointestinal diseases and problems and improve gut health (Zielińska et al., 2021).

Some fungi and edible mushrooms have been used as food, supplements, and medicine. Edible medicinal mushrooms are rich in nutrients and bioactive compounds with potential health benefits. Polysaccharides, a type of carbohydrate found in many mushrooms, have shown promise in modulating the gut microbiome and treating intestinal disorders. Several mushrooms, such as *Agrocybe cylindracea*, *Volvariella volvacea*, *Agaricus bisporus*, *Trametes versicolor*, and *Lentinus squarrosulus*, have been particularly studied for their potential gut health benefits but not yet reported in *Cordyceps militaris* (Álvarez-Mercado & Plaza-Diaz, 2022).

Cordyceps militaris is a natural medicinal fungus of ascomycetes with multiple biological functions, and several bioactive components with medicinal value can be extracted from *C. militaris*, including adenosine, cordycepin, ergosterol, carotenoids, mannitol, minerals, nucleosides and sterols, and several types of carbohydrates, such as monosaccharides, oligosaccharides and polysaccharides. These bioactive components have pharmacological effects such as antioxidant, anti-cancer, anti-proliferative, anti-microbial, antibacterial, antiviral, antifungal, hypoglycemic, and hypolipidemic (Wu et al., 2021).

2.4 Beta-glucan

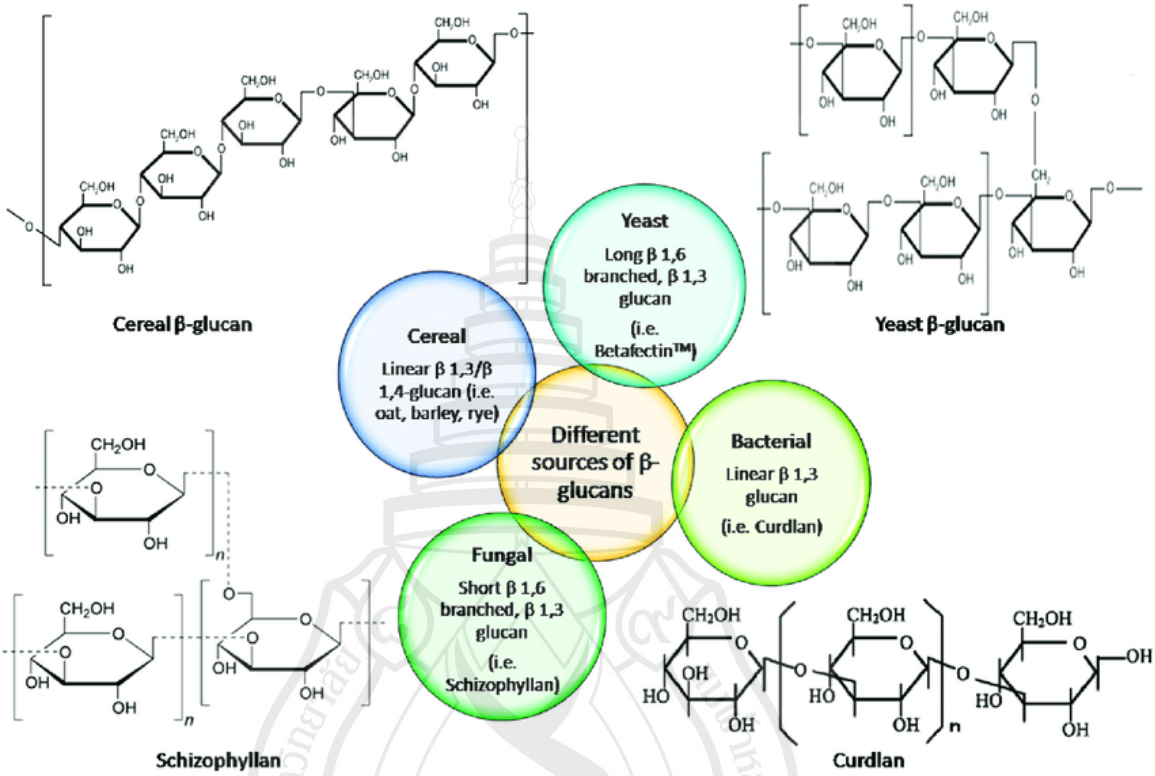
Beta-glucans are polysaccharides found in the cell walls of plants, fungi (including mushrooms), bacteria, algae, and cereals (Markowiak & Ślizewska, 2017). The soluble dietary fiber beta-glucan is found in the cell walls of certain plants. A fiber that dissolves in water and forms a sticky gel-like substance is referred to as soluble fiber (El Khoury et al., 2012). This means it absorbs water and swells in the digestive tract, slowing food passage through the intestine. As a result, beta-glucan can help in the stabilization of blood sugar levels, the promotion of heart health, and the stimulation of immune function (Bozbulut & Sanlier, 2019). The main sources of beta-glucan are grains such as wheat, oats, and rye, as well as mushrooms, seaweed, and certain types of yeast (Meena et al., 2013).

The cell walls of certain plants include a type of dietary fiber known as beta-glucan, which is soluble. A fiber that, when combined with water, results in the formation of a substance that is like a sticky gel is known as a soluble fiber (El Khoury et al., 2012). This indicates that it can absorb water and swelling within the digestive tract, so slowing the movement of food through the intestines. Because of this, beta-glucan has the potential to assist in the regulation of blood sugar levels, the enhancement of cardiovascular health, and the enhancement of immunological function (Bozbulut & Sanlier, 2019). The most common places to get beta-glucan are in cereal grains including wheat, rye, and oats, as well as in mushrooms, seaweed, and specific varieties of yeast (Meena et al., 2013).

Chemical structure of beta-glucan

Beta-glucans are composed of D-glucose subunits with a backbone of longer or shorter chains linked together with beta-1,3 or beta-1,4 glycosidic bonds. The distribution and length of D-glucose polymers with beta-1,3 or beta-1,4 glycosidic bonds vary, and most of the D-glucose branched chains are linked to a D-glucose backbone with beta-1,6 glycosidic bonds (Maheshwari et al., 2017). The macromolecular structure of beta-glucans varies significantly depending on the sources (Figure 2.1). The macromolecular structure of beta-glucans varies significantly depending on where they come from. There are two types of fungal glucans: linear and branched, -or -glycosidic links, as well as the many kinds of glycosidic linkages found in the same molecule, bind the molecules of certain monosaccharides, mainly glucose. Different linking types, branching degrees, molecular weights, and solubility profiles may be found in D-glucans derived from mushrooms. The beta-glucan of fungus is a blend of short branches joined by beta (1,6) and straight beta (1,3) glucan. The beta (1→3)-D-glucan backbone of the bacterial beta-glucan is straight and unbranched. The beta-glucan of fungi is a mixture of straight beta (1,3) glucan with short branches connected by beta (1,6) as shown in Table 2.1. The bacterial beta-glucan has a straight and unbranched beta (1,3)-D-glucan backbone (Du et al., 2019). The glucans extracted from *C. militaris* are mainly beta-(1,3) -glucan and beta-(1,6)-glucan. The structure of the main chain is composed of glucose molecules in a linear manner through the beta-(1,3)-glucoside bond. In addition to the main beta-(1,3)-linked glucose skeleton,

C. militaris beta-glucans often contain beta-(1,6)-linked side chain branches (Liu et al., 2020).



Source Mirończuk-Chodakowska et al. (2021)

Figure 2.1 Structure and branching degree of beta-glucan from different sources

Table 2.1 Examples of studied beta-glucans of various macrofungi.

Name of beta-glucan	Mushroom species	Structure	References
Lentinan	*Lentinula edodes*	1,3-1,6-beta-glucan	(Mirończuk-Chodakowska et al., 2021)
Pleuran	*Pleurotus ostreatus*	1,3-beta-glucan with galactose and mannose	

Table 2.1 (continued)

Name of beta-glucan	Mushroom species	Structure	References
Schizophyllan	*Schizophyllum commune*	1,3-1,6-beta-glucan	(Smiderle et al., 2014)
Grifolan	*Grifola frondosa*	1,6-1,3-beta-glucan 1,3-1,6-beta-glucan	
	Cordyceps. militaris	1,3-1,6-beta-glucan	(Liu et al., 2020)

2.5 Antioxidant Activity of Beta-glucan

Oxidative stress is characterized by elevated intracellular concentrations of oxygen radicals that induce damage to lipids, proteins, and DNA. Reactive oxygen species (ROS), which include superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, are among the most active oxygen free radicals (Schieber and Chandel 2014). To prevent oxidative stress, the majority of living organisms have developed enzymatic defenses, including superoxide dismutase (SOD), glutathione reductase (GR), non-enzymatic antioxidants (thioredoxin, vitamin C, vitamin E), and repair mechanisms (Wang et al., 2017). However, these endogenous antioxidant mechanisms are typically insufficient to avoid oxidative damage in living organisms. In order to protect the human body from the harmful effects of oxidative damage, it has been demonstrated that antioxidant additions that include molecules that either delay or block the oxidation of cellular substrates are effective.

