Phytochemical and Prebiotic Properties of Functional Mulberry Products Produced by Membrane-based Process

Thitirat Kaewsedam

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Functional Food and Nutrition
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ชื่อวิทยานิพนธ์ คุณสมบัติทางพฤกษเคมีและพรีไบโอติกของน้ำหม่อนเพื่อสุขภาพ
ที่ผลิตโดยกระบวนการเมมเบรน

ผู้เขียน นางสาว ฐิติรัตน์ แก้วศรีดำ

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ปีการศึกษา 2565

บทคัดย่อ

ผลหม่อน (*Morus alba* L.) เป็นผลไม้ที่มีประโยชน์ต่อสุขภาพของมนุษย์ หม่อนนั้นให้คุณประโยชน์มากมาย เช่น ลดคอเลสเตอรอล ความเสี่ยงต่อมะเร็ง และน้ำตาลในเลือด น้ำหม่อนเพื่อสุขภาพ (MBI) ผลิตได้จากการนำน้ำหม่อนเสริมด้วยไอโซมอลโตโอลิโกแซคคาไรด์ (isomaltooligosaccharide, IMO) และเสริมวิตามินและแร่ธาตุ นำมาผ่านการพาสเจอร์ไรส์แบบไม่ใช้ความร้อนด้วยไมโครฟิลเตรชันที่ใช้เมมบรนเส้นใยกลวงทำจากโพลีซัลโฟนที่มีขนาดรูพรุน 0.2 ไมครอน (μm) ภายใต้สภาวะการกรองที่มีความเร็วตามขวาง (cross flow velocity, CFV) ที่ 1.0 m/s อุณหภูมิ 20±2 องศาเซลเซียส ความดันขับ (transmembrane pressure, TMP) ที่ 0.8 bar น้ำหม่อนก่อนเข้าสู่กระบวนการไมโครฟิลเตรชัน มีการย่อยด้วยเอนไซม์เพคติเนสที่ความเข้มข้น 0.1% (V/V) ผลของการย่อยด้วยเอนไซม์ทำให้น้ำหม่อนมีความหนืดลดลงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเปรียบเทียบกับชุดการทดลองควบคุมที่ผ่านการย่อยด้วยเอนไซม์เพคติเนส ผลการทดลองพบว่า สารพฤกษเคมี (phytochemicals) เช่น กรดแอล-แอสคอร์บิก และ แอนโทไซยานิน ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) หลังผ่านการกรองด้วยไมโครฟิวเตรชัน พบว่าปริมาณสูงสุดของกรดแอล-แอสคอร์บิกและแอนโทไซยานินมีค่าเท่ากับ 14.19±0.06 มก./มล. และ 127.07±2.28 มก./กรัม ตามลำดับ ผลการศึกษาฤทธิ์การต้านอนุมูลอิสระ (DPPH และ FRAP) และ ปริมาณฟีนอลิกทั้งหมดของน้ำหม่อนเพื่อสุขภาพ พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ผลิตภัณฑ์สุดท้ายหลังผ่านกระบวนการไมโครฟิลเตรชันได้ผลิตภัณฑ์เครื่องดื่มน้ำผลไม้ใสและน้ำผลไม้เข้มข้น ผลการศึกษาการหมักของผลิตภัณฑ์ในระบบจำลองลำไส้แบบกะที่ใช้อุจจาระมนุษย์เป็นแหล่งจุลินทรีย์ลำไส้ เพื่อศึกษากรดไขมันสายสั้น (short-chain fatty acid, SCFAs) โดยใช้แก๊สโครมาโทกราฟีที่มีตัวตรวจวัดชนิดเฟลมไอออไนเซชัน (gas chromatography-flame ionization detection, GC-FID) นับแบคทีเรียลำไส้โดยวิธี next generation sequencing (NGS) และปริมาณฟีนอลิกโดย liquid chromatography-mass/mass spectrometry (LC-MS/MS) ผลการทดลองพบว่า ไอโซมอลโตโอลิโกแซคคาไรด์ที่เติมในน้ำหม่อนร้อยละ 2 และ 8 มีผลต่อการส่งเสริมการเจริญเติบโตของบิฟิโดแบคทีเรีย (bifidobacteria) เพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) ที่ระดับร้อยละ 5.03 และ

