METABOLIC PROFILING ANALYSIS OF THE *KOJI* AMAZAKE PRODUCT FERMENTED FROM THAI JASMINE RICE

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ABSTRACT

Koji amazake is a prebiotic fermented Japanese rice produced by koji mold originating in Japan. The benefits of the koji amazake include improved intestinal health, decreased cholesterol level, immunological function, etc. The high demand for Thai jasmine rice creates substandard grades, which can be fermented into koji amazake, boosting value while reducing waste. The objectives of this study were to find health benefit compounds in YoRice Thai jasmine rice amazake (JAS_AMA) metabolic profiling with a liquid chromatography quadrupole time-of-flight mass spectrometry instrument (LC-QTOF-MS/MS), comparing the results with Thai jasmine rice without fermentation overnight (JAS_bMIX), jasmine rice solution mixed with sucrose syrup (JAS_SYR), and commercial Japan koji amazake (JP_AMA). Candidate prebiotics identified through the LC-QTOF-MS/MS method were subsequently quantified via high-performance liquid chromatography with refractive index detection (HPLC-RID). The results of qualitative analysis of JAS_AMA have revealed a variety of compounds, including prebiotics such as isomaltooligosaccharide (IMOs), kojibiose, nigerose, other prebiotics, and other amino acid groups. This quantitative study found the IMOs to increase 5.95 times and the isomaltose to increase 2.62 times in JAS_AMA when compared with JAS_bMIX. Additionally, the *in vitro* study found the prebiotic effect in JAS_AMA. This study demonstrated that JAS_AMA contains the IMOs prebiotics that have the potential to promote human digestive health.

Keywords: Koji Amazake, Metabolic Profiling, Isomaltooligosaccharide

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ABBREVIATIONS AND SYMBOLS

JAS_AMA	Thai Jasmine Rice Amazake Sample Product
JAS_bMIX	Thai Jasmine Rice Before Mixed Fermentation
JAS_SYR	Thai Jasmine Rice Solution and Syrup Mixed
JP_AMA	Japanese Amazake Sample Product
IMO	Isomaltooligosaccharide
BS	*Bacillus subtills*
LP	*Lactobacillus plantarum*
EC	*Escherichia coli*
MRS (broth)	DeMan, Rogosa, and Sharpe broth. / Lactobacillus Broth
wMRS (broth)	Without carbohydrate source MRS broth
GLU (broth)	wMRS add glucose / MRS broth

CHAPTER 1

INTRODUCTION

1.1 Rationale and Background

Fermented foods are defined as “foods or beverages produced through controlled microbial growth and the conversion of food components through enzymatic action” (Rampelli et al., 2016). Most fermented foods are made to help preserve food or add flavor to make food more delicious. Nowadays, research on the gastrointestinal tract has become more active. As a result, fermented foods have been researched for their digestive health benefits (Dimidi et al., 2019). The effect of digestive health has been shown to be related to modulation of the gut microbiome.

The human gut microbiome consists of maximally 10^{14} commensal microorganisms that live in and around the human intestinal system; most of them live in the colon. They include bacteria, viruses, fungi, and protozoa (Gill et al., 2006). Their living affects personnel's health. If there is an abundance change, it will result in the host receiving special metabolic functions that are important for both health and disease. Inflammatory bowel diseases (Lucas López et al., 2017), inflammatory skin conditions like psoriasis and atopic dermatitis, autoimmune arthritis (Wu et al., 2010), type 2 diabetes (Qin et al., 2012), obesity (Turnbaugh et al., 2006), and atherosclerosis (Schäffler et al., 2016) have all been related by research studies to the composition and function of the intestinal microbiome in various disease states (Singh et al., 2017) and are also at risk for dangerous diseases like cardiovascular diseases (Schicho et al., 2015), and colorectal or bowel diseases (Brennan & Garrett, 2016). In China from 2015 to 2020, colorectal or bowel cancer was one of the top 5 cancer incidences, and it is expected to rise in the following years (Cao et al., 2021). Fermented foods may have positive impacts on health and disease via a variety of mechanisms. It can produce the probiotics and prebiotics that are so important for maintaining the gut microbiome.

Probiotics are living microorganisms. It usually resides in the gastrointestinal tract or is added to foods or products eaten that, when given in sufficient quantities, boost the host's health (Kumari et al., 2015). Consuming probiotics has been linked to several health benefits, including improved intestinal health, relief from lactose intolerance symptoms, improved immunological function, lower serum cholesterol, control of urogenital and respiratory tract infections, and a lower risk of colorectal cancer (Salminen et al., 2005). Colorectal cancer can be caused by, among other things, oxidative stress, which is one of the interesting causes of DNA damage in epithelial cells caused by certain gut microbes such as *Enterococcus faecalis* (de Almeida et al., 2018; Wang & Huycke, 2007). There is already a relationship between probiotics or gut microbiota equilibrium and the total of reactive oxygen species (ROS) (Louis et al., 2014).

Prebiotic compounds may therefore have a suitable impact on the probiotics' ability to persist in food products and the gastrointestinal tract. They are non-viable and non-digestible food ingredients that are metabolized selectively by beneficial intestinal bacteria and enhance their growth and/or activity (Pineiro et al., 2008). Prebiotic products are thought to represent a $6.05 billion global market (Grand View Research, 2020). The market value in September 2022 was supposed to be equal to 221,914 million baht in 2021 and anticipated to increase at a compound annual growth rate (CAGR) of 14.9 % per year from the year 2022 to 2030 in US. And also, the Asia Pacific was estimated to be worth USD 1.5 billion in 2020 and anticipated to develop at a CAGR of 15.6% from 2020 to 2028 (Curra, 2022). In Thailand Prebiotic products related to Thai white rice (Thai jasmine rice) are products that are fermented rice or rice flour mixed with probiotics to produce fermented products such as yogurts (Kumari et al., 2015). If we develop fermented foods derived from Thai-sourced raw ingredients. It's possible that it will improve Thai consumer gastrointestinal health. Additionally, this will increase the export of innovative Thai prebiotic products.

Amazake is an interesting selection because it is a sweet fermented rice that has a high potential to be developed as a healthful beverage or prebiotic beverage product from Japan. However, there are no similar products in Thailand that can compete with Japan koji amazake. Additionally, as amazake product is not an

alcoholic beverage, it can be sold to a wide range of consumers, from young children to the elderly (Kurahashi, 2021).

The health-benefiting ingredients being identified in this study, such as those from the prebiotics group, have the potential to become valuable components in future nutritional supplements or functional food formulations. This research serves as a guiding framework for developing health-oriented food products in Thailand. Specifically, it highlights the importance of utilizing locally available rice varieties, such as Thai jasmine rice, which is known for its distinctive flavor (Maleki et al., 2020)and robust antioxidant properties (Summpunn et al., 2022).

Therefore, this study was aimed at investigating the health-benefiting compounds in Thai jasmine rice amazake and evaluating the prebiotic components generated after rice fermentation. It also examines the potential of these prebiotics to promote probiotics with gut health-enhancing properties. This research provides a foundation for the further development of prebiotic products derived from Thai jasmine rice amazake.

1.2 Objectives

1.2.1 Explore nutritional, bioactive, and health benefit compounds in the Thai jasmine rice amazake.

1.2.2 Develop a computer program to analyze predicted formular data for health benefit compounds identification.

1.2.3 Quantify the amount of candidate compound in the Thai jasmine rice amazake.

1.2.4 Evaluate the activity of prebiotic compounds identified in the Thai jasmine rice amazake with various microorganisms using the *in vitro* technique.

1.3 Study Scope

In this study, metabolic profiles of products derived from Thai rice's amazake were explored, and candidate prebiotic functional ingredients were selected based on their liquid chromatography-mass spectrometry (LC-MS) profiles, analyzed from various perspectives using both tabular and graphical summaries. A constructed computer program was employed to calculate analytical metrics such as VIP scores and generate principal component analysis (PCA) plots. The amazake compound profiles were matched with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Python coding was used to facilitate the creation of Venn diagrams and other visualizations to review bioactive and health-benefit compounds in the amazake products. Additionally, high-performance liquid chromatography (HPLC) was used to absolutely quantify prebiotics's concentration in the samples. To assess the prebiotics's characteristics, *in vitro* tests were conducted. These prebiotics were tested with De Man–Rogosa–Sharpe (MRS) medium, without other carbohydrate sources, to evaluate their effects when combined with the selected candidate probiotics.

1.4 Expected Output

1.4.1 Various nutritional, bioactive, and health benefit compounds identified from LC-MS.

1.4.2 The level of prebiotic candidates in the Thai jasmine rice amazake was expected to be equal or greater than the Japanese rice amazake.

1.4.3 Prebiotic candidates from Thai jasmine rice amazake could improve probiotics and reduce the growth of pathogenic microbes.

1.5 Expected Outcome

1.5.1 The recognized health benefits of the Thai jasmine rice amazake.

1.5.2 The method for big data analysis of other health beverages using a mass spectrometer for health claim products.

1.5.3 Promotion of healthy beverage marketing and Thai jasmine rice fermented information in Thailand.

1.6 Impacts

1.6.1 A prebiotic beverage would be developed to promote a healthy gut microbiome among the Thai population, utilizing locally sourced functional ingredients and evidence-based fermentation methods.

1.6.2 Thai jasmine rice would contribute to the development of innovative healthy beverages and food products through alternative processing methods.

1.7 Operational Definition

1.7.1 Prebiotics: The active ingredients in foods or nutrient products that will affect gut microbiota and human colorectal health. They are not absorbed by the host organism's somatic cells and are not digested in the regular gastrointestinal tract. However, it is advantageous to promote the growth of the specific class of intestinal microbes (it means some microbes in gut microbiota) that are good for the human body (Gibson et al., 2004).

1.7.2 Metabolites: The intermediate products produced during metabolism, catalyzed by various enzymes that occur naturally within cells. In this study, it means microbes break down food such as rice, drugs, or chemicals, or human tissue such as fat or colorectal tissue. The entire collection of small-molecule chemicals present in a biological sample is referred to as the metabolome and may contain both endogenous metabolites, which are compounds produced by an organism naturally, and small-molecule chemicals as well as exogenous substances that an organism does not naturally produce ("NCI Dictionary of Cancer Terms," 2011; Tsoukalas et al., 2017; Zhang et al., 2013).

1.7.3 Koji amazake: A Japanese sweet and sour drink with no alcohol. It is a product that is both sweet and nutrient-rich, consisting mostly of prebiotic compounds. (Kurahashi, 2021).

1.7.4 Thai Jasmine rice (*Oryza sativa L.* KDML): Commonly known as "Khao-Hom-Mali" in Thai, this premium Thailand rice variety is characterized by its shiny white appearance (resembling jasmine flowers), soft texture, and distinctive aromatic fragrance when cooked. Recognized internationally for its superior quality, Thai Jasmine rice maintains high consumer demand and is classified as a premium agricultural product (Phanchaisri et al., 2007; Uawisetwathana et al., 2015).

1.8 Conceptual Framework

The analytical conceptual framework in this study is shown in Figure 1.1.

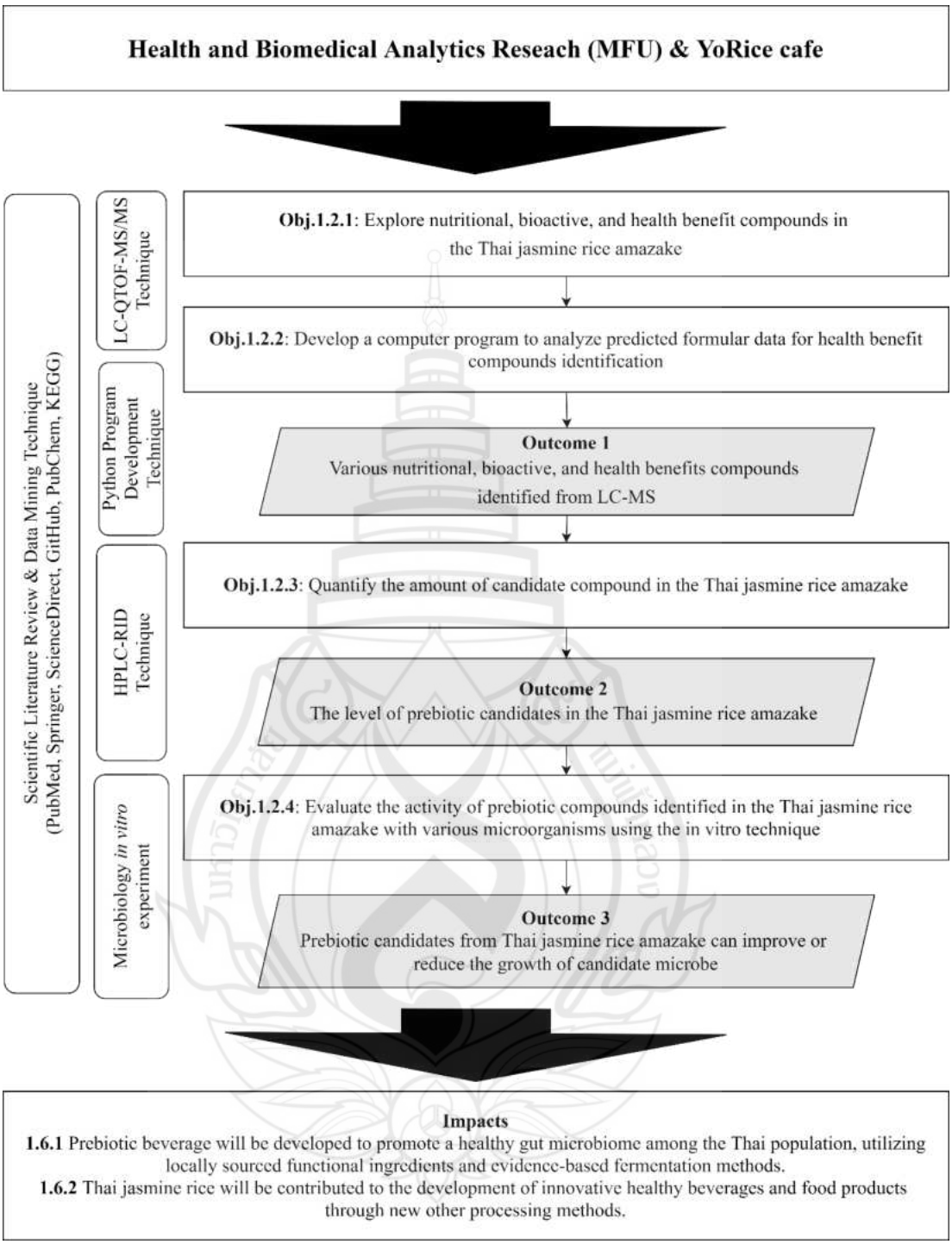


Figure 1.1 Conceptual Framework

CHAPTER 2

LITERATURE REVIEW

2.1 Gut Microbiota

The gut microbiota consists of thousands of microbial species, including tens of thousands of bacterial variants. Microbial density follows a gradient along the gastrointestinal tract, with the colon harboring the highest concentration of microorganisms, while significantly fewer populate the stomach and small intestine. The gut microbiota consists mainly of *Firmicutes* and *Bacteroidetes* phyla (Schmidt & Stallmach, 2005). Archaea bacteria and some eukaryotes, such as viruses and bacteria, are also microbes. There are many species in the intestines, including those from the flora kingdom. These microorganisms aid in the formation of barriers, promote the maintenance of the distal gastrointestinal tract, and stimulate epithelial cell regeneration by increasing the production of short-chain fatty acids (SCFAs), which aid in intestinal absorption (Eckburg et al., 2003).

Population and microbial diversity are primarily related to the feeding behavior of the host. Research has shown that the number and diversity of intestinal microflora affect intestinal pH and the secretion of compounds that affect host health as well. The study of the intestinal microbial kingdom is thus important because changes in intestinal populations are directly related to host health (Bibbò et al., 2016). This is because gut microbiota benefits by breaking down nutrients that the host mechanism cannot digest and releasing nutrients that the host can absorb and manipulate. It also inhibits the entry of pathogenic microorganisms into the intestines (Eggesbø et al., 2003; Huh et al., 2012; Sevelsted et al., 2015).

An imbalanced population of the gut microbiota has an incidence of various diseases or increases the risk of diseases, such as neurodegenerative disorders, including Alzheimer's disease (Hu et al., 2016), psychiatric disorders including depression disorder (Tian et al., 2022), schizophrenia (Li et al., 2021), autism spectrum (Wang et al., 2020), and attention-deficit hyperactivity disorder (Yang et al., 2023),

metabolic syndrome including obesity (Fei & Zhao, 2013), hyperglycemia or type 2 diabetes (Zhou et al., 2019), dyslipidemia (Wang et al., 2016), hypertension (Li et al., 2017), hyperuricemia (Guo et al., 2016), non-alcoholic fatty liver disease (Yuan et al., 2019), and cancer development including colorectal cancer. Most study treatment or cure methods involve replenishing the missing healthy microorganisms or, by definition, probiotics. Multiple studies have demonstrated the beneficial effects of probiotic combinations comprising *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Enterococcus faecalis*, and *Bacillus cereus*. These probiotics were shown to restore gut microbiome balance and improve immune responses while enhancing SCFA production, notably acetate, butyrate, and propionate, in patients with microbiota disruption following gastrectomy or chemoprevention therapy. These findings support their potential as adjuvant therapies for colorectal cancer patients undergoing such treatments (Huang et al., 2023; Zheng et al., 2019). The effects of prebiotics (fructooligosaccharides, xylooligosaccharides, polydextrose, and resistant dextrin) on colorectal cancer were investigated in a study by Xie et al. (2019). Prebiotic supplementation was recommended to enhance serum immunologic indicators in patients seven days prior to surgery. These findings underscore the significance of prebiotic and probiotic research in addressing gut microbiota imbalances.

2.2 Prebiotic, Probiotic, Synbiotic, and Postbiotic

There are four types of foods or supplements that affect the gut microbiota of host organisms: prebiotics, probiotics, synbiotics, and postbiotics (Li et al., 2021). The oldest definition of prebiotic by Gibson in 1995 is “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” (Gibson & Roberfroid, 1995). This definition is still current. However, the faculty has changed the definitions. In the year 2016, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined

“prebiotic” as “a substrate that is selectively utilized by host microorganisms, conferring a health benefit” (Gibson et al., 2017).

Prebiotics, among other food components, can be categorized using five fundamental characteristics, according to Wang (Wang, 2009). The first criterion is the assumption that prebiotics are not digested or are only partially digested in the upper digestive tract segments. The second requirement is that they eventually arrive in the colon, where they are selectively fermented by possibly advantageous bacteria (Macfarlane et al., 2007). The next requirement is when the fermentation results in the improvement of the immune system, an increase in stool mass, a moderate reduction in colonic pH, a decrease in nitrous end products and fecal enzymes, and an increase in the production of various SCFAs that are advantageous to the host (Koh et al., 2016). The fourth criterion is selective stimulation of intestinal bacteria growth and/or activity that may be associated with health protection and well-being (Gibson et al., 2004). The final classification criterion assumes that a prebiotic must be able to withstand food processing conditions while remaining unchanged, non-degraded, or chemically unaltered and available for bacterial metabolism in the intestine (Vallianou et al., 2020).

To summarize, prebiotics are a class of non-living food compounds, unlike probiotics and synbiotics. Postbiotics are non-living foods like prebiotics, but the different process of prebiotics is similar food, and postbiotics areinanimate microorganisms (Table 2.1) (Martyniak et al., 2021). When consumed, no metabolic pathway can digest or absorb this prebiotic nutrient. However, the gut microbiota can digest prebiotics and provide alternative compounds that benefit the human body and improve human health. Another is synbiotics, which are products that combine probiotics and prebiotics in a product. There are also certain types that are rarely discussed. Alternatively, the dead probiotic microbial cells and cell constituents are presently called “paraprobiotics” (Lakna, 2022).

Table 2.1: Difference between Prebiotic, Probiotic, and Postbiotic

Content	Prebiotic	Probiotic	Postbiotic
Description	A non-digestible food ingredient that promotes the growth of beneficial microorganisms in the intestines	Live bacteria and yeasts promoted as having various health benefits	A preparation of inanimate microorganisms and/or components that confers health benefit on host
Substrate	A compound in food, undigestible material that stimulates the growth of probiotics	*Lactobacillus, Bifidobacteria*, other microorganisms have health benefits	Metabolite or end product and microbial cell fragments/structures
Examples	Beta-glucan from oats and inulin from chicory root	Yogurt, Probiotic milk (Yakult)	SCFA*, Vitamins, Phenols

Note SCFA = Short Chain Fatty Acid

Source Kim & Park, (2021), Mao et al., (2021)

Recently, there has been an increasing interest in determining whether synbiotics (a combination of prebiotics and probiotics) and postbiotics (inanimate microorganisms and/or their components that provide a health benefit to the host) have good biological activities for the prevention of metabolic syndrome diseases (Salminen et al., 2021; Wu et al., 2021). Examples of diseases treated are obesity (Holmes et al., 2020), type 2 and gestational diabetes mellitus (Markowiak & Śliżewska, 2017), non-alcoholic fatty liver disease (Mao et al., 2021). By supplying energy to the gut microbiota, they continuously excrete bioactive molecules in the bowel lumen, some of which may translocate into the circulation and influence the metabolic process as specific ligands (Holmes et al., 2020) . In this process, intestinal

integrity is crucial because it prevents the transmission of toxic bacterial metabolites into the circulation (Jansma et al., 2021).

An example of a clinical trial of probiotics, prebiotics, symbiotics, and postbiotics in the past 2018–2023 is described in Table 2.2. The examples given are still only some of the studies in this scope. However, there are studies in various age groups, from newborn infants to elderly participants. The diseases of interest are diverse, ranging from simple diseases such as high blood pressure, obesity, and pneumonia to stress-related brain diseases. Some studies have significant improvements in treatment results that are all related to the actual modified probiotic group in participant gut microbiota. Due to Omic's advanced analytical technology, this study of large amounts of data is now closer to reality in terms of its application to improve our knowledge of the gut microbial ecosystem (Lagier et al., 2018). This fermented food study is therefore suitable to be the first step in research to be forwarded for further research.

Table 2.2 Example of Probiotics, Prebiotics, Synbiotics, and Postbiotics Used in the Treatment of Various Sample Groups.

	Main treatment used	**Groups**	**Gut microbes affected**	**Reference**
Probiotics	*Streptococcus thermophilus* [2], *Bifidobacterium bifidum* [1], *Bifidobacterium breve* [2], *Bifidobacterium longum* [1, 2], *Bifidobacterium infantis* [2], *Lactobacillus acidophilus* [2], *Lactobacillus plantarum* [2], *Lactobacillus paracasei* [2], and *Lactobacillus helveticus* [2]	Cognition impairment [2] (n=242) and depressive disorder [1] (n=53)	**(-)** *Eubacterium*, *Allisonella,* and *Prevotellaceae* [1] **(+)** Lactobacillus and Bifidobacteria genera [2] **= (-)** Cognition impairment and depressive disorder [1, 2]	[1] (Kim et al., 2021) [2] (Schaub et al., 2022)
	Bifidobacterium brave, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium longum, and Lacticaseibacillus rhamnosus	Necrotizing enterocolitis and neonatal sepsis (n=57).	**(+)** Bifidobacteria genera **(-)** *Lacticaseibacillus rhamnosus* **= (-)** Necrotizing enterocolitis and neonatal sepsis	(Samara et al., 2022)

Table 2.2 (continued)

	Main treatment used	**Groups**	**Gut microbes affected**	**Reference**
Probiotics	*Bifidobacterium longum*, *Bifidobacterium breve*, *Lactococcus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactobacillus crispatus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus casei*	Type 2 diabetes (n=365)	**(+)** *Bifidobacterium breve* **(-)** *Eggerthella lenta* **= (-)** Postprandial total cholesterol and low-density lipoprotein cholesterol	(Wang et al., 2022)
Prebiotics	Galactooligosaccharide	Autistic spectrum disorder (n=30)	**(+)** *Bacteroides*, *Roseburia*, *Akkermansia*, *Hespellia spp* (+) Amino acid (in fecal sample) ≈ **(-)** Nutrient malabsorption **(-)** *Prevotella* and *bifidobacteria.* = **(-)** Autistic spectrum disorder	(Grimaldi et al., 2018)
	Type IV resistant starch (RS) group, including RS maize/ RS potato/ RS tapioca-derived [3] Psyllium husk [4]	Constipated Patients and Excretion problem participants [3] (n=40), [4] (n=8)	**RS maize (-)** *Ruminococcus callidus*, *Agathobaculum butyriciproducens*, and *Adlercreutzia equolifaciens*; **RS tapioca (-)** unclassified genus of *Ruminococcaceae*, *Eubacterium hallii*, and *Clostridium viride*; **All RS (-)** constipation and excretion problems [3] **Psyllium husk (+)** *Lachnospira, Faecalibacterium*, *Phascolarctobacterium, Veillonella*, *Sutterella* = **(+)** fecal water **(-)** *Coriobacteria, Christensenella* = **(-)** Constipation and excretion problems [4]	[3.] (Deehan et al., 2020) [4.] (Jalanka et al., 2019)

Table 2.2 (continued)

	Main treatment used	Groups	Gut microbes affected	Reference
Prebiotics	Oats	Mildly Hypercholesterolemic (n=187)	**(+)***Akkermansia muciniphila* and *Roseburia*, and the relative abundance of *Dialister*, *Butyrivibrio*, and *Paraprevotella* **(-)** unclassified *f-Sutterellaceae* **(+)** *Bifidobacterium* = **(-)** LDL-C **(+)** *Enterobacteriaceae*, *Roseburia*, and *Faecalibacterium prausnitzii* = **(+)** Plasma butyric and valeric acid **(-) C**holesterol	(Xu et al., 2021)
Synbiotics	*Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, and trans-galactooligosaccharide [5] *Lactobacillus casei*, *Bifidobacterium breve*, and galactooligosaccharides (GOS) [6].	Obesity with type 2 diabetes [5] (n=20), [6] (n=72)	**(+)** *Bifidobacterium* and *Lactobacillus* = **(+)** Gut microbiota richness = **(-)** Obesity [5] **(+)** *Bifidobacterium* and total lactobacilli = **(+)** Acetic and butyric acids = **(-)** Obesity [6]	[5](Sergeev et al., 2020) [6](Kanazawa et al., 2021)
	Lactobacillus acidophilus, *Lactobacillus casei*, *Bifidobacterium lactis* and Inulin	Chronic kidney disease (n=34)	**(+)** *Bifidobacteria*, *Lactobacillus*, and *Subdoligranulum* = **(-)** Indoxyl sulfate, **(+)** Estimated glomerular filtration rate, and **(-)** C-reactive protein = **(-)** Chronic kidney disease	(Mitrović et al., 2023)
	Lactobacillus paracasei ssp. Fructooligosaccharide and Galactooligosaccharides	Healthy infant (n=89)	**(-)** *Klebsiella* = (+) *Bifidobacteria* = **(+)** Microbial degradation metabolites implicated in immune signaling and in the gut-lung and gut-skin axes.	(Sjödin et al., 2023)

Table 2.2 (continued)

	Main treatment used	Groups	Gut microbes affected	Reference
Postbiotics (Most are mixed with prebiotics)	Seafood sticks containing heat-inactivated *B. animalis* subsp. *lactis* CECT8145, omega-3, and inulin (prebiotics)	Abdominally obese individuals (Cardiometabolic risk factors) (n=114)	**(+)** Glycemic parameter = **(-)** *Alistipes finegoldii* and Ruminococcaceae family **(-)** Pulse pressure = **(-)** *Prevotella* 9-ASV0283 and Christensenellaceae family	(Companys et al., 2022)
	9:1 short-chain galactooligosaccharides (scGOS): long-chain fructooligosaccharides (lcFOS) and postbiotics derived from the Lactofidus™ fermentation process milk and oligosaccharide found in human milk, at a final level of ~25 mg/100 mL formula	Healthy infants (compare between prebiotics + postbiotic infant milk formula and only milk formula) (n=90)	*Bifidobacterium* levels = Corresponding activity (e.g., lactate levels). Several gut microbe groups Pre+postbiotics infant milk formula ≠ Infant milk formula group. and Pre+postbiotics infant milk formula ≈ Breast-fed infants	(Rodriguez-Herrera et al., 2022)
	Acetylated and butyrylated (form postbiotics) mix high amylose maize starch (prebiotics)	Hypertension (n=16)	**(+)** Microbial diversity and SCFA production **(-)** Systolic BP and Arterial stiffness	(Rhys-Jones et al., 2021)

Note "**(+)**": Increased / Significant Positive Correlation, "**(-)**": Reduced / Significant Negative Correlation, "=": Similar or Correlation, "≠": Significantly different, "≈": Possibly Similar or Correlation

2.3 Fermented Foods

2.3.1 Fermented Foods and The Gut Microbiota Health

Since prehistoric times, humans have included fermented foods in their diet. Pottery jars from 7000 BC that were used to ferment rice, honey, and fruit have been found in China. These vessels include some of the earliest evidence of the intentional use of fermentation (McGovern et al., 2004). In the same age, India had a symbol of endless bounty in numerous contexts, and the importance of milk, curds, buttermilk, and country butter was emphasized (Farnworth, 2008). It can be assumed that fermented milk products have been around since that era. In the early days, fermentation was primarily used for food preservation and creating new methods of food production all the time (Vitorino & Bessa, 2017).

As highlighted by Marco and team's review on the health benefits effects of fermented foods and beverages. The production of foods such as yogurt and cultured milk, wine and beer, sauerkraut and kimchi, and fermented sausage was initially valued because of their improved shelf life, safety, and organoleptic properties, but although only a limited number of clinical studies on fermented foods have been performed, there is evidence that these foods provide health benefits well beyond the starting food materials (Marco et al., 2017). This shows that there has been ongoing interest in study topics up until the present.

Rice represents Thailand's most economically significant agricultural product (Suebpongsang et al., 2020). A comprehensive review of rice-fermented products (Table 2.3) revealed that while Japanese amazake has been extensively studied, research on Thai rice fermentation remains limited. Notably, no prior studies have investigated the fermentation of Thai rice varieties into amazake, highlighting the novelty and scientific rationale for this investigation.

Table 2.3 Example of Rice-Fermented Product used for Improving Health in Clinical Trial Study

Products	Substrate	Microorganism	Groups	Gut microbes affected	Reference
Amazake	Brown rice	*Aspergillus oryzae*	Metabolic disorder (n=120)	**(+)** Clostridia class = **(-)** Inflammation **(+)** *Lactobacillales bacterium*, *butyrate-producing bacterium*, and *Firmicutes bacterium* = **(+)** Short-chain fatty acid product = **(-)** Metabolic disorder	(Akamine et al., 2022)
Amazake	Brown rice	*Aspergillus oryzae*	Severe motor and intellectual disabilities patient and has constipation symptoms (n=10)	**(+)** *Bifidobacterium* = **(-)** *constipation assessment scale*	(Kageyama et al., 2021)
Red yeast rice	Rice	*Monascus purpureus*	Caucasian patients with low cardiovascular risk (n=80)	**(-)** Cholesterol, triglycerides, LDL, intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, and sE-selectin = **(-)** Oral fat load	(Derosa et al., 2018)
Rice bran fermented	Rice bran	*Lentinus edodes*	Healthy adults (n=80)	**(+)** IFN-$\gamma \neq$ NK cell, cytokine level, IL-2, IL-4, IL-10, IL-12, and TNF-α	(Choi et al., 2014)

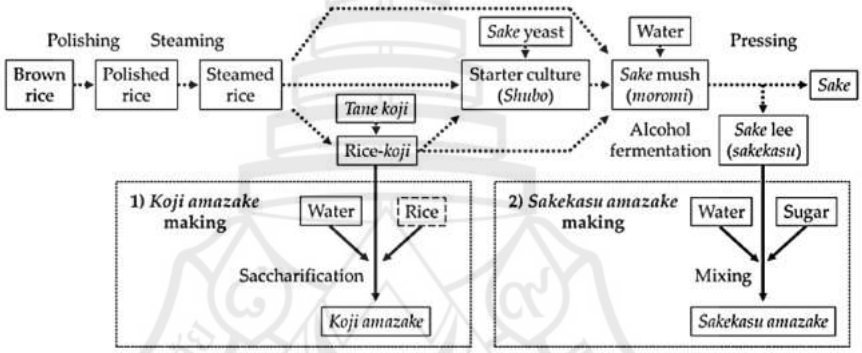
Table 2.3 (continued)

Products	Substrate	Microorganism	Groups	Gut microbes affected	Reference
Rice fermented	Rice	*Lactobacillus paracasei* CBA L74	Healthy children (n=377)	**(-)** Upper respiratory tract infections **(-)** Acute gastroenteritis	(Nocerino et al., 2017)
Rice flour fermented	Rice	*Lactobacillus paracasei* CBA L74	Infant severe atopic dermatitis (n=58)	**(-** no significant**)** Cytokines ≈ **(-)** Topical steroids uses	(D'Auria et al., 2021)
Sake-kasu	Steamed rice	*Aspergillus oryzae*	Community-dwelling physically active older adult (n=35)	**(+)** Visual selective attention and serum transthyretin	(Nagai et al., 2020)
Yellow yeast rice supplement	Steamed rice	*Aspergillus terreus*	Mild-to-Moderate Hypercholesterolemia (n=60)	**(-)** Total cholesterol, LDL, and apolipoprotein B100 = **(-)** Intrahepatic cholesterol availability through stimulating bile salt export pumps and inhibiting cholesterol biosynthesis	(Kang et al., 2022)

Note: "**(+)**": Increased / Significant Positive Correlation, "**(-)**": Reduced / Significant Negative Correlation, "=": Similar or Correlation, "≠": Significantly different, "≈": Possibly Similar or Correlation

2.3.2 Koji Amazake Product

Koji amazake is a sweet and sour Japanese drink with no alcohol. The process of producing amazake is using koji (*Aspergillus oryzae*) rice or leavening agent in inoculated rice and cooked rice, mixed with water, packed in fermentation tanks, and fermented at a temperature of 50 to 60°C. *A. oryzae* will release amylase to digest starch from rice to produce glucose. One example is having a mechanism for digesting other compounds into nutrients or substances that affect health. There are two main types of production processes. One type is made solely from koji, rice, and water, while another variety includes additional rice blends (Figure 2.1) (Kurahashi, 2021).