By scavenging reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, improving the activity of antioxidant enzymes, and blocking the damage pathway of peroxides and reactive oxygen species to the body, beta-glucan has a potent antioxidant effect that enhances the body's antioxidant capacity. This protects macrophages from free radical attack after radiation exposure and let them to

carry on operating normally. The existence of heterotrimeric hydrogen atoms, whose molecular and polymeric structures give increased free radical scavenging capability, may be the cause of beta-glucans strong antioxidant activity. These research' findings indicate that beta-glucan could have antioxidant properties and shield the body from the damaging effects of free radicals.

2.6 Technique for Determination Bacterial Growth in Culture

A bacterial cell can be quantified using total plate count, dry or wet weight per litre of sample, or optical density measurement of the sample. Generally, a common method for dertermination the total number of probiotic bacteria is used the total plate count. However, this technique is laborious and time-consuming. So far, the advancement technique using genome based has been reproted. Polymerase chain reaction (PCR) is a technique that enables the detection of specific genes in a variety of individual bacterial strains at relatively low concentrations (Cassoli et al., 2007) and quantitative PCR also has been used to monitoring in food contamination and pathogen diagnosis (Seifi et al., 2012).

2.7 Quantitative PCR (qPCR)

Quantitative PCR (qPCR), also known as real-time PCR or quantitative real-time PCR, is a PCR-based technique that combines the amplification of a target DNA sequence with the determination of its quantity in a reaction. This approach can calculate the beginning template concentration and so is a popular analytical tool for determining DNA copy number, viral load, SNP detection, and allele identification (Seifi et al., 2012). PCR is faster, more sensitive, and more specific than culture-based approaches, and it can detect subdominant populations even in the absence of a selective enrichment medium and other (dominant) populations. It can also detect dead or non-culturable cells. In recent years, quantitative PCR has been employed to quantify microbes by using genomic information.

This gene quantification technique has the potential to serve as an alternative to determining or quantifying an identification, enumerate of bacterial cells, or growth kinetics by using single or mixed culture (Sharkey et al., 2004). This provided evidence that a genomic assay based on qPCR can be used to effectively determine microbial kinetics, model practice, and the operation of bioreactors, which include complex mixed culture handling (Gieg et al., 2011).

2.8 Glucokinase Gene

Glucokinase (*GlcK*) is an important enzyme involved in glucose metabolism, specifically in the phosphorylation of glucose. It catalyzes the conversion of glucose to glucose-6-phosphate (G6P) using ATP as a phosphate donor. This reaction is the first step in the glycolytic pathway, where glucose is ultimately converted into pyruvate, producing energy in the form of ATP (Cáceres et al., 2007). Species-specific primers were extracted from the *GlcK* gene to identify *S. thermophilus.* This gene promotes glucose phosphorylation to form glucose-6-phosphate and initiates its entry into the glycolytic pathway, playing a central role in glucose metabolism of *S. thermophilus*. This enzyme is essential for the bacteria's energy production and efficient use of glucose as a carbon source.

2.9 Beta-galactosidase Gene

Escherichia coli is generally used as the negative control for prebiotic testing. The most common molecular targets include enzymes encoding genes e.g. beta-galactosidase and beta-D-glucuronidase present in the majority of *E. coli* strains using PCR. Particularly, beta-galactosidase is encoded by *LacZ* gene that hydrolyses the beta-glycoside. This enzyme is essential for the metabolism of lactose, a sugar present in milk. Specifically, beta-galactosidase catalyses the breakdown of lactose into its component sugars, glucose and galactose, allowing bacteria to use lactose as an energy source when other sources such

as glucose are scarce. The availability of lactose and glucose regulates operon activity, enabling *E. coli* to adapt to diverse nutritional conditions. Then, *LacZ* gene is a fundamental component of *E. coli*, serving as a valuable tool in molecular biology.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Bacterial Strains

This experiment was used *Streptococcus thermophilus* (strain TISTR 894) and the *Escherichia coli* (strain TISTR 527). In accordance with Rokana et al. (2018), *S. thermophilus* was inoculated in de Man Rogosa Sharpe (MRS) broth medium (Hi media, India). *E. coli* was incubated at 37 °C for 16-24 hours and maintained on nutrient broth (Hi media, India).

3.2 Bacterial Genomic DNA Extraction

After the bacteria were grown, the GF-1 Bacterial DNA Extraction Kit (Vivantis, Germany) was used to extract genomic DNA as described follows. An overnight culture of bacteria was cultivated in pellets (1-3 ml) and centrifuged at 6,000 rpm for 2 min at room temperature. The supernatant was entirely decanted. The particle was resuspended completely by pipetting up and down, and the cells were incubated at 37 °C for 20 min after 100 ml of Buffer R1 was added. Cells were pellet digested by centrifugation at 10,000 rpm for 3 min. The supernatant was entirely decanted. The pellets were resuspended in 180 ml of Buffer R2, and 20 ml of proteinase K (20 mg/ml) was added. Thoroughly combined and incubated at 60 °C for 20 min in a shaking water bath or with occasional combining every 5 minutes. Buffer BG was added in two volumes (approximately 400 µl) and mixed thoroughly by inverting the tube multiple times until a homogeneous solution was achieved. Incubated for an additional 10 min at 60 °C. The mixture is allowed to chill for approximately one minute. Then, 200 ml of absolute ethanol was added. Thoroughly and immediately combined. The sample was transferred to a column that was assembled in a

sterile 2 ml collection the tube that was provided. Centrifuge at 10,000 rpm for one minute. The column was centrifuged at 10,000 rpm for 1 min after being washed with 650 ml of Wash Buffer. Discard the flow through. Centrifuge the column at 10,000 rpm for 1 min to take out any remaining ethanol. The column was inserted into a sterile 1.5ml microcentrifuge tube. The column membrane was directly treated with 30 ml of preheated Elution Buffer (10mM Tris-HCl, pH8.5), TE buffer, or sterile water, and allowed to settle for 2 min. To elute DNA, centrifuge at 10,000 rpm for 1 min. DNA was maintained at either 4 °C or -20 °C. The GeneRay UV Spectophotometer was employed to determine the DNA concentration at A260 absorbance.

3.3 PCR and Gel Electrophoresis

The PCR reaction was performed in total volume (10 µl) consisted of 5 µl of DreamTaq Green PCR Master Mix (2X) (Thermofisher, USA), 2 µl of nuclease-free water, 1 µl of each primer, and 1 µl of a diluted 10X DNA template. The parameters for the reaction were as follows: initial denaturation at 95 ^{o}C for 2 min followed by 35 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 30 s, and 72 ^{o}C for 1 min; final extension at 72 ^{o}C for 15 min. Primer-BLAST, an online tool available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov), was used to create the primers that were employed. The information on these primers may be seen in Table 3.1. An agarose gel electrophoresis with a concentration of 1.5% was used to analyze the amplified products.

Table 3.1 Primers were used in the study

Bacterial strains	gene	Sequence (5'-3')	Size (bp)
S. thermophilus	*GlcK*	F - ACTGGGTGAACGTTGGGTTG R - ACCAATTTCCCCACCAGCAC	139
E. coli	*LacZ*	F - AAAATGGCTTTCGCTGCCTG R - AACGGGGGTACTGACGAAAC	122

3.4 Gene Cloning using TOPO Cloning Kit

The gene cloning was performed as described by Invitrogen's TOPO TA Cloning® Kits (Invitrogen, USA). PCR products were cloned into the TOPO vector. The resulting recombinant TOPO with the inserted PCR was then converted to competent *E. coli* DH5α by the heat shock method and cultivated for 24 hours on Luria-Bertani medium. The TIANprep Rapid Mini Plasmid Kit® (TIANGEN, China) was used after plasmid isolation. As previously mentioned, the isolated plasmid was examined utilizing gene primers and PCR methods. The recombinant TOPO plasmid was sent to First BASE Laboratories (Malaysia) for sequencing analysis.

3.5 Quantification of *GlcK* and *LacZ* Gene using qPCR

Five replicates of the quantitative PCR experiment using the primers were carried out using a CFX Manager Software in Real-Time Thermal Cyclers CFX96 TouchTM (Bio-Rad, Singapore). 1 µL of template DNA (25 ng), 2 µL of EvaGreen®, 0.2 µL of each forward and reversed *GlcK* and *LacZ* primers (0.5 µM), and nuclease-free water are the components of the reaction mixture, which has a final volume of 10 µL. The operating temperature was set at 95 ºC for 10 minutes, followed by 39 cycles of 95 ºC for 15 seconds, annealing at 58 ºC for 40 seconds, and 72 ºC for 30 seconds.