17.53 ตามลำดับ หลังการหมักเป็นเวลา 24 ชั่วโมง ในขณะที่แบคทีเรียกลุ่มแบคทีรอยด์ (bacteroides) ลดลงอย่างมีนัยสำคัญ ผลการวิเคราะห์กรดไขมันสายสั้น (SCFAs) ที่ผลิตขึ้นจากการหมักพบว่า ไอโซมอลโตโอลิโกแซคคาไรด์ที่เติมในน้ำหม่อนร้อยละ 2 และ 8 สามารถผลิตกรดโพรพิโอนิกและกรดบิวทิริกได้สูงสุดที่ความเข้มข้น 11.66 ±1.69 และ 13.68 ± 0.50 mM ที่เวลาการหมัก 24 ชั่วโมง สารเมตาบอไลซ์ที่เกิดจากการหมักสารฟีนอลิกในน้ำหม่อนที่พบมากที่สุดคือ 3-(2-hydroxyphenyl) propionic acid, 3,4-dihydroxybenzaldehyde, L-phenylalanine, aminocaproic acid, 3,4-dihydroxybenzaldehyde และ cholic acid ดังนั้นการเติมไอโซมอลโตโอลิโกแซคคาไรด์ในน้ำหม่อน สามารถส่งเสริมการเจริญเติบโตของบิฟิโดแบคทีเรียผลิตกรดไขมันสายสั้นชนิดโพรพิโอนิกและกรดบิวทิริก และผลิตสารเมตาบอไลซ์จากการหมักฟีนอลิกที่มีในน้ำหม่อน ดังนั้นเครื่องดื่มน้ำหม่อนที่เติมวิตามินดี 3 แคลเซียมแลกเตส พรีไบโอติกไอโซมอลโตโอลิโกแซคคาไรด์ และผ่านกระบวนการไมโครฟิวเตรชัน เป็นการเพิ่มคุณค่าของสารอาหารและประโยชน์ต่อสุขภาพ

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ABSTRACT

Mulberry (*Morus alba* L.) fruits are beneficial for human health. They provided many benefits such as lower cholesterol, cancer risk and blood sugar. In this study, functional mulberry juices (MBI) were produced by adding isomaltooligosaccharide (IMO) and mineral supplements and pasteurizing by microfiltration (MF). MF system used was 0.2 µm polysulfone hollow fiber and operated at constant crossflow velocity (CFV) of 1.0 m/s, temperature of 20±2°C and transmembrane pressure (TMP) of 0.8 bar. The mulberry juice was pre-treated by commercial pectinase 0.1% (V/V) before MF. The result showed that phytochemicals such as L-ascorbic acid and anthocyanin no significant difference ($p<0.05$) after microfiltration. The highest values of L-ascorbic acid and anthocyanin content were 14.19±0.06 mg/ml and 127.07±2.28 mg/g, respectively. In addition, the effect of antioxidant capacity (DPPH and FRAP) and total phenolic content of functional mulberry juice showed no significant difference ($p<0.05$) under the condition of 20±2°C. The final product after microfiltration step produced clarified juice and concentrated juice. Batch culture of MBI on short-chain fatty acids (SCFAs) by GC-FID, enumeration of fecal bacteria by NGS method and phenolic metabolites by LC-MS/MS were studied. The effect of IMO on beneficial bacteria (bifidobacterial) increased significantly ($P<0.05$) after 24h fermentation in MBI2 (5.03%) and MBI8 (17.53%), meanwhile the percentage of bacteroides after 24h decreased significantly. Moreover, studied on SCFAs analysis results showed that IMO can increase the production of propionic acid and butyric acid in the colon. The highest concentrations of propionic and butyric acid on MBI2 and MBI8 were increased by 11.66 ± 1.69, 13.68 ± 0.50 mM and 9.55 ± 1.01, 10.79 ± 0.33 mM, respectively at 48 h fermentation. LC-MS/MS method for the quantification of phenolic metabolites in human feces. The most abundant phenolic compounds were 3-(2-hydroxyphenyl)

propionic acid, 3,4-dihydroxybenzaldehyde, L-phenylalanine, aminocaproic acid, 3,4-dihydroxybenzaldehyde and cholic acid in functional mulberry juices. Thus, IMO can support the growth of bifidobacterial such as *Bifidobacterium scardovii, Bifidobacterium stercoris.*