Source Kurahashi, (2021)

Figure 2.1 Relationship between Koji Amazake (Non-Alcoholic Sweet Beverage) and the *Sake* (Alcoholic Beverage) Brewing Production Process.

Hakkaisan Brewery Co., Ltd., which distributes Koji amazake, has analyzed the nutritional value of the products according to Table 2.4. Amazake products were discovered to have high-energy nutrients. It also contains prebiotics that are associated with the gut microbiota (Kurahashi, 2021). Kurahashi et al. found that consuming koji amazake did not result in a significant difference in fecal short-chain fatty acid concentrations between experimental groups. However, *Blautia* abundance was significantly reduced and significantly increased the amount of *Bacteroides* abundance. Consuming koji amazake for at least three weeks increases the frequency of defecation (Kurahashi et al., 2021).

Table 2.4 Koji Amazake Nutritional Information (118 g per bottle)

Item	Koji amazake
Energy (Kcal)	127.4
Moisture (g)	86.4
Protein (g)	1.4
Fat (g)	0.4
Total Glycosylceramide (mg)	1.39
Glycosylceramide from *A. oryzae* (mg)	1.16
Glycosylceramide from rice (mg)	0.23
Ash (g)	0.1
Carbohydrate (CHO) (g)	29.7
Dietary fiber (g)	0.2
Available CHO (g)	29.5
Digestible CHO (g)	26.4
Glucose (g)	26.1
Maltose (g)	0.17
Trehalose (g)	0.14
Prebiotics (g)	3.12
Isomaltose (g)	1.96
Isomaltotiose (g)	0.10
Panose (g)	0.10
Sophorose (g)	0.66
Nigerose (g)	0.20
Kojibiose (g)	0.10

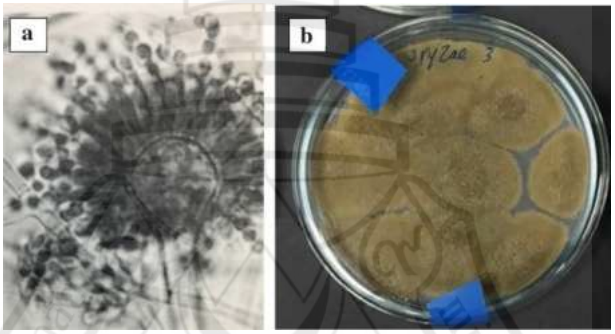
Source Kurahashi et al., (2021).

2.2.3 *Aspergillus oryzae*

A. oryzae is a *filamentous fungus,* or mold, used to incubate sweet potatoes and barley to produce an alcoholic beverage such as *sake* and *shochu*. If *A. oryzae* is

incubated with flour, yellow colonies are formed, known as “Yellow Koji” (Figure 2.2).

Fungi and mold, in general, are responsible for a wide range of diseases in the human body. *Aspergillus* species are also included. However, in history, *A. oryzae* was cultured and used to produce koji for food and beverage production in Japan. Later, the Japan Food and Drug Administration issued a certificate confirming that koji-derived foods were used to make *miso*, soy sauce, *sake*, amazake, vinegar*, kurozu*, fermented barley extract, and *shochu* that can be eaten safely if properly disinfected and correctly pasteurized (Kitagaki, 2021).



Note a. under microscope, b. Cultivated on Potato Dextrose Agar Medium
Source Daba et al., (2021)

Figure 2.2 Morphology of *Aspergillus Oryzae*

2.2.4 Production process and fermentation of Thai Koji amazake product

The production process at YoRice Café (Chiang Mai, Thailand) begins with cooked Japanese rice being inoculated with *A. oryzae* spores and mixed on a wooden tray. The mixture is covered with a cloth and incubated at 40°C for 3 days, resulting in the formation of koji. After the incubation, the koji is combined with broken rice in a 1:1 ratio, with water added, and stirred at 50°C for 1 day. The resulting koji amazake is then filtered (Figure 2.3), pasteurized, and prepared for sale in the market.

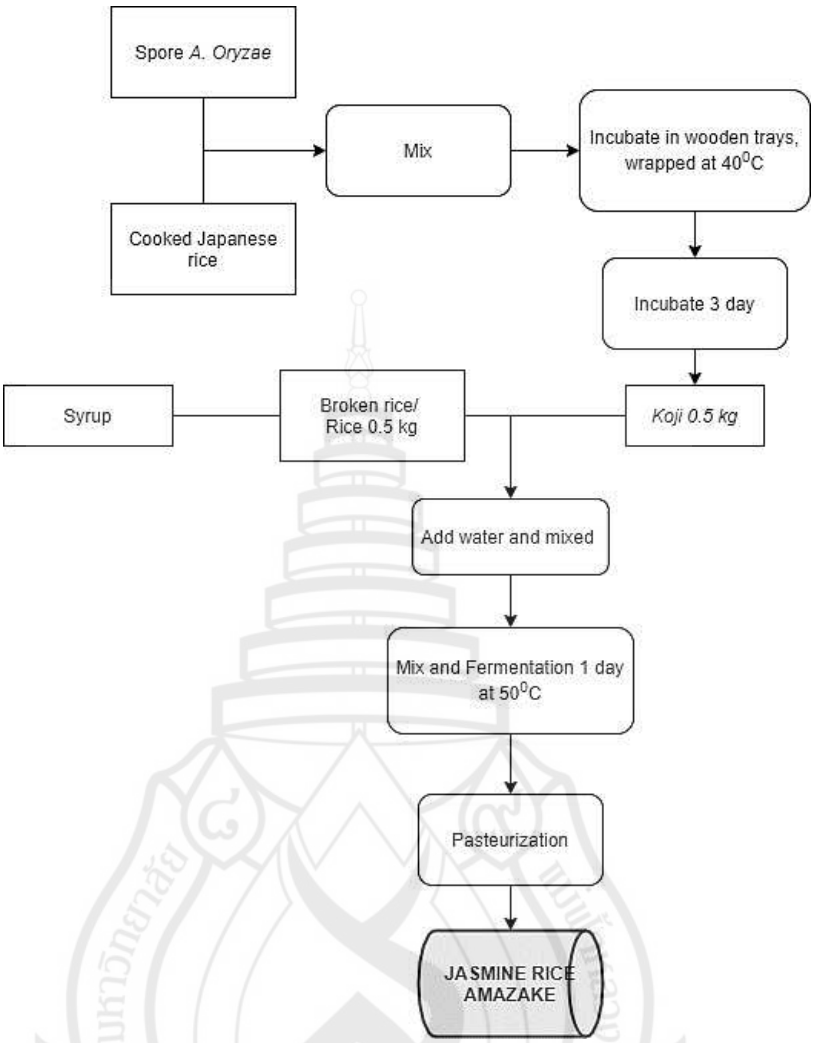


Figure 2.3. The Fermentation Process of Thai Koji Amazake Made in YoRice Café, Chiang Mai, Thailand

Information about the Japan amazake production process cannot be obtained within Japan due to marketing concerns, making it unclear whether the production method affects the compounds unique to this product. However, Oguro et al. provided experimental evidence showing that fermentation temperature impacts the glucose production of koji mold. Their research suggests that fermenting at 50°C for a period, followed by increasing the temperature to 70°C, results in higher levels of sugars and prebiotics compared to fermenting at a single temperature (Oguro et al., 2019).

Another previous study by Oguro et al. found that the germs and strains used for fermentation also had different yields (Oguro et al., 2017). As a result, it is possible

to conclude that the fermentation method, raw material, and temperature all have an impact on production quality.

2.4 Metabolite Profiling

To identify and measure the tiny molecules or metabolites that represent the final endpoint of changes and interactions between gene and protein expression, a field of research called “metabolomics or metabolite profiling” is now being developed. Some studies indicated that environmental factors would reflect biological metabolomics. The proteome, genome, and internal and external variables of the organism all influence the makeup of the compounds under study, as a study of phenotypes in biology (Tsoukalas et al., 2017; Zhang et al., 2013).

Within the pharmaceutical sector, metabolomics is the study of metabolites found in cells, biological compounds, tissues, or organisms. Metabolomics information is used to advance molecular medicine or precision medicine, transplantation monitoring, infant screening, pharmacology, and toxicology. Under genetic, dietary, and environmental factors, the number of discoveries increased in 2018, with global metabolite profiles totaling 3,000–20,000 records. Researching metabolomics in the medical field is intriguing since it provides the most thorough description of the patient's phenotypic condition when compared to other “Omics” data (Guijas et al., 2018).

Various high technologies and analyzing methods are necessary for the study of metabolomics (Table 2.5). For example, studies using mass spectrometry (MS), gas chromatography and mass spectrometry (GC-MS), liquid chromatography and mass spectrometry (LC-MS), capillary electrophoresis and mass spectrometry (CE-MS), Fourier transform ion cyclotron resonance and mass spectrometry (FTICR-MS), matrix-assisted laser desorption/ionization (MALDI), ion mobility spectrometry (IMS), Nuclear magnetic resonance (NMR) (Guijas et al., 2018), and next-generation sequencing (NGS) (Bao et al., 2021)

Table 2.5 Example of Instruments for Metabolomics Analysis Studies.

Article	Sample	Instruments	Software/Database/Method for Data Management	Reference
Identification of saliva metabolomic biomarkers of oral cancer screening	Saliva and tissue samples	CE-TOF-MS (Agilent)	Agilent ChemStation software for CE (A10.02) and Agilent MassHunter software for TOF-MS (B.02.00)	(Ishikawa et al., 2016)
Integrative analysis of the intestinal metabolome of childhood asthma	Fecal and blood samples	UPLC (Water, Milford, MA), MA)-LTQ or MS (ThermoFisher Scientific, Waltham, MA), and NGS (Illumina MiSeq)	AMDIS, AnalyzerPro/MatrixAnalyzer, CODA, MassFrontier, metAlign, Uppsala	(Bridgewater Br, 2014; Halket et al., 2005)
Fecal microbiome and metabolome differ in healthy and food-allergic twins	Fecal samples	NGS (Illumina MiSeq), RP/UPLC-MS/MS Ion mode ESI, HILIC/UPLC-MS/MS Ion mode ESI (UPLC use Waters ACQUITY UPLC)	Use the library in Metabolon Inc.	(Bao et al., 2021)
FODMAPs alter symptoms and the metabolome of patients with IBS: a randomised controlled trial	Urine and Stool samples	DI/LC-MS/MS (AbsolutIDQ p180 kit, Biocrates Life Sciences AG, Innsbruck, Austria), GC-MS (Agilent 5890 Series II) at The Metabolomics Innovation Center (Edmonton, AB), NGS (Illumina sequencing)	For metabolite analysis, they use library in The Metabolomics Innovation Center, and for microbiome, they use QIIME informatics	(Bouatra et al., 2013; McIntosh et al., 2017)
Particulate Matter Exposure and Stress Hormone Levels: A Randomized, Double-Blind, Crossover Trial of Air Purification	Blood and first time the patient urinates in morning	GC-TOF-MS (Agilent 7890A GC and Agilent 5875C TOF-MS), UPLC-QTOF-MS (Agilent 1290 UPLC and Agilent 6538 QTOF-MS)	ChromaTOF software and METLIN database	(H. Li et al., 2017)
Metabolomic biomarkers as strong correlates of Parkinson's disease progression	Plasma samples	Chromatography methods are LC and GC. And 3 separate mass spectrometers: UPLC-MS, OrbiElite system, and Trace UGC-DSQ-MS system (Thermo Scientific, Waltham, MA)	Quantify Individual Components in a Sample (QUICS) method	(Dehaven et al., 2010; LeWitt et al., 2017)

Table 2.5 (continued)

Article	Sample	Instruments	Software/Database/Method for Data Management	Reference
Metabolomics of Aerobic Exercise in Chronic Stroke Survivors: A Pilot Study	Blood samples	HPLC-MS (Thermo-Fusion, Thermo Fisher Scientific, Inc., San Diego, CA)	apLCMS Database	(Serra et al., 2019)
Plasma Ceramides, Mediterranean Diet, and Incident Cardiovascular Disease in the PREDIMED Trial	EDTA Plasma samples and	LC-QTOF-MS (4000 QTRAP, Applied Biosystems/Sciex)	SAS Software ver. 9.1.3 (SAS Institute)	(Rhee et al., 2011; Wang et al., 2017)
Quantile Normalization Approach for Liquid Chromatography Mass Spectrometry-based Metabolomics Data from Healthy Human Volunteers	Pre-dose and post-dose urine	LC-MS/MS analysis	Microsoft Excel 2010 create a matrix for principal component analysis (PCA), including box plot and quantile-quantile (Q-Q) plots.	(Lee et al., 2012)
Capillary Electrophoresis-Mass Spectrometry at Trial by Metabo-Ring: Effective Electrophoretic Mobility for Reproducible and Robust Compound Annotation	Plasma and Urine sample	Capillary zone electrophoresis (CE) coupled to mass spectrometry (MS) (Bremen, Germany)	Skyline software	(Drouin et al., 2020)
Validation of breath biomarkers for obstructive sleep apnea	Breath patient	SESI-HRMS (SESI is SEADM, Spain, and the mass spectrometer is AB Sciex, Concord, Ontario, Canada)	MATLAB R2020a and R 4.0.0 software	(Nowak et al., 2021)
Metabolic fingerprinting for diagnosis of fibromyalgia and other rheumatologic disorders	Blood samples	FT-IR, FT-Raman microspectroscopy, Ultra-HPLC (uHPLC) coupled to a photodiode array (PDA) and tandem (MS/MS)	Principal components analysis (PCA)	(Hackshaw et al., 2019)

Table 2.5 (continued)

Article	Sample	Instruments	Software/Database/Method for Data Management	Reference
Combination of UHPLC-MS/MS-molecular networking approach and FTICR-MS for the metabolic profiling of Saccharomyces cerevisiae	Yeast (Saccharomyces cerevisiae) metabolite extraction	UHPLC system (Dionex ultimate 3000 UPLC+, Thermo Scientific, San Jose, CA, USA), C18 silica-based column (Acquity UPLC HSS T3 1.8 -µm x 1.0 -mm x 100 mm, Waters Corporation, Milford, MA, USA), QTOF ESI analyzer (SYNAPT G2 HDMS, Water MS Technologies, Manchester, UK), FTICR instrument (SolariX XR FTMS, Bruker Daltonics)	MassLynx 4.1, MZMine2 software, GNPS fitted data	(Perruchon et al., 2021)
Proteomic and metabolomics study of Wax apple (*Syzygium samarangense*) fruit during ripening process	Fruit samples	MALDI-TOF MS/MS analysis (ultrafleXtreme Bruker Daltonics, Bremen, Germany)	MASCOT v. 3.5 database, NCBIProt taxonomy database, STRING v. 10.5	(Al-Obaidi et al., 2018)
Metabolomics Changes after Coffee Consumption: New Paths on the Block	Urine samples	UHPLC-TWIMS-QTOF analysis (ACQUITY I-Class, Waters, Wilmslow, UK) C18 HSS T3 ACQUITY column 2.1 x 100 mm, 1.7 µm particle size (Water, Milford, MA, USA)	UNIFI 1.8 data acquisition (Waters, Wilmslow, UK), QI Informatic identification software (Nonlinear Dynamics, Newcastle, UK), Principal components analysis (PCA)	(Favari et al., 2021)
Probiotic *Bifidobacterium longum* NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome	Urine samples	NMR (Bruker Biospin, Rheinstetten, Germany)	SIMCA-P+ software package (version 14.0; Umetrics AB, Umeå, Sweden)	(Pinto-Sanchez et al., 2017)

2.5 Metabolism of *Oryza sativa japonica* (Japanese rice)

Rice (*Oryza sativa L.*) is a staple food widely cultivated across Asia and internationally. It is classified into two primary subspecies: japonica and indica, which differ in morphological, agronomic, physiological, biochemical traits, and genomic structure (Yang et al., 2014). The most common rice grain colors are white, red, and black. However, in certain regions, such as Thailand, varieties of brown and purple rice are also grown (Fongfon et al., 2021; Oppong et al., 2021; Pang et al., 2018).

Du et al. used tandem mass spectrometry analysis using their fragment ions from the collision-induced dissociation (CID), and standard compounds were used as reference. The *Oryzae sativa* seedlings identified metabolites. A total of 34 compounds were discovered (Table 2.6) (Du et al., 2020). The KEGG Pathway Database searches for the *Oryza sativa japonica* plant and includes the main pathways: Glycolysis & Gluconeogenesis, Citrate Cycle (TCA Cycle), Phenylalanine Metabolism, Butanoate Metabolism, Starch and Sucrose Metabolism, and Phenylpropanoid Biosynthesis.

Table 2.6 Identified Metabolites in the *Oryza Sativa* Seedlings by EESI-MS and Check Molecular Weight in PubChem Database

No.	Compound	Molecular formula	Molecular weight (g/mol)
1	3-Methylbutyraldehyde	$C_5H_{10}O$	86.13
2	Beta-alanine	$C_3H_7NO_2$	89.09
3	4-Aminobutanoic acid	$C_4H_9NO_2$	103.12
4	Serine	$C_3H_7NO_3$	105.09
5	Betaine	$C_5H_{11}NO_2$	117.15
6	Threonine	$C_4H_9NO_3$	119.12
7	Nicotinic acid	$C_6H_5NO_2$	123.11
8	L-pyroglutamic acid	$C_5H_7NO_3$	129.11
9	Asparagine	$C_4H_8N_2O_3$	132.12

Table 2.6 (continued)

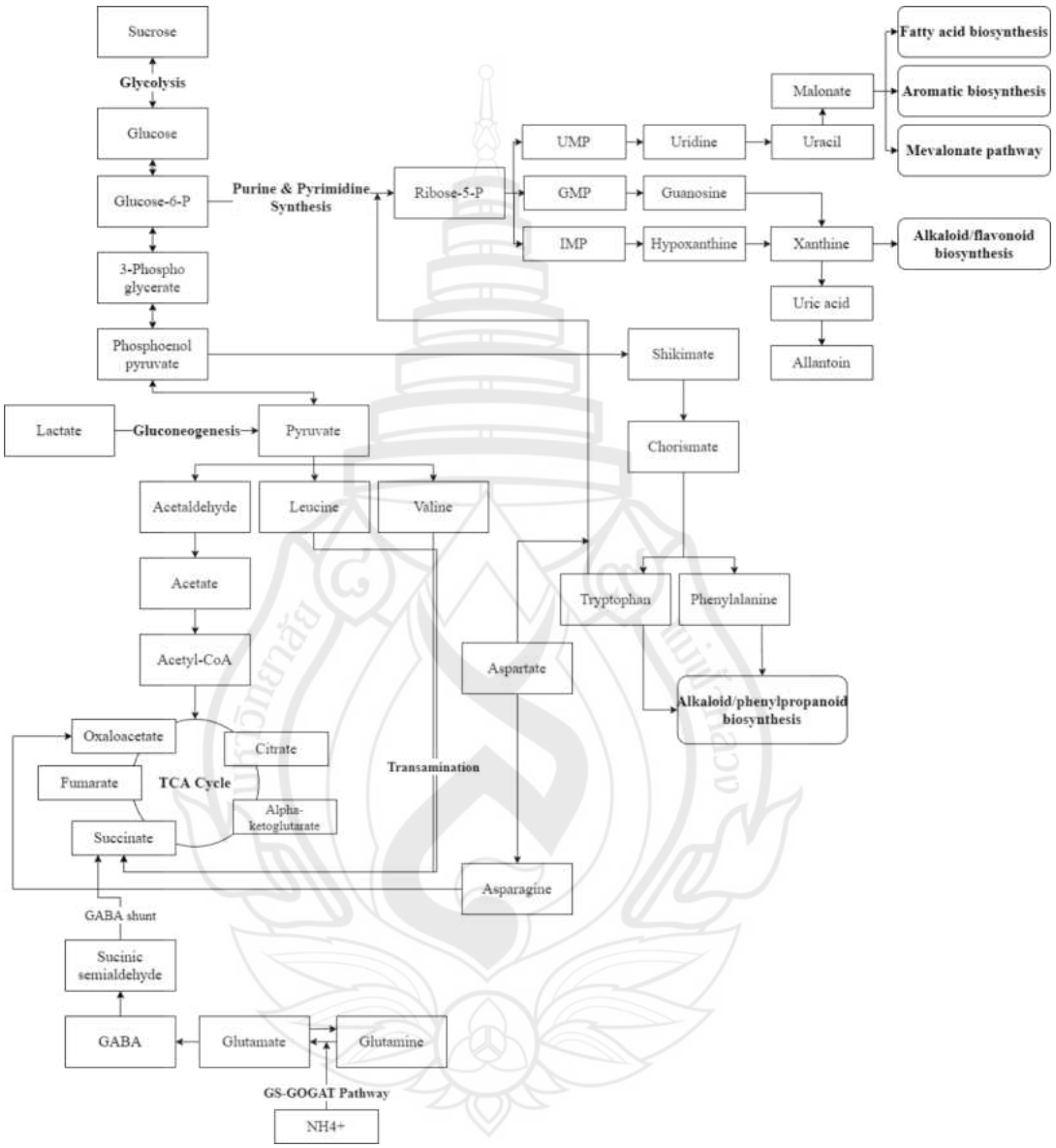
No.	Compound	Molecular formula	Molecular weight (g/mol)
10	Ornithine	$C_5H_{12}N_2O_2$	132.16
11	2-Aminobenzoic acid	$C_7H_7NO_2$	137.14
12	Tyramine	$C_8H_{11}NO$	137.18
13	Salicylic acid	$C_7H_6O_3$	138.12
14	Glutamine	$C_5H_{10}N_2O_3$	146.14
15	L-glutamic acid	$C_5H_9NO_4$	147.13
16	Cinnamic acid	$C_9H_8O_2$	148.16
17	Triethanolamine	$C_6H_{15}NO_3$	149.19
18	Protocatechuic acid	$C_7H_6O_4$	154.12
19	Tryptamine	$C_{10}H_{12}N_2$	160.22
20	P-coumaric acid	$C_9H_8O_3$	164.16
21	L-phenylalanine	$C_9H_{11}NO_2$	165.19
22	Gallic acid	$C_7H_6O_5$	170.12
23	Caffeic acid	$C_9H_8O_4$	180.16
24	Tyrosine	$C_9H_{11}NO_3$	181.19
25	Ferulic acid	$C_{10}H_{10}O_4$	194.18
26	Sinapic acid	$C_{11}H_{12}O_5$	224.21
27	Methyl jasmonate	$C_{13}H_{20}O_3$	224.30
28	Resveratrol	$C_{14}H_{12}O_3$	228.24
29	Naringenin	$C_{15}H_{12}O_5$	272.25
30	Kaempferol	$C_{15}H_{10}O_6$	286.24
31	Caffeoylmalic acid	$C_{13}H_{12}O_8$	296.23
32	Quercetin	$C_{15}H_{10}O_7$	302.23
33	Glutathione (oxidized)	$C_{10}H_{17}N_3O_6S$	307.33
34	Rutin	$C_{27}H_{36}O_{19}$	610.5

Source Du et al., (2020).

2.6 Metabolism of *Oryza sativa L.* KDML (Thai Jasmine Rice)

Thai jasmine rice is in greater demand on the global market; however, the annual production cannot keep up with this demand (Phanchaisri et al., 2007). Uawisetwathana et al. study interaction between brown planthopper and Jasmine rice.

Among these reported studies, various analytical platforms have been utilized to study the metabolome of plants with proton nuclear magnetic resonance (^{1}H NMR). Figure 2.4 shows the metabolic profile results for amino acid metabolism, secondary metabolite biosynthesis, and gluconeogenesis pathways (Uawisetwathana et al., 2015).

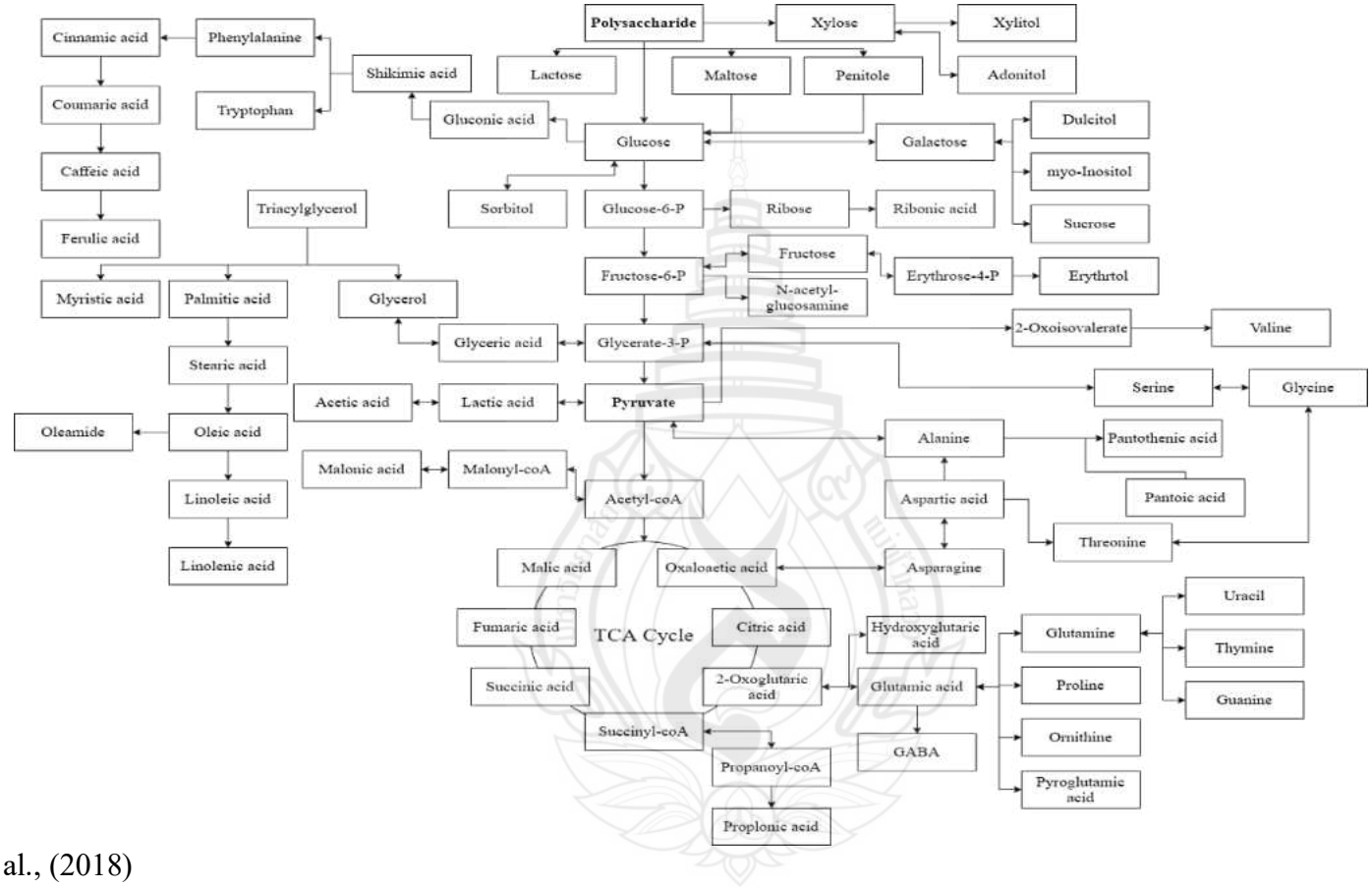


Source Uawisetwathana et al., (2015)

Figure 2.4. Thai Jasmine Rice Amino Acid Metabolism, Secondary Metabolite Biosynthesis, and Gluconeogenesis Pathways

2.7 Metabolism of *Aspergillus oryzae* (Koji)

To produce Korean bean paste (*Doenjang*), white *sake*, soy sauce, vinegar, and amazake, a fungus called koji is incubated with the *Aspergillus oryzae* fungus. To grow, the mold needs sugar, so they use saccharification through fermentation. Because of this, the product produces sugar as well as releases several enzymes: amylases, proteases, lipases, and cellulases associated with other metabolites (Figure 2.5). Other significant compounds with anti-tumor, anti-bacterial, and antioxidant activities are also present (Gil et al., 2018).



Source Gil et al., (2018)

Figure 2.5 Scheme of the Primary Metabolic Pathway in Koji Rice Fermented (KEGG Database)

CHAPTER 3

METHODOLOGY

3.1 Sample Collection

3.1.1 Amazake Samples

Thai amazake samples used in this study were kindly provided by YoRice Café, and Japanese amazake was commercially ordered. All samples were kept at -20°C until analysis. A total of 4 samples were used in this study, including (1) YoRice Thai Jasmine rice amazake (JAS_AMA), the product shown in Figure 3.1; (2) Thai jasmine rice without fermentation overnight (JAS_bMIX); (3) jasmine rice solution mixed with sucrose syrup (JAS_SYR); and (4) commercial Japanese koji amazake (JP_AMA). The process of the Thai amazake product is described in Figure 3.2A.

Figure 3.1. Thai Jasmine Rice Amazake Product from YoRice Café

3.1.2 Amazake Samples Preparation

Before being used for analysis, the samples were prepared using the amazake sample extract as explained in Figure 3.2B, following the protocol from Oguro et al. (Oguro et al., 2019) to prevent damage to the HPLC analyzer.

The amazake total sample of 10.0 ml was placed in a 50-ml tube (Conning, China) and centrifuged by a Universal 320 centrifuge (Hettich) at 2,300 x g for 5 min

to separate into layers. The supernatant of the solution was transferred into the 250-ml evaporating flask (Duran, Germany) for drying in the Rotary Evaporator Hel-Vop (Heidolph, Germany) until the sample was completely dried. The dried sample was then dissolved with 1.0 ml of 50% isopropanol (Merk, Germany) and finally diluted with 5-fold distilled deionized water (prepared by mixing a 1:4 ml sample-to-water ratio). The sample was then filtered with a 0.22-micron nylon syringe filter (ALWSCI Corp., China), obtaining approximately 4.0-4.5 ml of filtered sample. The 1.0 ml of the filtered sample was aliquoted and stored in a clean 1 ml glass vial (AIJIREN, China) at -20°C before analysis.