The threshold cycle (Cq) was plotted against the logarithm of known DNA concentrations using a series of 10-fold dilutions (10^1–10^8) of a plasmid encoding the *GlcK* and *LacZ* gene using real-time PCR to create the DNA standard curve.

For determine the copy numbers of the plasmid concentration, the number of copies of the target gene is calculated by the following equation:

$$\text{Plasmid copy} = \frac{6.02 \times 10^{23}\ (\text{copy/mol}) \times \text{plasmid DNA amount (g)}}{\text{Plasmid DNA length (bp)} \times 660\ (\text{g/mol/bp})}$$

To get a slope value, a scatter plot of the Cq readings against logarithmic plasmid concentrations was created by following equations:

$$\text{Cq} = (\log 10\ \text{starting copy}) \times \text{slope} + \text{y} - \text{intercept}$$

When the value C*q* is the cycle threshold, the slope is converted to the primer amplification efficiency (E) using the following formula:

$$\text{E} = \left(10^{\frac{-1}{\text{slope}}}\right) - 1$$

3.6 qPCR Sensitivity and Repeatability

To assess the sensitivity of DNA concentration, five ten-fold serial dilutions were performed from three different starting concentrations (10, 5, and 2 ng/µl) (Zhao et al. 2022). All tests were performed three times with the quantitative PCR procedure as described above, and a standard curve was created by plotting the Cq value against the logarithmic DNA concentration. The reaction efficiency was estimated using the formula provided above.

The repeatability of the test was assessed by repeating five samples at three different DNA concentrations (0.1, 1, and 10 ng/µL). The repeatability results were expressed as relative standard deviation (RSD), defined as the average Cq of the same

sample at various concentrations (Allen & Rutan, 2011). Here, the RSD of repeatability is an important reference standard in quantitative PCR experiments and should be less than or equal to 1% (Allen & Rutan, 2011).

3.7 Correlation between Quantitative PCR and Culture-Based Methods

After being cultured for the whole night at 37°C, *S. thermophilus* and *E. coli* were placed into new MRS and NB media with 1% bacterial medium. Measure the OD at 600 nm every hour after inoculation until it reaches 0.1. 5ml of bacterial cultures were then collected and processed individually. Using the complete plate count technique, CFU was determined by dilution 10-fold (from 10^1 to 10^6), and qPCR was conducted in five duplicates as outlined in section 3.3. Finally, the log DNA copy numbers and log CFU counts were plotted to display.

3.8 Fungal Strain and Cultivation

Cordyceps militaris was grown on potato dextrose agar (PDA) at 20 °C for 21 days. The mycelium was taken from the PDA and transferred into potato dextrose broth (PDB) was cultured at 25 °C with shaking at 150 rpm for 10 days.

3.9 Fungal Mycelia Preparation

The mycelium culture liquid was collected by using centrifugation, and the mycelium was then frozen for preservation. Following collection, all samples were freeze-dried for three days at -55 °C. Before extraction, the dried mycelium's weight was determined, and it was then ground into powder before storage.

3.10 Extraction of Beta-glucan

The ground mycelial powder of *C. militaris* was suspended in deionised water at a ratio of 1:20, w/v. The beta-glucan was extracted using a microwave oven operating at 800W for 5 minutes. After extraction, the mixture was centrifuged at 4500 rpm, 20 ºC for 20 minutes, then collect supernatant. After three days of freeze-drying at -55 °C, the supernatant was weighed and crushed into a powder for storing.

3.11 Determination of Beta-glucan using K-YBGL Beta-glucan Assay Kit

The K-YBGL beta-glucan Assay Kit (Yeast and Mushrooms) (Megazyme, Bray, Ireland) was used to measure beta-glucans. 0.4 ml of 2 M potassium hydroxide was mixed with 20 mg of the powder sample, and the mixture was vortexed for 10 seconds. Following 30 minutes of chilling in an ice bath, 40 µl of the Glucamix enzyme combination was added after 1.6 ml of 1.2 M sodium acetate buffer (pH 3.8) had been well mixed in. After two minutes of mixing in the ice water bath, move the tubes to a water bath that is set at 40 °C and incubate for 16 hours.Next, fill each tube with 10 ml of pure water and mix well.Use a bench centrifuge to centrifuge the tubes for 10 minutes at 3,000 rpm.Then, carefully place duplicate 0.1 ml aliquots of the sample in the bottom of glass test tubes. The reaction tubes, controls, standards, and reagent blanks were then filled with 4 ml of GOPOD reagent, incubated for 20 minutes at 40 °C, and the blank was measured at OD at 510 nm.

3.12 Probiotic and Prebiotic Test

3.12.1 Prebiotic Test

MRS Medium in 1/4 strength contration was used in the basal medium for the *S. thermophilus* culture, while 0.1% peptone and 0.1% yeast extract were present in the basal medium for the *E. coli* culture. These were utilized as negative control media. As an

active control media in the experiment, 1% lactose, inulin, commercial beta-glucan, and beta-glucan (extracted from *C. militaris*) were added to the base medium. Therefore, 1% lactose, inulin, commercial beta-glucan, and beta-glucan (extracted from *C. militaris*) were added to test tubes for each strain's growth. For every strain, the treatment was carried out three times. The suspensions were made with an OD_{600} concentration of 0.05 and inoculated with 1% v/v medium for each suspension. For 48 hours, the inoculants were shaken at 200 rpm and incubated at 37 °C. For the reducing sugar test and qPCR, 5 ml of cultures were extracted at 0, 2, 4, 8, 12, 24, 36, and 48 hours.

3.12.2 Prebiotic Index

According to Figueroa-González et al. (2019), the prebiotic index is the ratio of probiotic growth in prebiotics to probiotic growth in control carbohydrates. The prebiotic index higher than 1 indicates that carbohydrates have a positive effect on the growth of probiotics. If the prebiotic index is close to 1, it indicates that the effectiveness of the assessed carbohydrate is lower. The prebiotic index (I_{pre}) is calculated by the following formula:

$$I_{pre} = \frac{\text{CFU of probiotics in prebiotic carbohydrate}}{\text{CFU of probiotics in control carbohydrate}}$$

3.12.3 Prebiotic Activity Score

Figueroa-González et al. (2019) have described using the prebiotic activity score (PA) to evaluate the efficacy of the experimental treatments. To find out if the substrates helped the growth of probiotic strains, we compared the score to that of other species, in this case, *E. coli* strain TISTR 527. In this work, we used the cell count (CFU/ml) to calculate the prebiotic activity score, as illustrated below.

$$A_{pre} = \frac{(LogP_{24} - LogP_0)\ pre}{(LogP_{24} - LogP_0)\ lac} - \frac{(LogE_{24} - LogE_0)\ pre}{(LogE_{24} - LogE_0)\ lac}$$

where, A_{pre} is the score for prebiotic activity. Probiotic bacteria growth (CFU/ml) on lactose (Lac) and prebiotic supplements (Pre) from 0 hours (P_0) to 24 hours (P_{24}) is represented by logP, and *E. coli* growth (CFU/ml) on lactose (Lac) and prebiotic

supplements (Pre) is represented by logE. If the PA is less than 1, it indicates that the tested strain is growing less than the reference bacteria (*E. coli*) or less than the control carbohydrate when given a particular prebiotic. Conversely, probiotic bacterial growth will be supported by a substrate with a high prebiotic activity score (Duarte et al., 2017).

3.13 Antioxidant Activity Test

3.13.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging assay was adapted from Molyneux (2006). Following the dilution of beta-glucan extracts (5 µl) with 5 µl of water, 10 µl of 0.2 mM DPPH in methanol was added to a 96-well plate, and the mixture was then mixed. Before utilizing the microplate spectrophotometer to measure absorbance at 517 nm, the solutions were allowed to incubate at room temperature for a period of 30 minutes. According to Molyneux (2006), the studies were carried out in triplicate, and ascorbic acid was utilized as the positive control molecule.

To be able to assess the extent to which beta-glucan extract can eradicate DPPH free radicals, the inhibition % was calculated using the following formula:

$$\% \text{ inhibition} = \frac{A_{control} - A_{sample}}{A_{control}}$$

3.13.2 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS) Radical Scavenging Assay

ABTS solution was prepared by first oxidizing 2.45 mM of $K_2S_2O_8$ for 16 hours then the working ABTS solution was final concentration at 7 mM in H2O. The dilution of the extracts ten times, 10 µl of each sample solution was combined with 1 µl of ABTS sample solution. A measurement of the absorbance at 734 nm was taken after the reaction had been carried out for a period of 6 minutes at room temperature. The studies were carried out in triplicate, and ascorbic acid was utilized as the positive control (Shalaby & Shanab, 2013).