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LIST OF ABBREVATIONS

˚C	=	degree Celsius
µg	=	microgram
µl	=	microliter
µM	=	micromolar
ACE	=	angiotensin I-converting enzyme
aw	=	water activity
C3G	=	cyanidin-3-glucoside
CFV	=	cross flow velocity
d	=	day
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
FRAP	=	Ferric reducing antioxidant power
g	=	gram
GC-FID	=	gas chromatography-flame ionization detection
h	=	hour
IBD	=	inflammatory bowel disease
IMO	=	isomaltooligosaccharide
L	=	liter
LAB	=	lactic acid bacteria
LC-MS/MS	=	liquid chromatography-mass/mass spectrometry
M	=	molarity
m	=	month
mM	=	millimolar
mg	=	milligram
min	=	minute
ml	=	milliliter
N	=	normality
nm	=	nanometer
NCBI	=	the National Center for Biotechnology Information

NGS	=	next generation sequencing
RDA	=	recommended dietary allowance
TMP	=	transmembrane pressure
UHPLC	=	Ultra high liquid chromatography

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Mulberry fruits are one of the most famous and important tropical fruits that are consumed all over the world. The mulberry tree is a fast-growing deciduous in the Moraceae family. The mulberry fruit (*Morus nigra* L.) provides high levels of active ingredients, including phenols, anthocyanins, flavonoids, ascorbic acid, etc. Currently, the food and pharmaceutical industries use mulberries widely. Mulberry fruit mostly originates from regions including Southern Europe, the Middle East, Northern Africa, the Indian subcontinent, East Asia, and the Americas. Moreover, there are more than 1000 species of mulberry in the world (Khalifa *et al*., 2018). Most of the mulberry is consumed as fresh fruit, but it is inconvenient. Consequently, processed mulberry products such as juices are very popular. Today, thermal processing such as pasteurization, etc. used in most industries for shelf-stable food preservation. Pasteurization for foods, such as juices or foods, uses heat to destroy all pathogenic micro-organisms but not all. Thus, the products are kept at a cooling temperature to extend shelf-life. Commercial sterilization uses high temperature which can destroy most spore bacteria and has a shelf-life of up to 1-2 years, kept at room temperature. Abera (2019) reported that heat-sensitive components are remarkably lost during thermal processing. On the other hand, membrane technologies, and non-thermal processes are widely used in the food industry, such as in fruit juice processing. Microfiltration (MF) is used to clarify and sterilize juices in industries worldwide (Paola and Vélez, 2011). Moreover, microfiltration can reduce nutrient loss (e.g. vitamin C, anthocyanin and carotenoids), and reduce energy consumption.

Prebiotics such as isomaltooligosaccharide (IMO) produced from starch is an alternative functional carbohydrate in food ingredients, promoting health benefits. The Meiji Food Company is the largest IMO production in Japan (Nakakuki, 2003). IMO is also recognized by food products such as drinks, yogurt, and sweeteners. It has been reported that IMO can enhance mineral absorption in humans, and decrease of pathogenic bacteria in the gastrointestinal tract and colon cancer (Han *et al*., 2008).

Slavin (2013) reports that short-chain fatty acids (SCFAs) can reduce the number of pathogenic bacteria in the body with prebiotics. IMO, a type of prebiotic can potentially be used by probiotic consequently releasing short-chain fatty acids including acetic, butyric, and propionic acid. Likewise, it can control gastrointestinal transit. In this study, functional mulberry juice with supplementation of IMO was developed and shelf-life was extended using a non-thermal pasteurization process and its phytochemical and prebiotic properties were studied.

1.2 Review of literature

1.2.1 Mulberry

Mulberry (*Morus* spp.) is in the order Rosales, family Moraceae and genus Morus. Mulberries are distributed in many parts of the world. Zeng *et al*. (2015) reported that mulberry fruit includes eight Asian species consisting of (*M. alba*, *M. australis, M. cathayana, M. macroura, M. mongolica, M. nigra*, *M. notabilis and M. serrata*). Moreover, four species (*M. celtidifolia, M. insignis, M. microphylla*, and *M. rubra*) and one African species (*M. mesozygia*).

Currently, Thailand is the main producer and exporter of mulberry product (Choosung *et al*., 2022). White mulberry (*Morus alba*) and black mulberry (*Morus nigra*) are two species of mulberry fruit in Thailand. Mulberry fruit is usually consumed as fresh fruit and juices. Mulberry is an easy-to-grow and fast-growing fruit. Mulberries can grow in a variety of climates, such as the temperate to subtropical regions of the Northern Hemisphere and the tropical regions of the Southern Hemisphere and Southeast Asia. The bioactive ingredients of mulberry fruit are anthocyanins (cyanidin-3-rutinoside and cyanidin-3-glucoside) which are water-soluble bioactive ingredients of the polyphenol class. Chemically, anthocyanins are polyphenols and belong to a large class of secondary metabolites known as flavonoids (Zhang *et al*., 2019) and have several health benefits, such as reducing the risk of cholesterol, obesity, and liver disease. (Zhang *et al.*, 2018).