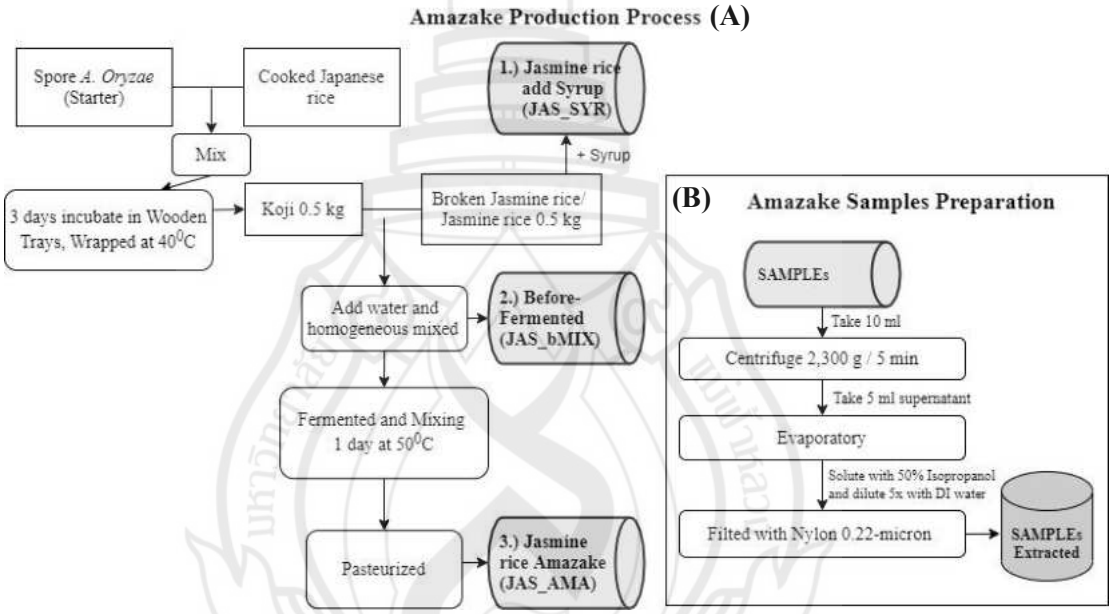


Figure 3.2. The YoRice café Thai Jasmine Rice Amazake Production Process (A) and Sample Preparation (B)

3.2 Characterization and Uniqueness of Metabolite Profiles with Liquid Chromatography Mass Spectrometry Technique

3.2.1 Liquid Chromatography Quadrupole Time of Flight Tandem Mass Spectrometry (LC-QTOF-MS/MS)

The analysis of the amazake sample was performed twice using LC-QTOF-MS/MS (Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry). The Agilent 1290 Infinity II/G6545B QTOF/MS (Agilent, Scientific and Technological Instruments Center, Mae Fah Luang University) instrument (Figure 3.3) and a standard C18 column were used to separate the compounds in the amazake sample. The detector provided qualitative and structural data for all compounds in the isolated samples. In addition to the HPLC principle, mass spectrometry was employed as an additional detector after compound separation. The compounds were introduced into the machine's spray, which enters a magnetic field in the mass spectrometer. The compounds were ionized by an electron beam, which was then processed by the mass analyzer, where the magnetic field arranges and processes the ions into spectral lines that represent the mass-to-charge ratio (m/z) of each ion.

Figure 3.3 LC-QTOF-MS/MS Agilent 1290 Infinity II/G6545B QTOF/MS Instrument

3.2.2 Data Collection

The results from the mass spectrometer, which generates large datasets, were screened for molecular data that deviates by more than 10 ppm (DB ppm) from the database. The identification uncertainty level (ILv.) of the data was categorized according to the study by Schymanski et al. (2014), based on the received data and corresponding ILv.: ILv.5: exact mass only; ILv.4: exact mass, predicted formula; ILv.3: exact mass, predicted formula, tentative compound; ILv.2: exact mass, predicted formula, tentative compound, probable structure; and ILv.1: exact mass, predicted formula, tentative compound, probable structure, and confirmed structure with a reference standard. During the data cleaning process, ILv.5 data, which are considered of poor quality and not useful for this analysis, were excluded first. Next, data with an error greater than 10 mDA (absolute value) were also excluded. Additionally, duplicate information from the same sample was eliminated by selecting the highest intensity peak (ion/min).

Data preparation and visualization were conducted using Microsoft® Excel 365, a suite of applications designed for data analysis and information management. To assist with analytical tasks that could not be performed by Excel, the researcher developed a custom analytical program using Python code (Appendix B). For more complex analyses, such as PCA plots and VIP score calculations, OriginLab® 2025 was used. The hardware used for all data processing includes a VivoBook ASUS laptop with an AMD Ryzen 3 3200U processor (2.60 GHz), 12.0 GB of RAM, an AMD Radeon™ Vega 3 Graphics GPU, and Windows 11.

3.2.3 Prebiotics Candidate Selection

Since the predicted compound names from the LC-QTOF-MS/MS instrument might differ in scope from those in the amazake sample. After cleaning the data, the predicted compound names were matched with the KEGG database, which includes pathways for two organisms: *Aspergillus oryzae* metabolism and *Oryza sativa japonica* metabolism. The list of pathways obtained from the KEGG database is presented in Table 3.1.

Table 3.1 Amount of Compound in Metabolism Pathway Information from KEGG Database

	Pathways list	***A. oryzae* profiles**	***O. sativa japonica* profiles**
1	Aflatoxin biosynthesis	27	0
2	Alanine, aspartate, and glutamate metabolism	28	28
3	alpha-Linolenic acid metabolism	44	44
4	Amino sugar and nucleotide sugar metabolism	116	116
5	Anthocyanin biosynthesis	0	66
6	Arachidonic acid metabolism	79	79
7	Arginine and proline metabolism	69	69
8	Arginine biosynthesis	23	23
9	Ascorbate and aldarate metabolism	57	57
10	Atrazine degradation	23	0
11	Benzoxazinoid biosynthesis	0	9
12	beta-Alanine metabolism	32	32
13	Betalain biosynthesis	0	23
14	Biosynthesis of siderophore	24	0
15	Biosynthesis of unsaturated fatty acids	74	74
16	Biosynthesis of various other secondary metabolites	67	0
17	Biosynthesis of various plant secondary metabolites	149	149
18	Biotin metabolism	29	29
19	Brassinosteroid biosynthesis	0	29
20	Butanoate metabolism	47	47
21	C5-Branched dibasic acid metabolism	34	34
22	Caffeine metabolism	22	22
23	Carbapenem biosynthesis	32	0
24	Carbon fixation in photosynthetic organisms	0	23
25	Carotenoid biosynthesis	123	123
26	Citrate cycle (TCA cycle)	20	20
27	Cutin, suberine and wax biosynthesis	0	25
28	Cyanoamino acid metabolism	45	45
29	Cysteine and methionine metabolism	66	66
30	D-Amino acid metabolism	68	68
31	Diterpenoid biosynthesis	0	124
32	Ether lipid metabolism	25	25
33	Fatty acid biosynthesis	58	58
34	Fatty acid degradation	48	48
35	Fatty acid elongation	40	40
36	Flavone and flavonol biosynthesis	0	51

Table 3.1 (continued)

	Pathways list	*A. oryzae* profiles	*O. sativa japonica* profiles
37	Flavonoid biosynthesis	0	74
38	Folate biosynthesis	58	58
39	Fructose and mannose metabolism	53	53
40	Galactose metabolism	46	46
41	Glucosinolate biosynthesis	0	77
42	Glutathione metabolism	36	36
43	Glycerolipid metabolism	38	38
44	Glycerophospholipid metabolism	56	56
45	Glycine, serine, and threonine metabolism	48	48
46	Glycolysis/Gluconeogenesis	31	31
47	Glycosaminoglycan degradation	4	4
48	Glycosylphosphatidylinositol (GPI) anchor biosynthesis	4	4
49	Glyoxylate and dicarboxylate metabolism	64	64
50	Histidine metabolism	47	47
51	Indole alkaloid biosynthesis	81	0
52	Indole diterpene alkaloid biosynthesis	33	0
53	Inositol phosphate metabolism	47	47
54	Isoquinoline alkaloid biosynthesis	0	129
55	Linoleic acid metabolism	28	28
56	Lipoic acid metabolism	13	13
57	Lysine biosynthesis	35	35
58	Lysine degradation	56	56
59	Mannose-type O-glycan biosynthesis	4	0
60	Methane metabolism	88	0
61	Monobactam biosynthesis	39	39
62	Monoterpenoid biosynthesis	0	61
63	N-Glycan biosynthesis	8	8
64	Nicotinate and nicotinamide metabolism	55	55
65	Nitrogen metabolism	19	19
66	One carbon pool by folate	9	9
67	Oxidative phosphorylation	16	16
68	Pantothenate and CoA biosynthesis	29	29
69	Penicillin and cephalosporin biosynthesis	18	0
70	Pentose and glucuronate interconversions	58	58
71	Pentose phosphate pathway	36	36
72	Phenylalanine metabolism	49	49

Table 3.1 (continued)

	Pathways list	***A. oryzae* profiles**	***O. sativa japonica* profiles**
73	Phenylalanine, tyrosine, and tryptophan biosynthesis	35	35
74	Phenylpropanoid biosynthesis	0	54
75	Phosphonate and phosphinate metabolism	55	55
76	Photosynthesis	0	11
77	Polyketide sugar unit biosynthesis	58	58
78	Porphyrin metabolism	137	137
79	Propanoate metabolism	41	41
80	Purine metabolism	101	101
81	Pyrimidine metabolism	64	64
82	Pyruvate metabolism	32	32
83	Riboflavin metabolism	24	24
84	Selenocompound metabolism	27	27
85	Sesquiterpenoid and triterpenoid biosynthesis	89	89
86	Sphingolipid metabolism	27	27
87	Starch and sucrose metabolism	37	37
88	Staurosporine biosynthesis	48	0
89	Steroid biosynthesis	57	57
90	Sulfur metabolism	33	33
91	Taurine and hypotaurine metabolism	24	24
92	Terpenoid backbone biosynthesis	46	46
93	Thiamine metabolism	31	31
94	Tropane, piperidine, and pyridine alkaloid biosynthesis	0	72
95	Tryptophan metabolism	83	83
96	Tyrosine metabolism	77	77
97	Ubiquinone and other terpenoid-quinone biosynthesis	71	71
98	Valine, leucine, and isoleucine biosynthesis	23	23
99	Valine, leucine, and isoleucine degradation	41	41
100	Vitamin B6 metabolism	29	29
101	Zeatin biosynthesis	39	39
	Total compound profiles	**4004**	**4387**

3.2.4 Workflow of Qualitative Study of the Amazake Product

In the qualitative study or collected characteristic and uniqueness study, there were many steps involved. Therefore, it is summarized in Figure 3.4.

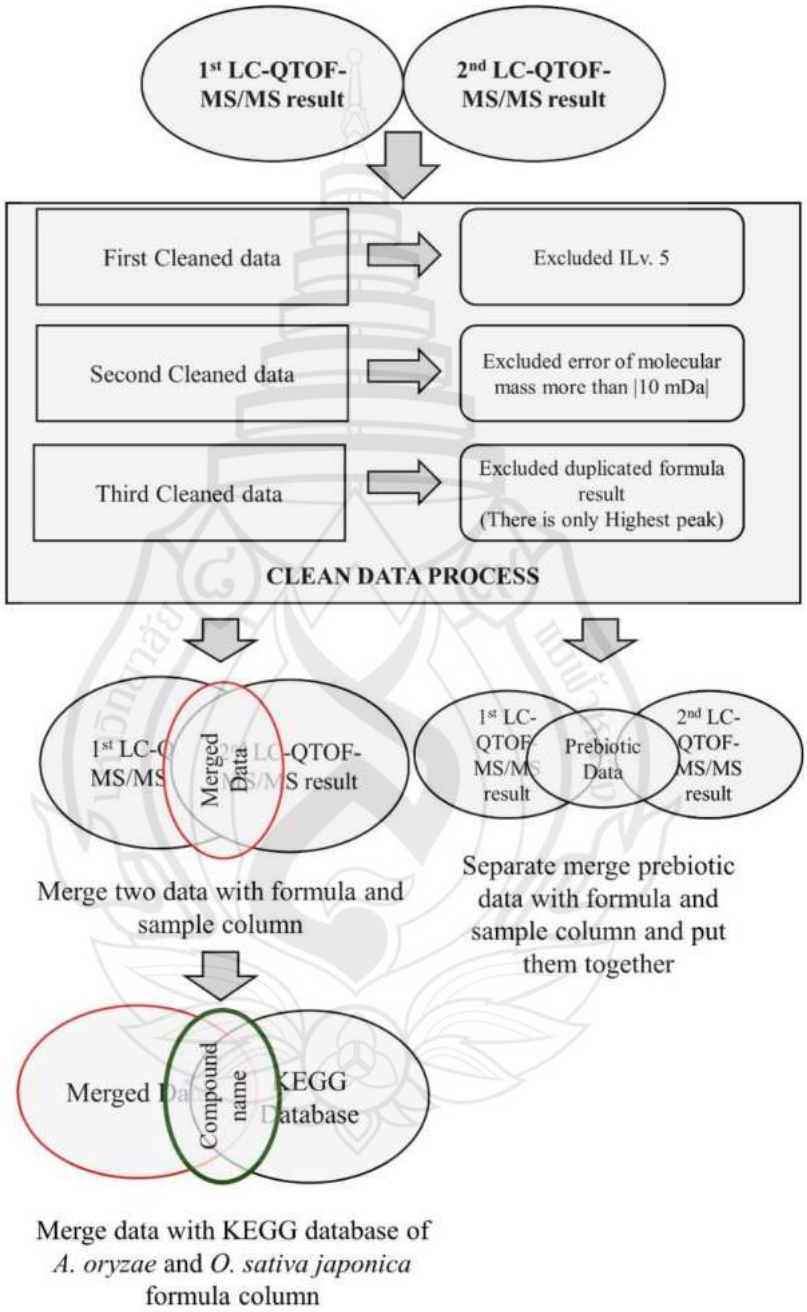


Figure 3.4 Workflow of The Collected Characteristic and Uniqueness of Amazake Study

3.3 Quantitative Analysis of Candidate Prebiotics with Liquid Chromatography Technique

3.3.1 High-performance Liquid Chromatography Refraction Index Detector

For the analysis of the characteristics and quantification of prebiotics in each amazake sample, an HPLC (High Performance Liquid Chromatography) instrument is shown in figure 3.5. The Water HPLC 600E refraction index (RI) detector (Scientific and Technology Instruments Center, Mae Fah Luang University) and an Asahipak NH2P-50 4.6 x 250 mm column (Shodex, United States) were employed to separate the compounds in the prepared samples. The separated flow of homogeneous compounds from the amazake samples and the standard of selected prebiotics were directed to the RI detector to quantify the candidate prebiotics identified.

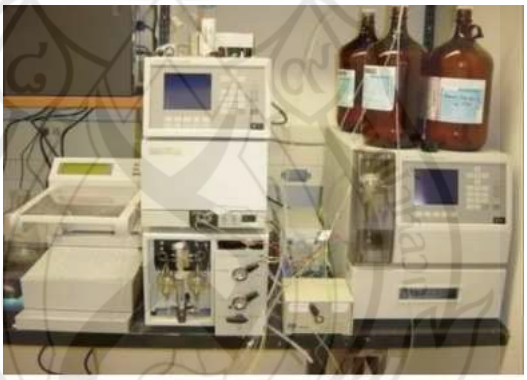


Figure 3.5 The Water HPLC 600E Refraction Index Detector Instrument

3.3.2 Sample Preparation and Instrument Setup

A 4 ml prepared sample was injected into the instrument using a 10 µl injection for a single analysis. Ribose was used as an internal standard in the sample to be injected. The instrument injects the sample of compressed liquid into the column, which contains a stationary phase of silica gel at 30°C. The sample was dissolved in a 60% acetonitrile (Merk, Germany) mobile phase. The compounds in the sample were suppressed through the column at different rates, with a flow rate of 0.8 ml/min and a total analysis time of 15 minutes per injection. The separation factor in this column relies on the acid-base state of the ion-exchange molecules interacting with the

stationary phase (Oguro et al., 2017). The signal intensity peak information is generated by the RI detector. To enhance measurement accuracy, an internal standard compound is added to determine the area under the signal intensity peak of interest.

3.4 Evaluated Effectiveness of Candidate Prebiotics with *In Vitro* Study

3.4.1 Candidate Microbial Selection and Preparation

Three candidate microbes were chosen to evaluate the effectiveness of the prebiotic property from amazake in this study. The characteristics of pure culture are described in Table 3.2. The two selected probiotics, *Lactobacillus plantarum* and *Bacillus subtilis*, are probiotics that are affected by IMOs (Fuhren et al., 2020; Gu et al., 2019) , and *Escherichia coli* is a negative control or pathogen strain obtained from an infected urine sample that has been tested as a representative of pathogens in various research studies, such as the Lueangprasert and Saelim study, which used this microbe as a representative fecal contamination pathogen in foods (Lueangprasert & Saelim, 2020).

Table 3.2 Growth of bacteria on different agar and incubated at 37°C overnight (24 hours)

Bacteria sp. (TISTR* Serial number)	**Gram**	**MRS**	
		Agar	**Broth**
Lactobacillus plantarum (TISTR-2074)	Positive	small, flat	Aerotolerant anaerobes
Bacillus subtilis (TISTR-1248)	Positive	slime (wet, shiny)	Obligate anaerobes
Escherichia coli (TISTR-527)	Negative	small, flat	Obligate anaerobes

Note TISTR for Thailand Institute of Scientific and Technological Research

3.4.2 Prebiotics Effectiveness Experimental Design

The experiments categorize the media used for cultivating the candidate microbial culture into four types: MRS nutrient without glucose (wMRS), wMRS with glucose, wMRS with amazake, and wMRS with IMOs. In other words, three different carbohydrate source broths are tested: glucose, amazake extract, and IMOs at a concentration of 20 g/L, all within the wMRS broth. Each sample contains 30 mL of broth in a 50 mL cotton-capped flask, and each set of samples was tested in triplicate. Additionally, two types of negative controls were included: MRS broth and wMRS broth without the candidate microbial inoculation. The wMRS broth consists of a mixture of beef extract, proteose peptone, yeast malt broth, Tween 20, citric acid monohydrate, ammonium acetate, sodium acetate, di-sodium hydrogen phosphate, magnesium sulfate anhydrous, and manganese (II) sulfate monohydrate, dissolved in distilled water. The same ingredients are used to prepare the broth, to which glucose or another carbohydrate source is added. Finally, the pH is adjusted to 6.5-7.0, and the broth is sterilized by autoclaving at 121°C for 15 minutes. Besides the negative control bottle, at other bottles inoculate 10 hours of candidate microbes (using the middle log phase of *E. coli*, *L. plantarum*, and *B. subtilis* from prelim data), including *E. coli*, *L. plantarum*, and *B. subtilis,* in a dilute for an approximate concentration of 3×10^8 cells by comparison with the McFarland turbidity standard.

In summary, as shown in Figure 3.6, the number of test sets depends on the number of candidate microbial tests. All test sets are placed in a shaker incubator (WiseCube™ WIS-10 Precise Shaking Incubator, DAIHAN, South Korea), with two test sets per round. Each round involves checking the turbidity nine times at the following time points: 0, 6, 9, 12, 15, 18, 24, 36, and 48 hours. The turbidity, which indicates microbial abundance, is measured by recording the absorbance at 560 nm using a GENESYS™ 180 UV-Vis spectrophotometer (Thermo Scientific, United States).

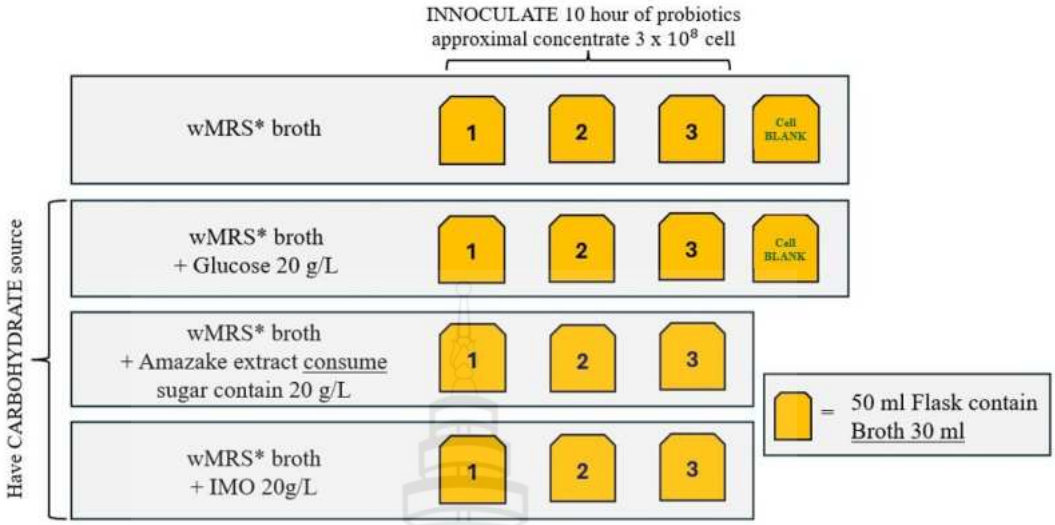


Figure 3.6 Broth Medium Experiment Setup for Determining Growth Parameters with Different Carbohydrate Sources for One Microbial Assay

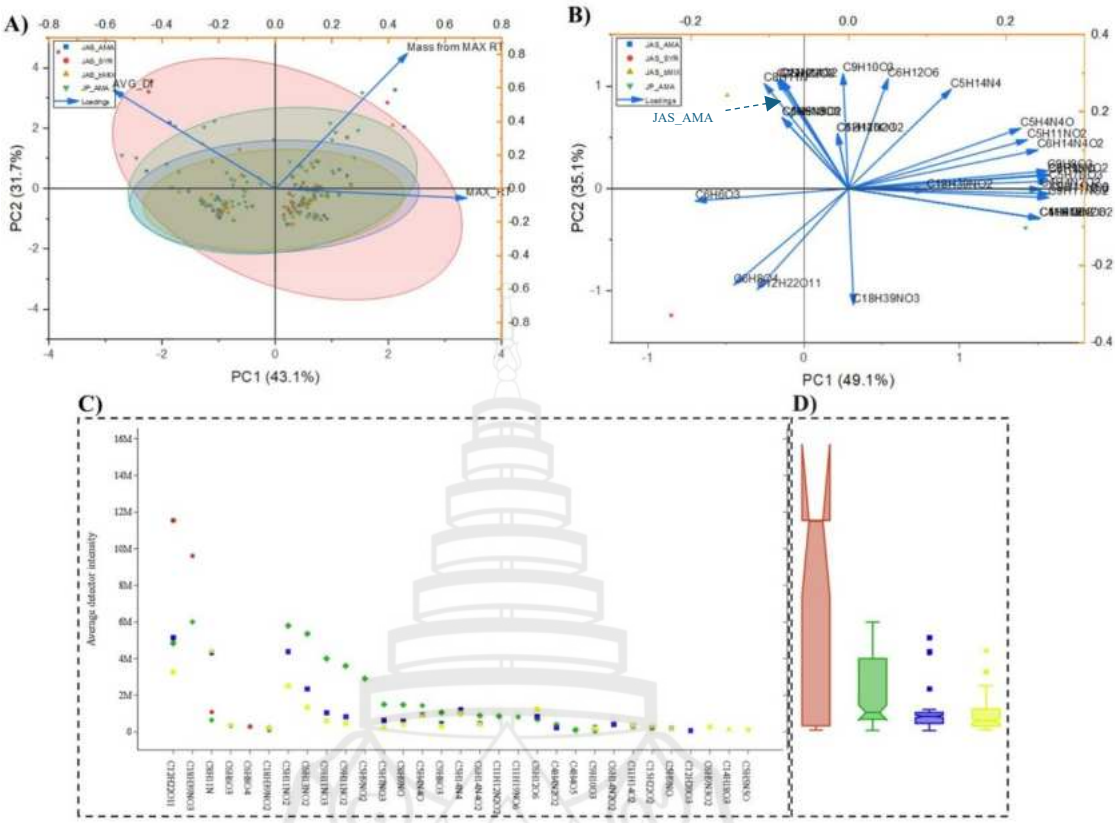
CHAPTER 4

RESULTS

4.1 Metabolite Profiles of The Koji Amazake Product Fermented from Thai Jasmine Rice

4.1.1 Mass Spectrometry Profiles Before Merge With KEGG

When the results of the first and second repeats of LC-QTOF-MS/MS analysis were merged with the same predicted formula column. There were up to 202 profiles. Distribution of metabolite results was displayed by PCA plot, as shown in Figure 4.1A. JAS_AMA and JAS_bMIX samples have similar average detector intensity, except for JP_AMA. Particularly, JAS_SYR is very different from the other samples. The results are quite consistent with the scatter plot results shown in Figure 4.1C-D. When averaged, RT and DI were considerably higher than the other samples, but the data were relatively few profiles. Figure 4.1B is a PCA loading plot between the predicted formula and the average detector intensity in each sample. The $C_{12}H_{22}O_{11}$ formula is uniquely interesting because it is possible that it is a disaccharide prebiotic.

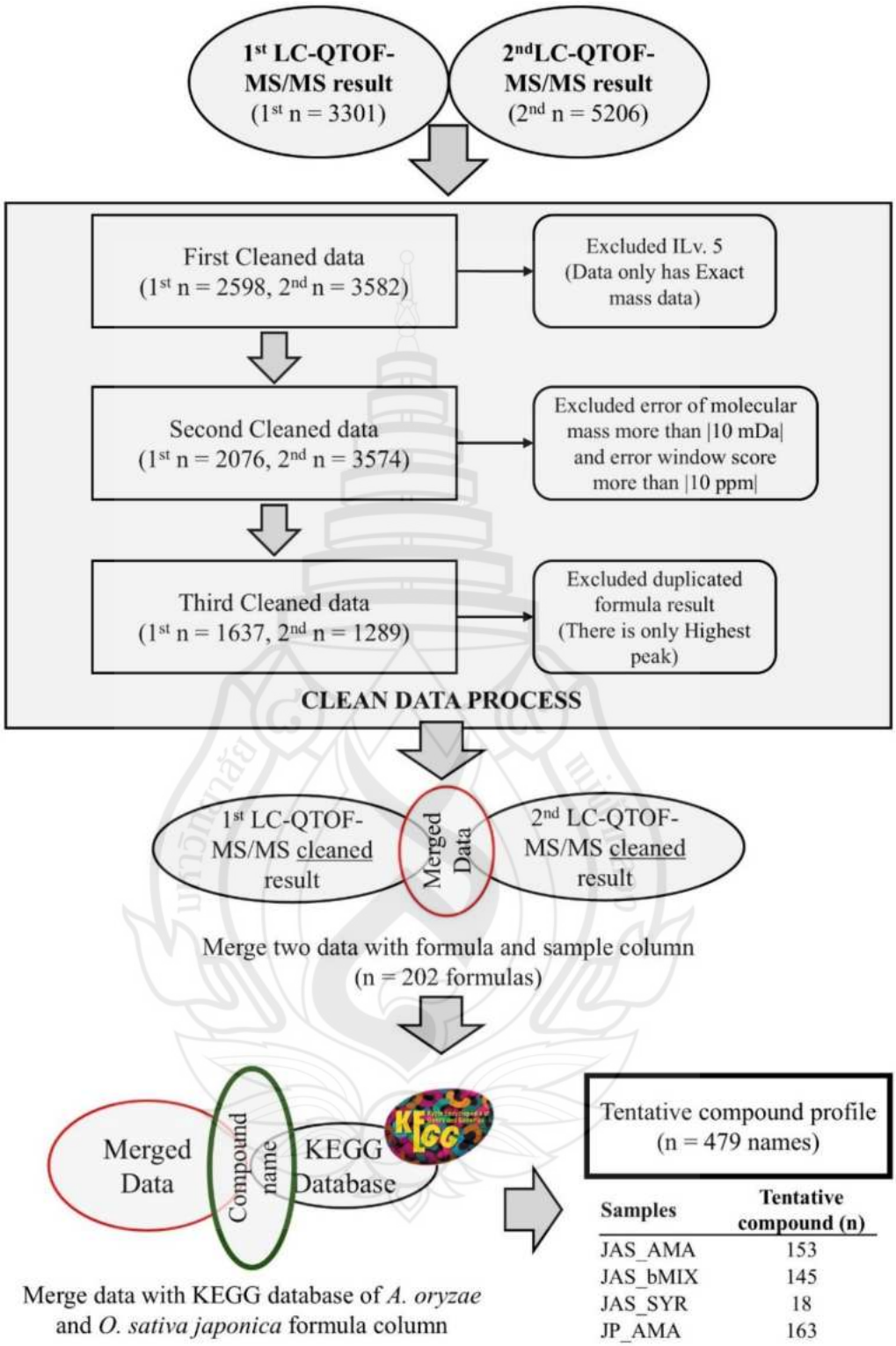


Note Blue is JAS_AMA sample, Yellow is JAS_bMIX, Red is JAS_SYR, and green is JP_AMA. In the B section, have a pointer to the JAS_AMA dot, because that difficult to find.

Figure 4.1 Analysis of Mass Spectrometry After Cleaning Profiles (A, The PCA plot of three variables, including Average of Detector Intensity (AVG_DI, ion/min), Maximum Retention time (MAX_RT, min), and Exact mass from MAX_RT data (Mass from MAX_RT); B, The PCA loading plot of all predicted formulas; C-D, The Scatter plot between MAX_RT (X) and AVG_DI (Y). D is the Box plot of median data each sample.

4.1.2 Mass Spectrometry Profiles After Merge with KEGG

Figure 4.2 presents the number of outcomes of data and the procedures used for managing it. By analyzing data from two replicate LC-QTOF-MS/MS results, it was for increasing the certainty of the MS results. There were three criteria for manual data cleansing: (1) excluding entries with an identification uncertainty level of 5 (i.e., those with only molecular mass results), (2) excluding entries with molecular mass discrepancies greater than ±10 mDa compared to instrument data, and (3) removing duplicate molecular formulas by retaining only the entry with the highest peak intensity. When the data cleaning was complete, the results of both rounds were intersected using the AMAS 1.2 program, and the obtained data were intersected with the KEGG data of *Oryzae sativa japonica* and *Aspergillus oryzae* metabolism data. The summary of detector intensity data is displayed in Figure 4.3. The cluster map indicated that galactose metabolism, D-amino acid metabolism, and phenylalanine metabolism were the most abundant pathway groups based on detector intensity counts. When considering these results alongside Figure 4.5C, it is evident that carotenoid biosynthesis and histidine metabolism had the highest VIP scores, as they were detected exclusively in the JAS_bMIX sample. This contrasts with the unique characteristics observed in the JP_AMA sample, such as the citrate cycle (TCA cycle) and alanine, aspartate, and glutamate metabolism, which had lower VIP scores. The lower scores can be attributed to the greater diversity of pathways in JP_AMA, as seen in the cluster map. Therefore, despite their lower intensity counts, carotenoid biosynthesis and histidine metabolism are considered highly unique or significant compared to other pathways. As shown in Figure 4.4, the extracted LC-QTOF-MS/MS data primarily consist of high-intensity peaks from small molecules, most of which align with the KEGG database, which predominantly contains small metabolite data. Since large molecules in the KEGG database often lack fixed compound names, the database used in this study included only a limited number of large molecules. Consequently, a substantial amount of data on large molecules was not captured, which partly explains why the Jasmine rice and syrup mixed sample contained significantly fewer detected compounds than the other samples.



Note Ilv.: Identification Uncertainty Level

Figure 4.2 Cleaned, Separated, and Merged KEGG Database Of LC-QTOF-MS/MS Raw Data Process to be Extracted Data

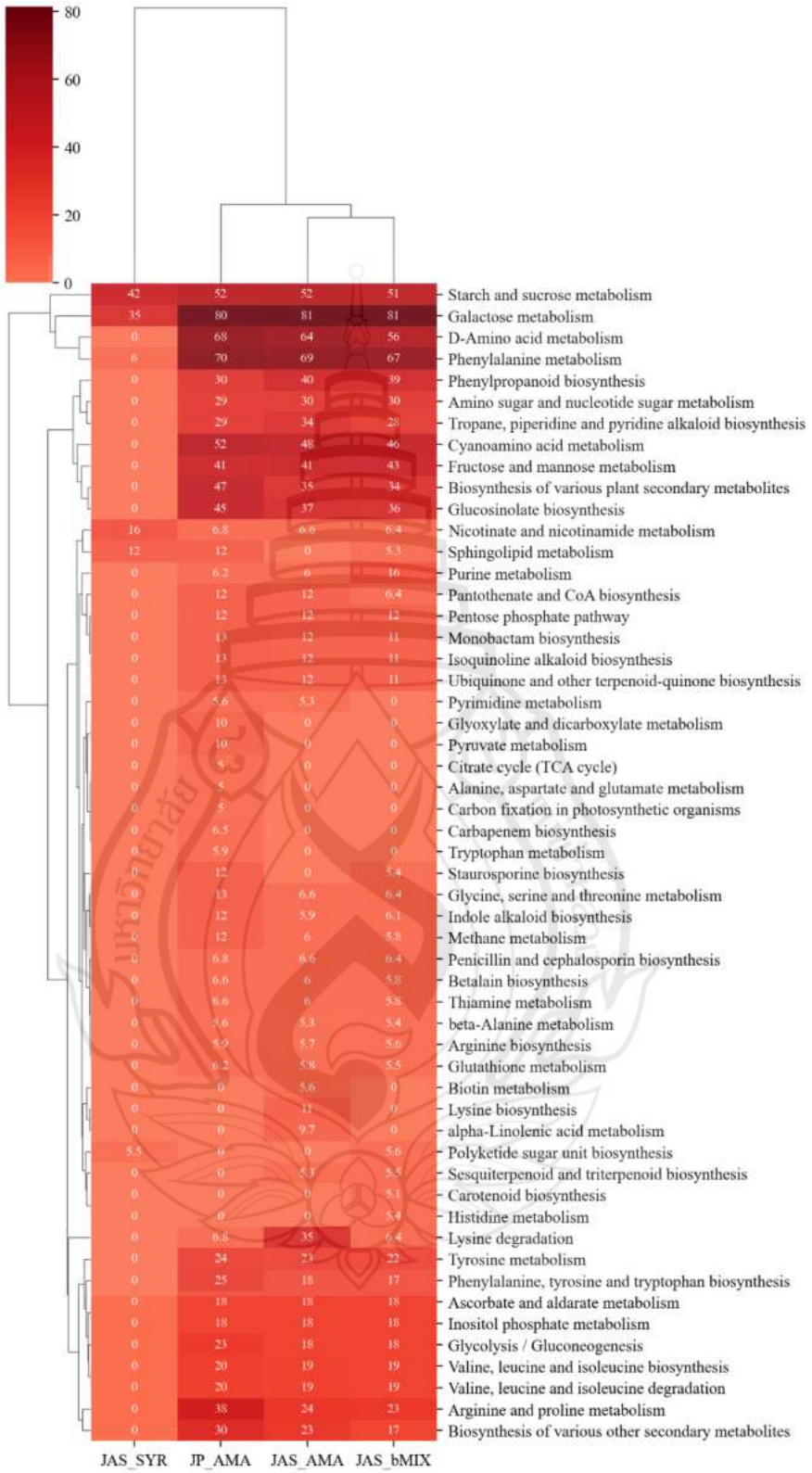


Figure 4.3 Clustermap of the Total Summary Detector Intensity (log10) Compare with KEGG Metabolic Pathway Lists in each sample.