To determine the free radical scavenging capacity of ABTS, the radical scavenging capacity % was calculated using the following formula:

$$\% \text{ Radical scavenging activity} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}}$$

3.14 Statistical Analysis

Experiments were conducted using three independent replicates of each sample. Each replication was analysed three times. The standard deviation was used to compute the intergroup error, and the curve was fitted using nonlinear least squares using the Levernberg-Marquardt technique.

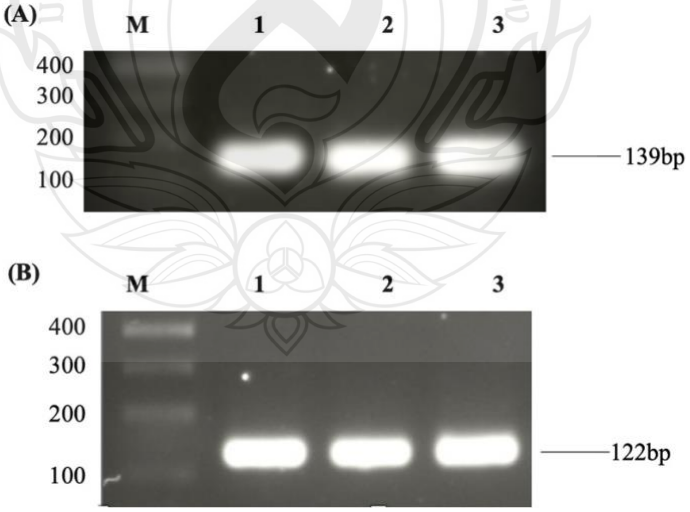
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Cloning of *GlcK* and *LacZ* Gene for qPCR Assay

4.1.1 Amplication of *Glck* Gene and *LacZ* Gene

This study was to generate the cloned for quantify bacterial growth during culture using qPCR technique, the two genes (*Glck* encoded for *S. thermophilus* and *LacZ* encoded by *E. coli*) that contribute bacterial growth was designed to use specific to detect S. *thermophilus* and *E. coli*, respectively. The bacterial genomes were collected, extracted DNA and then amplified using PCR. Here, we successfully amplify for the two genes. The results of PCR amplification using DNA extracted from *S. thermophilus* and *E. coli*, which amplified with *Glck*, 139 bp and *LacZ*, 122 bp of PCR product using specific primers (Figure 4.1). Then the two genes were cloned in TOPO vector system then to be quantified and generate standard curve using real time PCR.

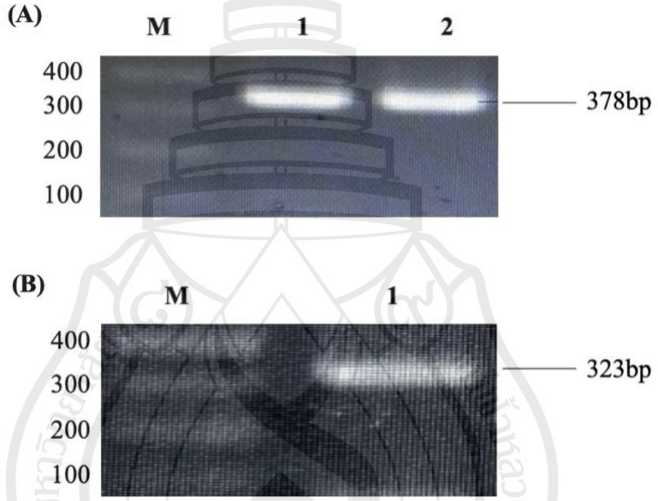


Note (A) *S. thermophilus* and (B) *E. coli.* M; 100 bp DNA ladder

Figure 4.1 1.5% of agrose gel electrophoresis of PCR product with *Glck* and *LacZ* gene

4.1.2 Optimization and Quantification of the *GlcK* and *LacZ* using qPCR

The *GlcK* and *LacZ* gene were amplified as described in section 4.3.1 and then were cloned into plasmid to be create a standard curve of gene copy number. Recombinant TOPO vectors of two genes were successfully transformed into *E. coli* DH5α competent cells using heat shock method. The positive of clones containing *Glck* or *LacZ* gene were amplified using PCR and analyzed using agarose gel electrophoresis, (Figure 4.2). The genes were confirmed using sequencing analysis. The results indicated that this gene was identical to *S. thermophilus* and *E. coli* (shown in supplementary data).



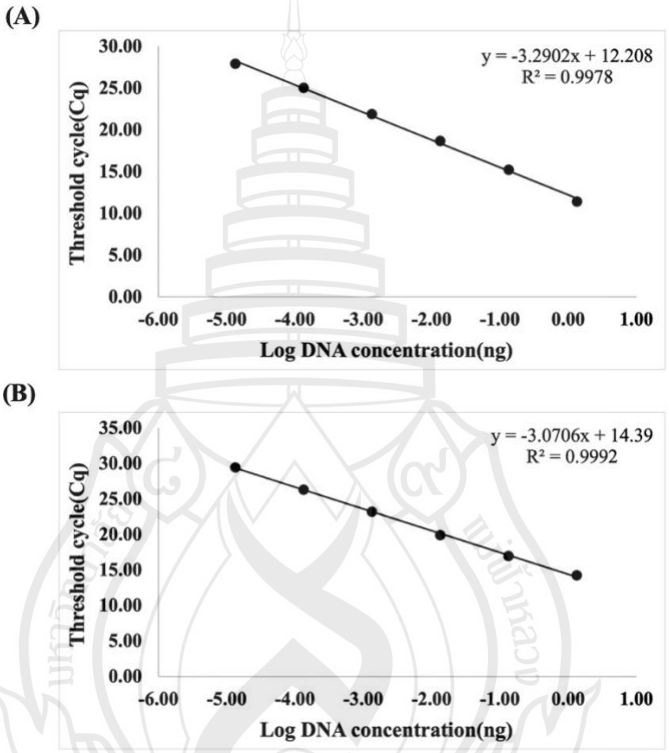
Note (A) *S. thermophilus* and (B) *E. Coli.* M; 100 bp, 1 and 2 represent TOPO TA® plasmid containing *Glck* gene (A). Lanes 1,2 and 3 present TOPO TA® plasmid containing *LacZ* gene (B)

Figure 4.2 1.5% agrose gel electrophoresis of PCR product with *Glck* and *LacZ* gene

4.2 Quantification of Gene Copy Number from the Bacterial Cultue of *S. thermophilus* and *E. coli* using qPCR

The standard curve was created by plotting the threshold cycle (Cq), which corresponds to the logarithm of the plasmid DNA concentration in each dilution. Three replicates of 10-fold dilutions of *S. thermophilus* and *E. coli* plasmid DNA ranging from

$3x10^3$ to $3x10^8$ DNA copies were processed to real-time PCR. Figure 4.3A shows that the linear correlation coefficient of the standard curve of *GlcK* gene was $R^2 = 0.99$ and the efficiency value was 97.2%. Figure 4.3B shows that the linear correlation coefficient of the standard curve of *LacZ* gene was $R^2 = 0.99$ and with the efficiency at 112%. The analysis of melting curve showed the specificity and accuracy of PCR-based quantification.



Note (A) *S. thermophilus* and (B) *E. coli*

Figure 4.3 Standard curve showing the log10 DNA amount (ng) vs threshold cycle of the real-time PCR method for 10-fold dilutions of *S. thermophilus* and *E. coli*

4.3 qPCR Sensitivity and Repeatability

To determine the sensitivity of this assay, quantitative PCR amplification was performed using serial dilutes with three initial nucleic acid solutions (10, 5, and 2 ng/µl, respectively). The results showed that the reaction efficiency values were 105%, 92.0% and 110%, and the R^2 values were 0.9686, 0.9921 and 0.9887, respectively (Figure 4.4).

All observed reaction efficiencies exceed 90%, which is in the optimal range (90-110%) (Broeders et al., 2014). A high R-square value indicates that the quantitative data measured has a good linear relationship with *GlcK* gene DNA concentration, which indicates that the sensitivity of quantitative PCR detection is more accurate. The results exhibited high sensitivity, detecting genomic DNA at 0.2 pg.