Figure 1. *Morus nigra* L. (Black mulberry)

Source: Miljkovic *et al*. (2014)

Figure 2. *Morus alba* L. (White mulberry)

Source: Miljkovic *et al.* (2014)

1.2.1.1 Classification of mulberry cultivars

In Asia, more than 68 species of the genus *Morus* (Jalaja *et al*., 2000) have been found. Mulberry fruit usually has four main kinds including the white mulberry (*Morus alba*), the Lu mulberry *(M. multicaulis*), the Mountain mulberry (*M. bombycis*), and the Guangdong mulberry (*M. atropurpurea*). The most popular species

of mulberry including the black mulberry (*Morus nigra*) and white mulberry (*M. alba*) (Vijayan *et al.*, 2011).

The following are the major subgroups and mulberry varieties

(1) *Morus nigra* L. (Moraceae): black mulberry can be found in Iran and Afghanistan (western Asia). *M. nigra* is mostly suited for places with summers. Moreover, black mulberry can resist low temperatures (≥-10°C). The size of mulberries is around 1.5-2.5 cm and 3 cm in diameter. Black mulberries are tasty, juicy and slightly sweet and sour than *Morus alba*.

(2) *Morus alba* L. (Moraceae): white mulberry is easy to grow. The characteristic of white mulberry is a medium-sized tree around 25-35 m. The size of mulberries is around 5 cm. White mulberries are fleshy and juicy. Moreover, it can be transformed into juices or beverages. *Morus alba* L. not only produce beverage but also use to rear silkworm and produce silk. Generally, white mulberry fruits can be grown in temperatures around 18 to 30°C and are easy to grow in poor soil (Zhou *et al*., 2015).

1.2.1.2 Nutritional value of Mulberry

The nutritional value of mulberries depends on several factors, such as the wide range of variety, ripeness, and rawness of the mulberry fruit. In addition to the variety and the level of rawness, environmental conditions play a critical role in the value of the mulberry fruit including soil nutrient content. Moreover, the moisture content in the atmosphere also regulates energy value and nutrient density. The energy value of the mulberries involves the content of carbohydrates, and protein. Mulberry contains some vitamins, organic acid, and other nutrients but it has low fat and is absent of cholesterol. By fresh weight, mulberry fruit consists of 9.8 percent of carbohydrates, 1.7 percent of fiber, 1.4 percent of protein, and 0.4 percent of fat. (Calín-Sánchez *et al*., 2013).

The varieties of these nutritional values of mulberries are shown in Table 1. Mulberries are high in vitamins and suitable for the preparation of low-fat diets, a portion of excellent food for people who want to lose weight. In addition,

mulberries are an important source of Iron. A cup of mulberry (100g) provides 23 percent of the Iron that the body needs daily. Iron benefits help to preserve several necessary functions in the human body such as energy and meditation, alimentary tract processes, the immune system, and the modulation of body temperature. Moreover, Iron-deficiency anemia can cause weakness, impaired immunity, heart oscillation, pale skin, and breathlessness. Mulberries are also an excellent source of vitamins. (1) vitamin A can protect against infection and facilitate vision, healthy eyes, healthy teeth, bones, and soft tissue. (2) vitamin B2 enhances the body to enzymatically digestion of food mainly carbohydrates, proteins, and fats to generate energy called ATP in the cells. (3) vitamin B3 might be decreasing of cholesterol levels in the blood along with enhancing brain performance. (4) vitamin B6 improves the immune system, maintains healthy blood vessels, and promotes brain health, heart health, and eye health. (5) vitamin B9 is necessary to produce red blood cells, enhances the mechanism involved in DNA replication, and also helps with tissue growth and cell function (6) Surprisingly, mulberry fruit provided a large amount of vitamin C, which is more than vitamin C in orange juice about 190%. Vitamin C generates an immune system for the human body. Moreover, it plays an important role in protecting against infection, improves the body to use of certain forms of iron, and aids in healing, and the growth of tissue, and ligaments. (7) vitamin E prevents coronary heart disease, supports immune function, prevents inflammation, improves eye health, and protects against cancer. Finally, (8) vitamin K is an important factor in bone health and helps produce four of the 13 proteins needed for blood clotting.