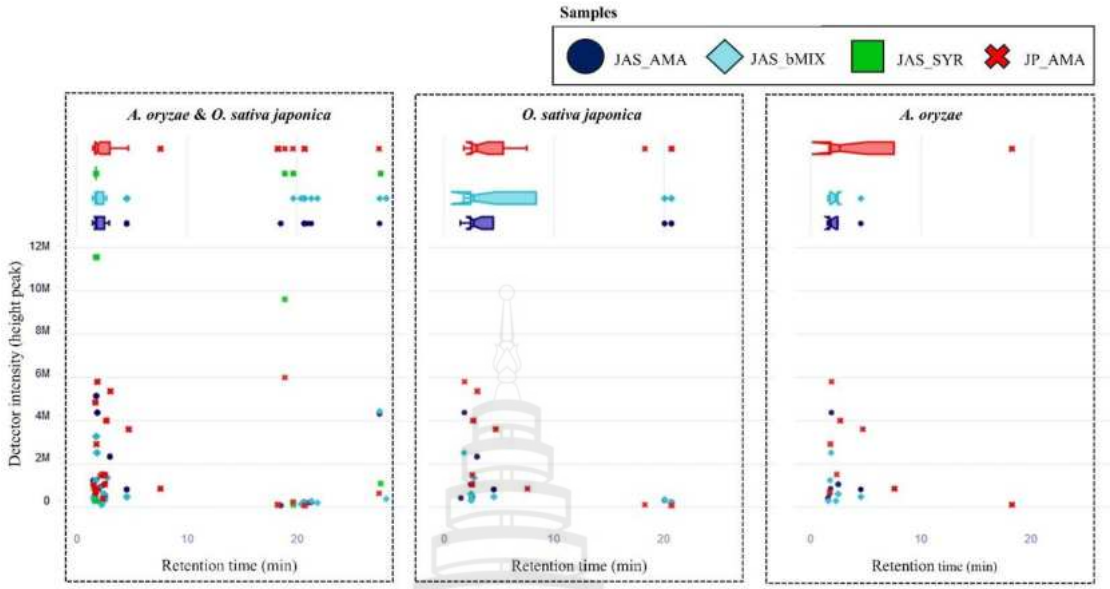
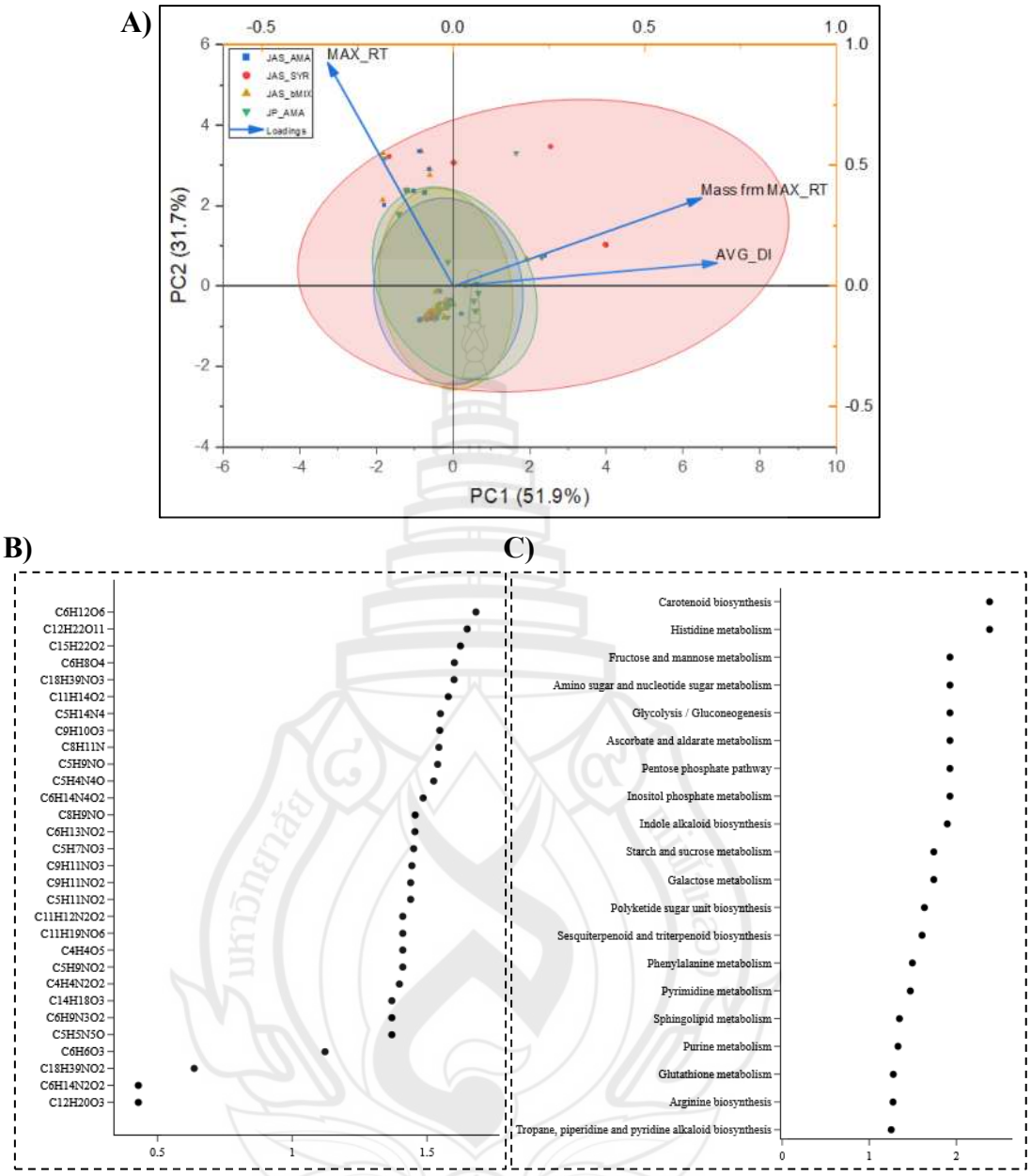


Figure 4.4 Scatterplot of the Tentative Compound Name of Mass Spectrometry Profiles Extracted Data, Grouped by KEGG Metabolic Profile Organism

When the overall profiles in Figure 4.5A were compared with the ellipse in Figure 4.1A of the PCA plot, it was observed that the retention time (RT) variable became smaller after merging with the KEGG data. This indicated that large-scale profiles had been removed following the integration with KEGG, further supporting the explanation in the previous paragraph regarding the exclusion of large molecules from the analysis.

Figure 4.5B presents the VIP scores of the predicted formulas for the extracted data based on detector intensity. The results revealed a prominent presence of essential amino acids. However, the prebiotic formula of $C_{12}H_{22}O_{11}$ is particularly notable, as it had the second-highest VIP score. This formula corresponds to possible compounds such as isomaltose, kojibiose, lactulose, and nigerose. The extracted data are presented in Table 4.1, which lists all tentative compound names classified under Identification Uncertainty Level 2 (ILv.2). These compounds had been previously reported in the metabolism of *Aspergillus oryzae* and Japanese rice, making their identification highly probable, though not at the level of absolute certainty (Identification Uncertainty Level 1, ILv.1). The hit values, which indicate the number of possible compounds matching this formula, along with molecular weight (Da) and retention time (min), are displayed across various sample groups, including JAS_AMA, JAS_bMIX, JAS_SYR, and JP_AMA.



Note (A) section Blue is JAS_AMA sample, Yellow is JAS_bMIX, Red is JAS_SYR, and green is JP_AMA

Figure 4.5 Analysis of Mass Spectrometry After KEGG Profiles Merged (A, The PCA plot of three variables including Average of Detector Intensity (AVG_DI, ion/min), Maximum Retention Time (MAX_RT, min), and Exact Mass from MAX_RT Data (Mass from MAX_RT); B, VIP Score of Predicted Formula Profile; C, Top 20 VIP Score of Metabolic Pathway Profiles)

Table 4.1 Tentative Compound Name After Filtered, Cleaned, and Merged KEGG Database Of LC-QTOF-MS/MS Raw Data in each Sample

Tentative compound name (ILv.2)	Formula hit data / Molecular weight (Da) and Retention time (min) each sample found			
	JP_AMA	JAS_AMA	JAS_bMIX	JAS_SYR
$C_{11}H_{12}N_2O_2$		Formula hit = 17		
L-Tryptophan	204.0904, [6.278]	N/A	N/A	N/A
$C_{11}H_{14}O_2$		Formula hit = 20		
Methyleugenol	N/A	178.0999, [19.528]	178.0994, [19.535]	N/A
Methylisoeugenol				
$C_{11}H_{19}NO_6$		Formula hit = 13		
Lotaustralin	261.1215, [1.845]	N/A	N/A	N/A
$C_{12}H_{20}O_3$		Formula hit = 15		
12-Oxo-9(Z)-dodecenoic acid	N/A	212.1404, [18.0275]	N/A	N/A
Traumatin				
$C_{12}H_{22}O_{11}$		Formula hit = 47		
alpha,alpha-Trehalose	342.1158, [1.661]	342.1154, [1.6845]	342.1158, [1.6965]	342.115, [1.6545]
alpha-D-Galactosyl-1D-myo-inositol				
Cellobiose				
Epimelibiose				
Isomaltose				
Lactose				
Levanbiose				
Maltose				
Melibiose				
Sucrose				
$C_{14}H_{18}O_3$		Formula hit = 20		
Strigolactone ABC-rings	N/A	N/A	234.1253, [19.854]	N/A
$C_{15}H_{22}O_2$		Formula hit = 25		
Germacrene A acid	N/A	234.1621, [20.818]	234.1617, [20.822]	N/A
$C_{18}H_{39}NO_2$		Formula hit = 13		
Sphinganine	301.2982, [19.205]	N/A	301.2971, [19.2325]	301.2972, [19.226]

Table 4.1 (continued)

Tentative compound name (ILv.2)	Formula hit data / Molecular weight (Da) and Retention time (min) each sample found			
	JP_AMA	**JAS_AMA**	**JAS_bMIX**	**JAS_SYR**
$C_{18}H_{39}NO_3$		Formula hit = 16		
Phytosphingosine	317.2933, [18.5135]	N/A	N/A	317.2926, [18.5265]
$C_4H_4N_2O_2$		Formula hit = 12		
Uracil	112.0282, [2.1725]	112.028, [2.112]	N/A	N/A
$C_4H_4O_5$		Formula hit = 10		
2-Hydroxyethylenedicarboxylate	132.0057, [17.822]	N/A	N/A	N/A
Oxaloacetate				
trans-2,3-Epoxysuccinate				
$C_5H_{11}NO_2$		Formula hit = 22		
4-Methylaminobutyrate	117.0799, [1.7765]	117.0796, [1.7735]	117.0798, [1.7535]	N/A
5-Aminopentanoate				
Betaine				
L-Valine				
$C_5H_{14}N_4$		Formula hit = 2		
Agmatine	130.1227, [1.4455]	130.1224, [1.427]	130.1226, [1.4365]	N/A
$C_5H_4N_4O$		Formula hit = 12		
Hypoxanthine	136.0394, [2.0345]	136.0391, [2.0375]	136.0394, [2.0355]	N/A
$C_5H_5N_5O$		Formula hit = 15		
8-Hydroxyadenine	N/A	N/A	151.0499, [2.0875]	N/A
Guanine				
$C_5H_7NO_3$		Formula hit = 13		
1-Pyrroline-4-hydroxy-2-carboxylate	129.0434, [2.1375]	129.0432, [1.6375]	129.0434, [1.9205]	N/A
5-Oxo-D-proline				
5-Oxoproline				
L-1-Pyrroline-3-hydroxy-5-carboxylate				
L-beta-Ethynylserine				
C_5H_9NO		Formula hit = 9		
(2R)-2-Hydroxy-2-methylbutanenitrile	N/A	99.0691, [19.4335]	99.0688, [20.358]	N/A

Table 4.1 (continued)

Tentative compound name (ILv.2)	Formula hit data / Molecular weight (Da) and Retention time (min) each sample found			
	JP_AMA	**JAS_AMA**	**JAS_bMIX**	**JAS_SYR**
$C_5H_9NO_2$		Formula hit = 15		
D-Proline	115.0642, [1.703]	N/A	N/A	N/A
$C_6H_{12}O_6$		Formula hit = 27		
1D-chiro-Inositol	180.0639, [1.669]	180.0634, [1.7745]	180.0639, [1.692]	N/A
alpha-D-Galactose				
alpha-D-Glucose				
beta-D-Fructose				
beta-D-Glucose				
D-Allose				
D-Fructose				
D-Galactose				
D-Glucose				
D-Mannose				
D-Tagatose				
L-Fuconate				
L-Galactose				
L-Gulose				
L-Rhamnonate				
L-Sorbose				
myo-Inositol				
scyllo-Inositol				
$C_6H_{13}NO_2$		Formula hit = 21		
L-Isoleucine	131.0955, [2.528]	131.0953, [2.511]	131.0955, [2.3815]	N/A
L-Leucine				
$C_6H_{14}N_2O_2$		Formula hit = 15		
(2R,3R)-3-Methylornithine	N/A	146.1059, [1.4855]	N/A	N/A
(2R,5S)-2,5-Diaminohexanoate				
(3S)-3,6-Diaminohexanoate				
(3S,5S)-3,5-Diaminohexanoate				
D-Lysine				
L-Lysine				

Table 4.1 (continued)

Tentative compound name (ILv.2)	Formula hit data / Molecular weight (Da) and Retention time (min) each sample found			
	JP_AMA	**JAS_AMA**	**JAS_bMIX**	**JAS_SYR**
$C_6H_{14}N_4O_2$		Formula hit = 11		
D-Arginine	174.1123, [1.513]	174.112, [1.521]	174.1123, [1.51]	N/A
L-Arginine				
$C_6H_6O_3$		Formula hit = 18		
Maltol	N/A	N/A	126.0313, [14.9265]	126.0319, [1.7295]
$C_6H_8O_4$		Formula hit = 17		
2,3-Dimethylmaleate	N/A	N/A	N/A	144.0423, [1.741]
2-Methyleneglutarate				
Methylitaconate				
$C_6H_9N_3O_2$		Formula hit = 18		
D-Histidine	N/A	N/A	155.0702, [1.5385]	N/A
L-Histidine				
$C_8H_{11}N$		Formula hit = 12		
Phenethylamine	121.09, [22.616]	121.0899, [24.001]	121.0896, [23.919]	121.0895, [27.5415]
C_8H_9NO		Formula hit = 16		
(E)-Phenylacetaldoxime	135.0692, [2.299]	135.0691, [2.0835]	135.0693, [2.097]	N/A
(Z)-Phenylacetaldehyde oxime				
2-Phenylacetamide				
$C_9H_{10}O_3$		Formula hit = 25		
3-(2-Hydroxyphenyl)propanoate	166.0638, [20.144]	166.0636, [20.1285]	166.0631, [20.125]	N/A
3-(3-Hydroxyphenyl)propanoic acid				
3-Methoxy-4-hydroxyphenylacetaldehyde				
Caffeyl alcohol				
Phenyllactate				
Tropate				
$C_9H_{11}NO_2$		Formula hit = 23		
D-Phenylalanine	165.0797, [3.738]	165.0794, [3.674]	165.0796, [3.6725]	N/A
L-Phenylalanine				

Table 4.1 (continued)

Tentative compound name (ILv.2)	Formula hit data / Molecular weight (Da) and Retention time (min) each sample found			
	JP_AMA	**JAS_AMA**	**JAS_bMIX**	**JAS_SYR**
$C_9H_{11}NO_3$	Formula hit = 24			
3-Amino-3-(4-hydroxyphenyl)propanoate L-Tyrosine N-Hydroxy-L-phenylalanine	181.0745, [2.3615]	181.0741, [2.26]	181.0746, [2.2635]	N/A
$C_9H_8O_3$	Formula hit = 18			
2-Hydroxy-3-phenylpropenoate 4-Coumarate Caffeic aldehyde cis-2-Hydroxycinnamate Phenylpyruvate trans-2-Hydroxycinnamate trans-3-Hydroxycinnamate	164.048, [2.2805]	164.0477, [2.28]	164.0479, [2.2475]	N/A

This KEGG comparative data table was compared with the sample using a Venn-diagram. When each sample of the Thai jasmine rice amazake represented different production processes, including JAS_AMA, JAS_bMIX, and JAS_SYR. Figure 4.6 and other Venn-diagram were different between other product, including JAS_AMA, JP_AMA, and JAS_SYR in Figure 4.7. Observable because the carbohydrate group was believed to be at the center of both Venn-diagrams, tentative molecules containing this group could be challenging. It was difficult to find the uniqueness of this group. Because they all had the same formula and many database hits.

In summary, from the results obtained, this study were interested in analyzing the group of all prebiotics that could be found. Unfortunately, there were still many analytical options more than the budget this study had, so we narrowed down our target by comparing the LC-QTOF-MS/MS data to the prebiotics that should be found in prebiotic products.

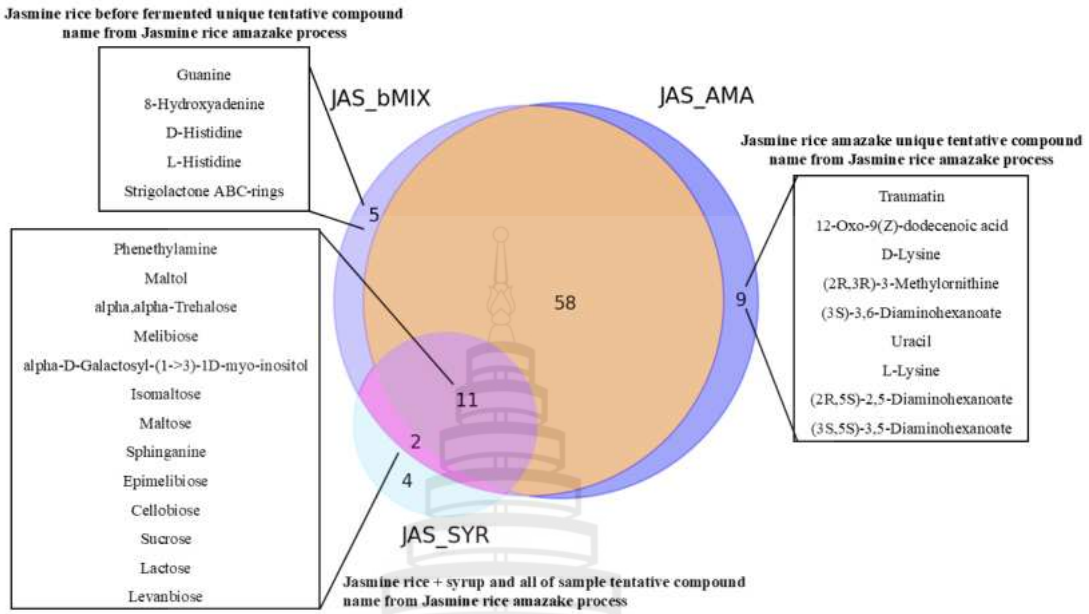


Figure 4.6 Venn-Diagram of Jasmine Rice Amazake Process (Jasmine Rice +Syrup (Light Blue), Jasmine Rice Koji Mixed Before Fermented (Blue-Sky), and Jasmine Rice Amazake (Blue) sample)

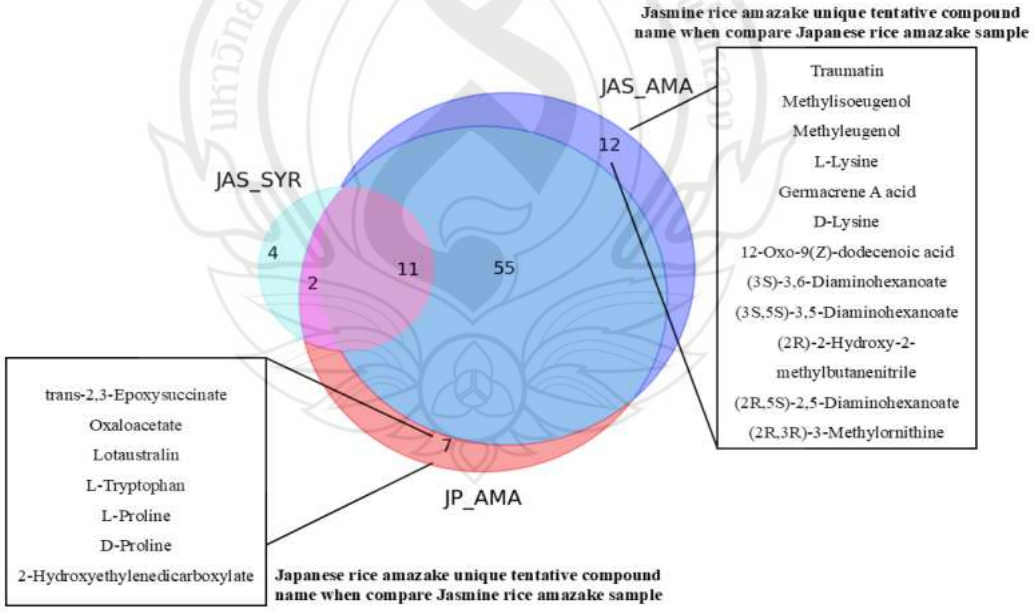


Figure 4.7 Venn-diagram compare between Amazake Product (Jasmine rice + syrup (Light blue), Jasmine rice amazake (Blue), and Japanese rice amazake (Red) sample)

4.1.3 Prebiotics Data Merged and Candidate Prebiotics Selected

Since the LC-QTOF-MS/MS results are large molecules and difficult to predict, they were compared with possible prebiotic data. The process was explained in Figure 4.8. The data cleaning process had been the same as in Topic 4.1.2. and intersect the data with the Prebiotic data. However, in this process, the data from both times will not be merged before doing this process. This is because this process needs data from both times to plan which prebiotic candidates to use in the next experiment.

The results were shown in Table 4.2. This research decided to choose the isomaltooligosaccharide group, which includes isomaltose, isomaltotriose, and isomaltotetraose. The isomaltotetraose appeared in the JAS_AMA and JAS_bMIX products, so it had been possible for it to be the prebiotic that was unique in the Thai jasmine rice amazake. Therefore, that was the main reason why we chose this group to confirm quantitative results.

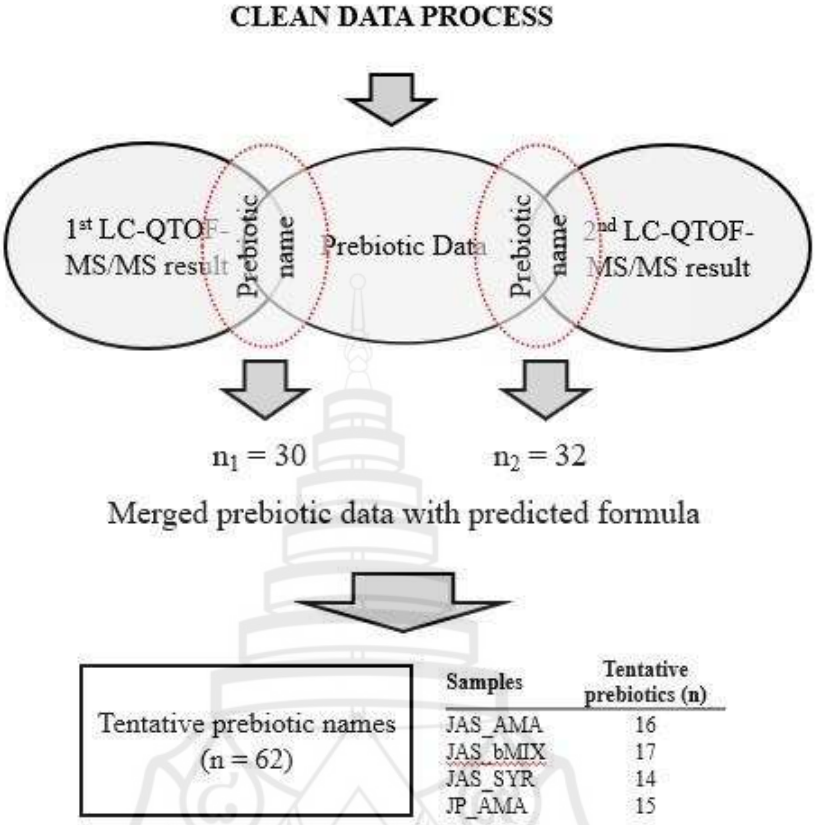


Figure 4.8 Merged Mass Spectrometry Data with Prebiotics Data Process

Table 4.2 Tentative Prebiotic Name After Filtering and Cleaning in each Sample

Tentative prebiotic name (ILv.2)	Formula hit data / Molecular weight (Da) and Retention time (min) each sample found			
	JP_AMA	**JAS_AMA**	**JAS_bMIX**	**JAS_SYR**
C12H22O11		Formula hit = 47		
Isomaltose	342.1153, [1.637]	342.1146, [1.623]	342.1154, [1.625]	342.1138, [1.544]
Kojibiose				
Lactulose				
Nigerese				
Polydextrose				
C12H24O12		Formula hit = 46		
Alpha-Sophorose	360.1269, [1.691]	N/A	360.1269, [1.901]	N/A
C18H32O16		Formula hit = 19		
Galactooligosaccharide	504.1659, [1.536]	504.1644, [1.521]	504.166, [1.528]	504.1694, [1.773]
Isomaltotriose				
Panose				
Raffinose				
C24H42O21		Formula hit = 165		
Isomaltotetraose	N/A	666.2222, [1.774]	666.2223, [1.678]	N/A
Mannanoligosaccharide				

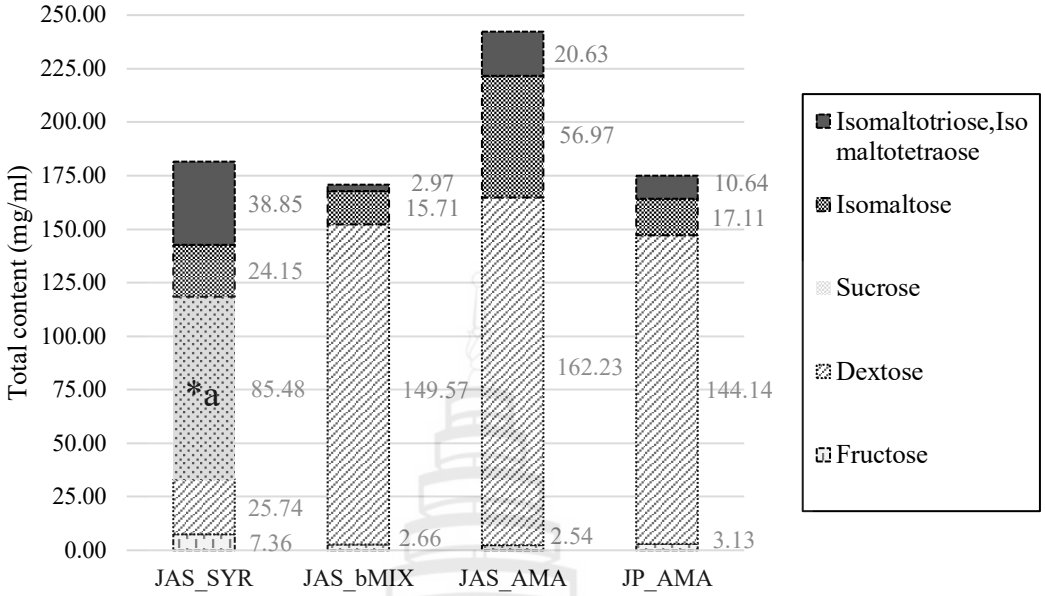
4.2 Quantification of Prebiotics and Effectiveness Analysis Results

4.2.1 Quantification Results of Selected Prebiotics by Liquid Chromatography

As expected, the results confirm the quantity displayed in Figure 4.9. The quantity of sugar in each sample, fructose in the JAS_SYR (7.36 mg/ml), had the highest concentration of sugar in the JAS_SYR sample because that sample had syrup mixed in. For the comparison between before and after mixing, there are quite a few differences. The sucrose sugar in the JAS_SYR (85.48 mg/ml) is different, which is a mixture of plain rice water and syrup. It could not be the same concentration as the rice that has been inoculated with *Aspergillus oryzae*, including JAS_AMA, JAS_bMIX, and JP_AMA. The sucrose was only found in the JAS_SYR sample, to which syrup was added, and it also represents that no sucrose was added to the amazake product samples to increase sweetness.

The quantity of prebiotics such as isomaltose and isomaltotriose/ isomaltotetraose was found to be a problem in the HPLC analytics. Isomaltotriose and isomaltotetraose had similar retention times. That was the reason why the result of isomaltotriose and isomaltotetraose was reported together. The isomaltose had increased concentration when comparing JAS_bMIX (15.71 mg/ml) and JAS_AMA (56.97 mg/ml). However, the concentration in JAS_bMIX (15.71 mg/ml) was lower than in sample JAS_SYR (24.15 mg/ml). Additionally, the JAS_SYR had the most isomaltotriose/isomaltotetraose (38.85 mg/ml).

The summary quantity of sugar and prebiotics contained, other than JAS_SYR, which contains the syrup, is that there is no chance that sucrose can be discovered in any other product. However, it is not unclear why JAS_SYR is, even though the IMOs group more than JAS_bMIX. It's possible because that, the JAS_SYR sample, uses sugar and boiling rice water. They had concentrated more than JAS_bMIX in the sample. When observed from other samples, it was found that the isomaltose group in JAS_AMA is more than in JAS_SYR. Therefore, the amazake fermentation of the Thai jasmine rice will provide a high concentration of the isomaltose/isomaltotriose/ isomaltotetraose prebiotic group, similar to the amazake from Japanese rice.



Note *a is sucrose can only be founded in JAS_SYR sample; other samples have sucrose area under the peak < 0.001

Figure 4.9 HPLC Result Confirm the Quantity of Sugar in Each Sample

4.2.2 Efficacy Results of Amazake-Containing Media on Probiotics Growth by *In Vitro* Growth Curve Analysis

There was a slight problem in controlling the temperature of the instrument during curing, as seen in Figure 4.10. Because The *L. plantarum* abundance is the good potential. Thus, this part repeated it one more time the next day to assure the potential of the *L. plantarum* abundance and incubator performance again. It could be observed the first day will not be the same as that on the second day, even if the similar starter candidate microbe has an abundance of 1.0 McFarland (amount 3.0 x 10^8 approximate bacterial suspension/ml), but the growth pattern sequence remains the same on the first day. Therefore, the results that can be used are not a comparison of which organism's growth is better, but rather a comparison of the maximum OD value of each sample compared to the sample without a carbohydrate source group (wMRS) and the sample broth containing the glucose or obtained as MaxOD - x_wMRS and MaxOD - x_Glu values according to Table 4.3. From the table, it could be found that the *B. subtilis* group did not grow more than wMRS (BS_wMRS = 1.4200 ± 0.0990, the same as BS_JAS_AMA = 1.3600 ± 0.0141, $P = 0.4406$ and BS_IMO = 1.4167 ± 0.1891, $P = 0.2236$). However, the result was that BS_JAS_AMA significantly grew faster than the other carbohydrate source groups when compared to the 12-hour stage in each other sample ($P < 0.02$).