Repeatability is a measure of accuracy or reproducibility because it indicates the consistency of results and the reliability of the method (McAlinden et al., 2015). Next, we tested the reliability of qPCR using three different DNA concentrations (0.1, 1, and 10 ng/l). Here, the repeatability results are expressed as RSD as described by Yuwono & Indrayanto, 2005. Here, our results revealed the RSD of the Cq averages for the three different DNA concentrations was 0.01% (Table 4.1) which showed a repeatability. Similarly, there are reports regarding the monitoring of probiotic growth in *L. rhamnosus*. The sensitivity was also 0.2 pg of genomic DNA, or 10^3 CFU/ml of bacterial strain. Relative standard deviations were less than 1% and 3%, respectively, in terms of repeatability and reproducibility (Zhao et al., 2022).

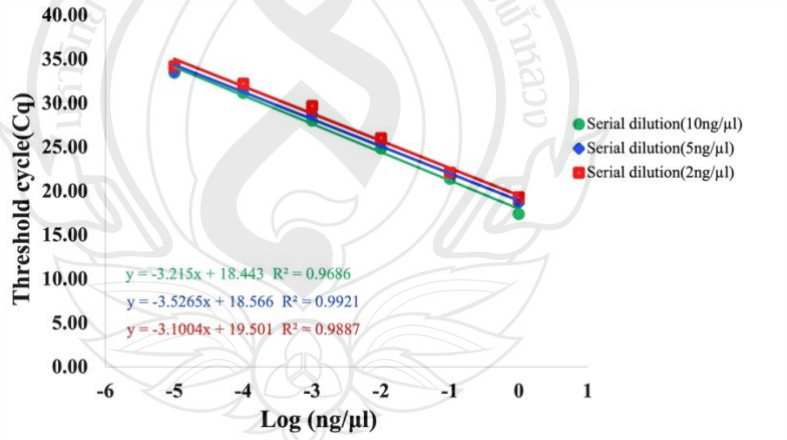


Figure 4.4 Calibration curves and sensitivity of the qPCR assay derived from DNA concentrations *GlcK* gene from 2, 5 and 10 ng/µl

Table 4.1 The repeatability of the qPCR method was assessed by calculating the relative standard deviation (RSD) of three different DNA concentrations

DNA concentration (ng/µl)	Cq					Mean Cq	RSD (%)
10	17.19	17.37	17.51	17.4	17.41	17.38	0.01
1	20.37	20.13	20.56	20.28	20.38	20.34	0.01
0.1	23.34	23.4	23.68	23.61	23.46	23.5	0.01

4.4 Correlation between Colony Forming Unit (CFU/ml) and Gene Copy Number of qPCR

To evaluate the correlation between CFU/ml and a copy number of gene copies *Glck* and *LacZ* in *S. thermophilus* and *E. coli* and the bacterial cell concentrations of *S. thermophilus* (10^1-10^6 CFU/ml) and *E. coli* (10^1-10^6 CFU/ml) were performed 2 methods by using qPCR and total plate count technique. The bacteria culture having the OD at 0.1 was used to make dilutions (see section 3.11). The correlation between *S. thermophilus* and *E. coli* gene copy number and bacterial cell count was subsequently established (Figure 4.5). These findings indicate a high degree of confidence in fitting the correlation between the two bacteria. However, compared to *S. thermophilus* and *E. coli* covered a wider range of cell detection. The qPCR result showed a detection at a lower copy number/ml, likely due to fastidious bacteria.

Here, *S. thermophilus* was detected from a logarithmic CFU of 1.69-6.56 and the logarithmic DNA copy number of 2.07-6.03 (Table 4.2). In addition, the logarithmic CFU (2.35–7.35) and logarithmic DNA copy number (2.61–7.08) were used to detect *E. coli* (Table 4.3). As show in Figure 4.4, the linear correlation coefficients of the standard curve of *S. thermophilus* were $R^2 = 0.989$, the efficiency value of 103%. The linear correlation coefficient of the standard curve of *E. coli* is $R^2 = 0.9912$, and the efficiency value is 112%.

Therefore, qPCR quantification was successfully to determine enumeration of the bacterial cell during culture.

This study demonstrates that qPCR provides a sensitive and specific approach for the detection and quantification of target DNA sequences. Nonetheless, qPCR is effective in detecting DNA; it is unable to distinguish between DNA from viable and non-viable cells (Janssen et al., 2016) . This limitation requires the application of culture-dependent techniques to verify the existence of live bacteria. This study was conducted and demonstrated the correlation with viable cells using total plate count and qPCR, revealing a significant association as stated in Table 4.2 and Figure 4.5. Recently, propidium monoazide (PMA) has emerged as a dye that is incapable of penetrating undamaged bacterial membranes (Submitted et al., 2021). PMA interacts with DNA when exposed to light, hence preventing its amplification in subsequent PCR assays. This dye has been used to differentiate between viable and non-viable bacterial cells. PMA-qPCR facilitates the accurate identification of live bacteria (Chen et al., 2021). Alternatively, the combination of PCR and the PMA approach, qPCR, provides a dependable method for accurately identifying and quantifying viable bacteria, proving beneficial for futher work (Guo et al., 2024).

Table 4.2 Quantification of *S. thermophilus* using the total plate count (Log CFU/ml) and qPCR (Log gene copy number) method

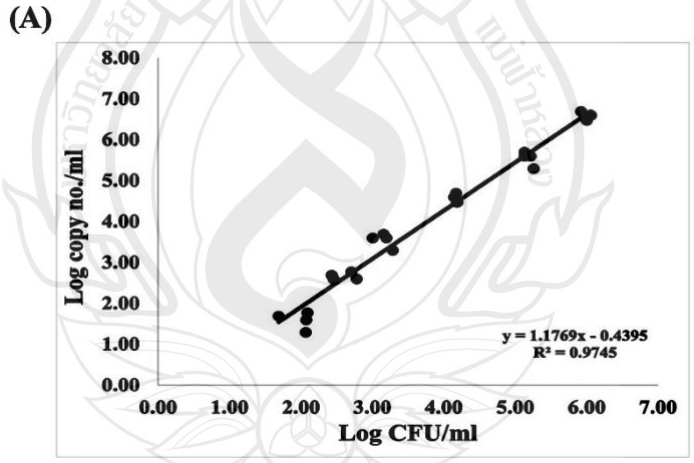
Bacterial cell concentration	**Average Cq**	**Average Log CFU/ml**	**Average Log copy number/ml**
10^1	19.95±0.09	6.56±0.06	6.03±0.03
10^2	22.91±0.14	5.63±0.05	5.16±0.04
10^3	26.23±0.02	4.52±0.06	4.18±0.01
10^4	29.51±0.18	3.50±0.14	3.21±0.05
10^5	31.83±0.42	2.63±0.05	2.53±0.12
10^6	33.37±0.03	1.69±0.07	2.07±0.01

Note All qPCR reactions and CFU were performed in three plicates (technical replicates)

Table 4.3 Quantification of *E. coli* using the total plate count (Log CFU/ml) and qPCR (Log gene copy number) method

Bacterial cell concentration	Average Cq	Average Log CFU/ml	Average Log copy number/ml
10^1	16.41±0.28	7.35±0.02	7.08±0.08
10^2	18.33±0.1	6.29±0.01	6.51±0.03
10^3	21.73±0.18	5.34±0.03	5.51±0.05
10^4	25.04±0.15	4.35±0.02	4.53±0.04
10^5	28.70±0.01	3.34±0.01	3.45±0.00
10^6	31.56±0.29	2.35±0.02	2.61±0.08

Note All qPCR reactions and CFU were performed in triplicates (technical replicates)



Note (A) *S. thermophilus* and (B) *E. coli*. The average Log10 copies/ml for three different assays was compared to the average Log10 CFU from three independent plating experiments. In all examples, error bars represent standard deviation. Linear regression between CFUs/ml and copies/ml as detected by qPCR for (A) *GlcK* and (B) *LacZ* gene

Figure 4.5 Standard curve of *GlcK* and *LacZ* was evaluated by qPCR and compared to CFUs/ml

(B)

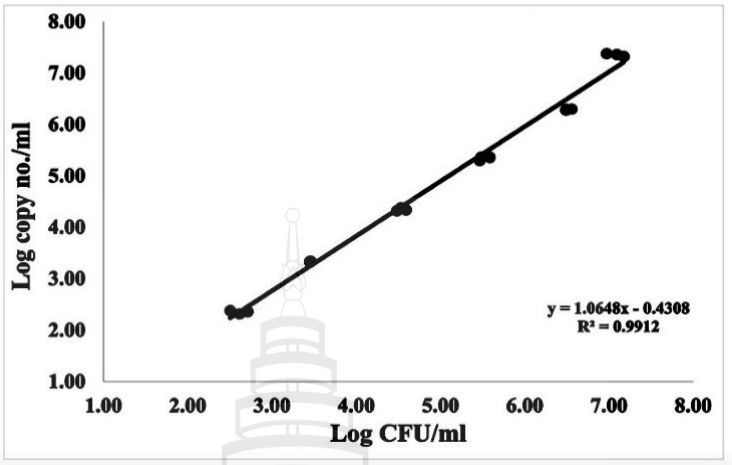


Figure 4.5 (continued)