Eventually, mulberries can provide several benefits to the human body. The main function is to maintain bone density, increased the immune system, and reduce cholesterol in the blood since they have high levels of protein, iron, vitamin C, fiber, calcium, and antioxidants. Nowadays, people can easily make healthy food mixed with mulberry to provide for their health.

Sánchez-Salcedo *et al*. (2015) described the levels of phenolic compounds in mulberry, which are higher than those in the fruit of berries such as blueberry, blackberry, raspberry, and strawberry. Mulberry also contains diverse

phenolic compounds including polyphenols, anthocyanins and flavonoids. So, the benefit of mulberry fruits is good sources of phenolic compounds.

Mulberry contains various organic compounds such as anthocyanin phenolics and flavonoids. The amount of such organic was detected are 23.0 mg/g gallic acid equivalents, 3.9 mg/g rutin and 0.87 mg/g cyanidin-3-gucoside reagent, respectively. There are several major flavonoids detected in mulberry mainly rutin (0.43 mg/g), morin (0.16 mg/g), quercetin (0.01 mg/g) and myricetin (0.01 mg/g). Jiang and Nie (2015) reported that mulberry provided many types of essential amino acids such as leucine, isoleucine, threonine, lysine, phenylalanine, tyrosine, valine, tryptophan, histidine, methionine and cysteine and seven non-essential amino acids such as arginine, serine, alanine, proline, glutamic acid, glycine and aspartic acid. In addition, minerals including potassium, calcium, magnesium, iron, sodium, zinc and copper in mulberry fruit were detected. Moreover, linoleic acid, myristic acid, stearic acid, palmitic acid and α-linoleic acid are also detected.

There are two different colors of mulberry fruit mainly dark and pale colors, which contained minerals and nutrients differentially. Pale color of mulberry species contains a higher amount of vitamin C compared with dark color. However, dark color-mulberry fruit has a higher total antioxidant capacity than pale color. In black mulberry, the succinic acid content is higher than tartaric acid concentration. In addition, the most organic acid in the mulberry species is malic acid, citric, tartaric, succinic, lactic, fumaric, and acetic acid (Gundogdu *et al.*, 2011).

Koyuncu *et al.* (2004) reported that malate, citrate, tartarate, and fumarate contents of black mulberry ranged from 35.4 to 198.5 mgg^{-1}, 5.5 to 23.4 mgg^{-1}, 2.95 to 5.55 mgg^{-1} and 0.015 to 0.033 mgg^{-1}, respectively. Therefore, mulberry fruit is high nutritional value and health benefits. The functional components of mulberry, are mainly anthocyanins, polysaccharides, phenols and flavonoids. These bioactive compounds play a vital role in the physiological activities of the human body.

Table 1. The nutritional value of various mulberries

Plant name	*Morus alba*	*Morus nigra*	*Morus laevigata* (large white fruit)	*Morus laevigata* (large blackfruit)
Moisture (g/100g FW)	81.72±2.25	82.40±3.85	81.48±1.87	78.03±3.22
Ash (g/100gDW)	0.57±0.11	0.50±0.08	0.46±0.06	0.87±0.12
Lipid (g/100gDW)	0.48±0.11	0.55±0.06	0.71±0.07	0.60±0.03
Protein (g/100gDW)	1.55±0.30	0.96±0.16	1.57±0.19	1.73±0.10
Fibre (g/100gDW)	1.47±0.15	11.75±1.21	0.57±0.14	0.81±0.12
TC (g/100gDW)	14.21±1.01	13.83±1.20	15.21±1.32	17.96±1.54
E (kcal/100 g DW)	67.36±3.22	64.11±2.45	73.51±3.46	84.22±4.00

FW: fresh weight; DW: dry weight; TC: total carbohydrates; E: energy value. Values (mean±SD) in the same column are not significantly different at $P<0.05$

Source: Imran *et al.* (2010)

Table 2. Mulberry nutrition profile

Nutrient	Value (per 100 g)	Thai RDA (%)
Energy	43 Kcal	2%
Carbohydrates	9.80 g	7.5%
Protein	1.44 g	2.5%
Total Fat	0.39 g	2%
Cholesterol	0 mg	0%
Dietary Fiber	1.7 g	4.5%
Folate	6 µg	1.5%
Niacin	0.620 mg	4%
Pyridoxine	0.050 mg	4%
Riboflavin	0.101 mg	8%
Vitamin A	25 IU	1%
Vitamin C	36.4 mg	61%
Vitamin E	0.87 mg	6%
Vitamin K	7.8 µg	6.5%
Calcium	39 mg	4%
Copper	0.06 mg	6.5%
Iron	1.85 mg	23%
Magnesium	18 mg	4.5%
Zinc	0.12 mg	1%