In the *L. plantarum* group, it is different. The carbohydrate source that the bacteria very significantly grows the best on is the Thai jasmine rice amazake (LP_wMRS = 1.0667 ± 0.1069 < LP_JAS_AMA = 2.100 ± 0.1054, $P < 0.000$) when compared to the group without a carbohydrate source, which is still more than that growth with glucose, but not significantly (LP_GLU = 1.9550 ± 0.1882 < LP_JAS_AMA = 2.100 ± 0.1054, $P = 0.163$). However, IMOs are still the last one, even though they can help promote growth (LP_wMRS = 1.0667 ± 0.1069 < LP_IMO = 1.5017 ± 0.0925, $P < 0.05$) when compared without a carbohydrate source group. The result is that glucose helps promote growth better than IMOs.

The *E. coli* was the last group, which represents the pathogen. It was clearly seen that the bacteria growth with the Thai jasmine rice amazake and IMOs had a significantly reduced growth rate than without a carbohydrate source (EC_wMRS =

2.5417 ± 0.1826 > EC_IMO = 2.0817 ± 0.1697, $P = 0.017$). The Thai jasmine rice amazake group has a little bit of an effect but does not significantly reduce the growth rate of *E. coli* when compared to the group without a carbohydrate source (EC_wMRS = 2.5417 ± 0.1826 > EC_JAS_AMA = 2.4317 ± 0.0071, $P = 0.364$).

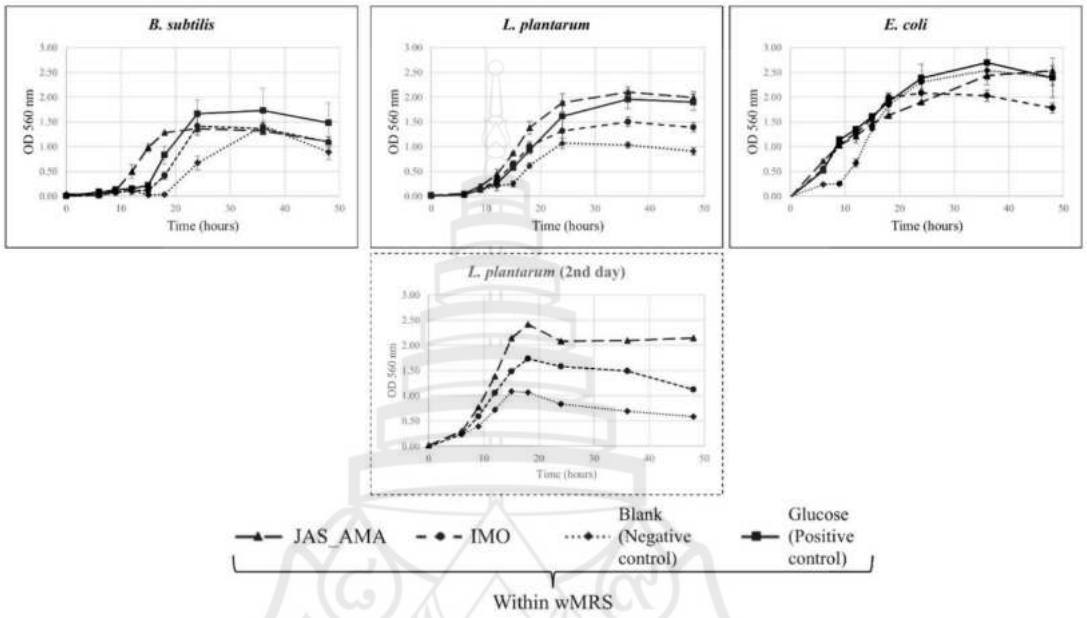


Figure 4.10 Growth Pattern in each Three Candidate Microbial Effectiveness and Test Prebiotics (JAS_AMA; Jasmine Rice Amazake, IMO; Isomaltooligo-saccharide, wMRS; Without Carbohydrate Source MRS Broth)

Table 4.3. The Result of Calculating the Maximum OD Between the Other Three Candidate Microbial Effectiveness (BS, *B. subtilis;* LP, *L. plantarum*; EC, *E. coli*; JA_AMA, Jasmine Rice Amazake; IMO, Isomaltooligosaccharide) with wMRS and Glucose Group

Sample	MaxOD	SD	MaxOD – x_wMRS	MaxOD – x_GLU
BS_IMO	1.4167	0.1891	-0.003	-0.313
BS_JAS_AMA	1.3600	0.0141	+0.247	-0.063
LP_IMO	1.5017	0.0925	+0.435	-0.453
LP_JAS_AMA	2.1000	0.1054	+1.033	+0.145
EC_IMO	2.0817	0.1697	-0.460	-0.615
EC_JAS_AMA	2.4317	0.0071	-0.110	-0.265

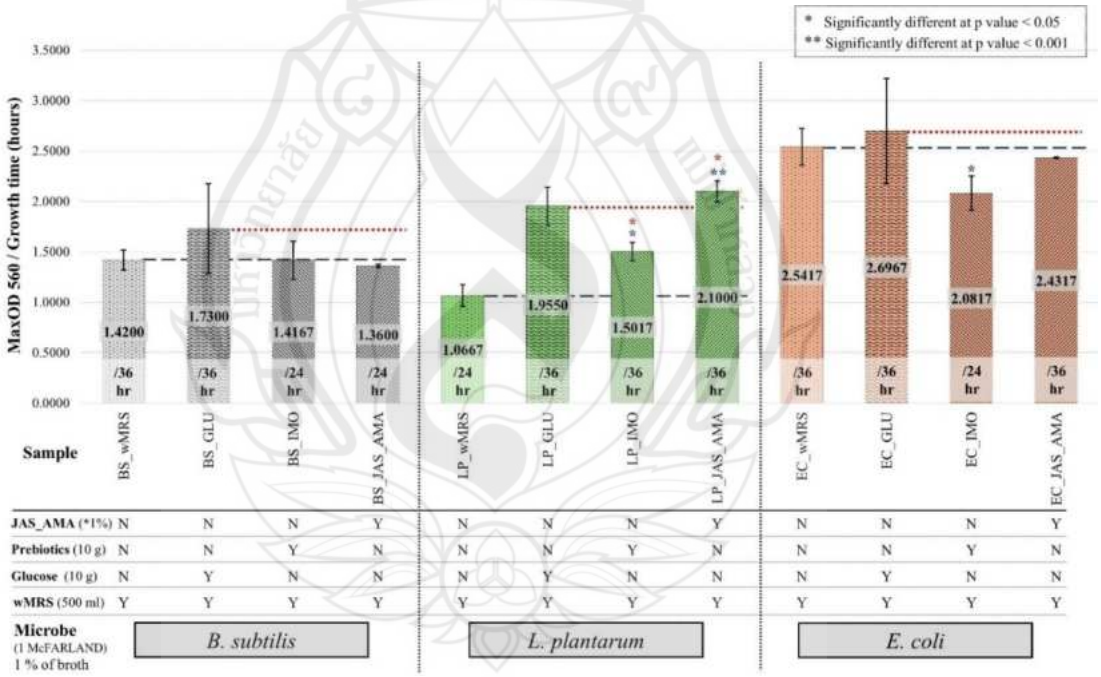


Figure 4.11 Histogram of Maximum OD in Each of The Three Candidate Microbial Effectiveness (BS, *B. subtilis;* LP, *L. plantarum*; EC, *E. coli*) with Sugar and Prebiotic uses Total Sugar Assumed to be 20 g/l (GLU, Glucose; JA_AMA, Jasmine Rice Amazake; IMO, Isomaltooligosaccharide; wMRS, Without Carbohydrate Source MRS broth)

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 Metabolite Profile

The components found in sugar beverages were explored in this study, which was considered highly challenging because all carbohydrate compounds were found to have molecular formulas and molecular weight profiles with several similar groups. A problem had been encountered with caramel formation in the LC-QTOF-MS/MS column and jet stream electrospray ionization mass spectrometry (ESI-MS). For this reason, the protocol for LC-QTOF-MS/MS analysis was adjusted to ensure suitability for the amazake product. This study was supported by laboratory work based on Oguro's metabolomic analysis, which had investigated the same objective (Oguro et al., 2017). From this experiment and textbook studies, it was understood that LC-QTOF-MS/MS could only be used for primary screening. However, it was expected that conducting the analysis multiple times would lead to more accurate identification of results.

When octadecylsilyl groups (ODS or C18) were used as the column type, an advantage was observed: the column remained undamaged and stable when employed for analyzing sweet or carbohydrate-based beverages. Unfortunately, this method was found unsuitable for the separation and demonstration of disaccharide and monosaccharide anomers (Eliasson & Ann-Charlotte, 2006).

The mass spectrometry profiles in Figure 4.1A and D (after data cleaning) revealed no clear reasons why the JAS_SYR sample contained higher amounts of compounds compared to other samples, including those with added koji. At this stage, only a hypothesis could be proposed: the JAS_SYR production process may have involved high-temperature boiled rice water mixed with syrup at a higher rice ratio than other samples. However, this explanation was considered unreliable unless the sample production was controlled and retested under standardized conditions.

When the metabolic pathway cluster map (Figure 4.3) was examined, tentative compounds were compared across samples. The koji-added samples (JP_AMA, JAS_AMA, and JAS_bMIX) showed overlapping metabolic pathways, particularly between JAS_AMA and JAS_bMIX. In contrast, JAS_SYR exhibited distinct profiles, suggesting that the elevated tentative compounds after fermentation originated primarily from koji mold metabolism rather than rice. Furthermore, tentative compounds were detected immediately after koji addition, with quantity variations likely linked to fungal fermentation or secondary metabolism.

Figure 4.4 had surprising results: the KEGG data scatter plot (middle panel) for *O. sativa japonica* was dominated by flavonoid metabolism and antioxidant-related compounds, with no JAS_SYR representation, possibly due to its limited analyte compounds. In Figure 4.5A (PCA plot), JAS_AMA displayed slightly higher detector intensity than JAS_bMIX, reinforcing that post-fermentation compound increases were driven more by koji activity than Thai jasmine rice alone.

This metabolite profile study had several limitations. Overly stringent data cleaning aimed at maximizing accuracy may have contributed to gaps in the dataset. Additionally, as a primary screening, the study did not prioritize direct comparisons between Thai jasmine rice (JAS_AMA) and Japanese rice (JP_AMA) amazake. Future comparative metabolite profiling should employ stricter sample collection and systematic data processing to strengthen conclusions.

Therefore, LC-QTOF-MS/MS was utilized for primary metabolomic screening, aligning with the methodology employed in the Oguro study. The tentative compounds identified in Table 4.1 required further validation. Figures 4.5B and C could be referenced for predicted formulas and metabolic pathway profiles to guide subsequent investigations. However, definitive confirmation of the compounds detected in JAS_AMA could not be immediately established.

The study was limited to hypothesizing that the $C_{12}H_{22}O_{11}$ disaccharide group (a potential prebiotic) might be of interest, though the possibility of sucrose contamination could not be ruled out. To address this concern, sucrose levels were monitored via HPLC (Figure 4.9), which revealed only three detectable sugars: glucose, sucrose, and fructose. Notably, fermented rice products were found to contain minimal fructose and sucrose a significant advantage, as excessive consumption of

these sugars has been linked to increased risks of (Stanhope et al., 2009) and type 2 diabetes (T2DM) (Malik et al., 2010). In contrast, glucose the predominant sugar in these products was observed to support human glucose metabolism and gut-brain homeostasis, though its intake should be regulated to prevent overconsumption (Bacharach et al., 2023; Zhao et al., 2021).

The unique compound profile of JAS_AMA was observed in Figure 4.1A, where it was found to differ significantly from JAS_SYR. Additionally, subtle distinctions between JAS_AMA and JAS_bMIX were noted in the ellipses representing maximum retention time and exact mass profiles. These differences, combined with variations in production processes, suggested that certain health-beneficial compounds might have been produced following koji integration. In Figure 4.6, several compounds with potential health benefits were identified, including: traumatic acid, which has been linked to the treatment of oxidative stress-related skin conditions (Jabłońska-Trypuć et al., 2016), lysine an essential amino acid critical for human health (Kurpad & Young, 2003), and uracil a pyrimidine nucleobase with no unique dietary effects.

Notably, lysine was detected exclusively in JAS_AMA (Figures 4.6 and 4.7). Further revealed other amino acids, such as isovaline, a rare amino acid with anticonvulsant properties (Shin et al., 2011), agmatine, which has been studied for therapeutic applications in Parkinson's disease (Zamanian et al., 2024); and Additional essential amino acids. However, the amino acid profile was limited compared to the Yamamoto and group study, which reported comprehensive free amino acids in both white and brown Japanese rice amazake (Yamamoto et al., 2011). This discrepancy might have resulted from stringent data cleaning, potentially excluding other amino acids. While the qualitative uniqueness of these amino acids could not be confirmed, the quantitative abundance in JAS_AMA remained an intriguing avenue for future research.

5.1.2 Prebiotics and Isomaltooligosaccharide Profile

The tentative prebiotic compounds identified through LC-QTOF-MS/MS analysis (Table 4.2, Figures 4.1B and 4.5B) were selected based on their $C_{12}H_{22}O_{11}$ molecular formula, which included kojibiose, nigerose, sophorose, panose, and isomaltose. These findings provided sufficient evidence to warrant further

investigation. Previous research by Kurahashi and group had reported the presence of these compounds in Japanese rice amazake, suggesting a high probability of their occurrence in the current study, though quantitative measurements were not obtained (Kurahashi et al., 2021).

Analysis of Table 4.2 revealed that sophorose was detected in both JAS_bMIX and JP_AMA samples, while isomaltotetraose was uniquely identified in the Thai jasmine rice amazake processes (JAS_AMA and JAS_bMIX). However, examination of Figures 4.1A and 4.1C indicated that Japanese rice amazake exhibited higher average detector intensity and more distinct exact mass profiles compared to JAS_AMA, suggesting potential quantitative and qualitative differences in the prebiotic profiles between the two rice varieties.

The prebiotic category selected for this investigation was isomaltooligo-saccharides (IMOs), primarily consisting of isomaltose, isomaltotriose, and panose (Banerjee et al., 2022). IMOs have been previously identified in various fermented products including miso, sake, soy sauce, beer, and honey (Tungland & Meyer, 2002), and have demonstrated potential benefits in managing metabolic disorders such as obesity (Singh et al., 2018) and type 2 diabetes (Um et al., 2023). While isomaltose has been specifically reported in amazake (Kurahashi, 2021), the current study found isomaltotriose and isomaltotetraose present in all amazake samples, including the non-fermented JAS_SYR group (Figure 4.9). Consequently, while unique prebiotic compounds were not identified exclusively in JAS_AMA, the prebiotic profiles were found to be similar to Japanese rice amazake, with only minor quantitative differences in IMOs observed between the samples.

The detection of both isomaltose and maltose in JAS_SYR (unprocessed rice) was unexpected, as these compounds are typically associated with fermented foods (Tennant & Hornbuckle, 1980). Structural analysis revealed that isomaltose and maltose share the same molecular formula ($C_{12}H_{22}O_{11}$) with 47 potential library matches (Table 4.1), highlighting the necessity for standardized measurements. This requirement was addressed through HPLC analysis, which confirmed the presence of isomaltose and IMOs in JAS_SYR.

The origin of these prebiotics in unprocessed Thai jasmine rice remains unexplained, as no previous studies have reported their natural occurrence. Analytical

challenges emerged during HPLC quantification, where isomaltotriose and isomaltotetraose could not be resolved due to nearly identical retention times, necessitating combined reporting. Results demonstrated a significant increase in isomaltose levels but markedly lower concentrations of isomaltotriose/isomaltotetraose compared to JAS_SYR. These findings suggest that conventional consumption of Thai jasmine rice or standard sugar might provide higher quantities of isomaltotriose/isomaltotetraose, though the underlying mechanisms remain unclear. Potential explanations may involve: 1) unique sample preparation methods for JAS_SYR, and 2) interference from abundant compounds observed in mass spectrometry profiles.

Kojibiose emerged as another potentially significant prebiotic compound, consistently detected at lower concentrations than isomaltose but in greater quantities than reported in Japanese rice amazake by Oguro and team (Oguro et al., 2019). This rare disaccharide has been previously identified in koji extract (Chaen et al., 2001), honey, and fermented rice products. Current research demonstrates kojibiose's prebiotic efficacy in selectively promoting beneficial gut microbiota, including *Bifidobacterium breve*, *Eubacterium aerofaciens*, *Peptostreptococcus productus*, Various *Lactobacillus*, and *Streptococcus* strains (Nakada et al., 2003; Onyango et al., 2020). Notably, animal studies have shown kojibiose may mitigate liver dysfunction in hyperglycemic conditions (Moisés Laparra et al., 2015). Similarly, nigerose exhibited potential therapeutic value through its demonstrated resistance to *Lactobacillus plantarum* infection (Biesiada et al., 2019), which can trigger inflammatory responses via IL-12 and IL-2-induced IFN-γ production. When administered in combination kojibiose, nigerose, and sophorose, these prebiotics demonstrate synergistic effects by selectively enhancing *Bifidobacterium* and *Bacteroides* populations and simultaneously inhibiting *Clostridium* strains (Murosaki et al., 1999).

5.1.3 Prebiotic Properties of Thai Jasmine Rice Amazake

As shown in Figure 4.11, *B. subtilis* (TISTR-1248) exhibited significantly greater growth in the BS_JAS_AMA group compared to other test conditions, though its proliferation remained lower than in the BS_GLU and BS_IMO groups, after which growth plateaued. Notably, the amazake substrate (Figure 4.10) supported faster microbial growth than other carbohydrate sources tested. The selection of *B. subtilis* (TISTR-1248) for this study was based on the absence of prior evidence regarding IMOs' effects on this strain. Existing literature has only documented its growth response to β-glucan, inulin, oligofructose, xylooligosaccharide, arabinoxylan-oligosaccharide (Rurangwa et al., 2009), and maltose (Tangney et al., 1992). These studies suggest that *B. subtilis* (TISTR-1248) thrives preferentially on disaccharides but shows limited utilization of oligosaccharides, potentially explaining the observed lack of IMOs-mediated growth enhancement. The growth patterns of BS_IMO and BS_JAS_AMA aligned with prior prebiotic studies, though this investigation could not assess the specific impact of isomaltose due to difficulties in procuring the compound for *in vitro* testing. Consequently, the influence of isomaltose on *B. subtilis* (TISTR-1248) remains unresolved and warrants further examination.

The effectiveness of IMOs in promoting growth differed significantly between *L. plantarum* (TISTR-2074) and *B. subtilis* (TISTR-1248). While *L. plantarum* exhibited optimal growth with LP_JAS_AMA surpassing even glucose IMOs remained the least effective carbohydrate source, despite still demonstrating some growth-promoting effects. These observations align with prior findings by Saulnier and team, where glucose outperformed IMOs in supporting lactic acid bacteria growth (Saulnier et al., 2008). Notably, Japanese rice amazake has been widely reported to enhance *Lactobacillus* and other lactic acid bacteria (LAB) strains, consistent with our results showing improved *L. plantarum* proliferation. This is particularly advantageous given *L. plantarum* is documented probiotic benefits, including: reduction of *Clostridium difficile* colonization in antibiotic-associated gut dysbiosis (Klarin et al., 2008), attenuation of stress-induced cortisol levels in young adults(Andersson et al., 2016), enhanced iron absorption in women of reproductive age (Hoppe et al., 2015), and alleviation of irritable bowel syndrome (IBS) symptoms (Ducrotté et al., 2012). Although IMOs showed limited efficacy compared to glucose or amazake substrates,

their role in modulating gut microbiota particularly in synergy with other prebiotics warrants further investigation.

In the final test group, *E. coli* (TISTR-527), a pathogenic coliform bacterium exhibited significantly reduced growth rates when cultured with EC_JAS_AMA or EC_IMO compared to cultures without carbohydrate supplementation. This inhibitory effect aligns with findings from Pi and group, where IMOs combined with Chinese herbs similarly suppressed *E. coli* proliferation while promoting *Bifidobacteria* spp., thereby improving gut health outcomes (Pi et al., 2022). The clinical relevance of this result is underscored by the pathogenic potential of *E. coli* TISTR-527, which has been isolated from human urine (Lueangprasert & Saelim, 2020), serves as a fecal contamination marker in food (Gözde & Emek, 2019), and causes watery diarrhea with severe cases progressing to cholera-like symptoms (e.g., vomiting, high fever) (Donnenberg, 2013). Critically, the observed growth suppression confirms that these prebiotics (JAS_AMA and IMOs) do not support pathogenic *E. coli* proliferation, reinforcing their potential as safe and beneficial dietary components for consumers.

5.1.4 Thai Jasmine Rice Amazake and Thai People Health Benefit

Diabetes and Metabolic Health Context: Thailand has the seventh-highest number of diabetic patients in the Western Pacific region (Chan et al., 2014), with a rapidly growing prevalence. In 2023 alone, 300,000 new cases were reported—a significant increase from 3.3 million cases in 2022 (Department of Disease Control, Ministry of Public Health, 2023). Alarmingly, screening data revealed that 5 million of 22 million Thais aged ≥35 years remain unscreened for diabetes.

Potential of Amazake for Metabolic Health: As demonstrated in this study, Thai jasmine rice amazake contains IMOs, which may help regulate metabolic disorders like T2DM and obesity. While Akamine and team showed that Japanese white and whole-grain rice amazake reduce inflammation and increase short-chain fatty acid production in metabolic syndrome patients, our findings suggest JAS_AMA could similarly benefit gut microbiota modulation in Thai populations though clinical validation is needed (Akamine et al., 2022).

Antibiotic Misuse and Gut Microbiome Rescue: Thailand's rampant over-the-counter antibiotic availability (Sommanustweechai et al., 2018; Sumpradit et al.,

2015) disrupts gut microbiome homeostasis (Manichanh et al., 2010). Notably, JAS_AMA's prebiotic efficacy (evidenced by its promotion of *L. plantarum* a keystone probiotic for antibiotic-associated dysbiosis recovery) positions it as a promising dietary intervention. When combined with probiotics or synbiotics (Dahiya & Nigam, 2023), it may mitigate antibiotic-induced gut damage.

5.2 Conclusion

5.2.1 The study program discovered that Thai jasmine rice amazake products contained good nutrition in the form of carbs and amino acids. Importantly we discovered that Thai jasmine rice amazake has substances that are beneficial to health, such as disaccharide prebiotics, essential amino acids, and antioxidant amino acids. Regretfully, this study's metabolite profile is only suitable for primary screening. Nonetheless, this investigation could identify nutritional profiles and carbohydrate prebiotics in Thai jasmine rice amazake.

5.2.2 One of the prebiotic groups, the isomaltoligosaccharide group (IMOs), was subjected to quantitative examination during analytics. That could be observed from the comparison of the Thai jasmine rice before (JAS_bMIX) and after fermentation by A. oryzae pasteurized (JAS_AMA) to be the Thai jasmine rice amazake product. The Japanese rice amazake sample product with A. oryzae ability contained the same quantity of isomaltose, isomaltotriose, or isomaltotetraose as the Thai jasmine rice amazake.

5.2.3 The efficacy of Thai jasmine rice amazake as a prebiotic was demonstrated in this investigation. The Thai jasmine rice amazake had a similar effect to the prebiotics IMOs mix MRS medium, but IMOs had a greater impact because it could increase the growth of the *Lactobacillus plantarum* (TISTR-2074) probiotic group and potentially inhibit the growth of *Escherichia coli* (TISTR-527), which represents the pathogen group.

5.3 Suggestion

5.3.1 Research Protocol Suggestion

5.3.1.1 C18 type is not advised for use as an analytical sweetness liquid in the LC-QTOF-MS/MS column. This investigation effectively employed Asahipak NH2P-50 (Shodex, U.S.) silica-based amino columns for qualitative assessment.

5.3.1.2 It would be simpler to use a centrifugal filter with the same type and size of filter pore or use a 25-mm 0.22-micron Nylon syringe filter for the preparation of amazake sample. 13-mm type, which is very easily stuck, was used in this study. Alternative should we suggest using a larger filter pore.

5.3.1.3 Making the McFarland standard should be based on trustworthy criteria or use a product that has certification, even though this is just a preliminary estimate. However, there isn't much of a difference if you do it yourself.

5.3.1.4 Choose a pathogen that grows well in MRS medium could be a much better indicator of the prebiotic's effectiveness between *L. plantarum* more than compared with *E. coli*.

5.3.2 Preparation for Koji Metabolite Profiling Study

The LC-QTOF-MS/MS used to metabolite profiling analytics should have standard compounds, all candidate compounds can be found in a literature review. Too much preparation is needed because of the much more amount of standard compounds to be used and the LC-QTOF-MS/MS instrument service budget. Otherwise, regardless of how you have the analytics program performs. The results obtained from this LC-QTOF-MS/MS the advanced instrument will only be primary screening. Because the obtained information has not been verified correctly.

5.3.3 Bias of Thai Jasmine Rice and Japanese Rice Amazake Product

Thai jasmine rice and Japanese rice amazake should be prepared by yourselves for this study. This research must use that YoRice café or only one Thai amazake manufactured products for analysis because its objective is to enhance the Thai products that are now available. This results in the inability to confirm whether the obtained product has a normal process as reported to the research team. This research is therefore about comparing the original products from Japan with our products instead. However, we are aware that doing is a little bit biased.

5.4 Limitation

The use of a non-specialized chromatographic column for sugar-based product analysis, the lack of quantitative measurements for all metabolites identified by MS, and the smaller range of microbial organisms used for the prebiotics effective assay which needs to have included a wider range of microbial organisms to produce more thorough and accurate results are the main limitations of this study. Furthermore, the reliability and reproducibility of the results may have been impacted by variability induced by the somewhat uncontrolled production procedures of the products under analysis.

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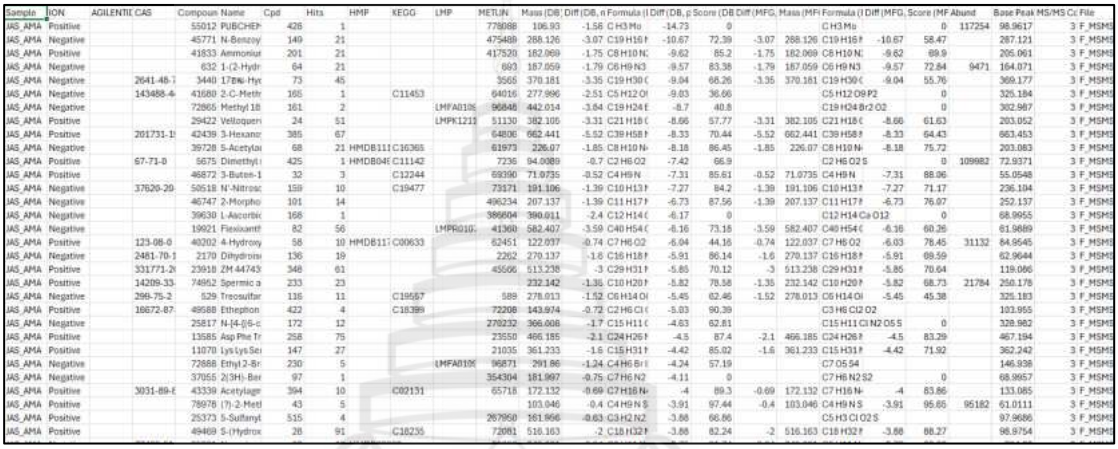
APPENDIX A

LC-QTOF-MS/MS RAW RESULT DATA



Sample	ION	AGILENTID	CAS	Compoun	Name	Cpd	Hits	HMP	KEGG	LMP	METLIN	Mass (DB	Diff (DB, n	Formula (I	Diff (DB, p	Score (DB	Diff (MFG,	Mass (MF	Formula (I	Diff (MFG,	Score (MF	Abund	Base Peak	MS/MS Cc	File
IAS_AMA	Positive			55012	PUBCHEM	428	1				778088	106.93	-1.56	C H3 Mo	-14.73	0			C H3 Mo		0	117254	98.9617	3	F_MSMS
IAS_AMA	Negative			45771	N-Benzoy	149	21				475489	288.126	-3.07	C19 H16 I	-10.67	72.39	-3.07	288.126	C19 H16 I	-10.67	58.47		287.121	3	F_MSMS
IAS_AMA	Positive			41833	Ammoniu	201	21				417520	182.069	-1.75	C8 H10 N(	-9.62	85.2	-1.75	182.069	C8 H10 N(	-9.62	89.9		205.061	3	F_MSMS
IAS_AMA	Negative			632	1-(2-Hydr	64	21				693	187.059	-1.79	C6 H9 N3	-9.57	83.38	-1.79	187.059	C6 H9 N3	-9.57	72.84	9471	164.071	3	F_MSMS
IAS_AMA	Negative		2641-48-7	3440	17β-Hy	73	45				3565	370.181	-3.35	C19 H30 (	-9.04	68.26	-3.35	370.181	C19 H30 (	-9.04	55.76		369.177	3	F_MSMS
IAS_AMA	Negative		143488-4	41680	2-C-Meth	165	1		C11453		64016	277.996	-2.51	C5 H12 O	-9.03	36.66			C5 H12 O9 P2		0		325.184	3	F_MSMS
IAS_AMA	Negative			72865	Methyl 18	161	2			LMFA010	96846	442.014	-3.64	C19 H24 E	-8.7	40.8			C19 H24 Br2 O2		0		302.987	3	F_MSMS
IAS_AMA	Positive			29422	Velloquer	24	51			LMPK121	51130	382.105	-3.31	C21 H18 (	-8.66	57.77	-3.31	382.105	C21 H18 (	-8.66	61.63		203.052	3	F_MSMS
IAS_AMA	Positive		201731-1	42439	3-Hexano	385	67				64806	662.441	-5.52	C38 H58 I	-8.33	70.44	-5.52	662.441	C38 H58 I	-8.33	64.43		663.453	3	F_MSMS
IAS_AMA	Negative			39728	S-Acetylat	68	21	HMDB111	C16365		61973	226.07	-1.85	C8 H10 N4	-8.18	86.45	-1.85	226.07	C8 H10 N4	-8.18	75.72		203.083	3	F_MSMS
IAS_AMA	Positive		67-71-0	5675	Dimethyl	425	1	HMDB04	C11142		7236	94.0089	-0.7	C2 H6 O2	-7.42	66.9			C2 H6 O2 S		0	109982	72.9371	3	F_MSMS
IAS_AMA	Positive			46872	3-Buten-1	32	3		C12244		69390	71.0735	-0.52	C4 H9 N	-7.31	85.61	-0.52	71.0735	C4 H9 N	-7.31	88.06		55.0548	3	F_MSMS
IAS_AMA	Negative		37620-29	50518	N'-Nitrosc	159	10		C19477		73171	191.106	-1.39	C10 H13 I	-7.27	84.2	-1.39	191.106	C10 H13 I	-7.27	71.17		236.104	3	F_MSMS
IAS_AMA	Negative			46747	2-Morpho	101	14				496234	207.137	-1.39	C11 H17 I	-6.73	87.56	-1.39	207.137	C11 H17 I	-6.73	76.07		252.137	3	F_MSMS
IAS_AMA	Negative			39630	L-Ascorbi	168	1				386604	390.011	-2.4	C12 H14 (	-6.17	0			C12 H14 Ca O12		0		68.9955	3	F_MSMS
IAS_AMA	Negative			19921	Flexixanth	82	56			LMPR010	41360	582.407	-3.59	C40 H54 (	-8.16	73.18	-3.59	582.407	C40 H54 (	-8.16	60.26		61.9889	3	F_MSMS
IAS_AMA	Positive		123-08-0	40202	4-Hydroxy	58	10	HMDB11	C00633		62451	122.037	-0.74	C7 H6 O2	-6.04	44.16	-0.74	122.037	C7 H6 O2	-6.03	78.45	31132	84.9545	3	F_MSMS
IAS_AMA	Negative		2481-70-1	2170	Dihydroisa	136	19				2262	270.137	-1.6	C16 H18 I	-5.91	86.14	-1.6	270.137	C16 H18 I	-5.91	69.59		62.9644	3	F_MSMS
IAS_AMA	Positive		331771-2	23918	ZM-44743	348	61				45566	513.238	-3	C29 H31 I	-5.85	70.12	-3	513.238	C29 H31 I	-5.85	70.64		119.086	3	F_MSMS
IAS_AMA	Positive		14209-33	74862	Spermic a	233	23					232.142	-1.35	C10 H20 I	-5.82	78.58	-1.35	232.142	C10 H20 I	-5.82	68.73	21784	250.178	3	F_MSMS
IAS_AMA	Negative		299-75-2	529	Treosulfar	116	11		C19557		589	278.013	-1.52	C6 H14 O	-5.45	62.46	-1.52	278.013	C6 H14 O	-5.45	45.38		325.183	3	F_MSMS
IAS_AMA	Positive		18672-87	48688	Ethephon	422	4		C18399		72208	143.974	-0.72	C2 H6 Cl (	-5.03	90.39			C3 H6 Cl2 O2				103.955	3	F_MSMS
IAS_AMA	Negative			25817	N-[4-(6-c	172	12				270232	366.008	-1.7	C15 H11 (	-4.63	62.81			C15 H11 Cl N2 O5 S		0		328.982	3	F_MSMS
IAS_AMA	Positive			13585	Asp Phe Tr	258	75				23550	466.185	-2.1	C24 H26 I	-4.5	87.4	-2.1	466.185	C24 H26 I	-4.5	83.29		467.194	3	F_MSMS
IAS_AMA	Positive			11070	Lys Lys Se	147	27				21035	361.233	-1.6	C15 H31 I	-4.42	85.02	-1.6	361.233	C15 H31 I	-4.42	71.92		362.242	3	F_MSMS
IAS_AMA	Negative			72888	Ethyl 2-Br	230	5			LMFA010	96871	291.86	-1.24	C4 H6 Br I	-4.24	57.19			C7 O5 S4				146.938	3	F_MSMS
IAS_AMA	Negative			37055	2(3H)-Ber	97	1				354304	181.997	-0.75	C7 H6 N2	-4.11	0			C7 H6 N2 S2		0		68.9957	3	F_MSMS
IAS_AMA	Positive		3031-89-E	43339	Acetylage	394	10		C02131		65718	172.132	-0.69	C7 H18 N+	-4	89.3	-0.69	172.132	C7 H18 N+	-4	83.86		133.085	3	F_MSMS
IAS_AMA	Positive			78978	(?)-2-Met	43	5					103.046	-0.4	C4 H9 N S	-3.91	97.44	-0.4	103.046	C4 H9 N S	-3.91	95.65	95182	61.0111	3	F_MSMS
IAS_AMA	Positive			25373	5-Sulfanyl	515	4				267950	161.966	-0.63	C3 H2 N2	-3.88	66.86			C5 H3 Cl O2 S				97.9686	3	F_MSMS
IAS_AMA	Positive			49469	S-(Hydrox	28	91		C18235		72081	516.163	-2	C18 H32 I	-3.88	82.24	-2	516.163	C18 H32 I	-3.88	88.27		98.9754	3	F_MSMS

Source: (We cannot copy all data to this appendix; you can observe at my drive.) https://drive.google.com/drive/folders/1h6_fbcaW_L5lfC3R_abw3DjBh9kDrZpl?usp=drive_link

Note:

Sample*: Name of samples

ION: Change or Mode of ionization techniques (protonated and non-protonated analytes)

AGILENTID, CAS, Compound ID: ID of chemical compounds

Name: Tentative compound names

Cpd: Compound number can be found

Hits: Number of possible compounds in one formula

HMP, KEGG, LMP, METLIN: Mass database including Human Metabolism, Kyoto Encyclopedia of Genes and Genomes, Lipid Map, and Metlin database ID

X (DB): X data reference from database

Y (MFG): Y data generated from machine or LC-QTOF-MS/MS instrument

Abund: Abundance under the peak of LC instruments

Base Peak: m/z of most abundance on, arbitrarily assigned a relative abundance of 100%

MS/MS Count: Count of MS to be used

End: Point of retention time to stop MS/MS analysis

Predicted_formula**: Chemical formular from LC-QTOF-MS/MS can be predicted (**We use this data to merge with KEGG profiles).