4.5 Dertermination of Beta-glucan Content in *C. militaris* Mycelium

Dried mycelium (1 g) of *C. militaris* was extracted with distilled water using microwave extraction method (see section 3.3). The amount of beta-glucan was analyzed using enzyme hydrolysis as described by Megazyme. It was found that this method could extract beta-glucan of *C. militaris* with efficiency at 5% and obtained 4.29±0.1% as shown in Table 4.4. This result revealed the amount commonly found since the beta-glucan in fungal about 4.02±0.02% and 5.02±0.71%, respectively (Hwang et al., 2018; Morales et al., 2019).The selection of extraction method is pivotal in determining both the yield and quality of the extracted beta-glucan. Traditional extraction techniques, such as hot water extraction and acid or alkali extraction, yield different results compared to advanced methodologies, including microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), and enzyme-assisted extraction (EAE), each of which affects the efficiency and specificity of beta-glucan. These extraction methods has been suggested that influenced by several parameters, including the extraction method, purification technique, source of beta-glucan, and the specific fungal species (Chadwick, 2007).The composition of beta-glucan varies by source, impacting its solubility and extractability.

Different fungi display distinct properties regarding beta-glucan content, molecular weight, and structural characteristics, leading to variations in extractability (Zhu et al., 2016).

Table 4.4 Beta-glucan content from the mycelium of *C. militaris* using microwave extraction method and other edible fungi

Fungi	Yield of crude extact (g/100g)	Beta-glucan Content (%)	References
Cordyceps militaris	5.8±0.31	4.29±0.1	This study
Ganoderma lucidum	9.69±1.12	4.02±0.02	(Hwang et al., 2018)
Lentinula edodes	7.71±1.09	5.02±0.71	(Morales et al., 2019)

Note Values shown are the means ± standard deviation of the mean

4.6 Antioxidant Activity of Beta glucan Extracted from *C. militaris*

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Several studies have reported that mushrooms and their crude extracts, or beta-glucans, have strong antioxidant activity. However, there are few comparative studies on the antioxidant activity of beta-glucan in *C. militaris*. The DPPH radical assay is a stable, qualitative solid free radical, is a typical in vitro antioxidant evaluation method, has been used to determine the ability of a sample to clear free radicals, by providing a proton to form a stable DPPH molecule, its color changes from purple to light yellow.

According to Nandi et al. (2014) research, water-soluble glucan (PS), an immune-enhancing antioxidant, is isolated from the alkaline extract of *Russula albonigra* (Krombh.) Fr, an edible fungus, and its antioxidant mechanism is due to the polysaccharide providing hydrogen, binding with free radicals to form stable free radicals, thereby terminating the free radical chain reaction.

We conducted antioxidant activity experiments using beta-glucan extracted from *C. militaris* at an extraction concentration of 0.05 g/ml. As shown in Table 4.5, the DPPH

scavenging ability of beta-glucan extracted from *C. militaris* was 86.94%, while that of beta-glucan extracted from other three fungi (*Agaricus bisporus*, *Lentinula edodes* , *Aspergillus brasiliensis*) was 75.5%, 58.36% and 66.6% (Afiati et al., 2019; Ashraf Khan et al., 2017), respectively. In comparison, beta-glucan in *C. militaris* had the highest DPPH scavenging ability, and the higher the inhibition rate, the stronger the antioxidant activity. Therefore, the antioxidant activity of beta-glucan extracted from *C. militaris* has been reported that show highly relative to other beta-glucans as described by Miao (Miao et al., 2022).

Table 4.5 Antioxidant activity of beta-glucan extracted from *C. militaris* using DPPH method other edible fungi

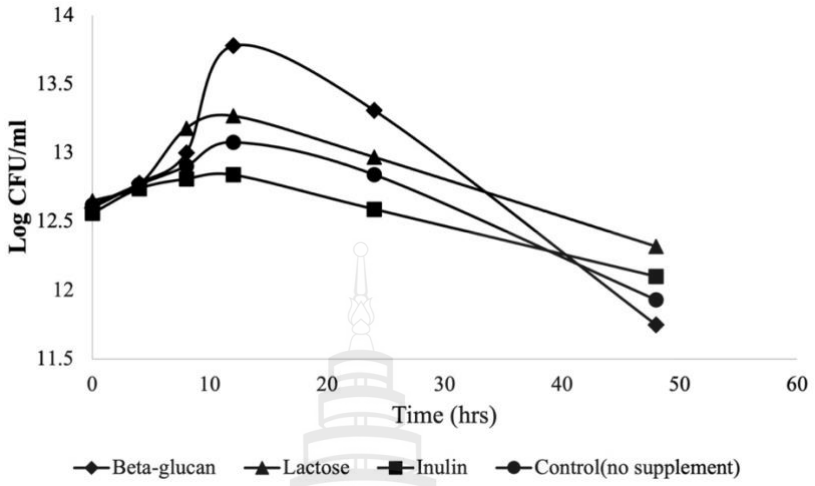
Fungi	% inhibition	References
Cordyceps militaris	86.94	This study
Agaricus bisporus	75.5	(Ashraf Khan et al., 2017)
Lentinula edodes	58.36	(Afiati et al., 2019)
Aspergillus brasiliensis	66.6	(Ashraf Khan et al., 2017)

4.7 qPCR Assay in Prebiotic Index

To investigate the detection of *S. thermophilus* and the activity of the probiotic, a qPCR method was designed, optimized, and validated. In this experiment, the copy number of *S. thermophilus* can be detected by using qPCR to calculate cell number (see section 4.3), and then the growth curve of *S. thermophilus* was made from cell number (Figure. 4.6). To assess the prebiotic index, we can add different prebiotic carbohydrates to culture media and cultivate bacteria for 24 hours. We then compare the growth of the bacteria with and without the supplement, determining ratios that exceed 1. This will suggest the prebiotic could help the microorganism by the prebiotic that was tested compared to the inulin as the standard carbohydrate as a prebiotic.

Figure 4.6 indicates that *S. thermophilus* reached to maximum cell density after 12 hours of growth on media supplemented with 1% (w/v) prebiotics. So calculated the prebiotic index using the bacterial counts at 12 hours with lactose as a positive control and as can be seen in Table 4.6, the prebiotic index of beta-glucan in *C. miliataris* revealed more than 1, indicating that beta-glucan show the potential to promote the growth of *S. thermophilus* compared to the lactose. However, the prebiotic index of inulin showed less than 1, indicating that inulin could not assist this bacterial strain.

As shown in Figure 4.6, the growth rate of *S. thermophilus* was the highest in the medium containing beta-glucan but not well in the medium containing inulin. So far, the beta-glucan does not many to report about the role in this bacterial strain. Some studies suggested that the addition of oat flour improved the viability of *S. thermophilus*, which can be attributed to the presence of the soluble dietary fiber beta-glucan, and the addition of oat beta-glucan enhanced the viability of the probiotic strains in the fermented product (Whitehead et al., 2014). Consistenly, the results have been reported that the addition of barley beta-glucan to probiotic low-fat yogurt enhanced the viability of probiotics during storage (Elsanhoty et al., 2018). In addition, the beta-glucan contribute to maintain viability of *S. thermophilus* and *Lactobacillus acidophillus* and *L. bifidum* during storage (Lazaridou et al., 2014). Moreover, *S. thermophilus* can be induced the beta-glucan by upregulating the expression of the L-lactate dehydrogenase gene (*ldhL*) to enhance lactic acid production (Goncerzewicz et al., 2016). Beta-glucan from *Pleurotus ostreatus* aqueous extract can promote growth. Since *S. thermophillus* lacks the enzyme glucanase to break down glucan, the findings suggested that the bacteria could grow with beta-glucan, this could be more for further study.