Source: Jan *et al.* (2021)

1.2.2 Mulberry juice

At present, consumers have choices of healthy foods. Therefore, Mulberry is one of the fruits that provide low calories and high nutrients. Moreover. all essential amino acid required for human was detected in mulberry. Therefore, mulberry fruit could be a good protein source. Mulberries are a major source of minerals, especially potassium, followed by phosphorus and calcium. On the other hand, lack of vitamin D3 (Gundogdu *et al.,* 2011). The benefits of mulberry juice, high content of vitamin C in fresh mulberry at 36.4 mg/100g of fresh mulberry. Mulberry also provides many vitamins mainly vitamin A (Thiamine), vitamin B (Riboflavin, Niacin/Nicotinamide,

Pyridoxine and Folic acid), vitamin E and vitamin K. Moreover, 4 tocopherols were detected in mulberry including γ-Tocopherol and β-tocopherol (0.245 mg/g), δ-tocopherol (0.074 mg/g) and α-tocopherol (0.004 mg/g) (Yang *et al.*, 2010). Consequently, the mulberry fruit had an impact on human health.

Mulberry juice is a good source of polyphenols. Polyphenols are the largest group of phytochemical substances and their structural features are similar to flavonoids such as anthocyanins, flavones, catechins, and isoflavonoids. Hence, Foods or beverages are rich sources of polyphenol and is related to reduced risk of cardiovascular diseases, cancer and neurodegeneration (Del Rio *et al.*, 2013). Phenolic compounds are present in mulberry shown in Figure 3.

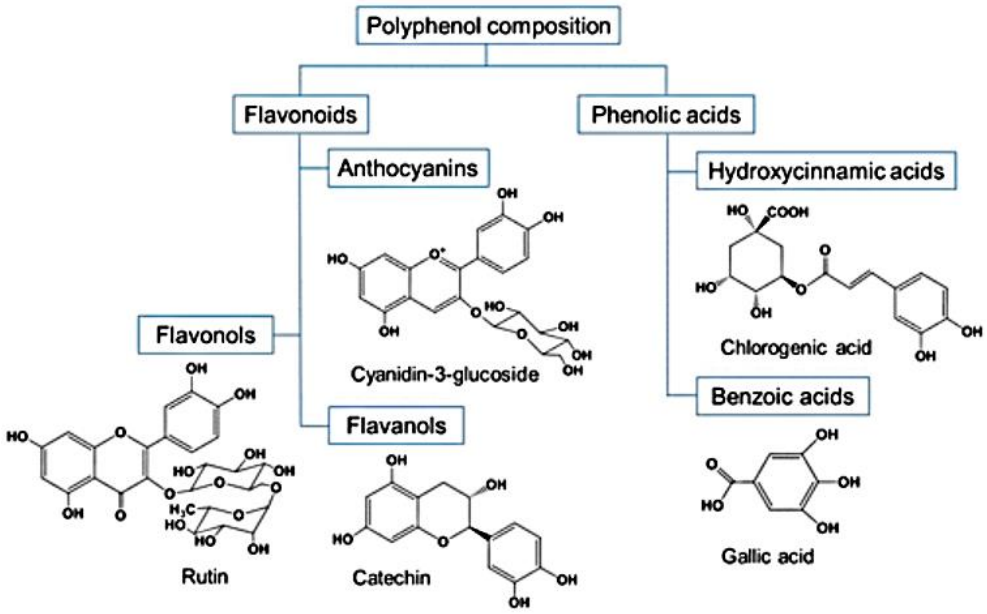

Figure 3. Chemical structures of the polyphenol compounds in mulberry fruit

Source: Yuan and Zhao (2017)

Cerrillo *et al*. (2015) reported that fruit juices fortified with vitamins C, D and zinc reduced symptoms of fatigue and have been proven to strengthen immune systems in the body. For the fruit juice industry, the latest trend focuses on fruit juices that consist of a variety of ingredients, such as vitamins, minerals, antioxidants,

probiotics, prebiotics and active compounds (BACs such as polyphenols, carotenoids, chlorophyll, tannins, etc.)