Height**: Height of precursor peak (**We use this parameter to be Detector Intensity)

After this column is summarized of LC-QTOF-MS/MS analysis in the other profiles and Lib/DB: Library fragmentation pattern data in the instrument can be predicted by compound and Score (Lib) = % of fit the library fragmentation pattern data

APPENDIX B

AMAS PROGRAM VERSION 1.2 INFORMATION

1. Constructed Data Analytics Program Using Python Programming

This part of the study aimed to 1) construct a program helping to analyze complex data from different sources and 2) evaluate the program's usefulness by utilizing the data resources from metabolite profiling of amazake-related products. To achieve the objectives, a program was constructed by coding using the Python language together with its related libraries. Later, data resources collected by mass spectrometry, public bioinformatic databases, and selected specific databases were employed on the program. Finally, data visualization related to the analyzed results was demonstrated. After the program is developed, this program will be tested by elemental data in Table B1. The table will assume three samples, including A, B, and C, and inside will consist of a list of atom names and their quantities. Samples A and B have the same list of atom names. Only sample C has a different list of atom names. The amount detected will be a random 1-100; only sample B has 3 atoms, setting the amount detected to a random 1-20. That is, we set sample B to have a lower amount than the other samples and set sample C to have a different type of atoms than the other samples. To test the program is working and reports the results as we set. Additionally, in other tests of the program's merging function, we have produced a list of atoms complete names together with their abbreviations as atom names.

1.1 AMAS (version 1.2) program

The constructed program in this research was called AMAS version 1.2. The program interface was written for data analytics using Python code and libraries. The interface is shown in Figure B1. By using the Streamlit package for a web-based interface, the left sidebar is used to input data files (.csv) that include analytic models, variable columns, and functions to be analyzed. The main interface on the right side is for some choice selections and progression display. This program could be compatible with all browsers, including Firefox, Chrome, and Microsoft Edge.

Table B1 Elemental Data, The Example Data for Calibration Analytic Program

Sample	Atom	Atomic weight	Amount detected
A	Sc	44.956	35.97557
A	Ti	47.967	90.46646
A	V	50.942	97.33848
A	Cr	51.996	47.30301
A	Mn	54.338	95.45414
A	Fe	55.845	32.12786
B	Sc	44.956	61.49639
B	Ti	47.967	68.50199
B	V	50.942	16.5804
B	Cr	51.996	12.61238
B	Mn	54.338	14.32755
B	Fe	55.845	81.05887
C	Sc	44.956	95.73021
C	Ti	47.967	22.4295
C	H	1.008	39.13212
C	N	14.007	76.02734
C	O	15.999	83.60874

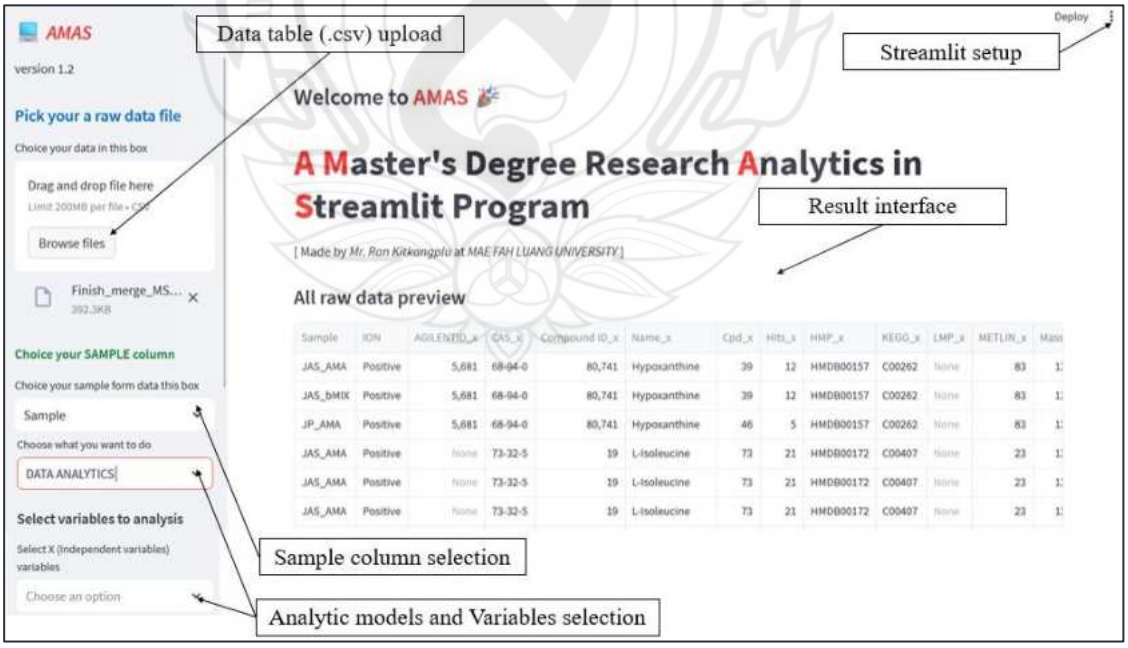


Figure B1 Interface of AMAS Program Version 1.2 (16/07/2024)

This program contained two main functions: “Data Analytics” and “Data Merge.” For the “Data Analytics” function, there are 7 functions that can be chosen, including 1) histogram, 2) scatter plot, 3) heatmap, 4) cluster heatmap, 5) correlation table analysis (such as Pearson, Kendall, or Spearman correlation), 6) Kruskal-Wallis H test (F value or P value), and 7) Venn diagram. Not all functionalities, however, may be employed in this study. This AMAS program was not for publication but for certain purposes such as verification data and visualization data (Figure B2).

For the “Data Merge” function, the primary data set could be LC-MS or another MS data set (containing a key column), and the secondary data could be other data (a .csv file) from the database containing the same keyed column (predicted formula). The new (merged) dataset must be downloaded and continued for the “Data Analytics” function.

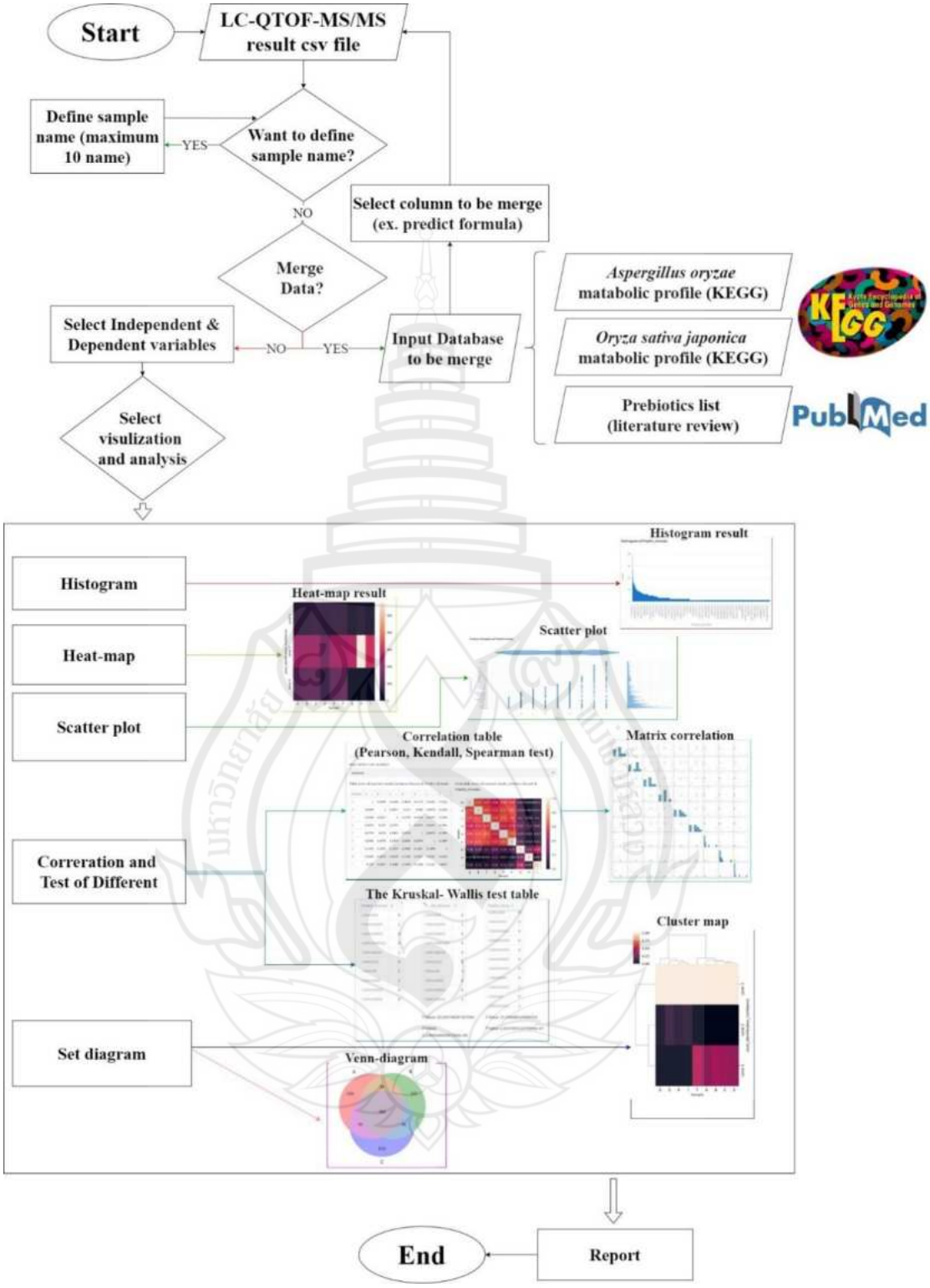


Figure B2 AMAS Version 1.2 (30/07/2024) Workflow Application

1.2 AMAS (version 1.2) program validation

1.2.1 Data file or MS result file upload section: this program uses comma-separated value file (.csv) data for analytics. Therefore, the Pandas package was used to read and manage the input data on the sidebar. The program was set to only upload data in .csv format to prevent errors after any changes were made to the primary data from the Excel result table file. The code used and the table display are as shown in Figure B3. By using an *elemental data* example from Table B1, the program can successfully upload a .csv file to the analytical program.

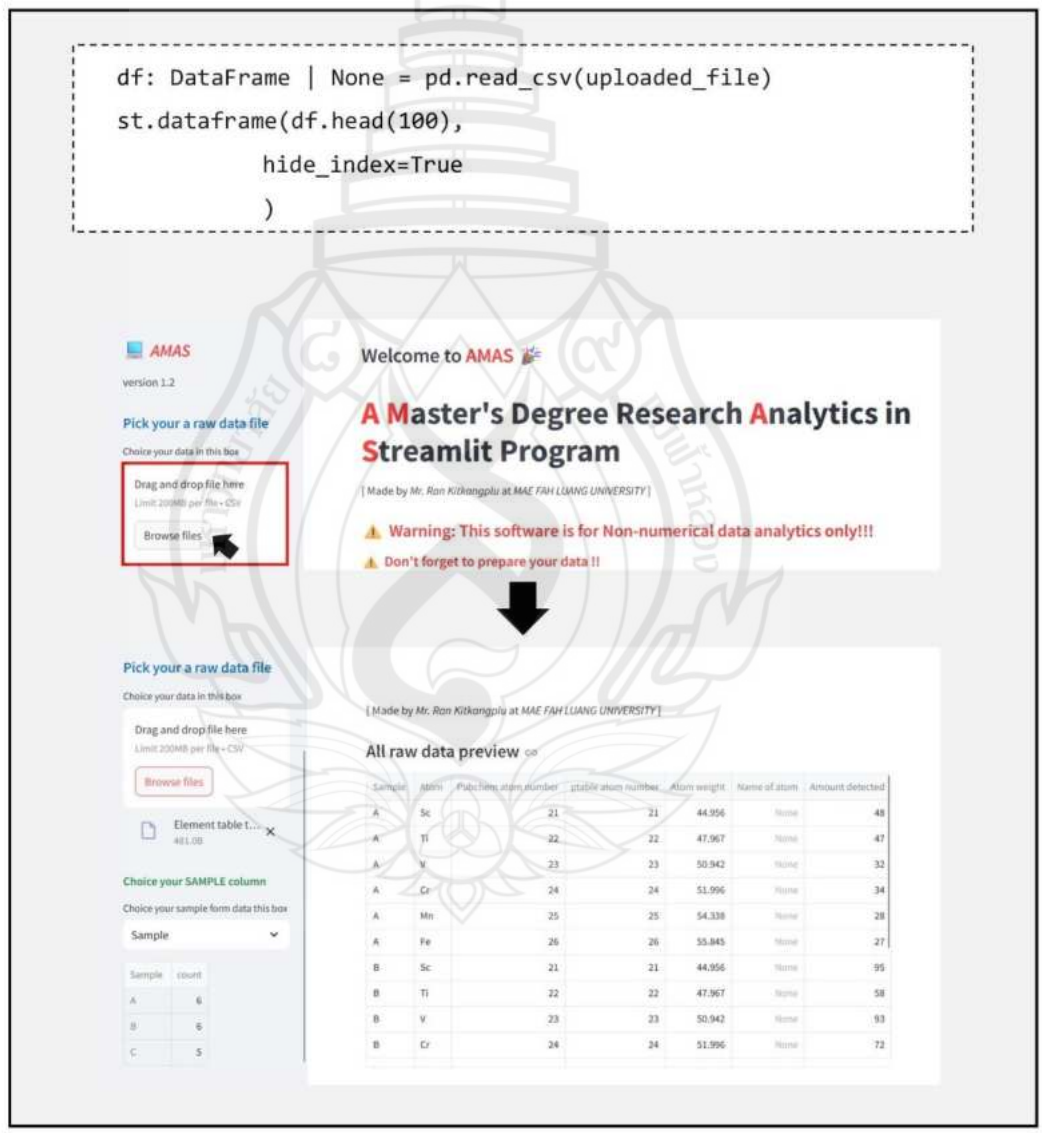


Figure B3 Key Part of Code and Interface of Data After Input Data

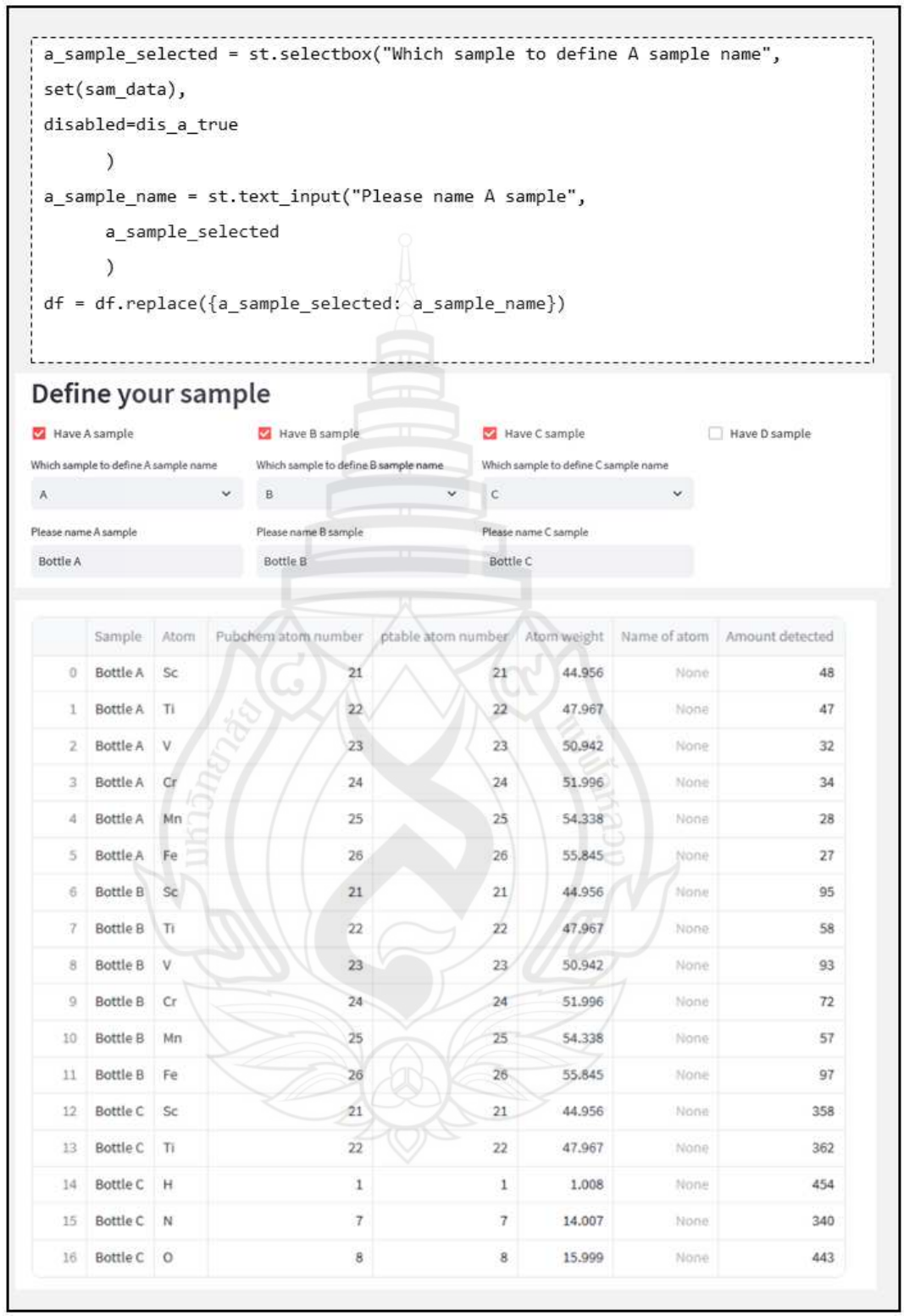
1.2.2 Named and renaming of data samples section: sometimes the old sample names were required to be edited for better comparison. Therefore, the "*Define your sample*" section was set up with the Streamlit package to provide the name changing over the old name, and there would show the overwritten name samples. The code used and the table display are as shown in Figure B4. In the *Elemental Data* example, the program could change the names of "A," "B," and "C" samples to be "Bottle A," "Bottle B," and "Bottle C" successfully.

1.2.3 Selected variables section: to filter the variables being used for analytics, the program therefore can select more than one variable in the left sidebar of the program. Linked to the analytics part, the chosen variables would be shown on the main screen for other variables setting to analysis. The result could show in Figure B5 that the *elemental data* example variables were displayed for variable selection on the sidebar and could be chosen from the set of variables for the next analytics on *variable setting*.

1.2.4 Histogram plot section: there are many types of histograms that could be displayed, including maximum values, average values, and minimum values from any X variables and any Y variables selected from the *Variables setting*. The Plotly.express package could create a histogram figure, for example, averaging data between the *Atom* and *Amount detected* variables in each sample. The packages could plot displays as shown in Figure B6. In this *elemental data* example, the program could represent the average of the *amount detected* by each sample, separated by the *atom* variable.

1.2.5 Scatter plot section: to examine the distribution of the data from the scalar variables, a scatter plot could be employed for the analytics. By using the X-Y option from the histogram in Topic 4.1.2.4, the additional Z-axis dimension could be expanded for further analytics and displayed in the form of additional dot sizes. The program used the Plotly Express package to create a scatter plot. For example, a scatter plot between the *PubChem atom number* and the amount *detected* variable and adding the size of the dot of the *atom weight* data can plot displayed in Figure B7. The *Elemental Data* example program could display the size of the dot arranged to be similar to the data of the *Atom weight* as we set.

```
a_sample_selected = st.selectbox("Which sample to define A sample name",
set(sam_data),
disabled=dis_a_true
      )
a_sample_name = st.text_input("Please name A sample",
      a_sample_selected
      )
df = df.replace({a_sample_selected: a_sample_name})
```



	Sample	Atom	Pubchem atom number	ptable atom number	Atom weight	Name of atom	Amount detected
0	Bottle A	Sc	21	21	44.956	None	48
1	Bottle A	Ti	22	22	47.967	None	47
2	Bottle A	V	23	23	50.942	None	32
3	Bottle A	Cr	24	24	51.996	None	34
4	Bottle A	Mn	25	25	54.338	None	28
5	Bottle A	Fe	26	26	55.845	None	27
6	Bottle B	Sc	21	21	44.956	None	95
7	Bottle B	Ti	22	22	47.967	None	58
8	Bottle B	V	23	23	50.942	None	93
9	Bottle B	Cr	24	24	51.996	None	72
10	Bottle B	Mn	25	25	54.338	None	57
11	Bottle B	Fe	26	26	55.845	None	97
12	Bottle C	Sc	21	21	44.956	None	358
13	Bottle C	Ti	22	22	47.967	None	362
14	Bottle C	H	1	1	1.008	None	454
15	Bottle C	N	7	7	14.007	None	340
16	Bottle C	O	8	8	15.999	None	443

Figure B4 Key Part of Code and Interface of Data After Define Name

```
x_selected_vars = st.sidebar.multiselect("Select X (Independent variables) variables",
                                         variables
                                         )
x_data = pd.DataFrame(df,
                      columns=x_selected_vars
                      )
option_xv = st.selectbox("Select your X variable to show in Histogram",
                         x_selected_vars
                         )
st.write('X variables selected:',
         option_xv
         )
st.dataframe(x_data)
```

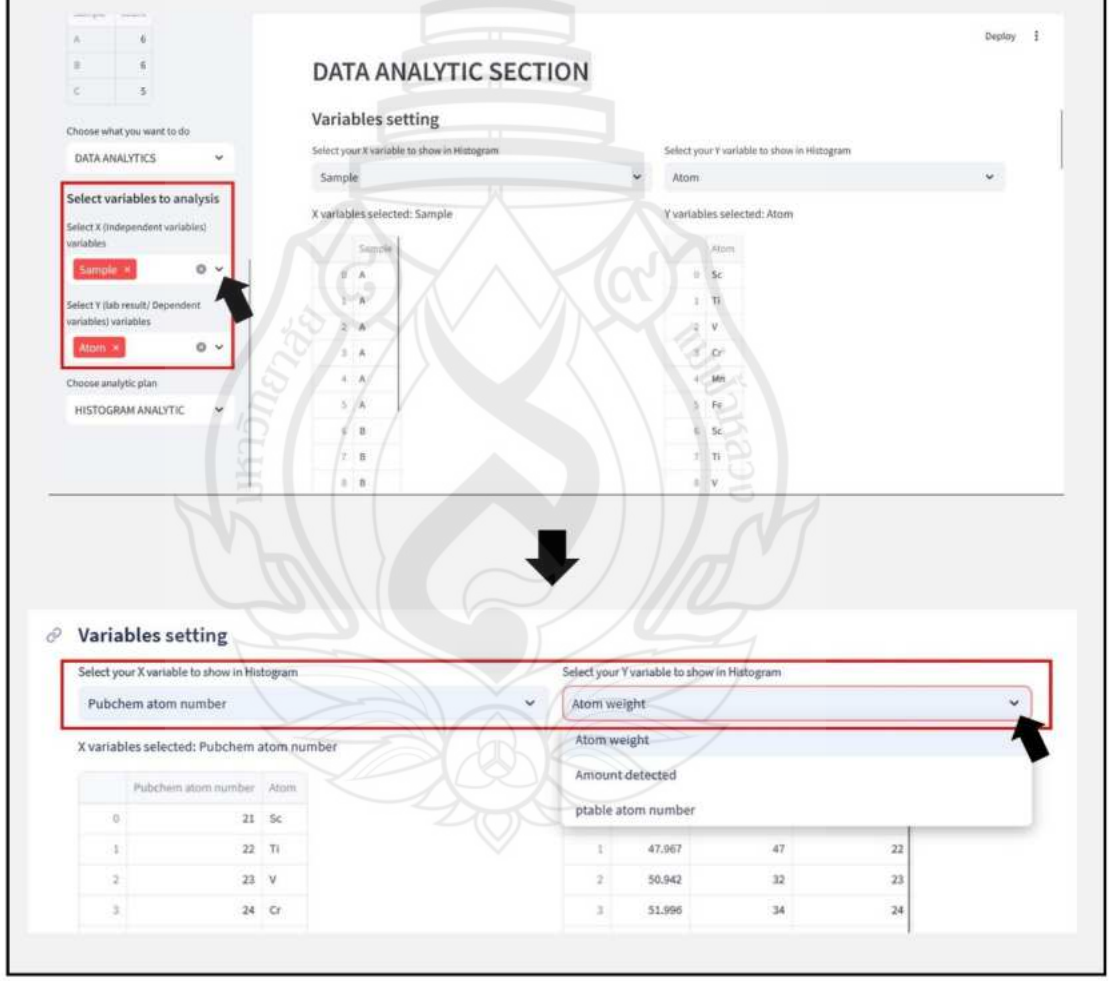


Figure B5 Key Part of Code and Interface of Select Variable to be Analytics

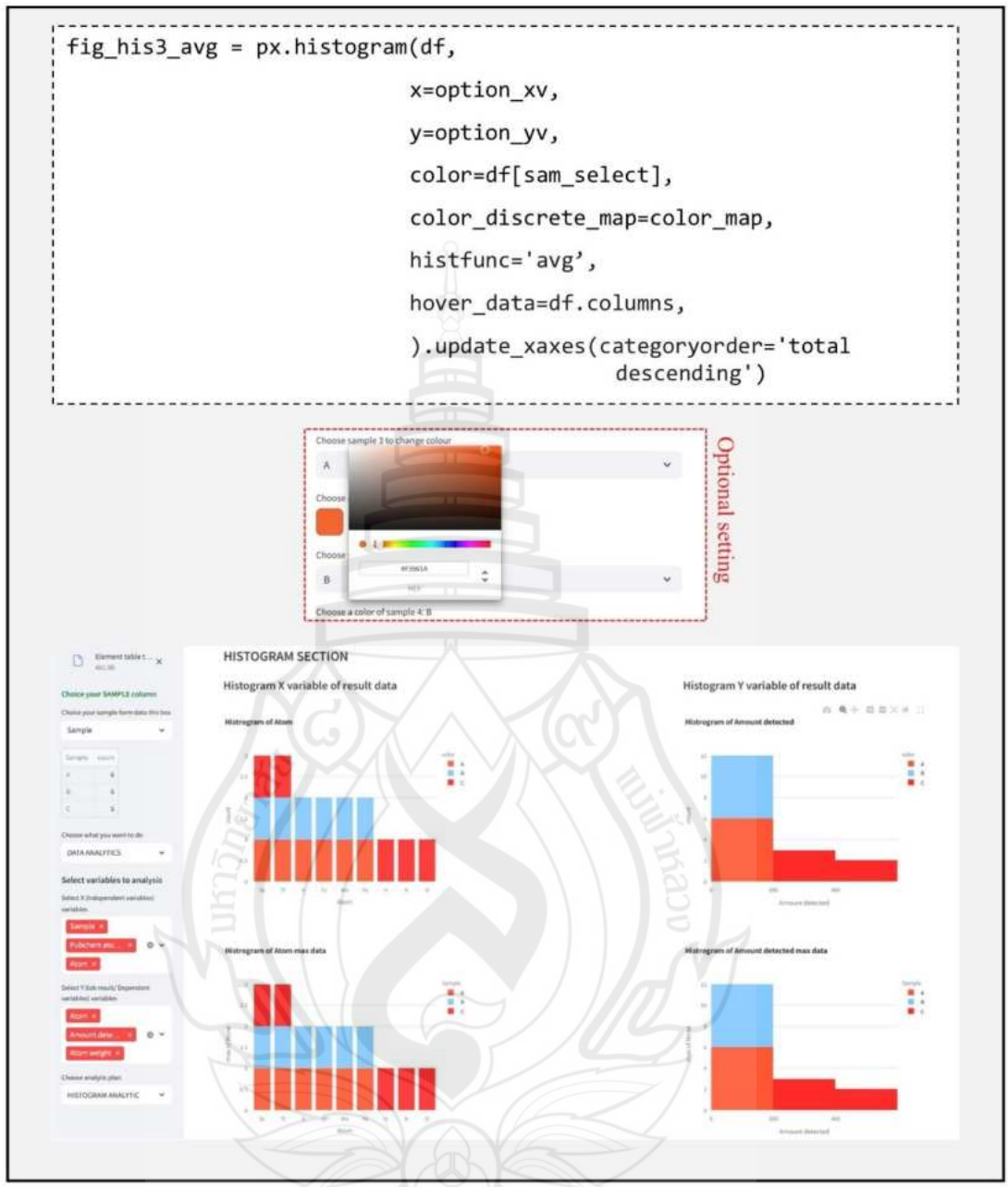


Figure B6 Key Part of Code and Interface of Histogram Amount Detected and Atom Data.

```
fig_his3_scatterz = px.scatter(df,
                               x=option_xv,
                               y=option_yv,
                               size=option_zv,
                               color=df[sam_select],
                               symbol=df[sam_select],
                               color_discrete_map=color_map,
                               marginal_x="rug",
                               marginal_y="box",
                               )
```

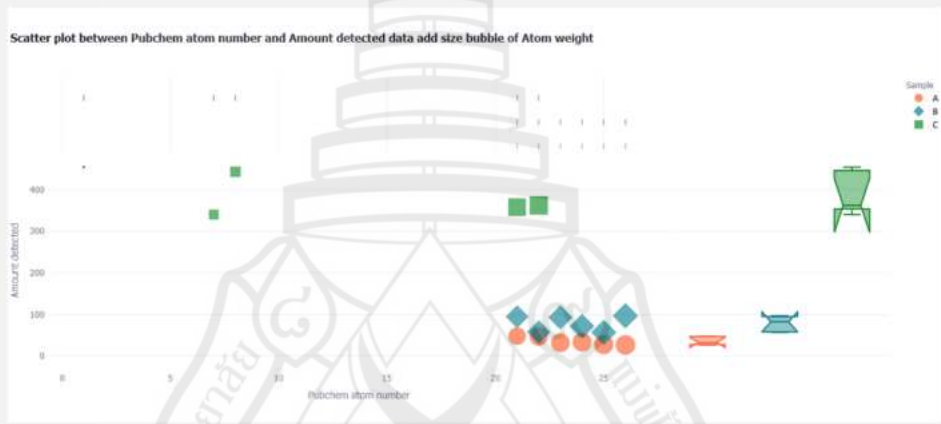


Figure B7 Key Part of Code and Interface of Scatter Plot Between Pubchem Atom Number and Amount Detected and Add Size of Dot Atom Weight Data

1.2.6 Correlation section: correlation was used for scalar-type data variable analysis. The program could automatically convert each analyzed variable to Z-score data and summarize it as a simple overview with either a selectable "Pearson," "Kendall," or "Spearman" correlation plot and a more intuitive heatmap plot. Given an example of a comparison between a sample and the *atom weight* data with a Pearson score, the result is shown in Figure B8. The *Elemental Data* example program could identically display the Pearson score from samples of mocked-up Bottle A and Bottle B (Pearson coefficient = 1.00) as expected because we entered the same atom weight data in the Bottle A and Bottle B samples. Only Bottle C differed in fewer ways than those two samples (Pearson coefficient = -0.63). The result showed success, as we expected.