Note All experiments were performed in three replicates

Figure 4.6 The growth curve of *S. thermophilus* with different supplements

Table 4.6 Prebiotic index derived from the cultivation of *S. thermophilus* and *E. coli* with and without supplement in 12 hours

Substrate	Prebiotic index
Beta-glucan extracted from *C. militaris*	3.21±0.19
Inulin	0.37±0.02
Control (without supplement)	0.65±0.08

CHAPTER 5

CONCLUSION

Beta-glucan has become an interestingly functional food, in particular its prebiotic properties. This study successfully developed a method to evaluate the bacterial growth on the prebiotic test, replacing the conventional culture-dependent assays. Here, use a specific gene of *S. thermophilus*, the glucose kinase gene (*GlcK*), and *E. coli*, the beta-galactosidase gene (*LacZ*), by using quantitative PCR. Comparison of bacterial growth derived from qPCR and plate count methods showed no significant variation ($p<0.05$). Hence, qPCR-based methods facilitated reliable quantification of *S. thermophilus* and *E. coli* in growth determination during culture. The detection of bacterial growth revealed a correlation between the log CFU/m (1.69-6.56) and the log DNA copies (2.07-6.03) in *S. thermophillus* and log CFU/ml (2.35-7.35) and the log DNA copies (2.61-7.08) in *E. coli.* The study revealed the potential of beta-glucan from *C. milataris* as a prebiotic ingredient, showing that it has antioxidant activity and prebiotic property with a high prebiotic index of 3.21. The qPCR assay has the potential to accurate and quickly detect the growth kinetics of prebiotic and probiotic activity in a wide range of substrates.

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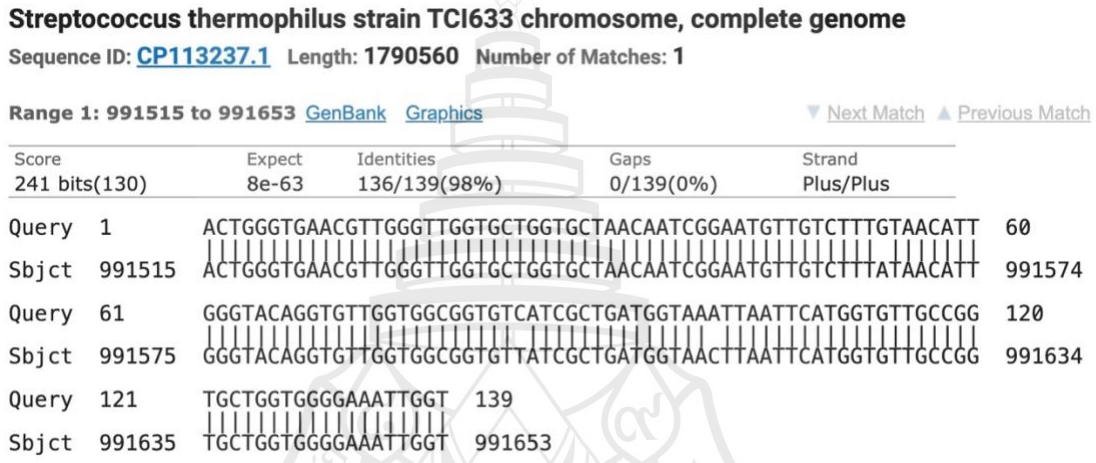
APPENDIX A

OPTIMIZATION AND QUANTIFICATION OF THE *GlcK* AND *LacZ* USING qPCR



Streptococcus thermophilus strain TCI633 chromosome, complete genome

Sequence ID: CP113237.1 Length: 1790560 Number of Matches: 1

Range 1: 991515 to 991653 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
241 bits(130)	8e-63	136/139(98%)	0/139(0%)	Plus/Plus

```
Query  1       ACTGGGTGAACGTTGGGTTGGTGCTGGTGCTAACAATCGGAATGTTGTCTTTGTAACATT  60
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Sbjct  991515  ACTGGGTGAACGTTGGGTTGGTGCTGGTGCTAACAATCGGAATGTTGTCTTTATAACATT  991574

Query  61      GGGTACAGGTGTTGGTGGCGGTGTCATCGCTGATGGTAAATTAATTCATGGTGTTGCCGG  120
               |||||||||||||||||||||||| |||||||||||||| ||||||||||||||||||||
Sbjct  991575  GGGTACAGGTGTTGGTGGCGGTGTTATCGCTGATGGTAACTTAATTCATGGTGTTGCCGG  991634

Query  121     TGCTGGTGGGGAAATTGGT  139
               ||||||||||||||||||||
Sbjct  991635  TGCTGGTGGGGAAATTGGT  991653
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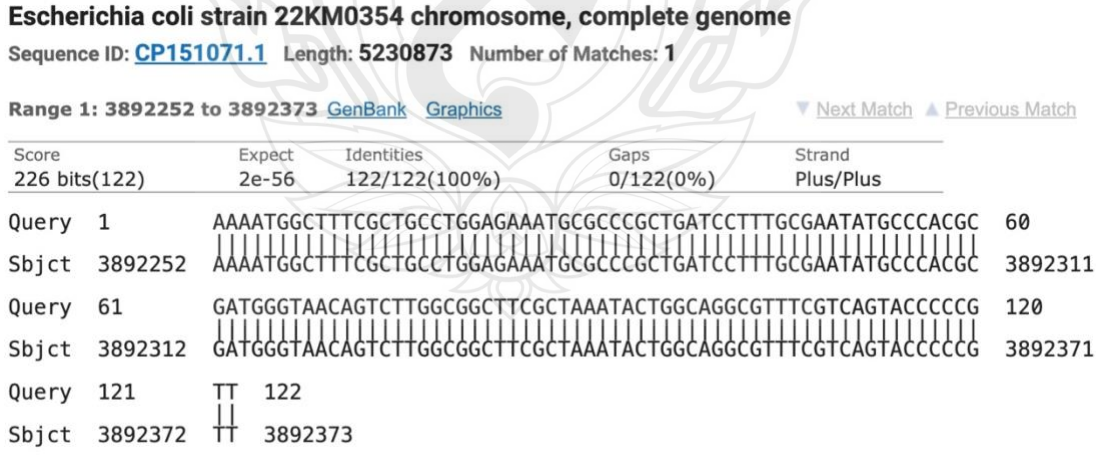
Figure A1 *GlcK* Gene Sequences Compared with the Database in the NCBI using BLAST Tool



Escherichia coli strain 22KM0354 chromosome, complete genome

Sequence ID: CP151071.1 Length: 5230873 Number of Matches: 1

Range 1: 3892252 to 3892373 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
226 bits(122)	2e-56	122/122(100%)	0/122(0%)	Plus/Plus

```
Query  1       AAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3892252 AAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGC  3892311

Query  61      GATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3892312 GATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCG  3892371