In addition, fruit juices with added synbiotics (probiotics and prebiotics) such as lactic acid bacteria, *Bifidobacterium*, *Lactobacillus*, inulin, fructooligosaccharides (FOS) are becoming widespread beverages. Normally, functional juice means adding vitamins, minerals, prebiotics, probiotics, flavonoids or fatty acids. For example, some fruit juices rich in vitamin C, such as orange juice, are considered beneficial. Vitamin C is an imperative antioxidant

1.2.3 Enzymatic treatment

Normally, the fruit is composed of a cell wall of polysaccharides such as cellulose, hemicellulose and pectin. Pectinases, a group of enzymes can break down the pectin in juice, change the solubility and help to exert a clarifying action on fruit juices. The uses of pectinase depend on the relative activity and the conditions of an enzyme. For industries, fruit juices are usually treated with enzyme to increase filtration performance. Carneiro *et al.* (2002) reported that the suspended solid content and the viscosity were reduced in the juice and they treated 0.1% (v/v) of pectinases (Pectinex SP-L and Celulast, from Novo Nordisk) at 30°C for 60 min before the process.

1.2.4 Preparation of mulberry juice

Ripe mulberries are washed and soaked and then drained. After that mulberry fruits are squeezed by a blender. The total soluble solid (TSS) is approximately 7.00 ºBrix and pH of the mulberry juice is 3.31-3.35. Before the process of microfiltration, the fresh mulberry juice is stored at 4°C and then the mulberry juice was treated with 0.1% (v/v) commercial pectinase at room temperature for 2 hours (Carneiro *et al*., 2002).

1.2.5 Technologies for shelf-life extension of fruit juice

In most industries, fruits are further processed into products such as jams, juices, or juice concentrates for the global market. This is because most fruits have a pH of less than 4.5 (pH<4.5). Although preserving food to extend its life is popular, using heat to extend the life of a product can destroy its chemical, physical, and phytochemical properties. (Subhan *et al*., 2020).

Effect of thermal processing on nutritional deterioration

The heat treatments of food or juice lead to affect the final product quality. Although the safety of microorganisms and chemicals is widespread during food production. However, the most important aspect of acceptance for the food industry is a sensory evaluation for acceptance or rejection. Usually, food processing methods such as pasteurization and sterilization are the most common industrial methods.

Pasteurization is a method of food preservation using a little heat (60-80 °C). Pasteurization is intended to inhibit microorganisms and enzyme activity. On the other hand, sterilization eliminates the spores.

Sterilization in fruit juices is widely used. Sterilization uses a higher temperature than pasteurization, lasting around 1 minute at 100-110 °C. On the other hand, sterilization has disadvantages such as product viscosity and difficulty in handling. Mainly, the packaging is put in sterile packaging.

In the fruit juice industry, this can be done at approximately 60–75°C for half an hour. The second step is pasteurization by using a temperature of 80°C for 15 minutes or more. It also depends on the size of the package and finally lowers the temperature to room temperature. HTST (High-temperature short-time) pasteurization is a heat-processing method that uses high temperatures of around 70–100 °C for a short time. Petruzzi *et al*. (2017) reported that apple juice was used at approximately 95°C–98°C for 30 s in the juicing process. Another processing method, ultrahigh temperature

(UHT), is the process of heat processing food at very high temperatures. It can be used in juices as well.

Thermal treatment: high temperature-short time (HTST)

The advantages of HTST for different types of enzymes

(1) The apple juice samples consisting of pectin methylesterase (PME) and polyphenol oxidase (PPO) had 95.3% and 90.9% reductions, respectively (Aguilar-Rosas *et al.,* 2013).

(2) HTST treatment reduced PPO, peroxidase (POD), and PME in apricot juice. (Huang *et al*., 2013).

(3) HTST treatment reduced pectin methylesterase and peroxidase in pummelo juice (Gao *et al.,* 2015).

(4) Fruit juice processing using the HTST method has beneficial effects on many nutrients, such as total phenolics, ferulic acid and epicatechin. Moreover, to preserve color shades in the juice (Mena *et al.,* 2013), and also on nutrients in fruit juices (Cerrillo *et al*., 2015).

The disadvantages of HTST on phytochemicals properties

(1) HTST treatment decreased citric acid and L-ascorbic acid in grapefruit juice (Igual *et al*., 2010).

(2) The effect of HTST reduced vitamin C in lemon juice (Mena *et al.,* 2013).