1.2.7 Heatmap and clustermap section: heatmap could be employed when multivariable were selected for visualization for data frequency (counts) distribution or correlation of the data, while clustermap had included cluster analysis for variable or sample grouping. In the program, we chose to use the Seaborn package to create a cluster map. Frequently in this *Elemental Data* example, the program can clearly represent correlations between Bottle A and Bottle C, which were separated into other groups (Figure B9). Additionally, the program can use this plot to link data correlations to make it easier to display. We let the program display the original variables set in Topic 4.1.2.6. The results were shown in Figure B8.

1.2.8 Venn-diagram section: the Venn diagram was useful when differentiation of samples was required based on a variable with a count value. In this program, 2- or 3-sample differentiation was applicable. It was for divided data groups to display clear images, and the group data contained in each set was downloaded for further analytics. The program could select samples that will use the set of variable values to plot the Venn diagram. For example, we chose to view variables as *Atom* columns for selecting and comparing 3 samples, Bottle A, B, and C. The result had been shown in the two rings (as A and B are identical samples) shown in Figure B10. The Venn diagram circles overlapped in these two samples. Comparing Bottle C to A and B, there are two atoms that are identical while 3 atoms are different. It also represents the correct atoms that are the same as the setting in the elemental data table.

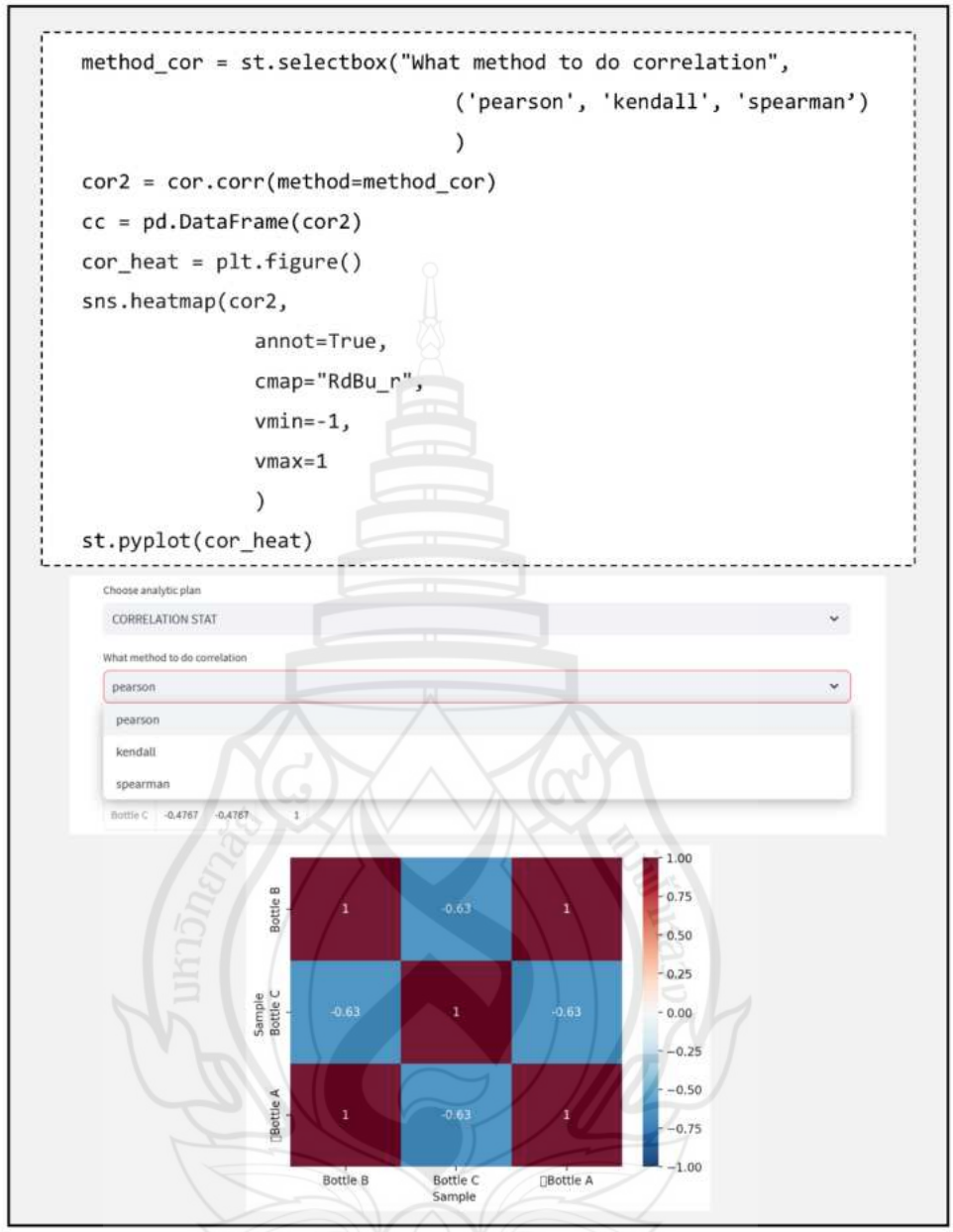


Figure B8 Key Part of Code and Interface of Heatmap between Pubchem Atom Number in each Sample

```
method_cor = st.selectbox("What method to do correlation",
                          ('pearson', 'kendall', 'spearman')
                          )
cor2 = cor.corr(method=method_cor)
cc = pd.DataFrame(cor2)
fig_cor = sns.clustermap(cc,
                         annot=True,
                         row_cluster=True,
                         vmin=-1,
                         vmax=1,
                         center=0,
                         cmap = "RdBu_r"
                         )
st.pyplot(fig_cor)
```

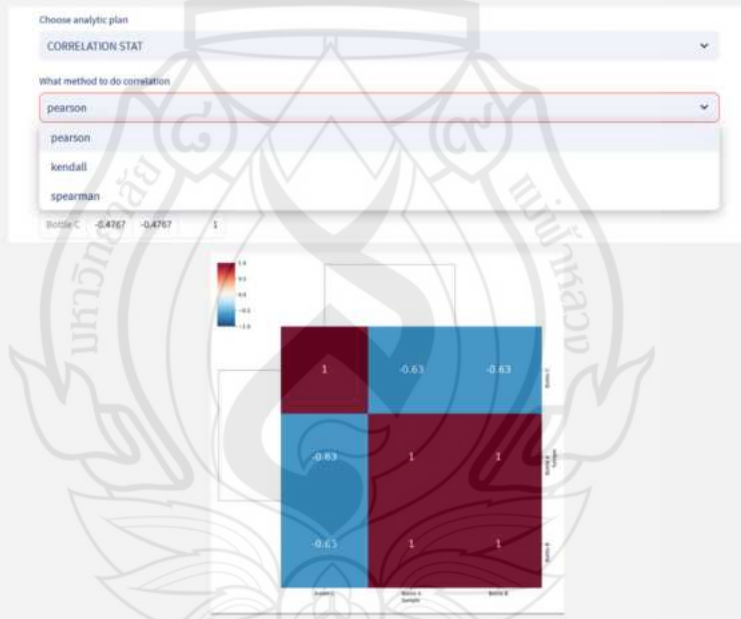


Figure B9 Key Part of Code and Interface of Cluster-Heatmap between Pubchem Atom Number in Each Sample

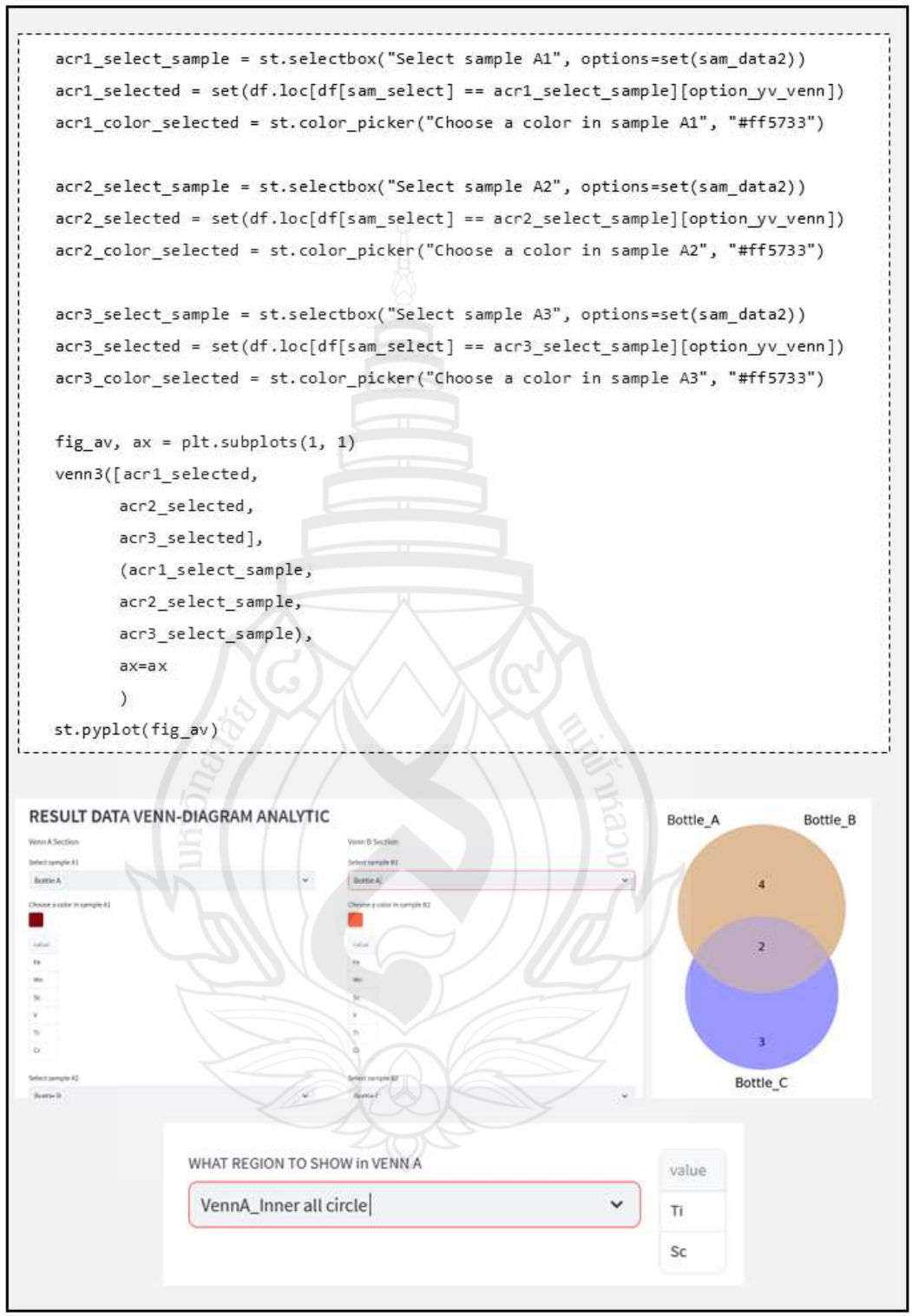


Figure B10 Key Part of Code and Interface of Venn-Diagram Option Interface and Venn-Diagram Result of Atomic in each Sample

1.2.9 Data Merge function: It combines the second table into the first table by selecting the box for overlaying the same columns (key column) of the two tables, expanding other possible predicting substances found in the other/additional database. In this example of *elemental data*, an additional database with the same key column and other columns with full name (*name of atom*) data could merge with the primary database. The results are shown in Figure B11. In this *Elemental Data* example, merge with the *Name of Atom* table correctly, and it can be downloaded for further analysis.

```
choice_how_merge = st.selectbox('HOW TO MERGE THIS DATA', ["inner",
"outer", "left", "right"])
        mdf = pd.merge(df,
                       df2,
                       how=choice_how_merge,
                       on=choice_merge
                       )
```

MERGE RESULT

	+ Sample	Atom	Pubchem atom number	ptable atom number	Atom weight	Name of atom_x	Amount detected	Name of atom_y	Reference
0	A	Sc	21	21	44.956	None	48	Scandium	Periodic table
3	A	Ti	22	22	47.967	None	47	Titanium	Periodic table
6	A	V	23	23	50.942	None	32	Vanadium	Periodic table
8	A	Cr	24	24	51.996	None	34	Chromium	Periodic table
10	A	Mn	25	25	54.338	None	28	Manganese	Periodic table
12	A	Fe	26	26	55.845	None	27	Iron	Periodic table
1	B	Sc	21	21	44.956	None	95	Scandium	Periodic table
4	B	Ti	22	22	47.967	None	58	Titanium	Periodic table
7	B	V	23	23	50.942	None	93	Vanadium	Periodic table
9	B	Cr	24	24	51.996	None	72	Chromium	Periodic table

DOWNLOAD MERGE TABLE

Figure B11 Key Part of Code and Interface of Merge with the Name of Atom Table

APPENDIX C

HPLC CARBOHYDRATE MEASUREMENT INFORMATION

Sample name meaning:

Th-Placebo = JAS_SYR sample 10-fold dilute form extracted sample (10% of extract sample)

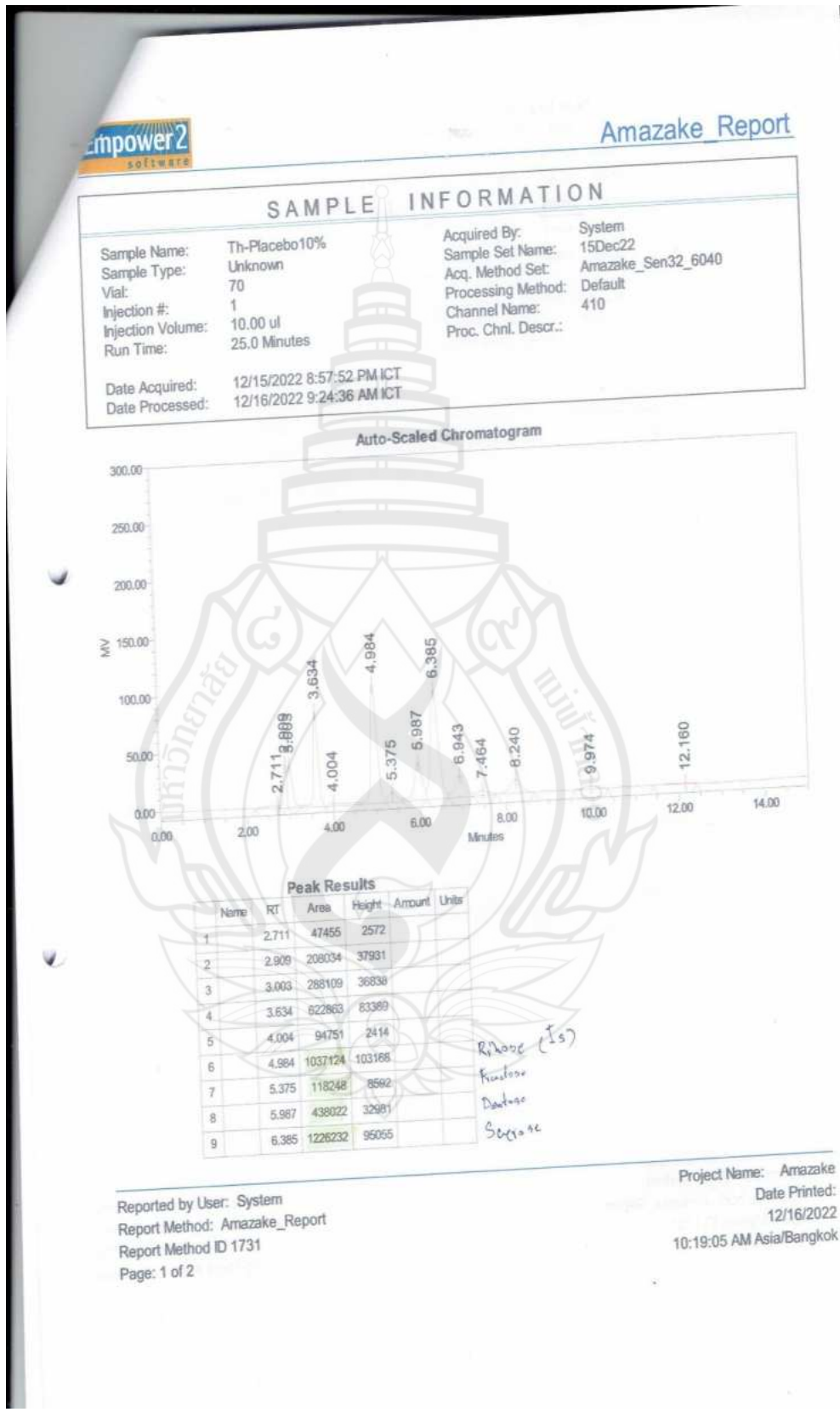
Th-Before = JAS_bMIX sample 10-fold dilute form extracted sample (10% of extract sample)

Th-Product = JAS_AMA sample 10-fold dilute form extracted sample (10% of extract sample)

[Blank] = JP_AMA sample (We accidentally included the product name, so we censored it.) 10-fold dilute form extracted sample (10% of extract sample)

Mix = Mix standard (2-fold dilution 5 times)

Figure C1 HPLC sample result (10-fold dilution)



Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Th-Placebo10%	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	15Dec22
Vial:	70	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 8:57:52 PM ICT		
Date Processed:	12/16/2022 9:24:36 AM ICT		

Auto-Scaled Chromatogram

Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.711	47455	2572		
2		2.909	208034	37931		
3		3.003	288109	36838		
4		3.634	622863	83389		
5		4.004	94751	2414		
6		4.984	1037124	103168		
7		5.375	118248	8592		
8		5.987	438022	32981		
9		6.385	1226232	95055		

Ribose (IS)
Fructose
Dextrose
Sucrose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

Project Name: Amazake
Date Printed:
12/16/2022
10:19:05 AM Asia/Bangkok

Peak Results

	Name	RT	Area	Height	Amount	Units
10		6.943	297160	18343		
11		7.464	92857	4575		
12		8.240	231065	12077		
13		9.974	54761	2709		
14		12.160	18864	662		

Maltose

Maltotriose, tetraose

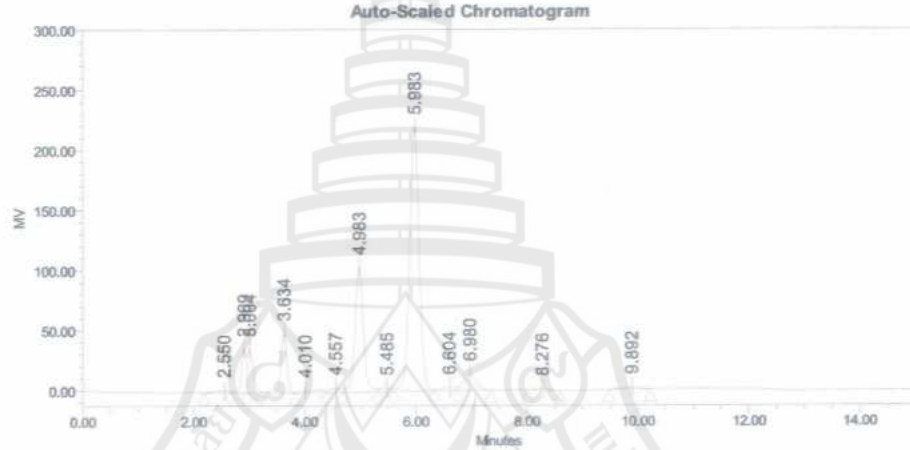
Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Th-Before10%	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	15Dec22
Vial:	71	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 9:24:02 PM ICT		
Date Processed:	12/16/2022 9:25:27 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.550	27376	1658		
2		2.909	237447	36620		
3		3.004	245746	36078		
4		3.634	377153	49179		
5		4.010	55812	1892		
6		4.557	43604	3497		
7		4.983	1069374	103245		
8		5.485	44060	2656		
9		5.983	2624331	219260		

Ribose (ไม่มี)
Fructose
Dextrose

Peak Results

	Name	RT	Area	Height	Amount	Units
10		6.604	48444	2645		
11		6.980	199297	13555		
12		8.276	18204	1140		
13		9.892	37049	1832		

Maltose
maltotriose

Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Th-Product10%	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	15Dec22
Vial:	72	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
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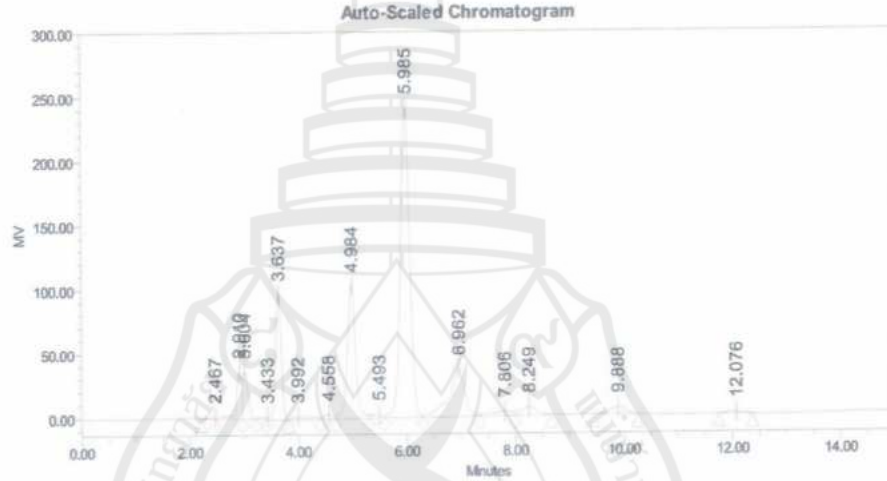
Date Processed:	12/16/2022 9:26:30 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.467	9427	1032		
2		2.910	264707	37267		
3		3.004	251676	38312		
4		3.433	20179	1724		
5		3.637	696957	97885		
6		3.992	55710	1958		
7		4.558	41014	3310		
8		4.984	1088318	103334		
9		5.493	42725	2705		

Ribose (IS)
Fructose

Peak Results

	Name	RT	Area	Height	Amount	Units
10		5.985	2896913	241475		
11		6.962	735668	37001		
12		7.806	89345	3348		
13		8.249	128767	6564		
14		9.888	113146	5565		
15		12.076	24113	1145		

Dextrose
Maltose
maltotriol/Tetraose

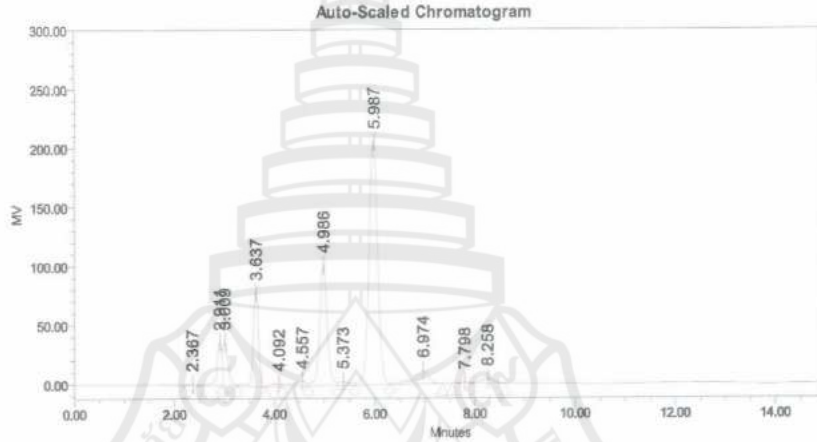
Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Marukome	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	15Dec22
Vial:		Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 11:08:50 PM ICT		
Date Processed:	12/16/2022 9:30:21 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.367	9282	749		
2		2.911	253434	36705		
3		3.009	265969	36829		
4		3.637	589008	79564		
5		4.092	31462	2046		
6		4.557	77797	3678		
7		4.986	1044644	101890		
8		5.373	50544	2905		
9		5.987	2470707	206514		

Ribose (Tg)
Fructose
Dextrose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

Project Name: Amazake
Date Printed:
12/16/2022
10:23:27 AM Asia/Bangkok

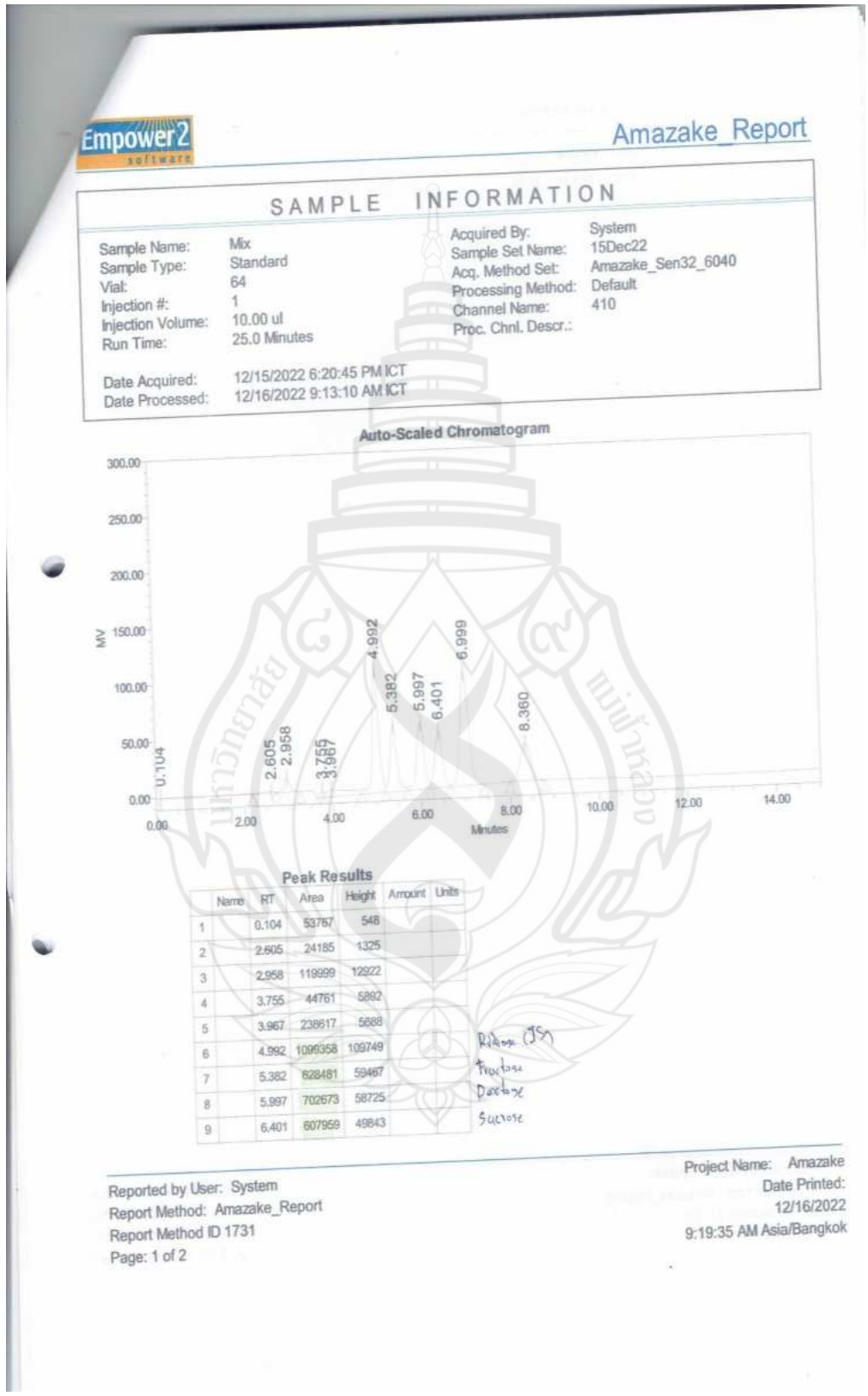
Peak Results

	Name	RT	Area	Height	Amount	Units
10		6.974	212044	11158		
11		7.798	12256	798		
12		8.258	63732	3541		

Maltose

Maltotriose / tetra ose

Figure C2 Mix standard (2-fold dilution)



Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Mix	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	15Dec22
Vial:	64	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 6:20:45 PM ICT		
Date Processed:	12/16/2022 9:13:10 AM ICT		

Auto-Scaled Chromatogram

Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.104	53757	548		
2		2.605	24185	1325		
3		2.958	119999	12922		
4		3.755	44761	5892		
5		3.967	238617	5688		
6		4.992	1099358	109749		
7		5.382	628481	59467		
8		5.997	702673	58725		
9		6.401	607959	49843		

Ribose (IS)
Fructose
Dextrose
Sucrose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

Project Name: Amazake
Date Printed: 12/16/2022
9:19:35 AM Asia/Bangkok

Peak Results

	Name	RT	Area	Height	Amount	Units
10		6.999	1360996	101593		
11		8.360	530624	33193		

maltose
maltotriose / tetraose

Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Mix/2	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	15Dec22
Vial:	65	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
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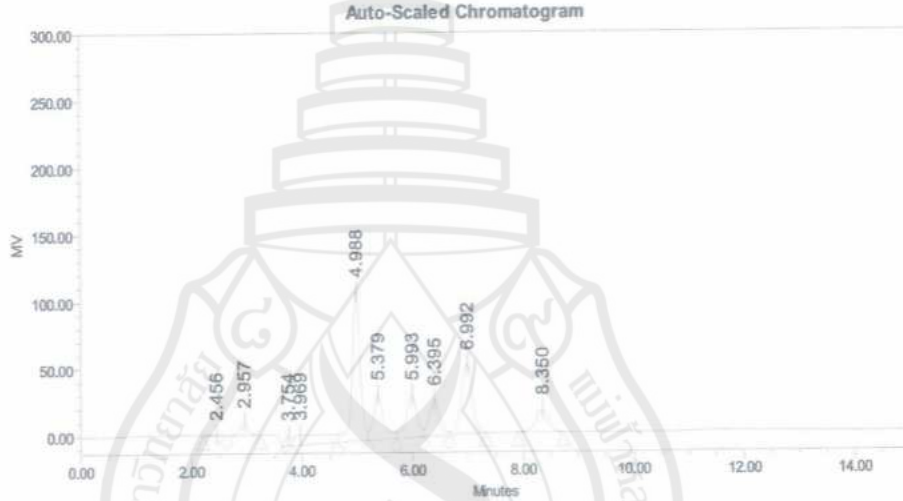
Date Processed:	12/16/2022 9:21:00 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units	
1		2.456	9246	594			
2		2.957	87217	9464			
3		3.754	59194	7654			
4		3.989	331590	7541			
5		4.988	1168764	112102			Ribose (IS)
6		5.379	428631	33316			Fructose
7		5.993	425254	31751			Dextose
8		6.395	350186	26708			Sucrose
9		6.992	705178	51751			Maltose

Peak Results

	Name	RT	Area	Height	Amount	Units
10		8.350	268540	16699		

maltotriose

Empower2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Mix/4	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	15Dec22
Vial:	66	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 7:13:08 PM ICT		
Date Processed:	12/16/2022 9:21:47 AM ICT		

Auto-Scaled Chromatogram

Peak Results

	Name	RT	Area	Height	Amount	Units	
1		2.375	8182	522			
2		2.961	72708	7414			
3		3.759	55807	7355			
4		3.988	323497	7533			
5		4.991	1125877	106756			Ribose (IS)
6		5.383	257733	18560			Fructose
7		5.997	250249	16853			Dextrose
8		6.400	189889	13882			Sucrose
9		6.998	352468	25821			Maltose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