Query  121     TT  122
               ||
Sbjct  3892372 TT  3892373
```

Figure A2 *LacZ* Gene Sequences Compared with the Database in the NCBI using BLAST Tool

APPENDIX B

QUANTIFICATION OF GENE COPY NUMBER FROM THE BACTERIAL CULTURE OF *S. thermophilus* AND *E. coli* USING qPCR

Table B1 Calculation of Plasmid Concentration and DNA Copy number of *GlcK* Gene

Plasmid concentration	Mass of a single plasmid	DNA copy number
1.34E-06		3.00E+11
1.34E-07		3.00E+10
1.34E-08		3.00E+09
1.34E-09		3.00E+08
1.34E-10	4.4684E-18	3.00E+07
1.34E-11		3.00E+06
1.34E-12		3.00E+05
1.34E-13		3.00E+04
1.34E-14		3.00E+03

Table B2 Calculation of Plasmid Concentration and DNA Copy number of *LacZ* Gene

Plasmid concentration	**Mass of a single plasmid**	**DNA copy number**
1.32E-06		3.00E+11
1.32E-07		3.00E+10
1.32E-08		3.00E+09
1.32E-09		3.00E+08
1.32E-10	4.4081E-18	3.00E+07
1.32E-11		3.00E+06
1.32E-12		3.00E+05
1.32E-13		3.00E+04
1.32E-14		3.00E+03

Table B3 Ten-fold Dilution used for Quantitative *GlcK* Gene by qPCR

Source of plasmid DNA for dilution	**Initial conc. (g/µl)**	**Vol. of plasmid DNA (µl)**	**Vol. of diluent (µl)**	**Final conc.in (g/µl)**	**Log DNA conc. (ng)**
stock	3.27E-07	20	0	3.27E-07	N/A
Dilution1	3.27E-07	10	90	3.27E-08	N/A
Dilution2	3.27E-08	3.6	96.4	1.34E-09	0.13
Dilution3	1.34E-09	10	90	1.34E-10	-0.87
Dilution4	1.34E-10	10	90	1.34E-11	-1.87
Dilution5	1.34E-11	10	90	1.34E-12	-2.87
Dilution6	1.34E-12	10	90	1.34E-13	-3.87
Dilution7	1.34E-13	10	90	1.34E-14	-4.87

Table B4 Ten-fold Dilution used for Quantitative *LacZ* Gene by qPCR

Source of plasmid DNA for dilution	**Initial conc. (g/µl)**	**Vol. of plasmid DNA (µl)**	**Vol. of diluent (µl)**	**Final conc.in (g/µl)**	**Log DNA conc. (ng)**
stock	5.47E-07	20	0	5.47E-07	N/A
Dilution1	5.47E-07	10	90	5.47E-08	N/A
Dilution2	5.47E-08	2.4	97.6	1.32E-09	0.13
Dilution3	1.32E-09	10	90	1.32E-10	-0.87
Dilution4	1.32E-10	10	90	1.32E-11	-1.87
Dilution5	1.32E-11	10	90	1.32E-12	-2.87
Dilution6	1.32E-12	10	90	1.32E-13	-3.87
Dilution7	1.32E-13	10	90	1.32E-14	-4.87

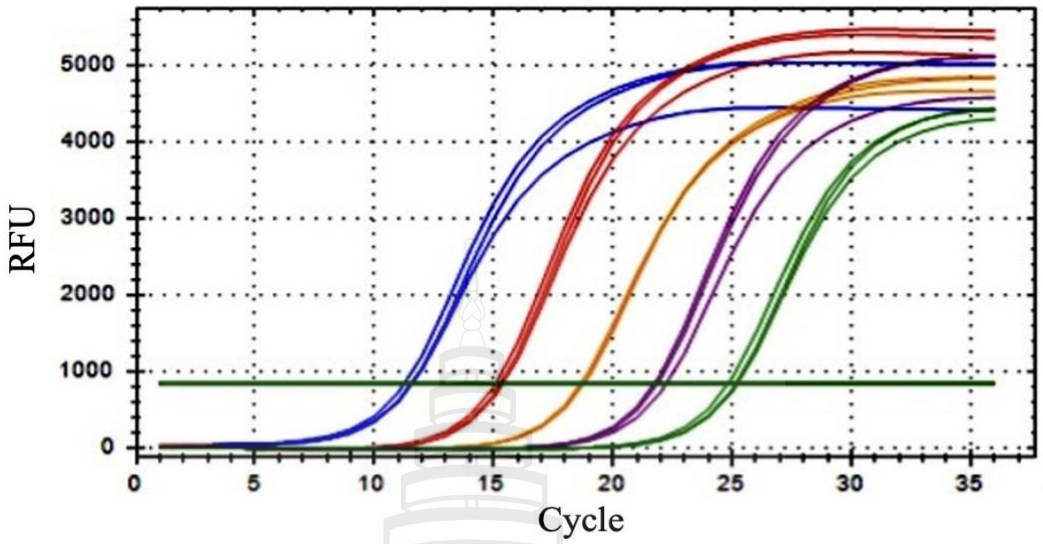


Figure B1 A Plot of Relative Fluorescence units Corresponding to Cycle number of the Amplification (*GlcK* Gene)

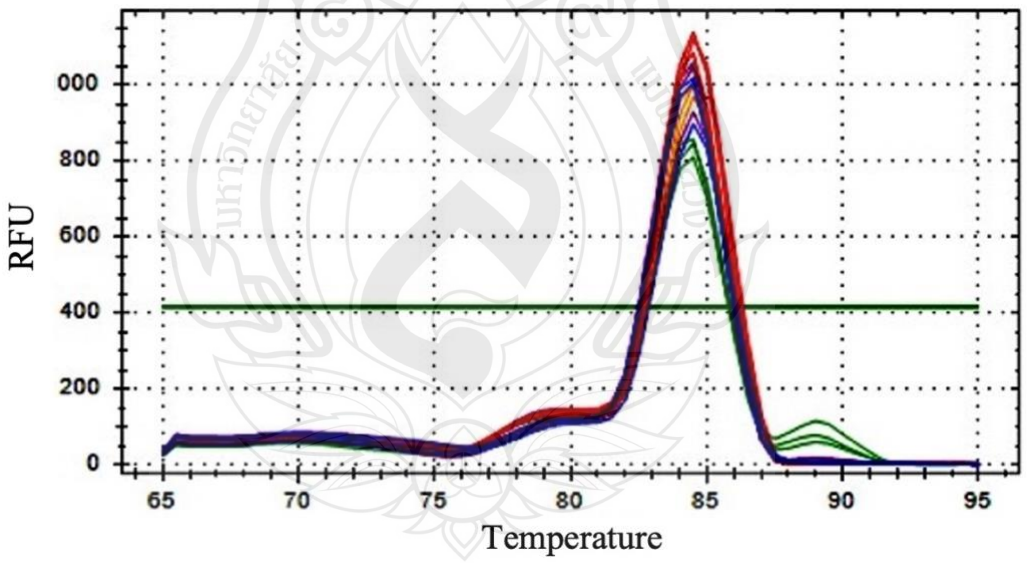


Figure B2 A Plot of Relative Fluorescence units Corresponding to Cycle number of the Melt Peak (*GlcK* Gene)

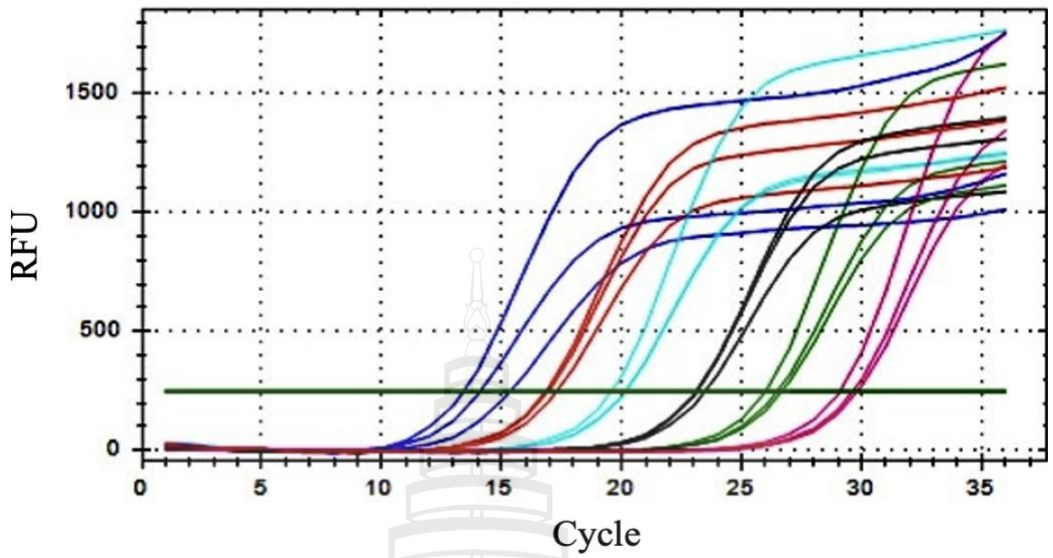


Figure B3 A Plot of Relative Fluorescence units Corresponding to Cycle number of the Amplification (*LacZ* Gene)

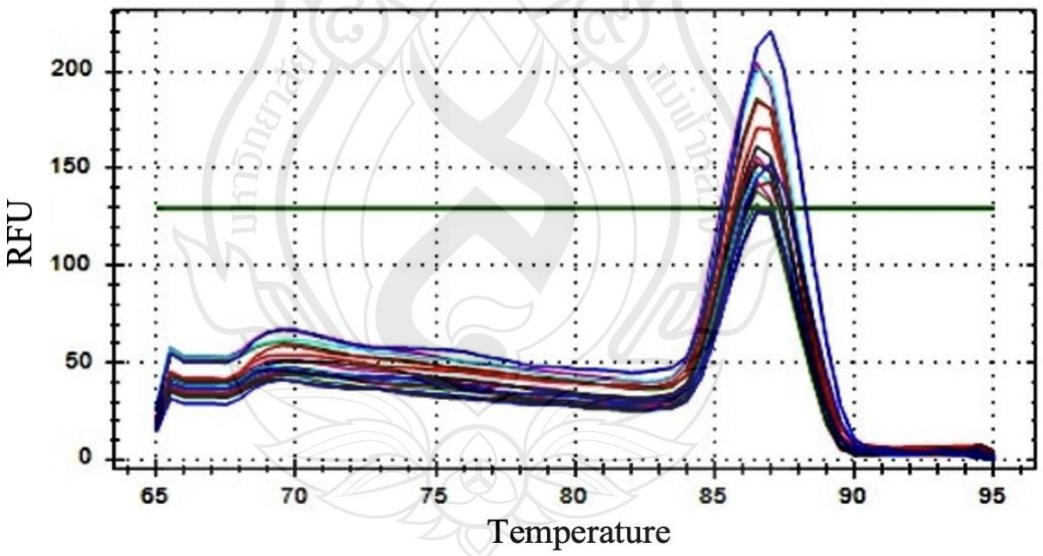


Figure B4 A Plot of Relative Fluorescence units Corresponding to Cycle number of the Melt Peak (*LacZ* Gene)

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