Project Name: Amazake
Date Printed:
12/16/2022
9:48:06 AM Asia/Bangkok

Peak Results

	Name	RT	Area	Height	Amount	Units
10		8.356	130114	8229		

Maltotriose / tetraose

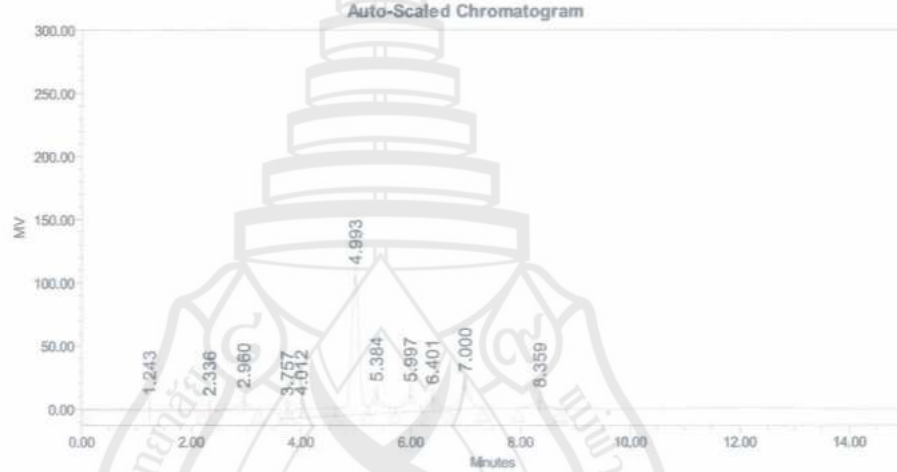
Empower2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Mix/6	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	15Dec22
Vial:	67	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 7:39:20 PM ICT		
Date Processed:	12/16/2022 9:22:26 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.243	80403	844		
2		2.336	3751	410		
3		2.960	89555	6561		
4		3.757	53586	7008		
5		4.012	316637	7156		
6		4.993	1158879	109039		
7		5.384	231888	14583		
8		5.997	195300	12687		
9		6.401	148881	10176		

Ribose (IS)
Fructose
Dextrose
Sucrose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

Project Name: Amazake
Date Printed:
12/16/2022
9:49:06 AM Asia/Bangkok

Peak Results

	Name	RT	Area	Height	Amount	Units
10		7.000	254064	18107		
11		8.359	92594	5750		

Maltose

Maltotriose / Isomaltose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 2 of 2

Project Name: Amazake
Date Printed:
12/16/2022
9:49:06 AM Asia/Bangkok

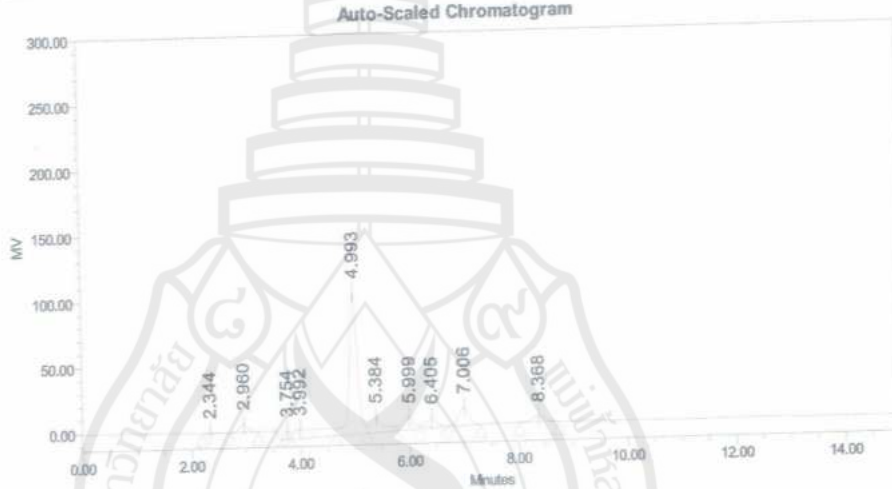
Empower 2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Mix/8	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	15Dec22
Vial:	68	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 8:05:31 PM ICT		
Date Processed:	12/16/2022 9:22:50 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.344	6039	400		
2		2.960	59102	6216		
3		3.754	49935	6490		
4		3.992	293721	6579		
5		4.993	1133592	106776		
6		5.384	193476	11452		
7		5.999	142666	9552		
8		6.405	111084	7583		
9		7.006	183668	13365		

Ribose (IS)
Fructose
Dextose
Sucrose
Maltose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

Project Name: Amazake
Date Printed:
12/16/2022
9:50:25 AM Asia/Bangkok

Peak Results

	Name	RT	Area	Height	Amount	Units
10		8.368	69534	4349		

Maltotriol / tetraose

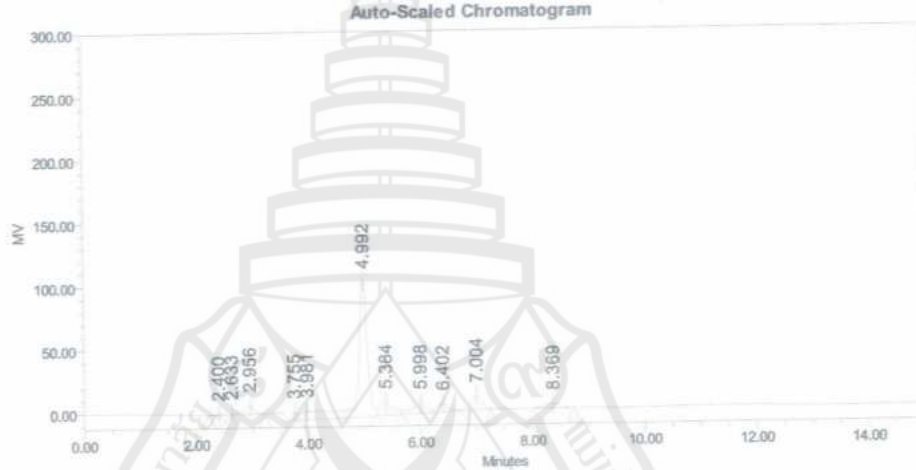
Empower2 software

Amazake_Report

SAMPLE INFORMATION

Sample Name:	Mix/10	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	15Dec22
Vial:	69	Acq. Method Set:	Amazake_Sen32_6040
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	410
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	
Date Acquired:	12/15/2022 8:31:41 PM ICT		
Date Processed:	12/16/2022 9:23:35 AM ICT		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.400	2444	393		
2		2.633	9748	924		
3		2.956	77442	6779		
4		3.755	48414	6295		
5		3.981	281656	6521		
6		4.992	1115562	106844		
7		5.384	158430	10017		
8		5.998	148441	8206		
9		6.402	95125	6412		

Ribose
Fructose
Dext-sc
Sucrose

Reported by User: System
Report Method: Amazake_Report
Report Method ID 1731
Page: 1 of 2

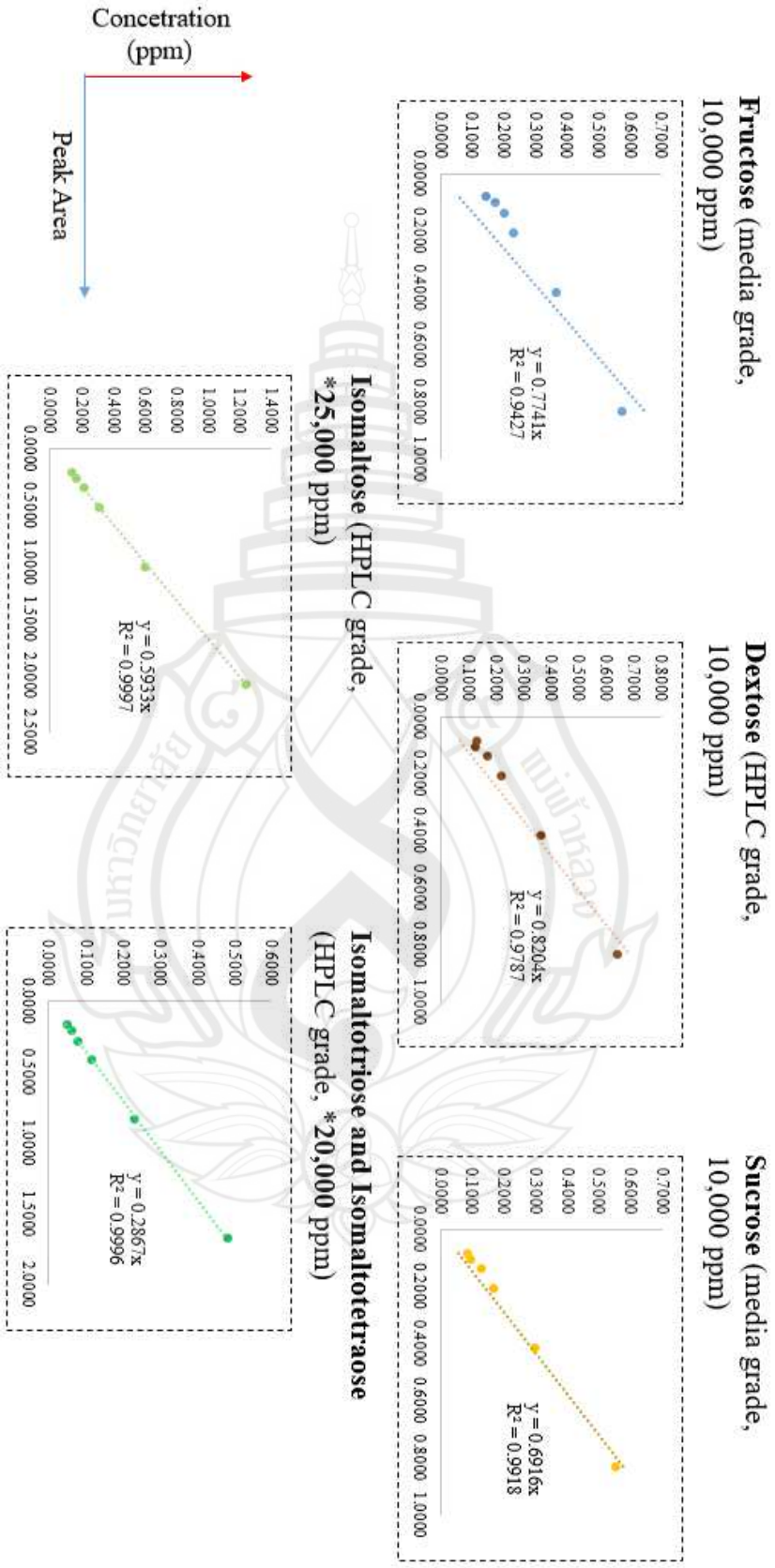
Project Name: Amazake
Date Printed:
12/16/2022
9:51:07 AM Asia/Bangkok

Peak Results

	Name	RT	Area	Height	Amount	Units
10		7.004	149766	10870		
11		8.369	57317	3540		

Maltose
Maltotriose/Tetraose

Figure C3 The component standard curve (for calculated the quantitative)



APPENDIX D

IN VITRO RESULT INFORMATION

1. Growth Curves of Selected Bacteria

Before starting the prebiotics effectiveness test. The growth characteristics of all three bacteria were observed after pre-experimental fermentation in MRS media first. To estimate the mid-period of the same log phase (in this experiment, it is a rough experiment; no estimate of the injected organism, such as McFarland standards, was made before starting this step). For the prebiotics effectiveness test, the inoculum is at equal times and in equal quantities to reduce confounding variables.

The results of the experiment revealed three things (represented in Figure D1). First, the results shown can estimate the log phase to be 10-11 hours. Second, 24 hours is not enough to observe the death phase of the candidate microbe. Finally, the temperature of the shaking incubator is uncontrollable; sometimes the temperature varies more than 37°C around 4-5°C, but not every time because this shaking incubator is an old instrument.

That was necessary to modify the experimental plan as a result. First, the observation period was changed to 48 hours. Because of the heating-up temperature, observation should be more intense in the period between 8 and 24 hours. Second, there will be no comparison of time and amount of candidate microbes grown. But it will be a comparison of individual microbes to observe if they grow better in broth with prebiotics than with normal MRS broth (glucose source) or wMRS broth (MRS without carbohydrate source). In order to prevent the result of the experiment from being impacted by the instrument's temperature control issue.

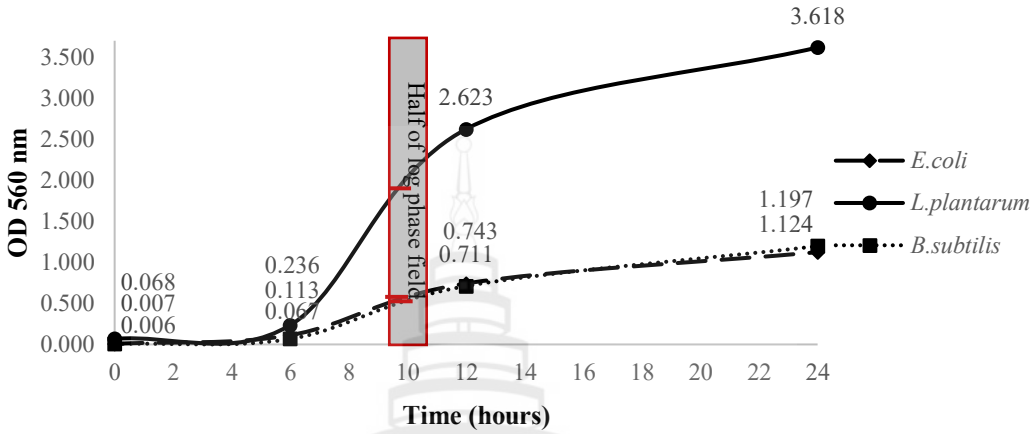


Figure D1 Result of Pre-Experimental Fermented Three Candidate Microbes Growth with MRS Broth to Find the Half of Log Phases

2. Standardization of Sugar Contents Among Testing Media

The preparation of Amazake mixed with MRS nutrition samples is unique in that the liquid sample is utilized for mixing. Therefore, to make the amount of sugar added to the MRS nutrient the same as the other samples at 20 grams. For the Amazake extract amount of carbohydrate approximation, we used the phenol sulfuric technique and measured reaction color with absorbance index at wavelength 490 nm. To make a standard curve of glucose dilution and calibrate the carbohydrate source group to have an amount of carbohydrate approximately the same as 20 grams of glucose at Figure D2

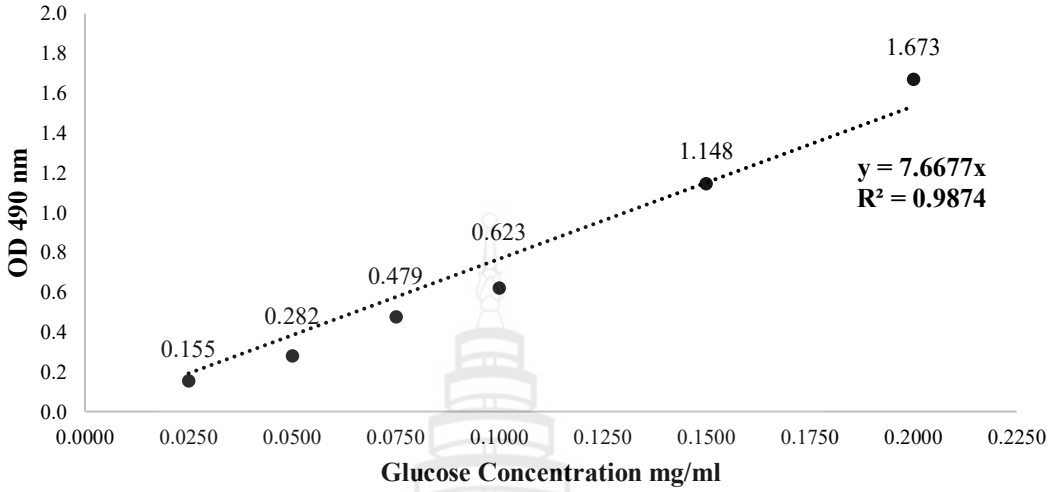


Figure D2 Calibration Curve of Standard Glucose Ranging 0.025-0.2 mg/ml by Phenol Sulfuric Method

Table D1 First time prebiotics effectiveness test *B. subtilis* and *L. plantarum* (14/05/2024)

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
BS_GLU	0	7:30:00 (14/5/67)	nan	0.009	nan	nan	1	0.0090	nan	nan
BS_GLU	6	13:30:00 (14/5/67)	nan	0.014	0.01	0.025	5	0.0700	0.0500	0.1250
BS_GLU	9	16:30:00 (14/5/67)	0.008	0.014	0.02	0.041	5	0.0700	0.1000	0.2050
BS_GLU	12	19:35:00 (14/5/67)	0.034	0.023	0.024	0.044	5	0.1150	0.1200	0.2200
BS_GLU	15	22:30:00 (14/5/67)	0.001	0.038	0.034	0.056	5	0.1900	0.1700	0.2800
BS_GLU	18	1:30:00 (15/5/67)	0.005	0.134	0.158	0.206	5	0.6700	0.7900	1.0300
BS_GLU	24	7:35:00 (15/5/67)	0.003	0.303	0.297	0.397	5	1.5150	1.4850	1.9850
BS_GLU	36	19:35:00 (15/5/67)	0.011	0.308	0.282	0.448	5	1.5400	1.4100	2.2400
BS_GLU	48	7:40:00 (16/5/67)	0.008	0.255	0.244	0.389	5	1.2750	1.2200	1.9450
BS_JA_AMA	0	7:30:00 (14/5/67)	nan	0.038	nan	nan	1	0.0380	nan	nan
BS_JA_AMA	6	13:30:00 (14/5/67)	nan	0.007	0.008	0	5	0.0350	0.0400	0.0000
BS_JA_AMA	9	16:30:00 (14/5/67)	0.008	0.016	0.025	0.02	5	0.0800	0.1250	0.1000
BS_JA_AMA	12	19:35:00 (14/5/67)	0.034	0.08	0.089	0.131	5	0.4000	0.4450	0.6550
BS_JA_AMA	15	22:30:00 (14/5/67)	0.001	0.179	0.21	0.2	5	0.8950	1.0500	1.0000
BS_JA_AMA	18	1:30:00 (15/5/67)	0.005	0.255	0.259	0.397 (over)	5	1.2750	1.2950	nan
BS_JA_AMA	24	7:35:00 (15/5/67)	0.003	0.27	0.274	0.456 (over)	5	1.3500	1.3700	nan
BS_JA_AMA	36	19:35:00 (15/5/67)	0.011	0.253	0.274	0.422 (over)	5	1.2650	1.3700	nan
BS_JA_AMA	48	7:40:00 (16/5/67)	0.008	0.205	0.234	0.363 (over)	5	1.0250	1.1700	nan
BS_IMO	0	7:30:00 (14/5/67)	nan	0	nan	nan	1	0.0000	nan	nan
BS_IMO	6	13:30:00 (14/5/67)	nan	0	0	0.011	5	0.0000	0.0000	0.0550
BS_IMO	9	16:30:00 (14/5/67)	0.008	0.015	0.01	0.021	5	0.0750	0.0500	0.1050

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
BS_IMO	12	19:35:00 (14/5/67)	0.034	0.028	0.009	0.028	5	0.1400	0.0450	0.1400
BS_IMO	15	22:30:00 (14/5/67)	0.001	0.014	0.036	0.017	5	0.0700	0.1800	0.0850
BS_IMO	18	1:30:00 (15/5/67)	0.005	0.071	0.098	0.077	5	0.3550	0.4900	0.3850
BS_IMO	24	7:35:00 (15/5/67)	0.003	0.262	0.261	0.327	5	1.3100	1.3050	1.6350
BS_IMO	36	19:35:00 (15/5/67)	0.011	0.285	0.246	0.286	5	1.4250	1.2300	1.4300
BS_IMO	48	7:40:00 (16/5/67)	0.008	0.224	0.196	0.236	5	1.1200	0.9800	1.1800
BS_wMRS	0	7:30:00 (14/5/67)	nan	0	nan	nan	1	0.0000	nan	nan
BS_wMRS	6	13:30:00 (14/5/67)	nan	0	0.005	0.003	5	0.0000	0.0250	0.0150
BS_wMRS	9	16:30:00 (14/5/67)	0.008	0.007	0.012	0.021	5	0.0350	0.0600	0.1050
BS_wMRS	12	19:35:00 (14/5/67)	0.034	0.006	0.021	0.038	5	0.0300	0.1050	0.1900
BS_wMRS	15	22:30:00 (14/5/67)	0.001	0.004	0.003	0.008	5	0.0200	0.0150	0.0400
BS_wMRS	18	1:30:00 (15/5/67)	0.005	0.002	0.005	0.009	5	0.0100	0.0250	0.0450
BS_wMRS	24	7:35:00 (15/5/67)	0.003	0.114	0.153	0.212 (over)	5	0.5700	0.7650	nan
BS_wMRS	36	19:35:00 (15/5/67)	0.011	0.27	0.298	0.397 (over)	5	1.3500	1.4900	nan
BS_wMRS	48	7:40:00 (16/5/67)	0.008	0.156	0.199	nan	5	0.7800	0.9950	nan
LP_GLU	0	7:30:00 (14/5/67)	nan	0.015	nan	nan	1	0.0150	nan	nan
LP_GLU	6	13:45:00 (14/5/67)	nan	0.009	0	0.008	5	0.0450	0.0000	0.0400
LP_GLU	9	16:50:00 (14/5/67)	0.008	0.032	0.019	0.028	5	0.1600	0.0950	0.1400
LP_GLU	12	19:55:00 (14/5/67)	0.034	0.05	0.051	0.054	5	0.2500	0.2550	0.2700
LP_GLU	15	22:45:00 (14/5/67)	0.001	0.111	0.12	0.115	5	0.5550	0.6000	0.5750
LP_GLU	18	1:45:00 (15/5/67)	0.005	0.193	0.196	0.167	5	0.9650	0.9800	0.8350
LP_GLU	24	7:50:00 (15/5/67)	0.003	0.38	0.328	0.258	5	1.9000	1.6400	1.2900

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
LP_GLU	36	19:50:00 (15/5/67)	0.011	0.399	0.424	0.35	5	1.9950	2.1200	1.7500
LP_GLU	48	8:05:00 (16/5/67)	0.008	0.386	0.408	0.344	5	1.9300	2.0400	1.7200
LP_JA_AMA	0	7:30:00 (14/5/67)	nan	0.01	nan	nan	1	0.0100	nan	nan
LP_JA_AMA	6	13:45:00 (14/5/67)	nan	0.013	0.001	0.019	5	0.0650	0.0050	0.0950
LP_JA_AMA	9	16:50:00 (14/5/67)	0.008	0.038	0.029	0.05	5	0.1900	0.1450	0.2500
LP_JA_AMA	12	19:55:00 (14/5/67)	0.034	0.08	0.072	0.113	5	0.4000	0.3600	0.5650
LP_JA_AMA	15	22:45:00 (14/5/67)	0.001	0.176	0.172	0.173	5	0.8800	0.8600	0.8650
LP_JA_AMA	18	1:45:00 (15/5/67)	0.005	0.294	0.287	0.246	5	1.4700	1.4350	1.2300
LP_JA_AMA	24	7:50:00 (15/5/67)	0.003	0.393	0.403	0.335	5	1.9650	2.0150	1.6750
LP_JA_AMA	36	19:50:00 (15/5/67)	0.011	0.422	0.44	0.398	5	2.1100	2.2000	1.9900
LP_JA_AMA	48	8:05:00 (16/5/67)	0.008	0.407	0.415	0.37	5	2.0350	2.0750	1.8500
LP_IMO	0	7:30:00 (14/5/67)	nan	0	nan	nan	1	0.0000	nan	nan
LP_IMO	6	13:45:00 (14/5/67)	nan	0	0.007	0.011	5	0.0000	0.0350	0.0550
LP_IMO	9	16:50:00 (14/5/67)	0.008	0.021	0.025	0.033	5	0.1050	0.1250	0.1650
LP_IMO	12	19:55:00 (14/5/67)	0.034	0.066	0.06	0.065	5	0.3300	0.3000	0.3250
LP_IMO	15	22:45:00 (14/5/67)	0.001	0.116	0.141	0.133	5	0.5800	0.7050	0.6650
LP_IMO	18	1:45:00 (15/5/67)	0.005	0.196	0.222	0.185	5	0.9800	1.1100	0.9250
LP_IMO	24	7:50:00 (15/5/67)	0.003	0.312	0.275	0.202	5	1.5600	1.3750	1.0100
LP_IMO	36	19:50:00 (15/5/67)	0.011	0.312	0.31	0.279	5	1.5600	1.5500	1.3950
LP_IMO	48	8:05:00 (16/5/67)	0.008	0.292	0.283	0.257	5	1.4600	1.4150	1.2850
LP_wMRS	0	7:30:00 (14/5/67)	nan	0.005	nan	nan	1	0.0050	nan	nan
LP_wMRS	6	13:45:00 (14/5/67)	nan	0.002	0.009	0.005	5	0.0100	0.0450	0.0250

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
LP_wMRS	9	16:50:00 (14/5/67)	0.008	0.018	0.021	0.033	5	0.0900	0.1050	0.1650
LP_wMRS	12	19:55:00 (14/5/67)	0.034	0.024	0.06	0.036	5	0.1200	0.3000	0.1800
LP_wMRS	15	22:45:00 (14/5/67)	0.001	0.044	0.063	0.041	5	0.2200	0.3150	0.2050
LP_wMRS	18	1:45:00 (15/5/67)	0.005	0.126	0.132	0.112	5	0.6300	0.6600	0.5600
LP_wMRS	24	7:50:00 (15/5/67)	0.003	0.202	0.238	0.2	5	1.0100	1.1900	1.0000
LP_wMRS	36	19:50:00 (15/5/67)	0.011	0.204	0.219	0.196	5	1.0200	1.0950	0.9800
LP_wMRS	48	8:05:00 (16/5/67)	0.008	0.169	0.197	0.177	5	0.8450	0.9850	0.8850

Table D2 Second time prebiotics effectiveness test *E. coli* and *L. plantarum* (17/05/2024)

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
EC_GLU	0		-0.032	-0.022	nan	nan	1	-0.0220	nan	nan
EC_GLU	6		-0.004	0.079	0.11	0.118	5	0.3950	0.5500	0.5900
EC_GLU	9		-0.001	0.209	0.198	0.273	5	1.0450	0.9900	1.3650
EC_GLU	12		-0.005	0.238	0.24	0.332	5	1.1900	1.2000	1.6600
EC_GLU	15		-0.001	0.294	0.277	0.396	5	1.4700	1.3850	1.9800
EC_GLU	18		0.002	0.32	0.333	0.489	5	1.6000	1.6650	2.4450
EC_GLU	24		-0.001	0.474	0.425	0.529	5	2.3700	2.1250	2.6450
EC_GLU	36		0.022	0.52	0.446	0.652	5	2.6000	2.2300	3.2600
EC_GLU	48		0.006	0.441	0.39	0.604	5	2.2050	1.9500	3.0200
EC_JA_AMA	0		-0.032	-0.024	nan	nan	1	-0.0240	nan	nan
EC_JA_AMA	6		-0.004	0.146	0.159	0.12	5	0.7300	0.7950	0.6000
EC_JA_AMA	9		-0.001	0.218	0.21	0.187	5	1.0900	1.0500	0.9350
EC_JA_AMA	12		-0.005	0.245	0.251	0.23	5	1.2250	1.2550	1.1500
EC_JA_AMA	15		-0.001	0.307	0.291	0.286	5	1.5350	1.4550	1.4300
EC_JA_AMA	18		0.002	0.348	0.312	0.318	5	1.7400	1.5600	1.5900
EC_JA_AMA	24		-0.001	0.376	0.432	0.33	5	1.8800	2.1600	1.6500
EC_JA_AMA	36		0.022	0.451	0.59	0.418	5	2.2550	2.9500	2.0900
EC_JA_AMA	48		0.006	0.508	0.506	nan	5	2.5400	2.5300	nan
EC_IMO	0		-0.032	-0.02	nan	nan	1	-0.0200	nan	nan
EC_IMO	6		-0.004	0.072	0.138	0.12	5	0.3600	0.6900	0.6000
EC_IMO	9		-0.001	0.205	0.194	0.229	5	1.0250	0.9700	1.1450

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
EC_IMO	12		-0.005	0.225	0.247	0.289	5	1.1250	1.2350	1.4450
EC_IMO	15		-0.001	0.296	0.305	0.343	5	1.4800	1.5250	1.7150
EC_IMO	18		0.002	0.371	0.408	0.416	5	1.8550	2.0400	2.0800
EC_IMO	24		-0.001	0.386	0.41	0.453	5	1.9300	2.0500	2.2650
EC_IMO	36		0.022	0.369	0.41	0.437	5	1.8450	2.0500	2.1850
EC_IMO	48		0.006	0.324	0.366	0.378	5	1.6200	1.8300	1.8900
EC_wMRS	0		-0.032	-0.012	nan	nan	1	-0.0120	nan	nan
EC_wMRS	6		-0.004	0.039	0.045	0.059	5	0.1950	0.2250	0.2950
EC_wMRS	9		-0.001	0.053	0.052	0.045	5	0.2650	0.2600	0.2250
EC_wMRS	12		-0.005	0.129	0.137	0.134	5	0.6450	0.6850	0.6700
EC_wMRS	15		-0.001	0.27	0.268	0.277	5	1.3500	1.3400	1.3850
EC_wMRS	18		0.002	0.356	0.361	0.385	5	1.7800	1.8050	1.9250
EC_wMRS	24		-0.001	0.445	0.455	0.484	5	2.2250	2.2750	2.4200
EC_wMRS	36		0.022	0.51	0.471	0.544	5	2.5500	2.3550	2.7200
EC_wMRS	48		0.006	0.461	0.439	0.536	5	2.3050	2.1950	2.6800
LP_JA_AMA	0		-0.032	0.016	nan	nan	1	0.0160	nan	nan
LP_JA_AMA	6		-0.004	0.059	nan	nan	5	0.2950	nan	nan
LP_JA_AMA	9		-0.001	0.154	nan	nan	5	0.7700	nan	nan
LP_JA_AMA	12		-0.005	0.277	nan	nan	5	1.3850	nan	nan
LP_JA_AMA	15		-0.001	0.428	nan	nan	5	2.1400	nan	nan
LP_JA_AMA	18		0.002	0.483	nan	nan	5	2.4150	nan	nan
LP_JA_AMA	24		-0.001	0.416	nan	nan	5	2.0800	nan	nan

Sample	Time (hours)	Analysis time	Blank	Raw data OD 560 nm*			x Fold	Dilute calculation (x-fold dilute)*		
				1	2	3		1	2	3
LP_JA_AMA	36		0.022	0.418	nan	nan	5	2.0900	nan	nan
LP_JA_AMA	48		0.006	0.429	nan	nan	5	2.1450	nan	nan
LP_IMO	0		-0.032	-0.019	nan	nan	1	-0.0190	nan	nan
LP_IMO	6		-0.004	0.048	nan	nan	5	0.2400	nan	nan
LP_IMO	9		-0.001	0.118	nan	nan	5	0.5900	nan	nan
LP_IMO	12		-0.005	0.211	nan	nan	5	1.0550	nan	nan
LP_IMO	15		-0.001	0.296	nan	nan	5	1.4800	nan	nan
LP_IMO	18		0.002	0.346	nan	nan	5	1.7300	nan	nan
LP_IMO	24		-0.001	0.315	nan	nan	5	1.5750	nan	nan
LP_IMO	36		0.022	0.298	nan	nan	5	1.4900	nan	nan
LP_IMO	48		0.006	0.224	nan	nan	5	1.1200	nan	nan
LP_wMRS	0		-0.032	-0.002	nan	nan	1	-0.0020	nan	nan
LP_wMRS	6		-0.004	0.047	nan	nan	5	0.2350	nan	nan
LP_wMRS	9		-0.001	0.078	nan	nan	5	0.3900	nan	nan
LP_wMRS	12		-0.005	0.143	nan	nan	5	0.7150	nan	nan
LP_wMRS	15		-0.001	0.216	nan	nan	5	1.0800	nan	nan
LP_wMRS	18		0.002	0.213	nan	nan	5	1.0650	nan	nan
LP_wMRS	24		-0.001	0.166	nan	nan	5	0.8300	nan	nan
LP_wMRS	36		0.022	0.138	nan	nan	5	0.6900	nan	nan
LP_wMRS	48		0.006	0.116	nan	nan	5	0.5800	nan	nan

CURRICULUM VITAE

NAME	Ran Kitcangplu
EDUCATIONAL BACKGROUND	
July 2020	Bachelor of Science Biological Science Kasetsart University, Kamphaeng Saen Campus
WORK EXPERIENCE	
Aug 2024 – Dec 2024	Community Primary Data Collector Mae Fah Luang University
June 2021 – July 2022	Researcher Assistant Mae Fah Luang University
May 2019 – July 2019	Intern Scientist Blood research center, Thalassemia Team/Unit, Naresuan University Hospital