(3) Finally, HTST reduced of total phenolic compounds in pear juice (Jiménez-Aguilar *et al*., 2015)

Thermal treatment: Low-Temperature Long Time (LTLT)

LTLT (Low-Temperature Long Time) is a method of processing at approximately 62–65°C for more than 30 minutes. After heat treatment, food or beverages must be kept at refrigerated temperatures (4 °C). LTLT can destroy pathogenic bacteria and inhibit the activity of lipase or inactivate some enzymes (Miller

and Silva 2012). This includes several enzymes such as polyphenol oxidase (PPO), peroxidase (POD), pectin esterase (PE), and polygalacturonase (PG) (Marszałek and Mitek, 2012).

The advantages of LTLT treatment for different types of enzymes

(1) Pectin methylesterase (PME) is one of the first enzymes to interact with pectin. De Carvalho *et al*. (2015) studied mombin juice. The result showed that the LTLT method decreased PME by more than 80%. In addition, (Swami Hulle and Srinivasa Rao, 2016; Saeeduddin *et al*., 2015; Rodríguez-Verástegui *et al.*, 2016) also tested the effect of the LTLT method on carrot, tomato, and pear juices.

(2) Catechol oxidase or polyphenol oxidase (PPO), an oxidative catalytic enzyme, is the main causative agent of browning. The effect of using the LTLT method in fruit juices such as longan juice, pear juice, and litchi juice was studied by (Swami Hulle and Srinivasa Rao, 2016; Rodrguez-Verástegui *et al.*, 2016; Saeeduddin *et al.*, 2015; Chaikham and Apichartsrangkoon, 2012). The PPO value was reduced by approximately 80%.

(3) Peroxidase (POD), a type of enzyme also found in plants, the effect of the LTLT method in fruit juices was to study the reduction of POD enzymes in mombin juice, pear juice and smoothie. The result showed that there was approximately 70-90% reduction. (Swami Hulle and Srinivasa Rao, 2016; Saeeduddin *et al*., 2015; Rodríguez-Verástegui *et al*., 2016).

(4) Lipoxygenases (LOX) are enzymes that catalyze the oxidation of unsaturated fatty acids. The effect of the LTLT method reduced LOX by approximately 50%.

The disadvantages of LTLT on phytochemicals properties

LTLT (Low-Temperature Long Time) is the most widely used method. However, the effects of the LTLT method, including antioxidant compounds and vitamins that are sensitive to damage by heat (vitamin C), were reduced. For example, the LTLT method results showed a significant decrease in L-ascorbic acid and antioxidant capacity ($p<0.05$) (Silva *et al.*, 2019).

(1) The degradation of vitamin C in amla juice (Bansal *et al*., 2015), coconut/lemon/litchi juices (Jayachandran *et al*., 2015), apple juice (Radziejewska-Kubzdela and Biegańska-Marecik, 2015), grapefruit juice (Uckoo *et al*., 2013).

(2) The thermal effect of the LTLT method resulted in a reduction of anthocyanins belonging to the flavonoid group as phytochemicals in pomegranate, berry juice, and Gulab Jamun (Shaheer *et al.,* 2014; Brauch *et al.,* 2016; Pala and Toklucu, 2011).

(3) Peach juice and pindo palm (jelly jelly) contain carotenoids as important pigments. The effect of using the LTLT method significantly reduced the value of carotenoids (Oliveira *et al.,* 2012; Jachna *et al.,* 2016).

(4) The reduction of antioxidant capacity asparagus (Chen *et al.,* 2015), orange juice (Velázquez-Estrada *et al.,* 2013), pear juice (Saeeduddin *et al.,* 2015)

In the same way, LTLT treatment also has a negative effect on other juices, such as color changes. (Andrés *et al*., 2016), coconut/nannari beverage (Kathiravan *et al*., 2014), grapefruit (Uckoo *et al*., 2013), litchi(Guo *et al*., 2011), spinach and sweet lime juice (Khandpur and Gogate, 2015), mango juice (Tribst *et al*., 2011). Moreover, the losses in physicochemical properties in cactus juice (Deboni *et al*., 2014), litchi juice (Guo *et al*., 2011), mango juice (Santhirasegaram *et al*., 2015), and watermelon juice (Liu *et al*., 2012). Finally, the effect of LTLT treatment on the flavor of longan juice and blueberry juice is discussed. (Simunek^ *et al*., 2013)

1.2.5.1 Non-thermal pasteurization by microfiltration

Nowadays, non-thermal technology, such as the membrane process plays important role in the industrial separation process. The main reason for using membrane technology is to avoid heat processing leading to reduce nutrient loss or vitamins are highly sensitive to damage. The advantages of membrane technology, such as energy saving and enzymes can be reduced. On the other hand, membrane filtration requires high-cost, cleaning. At present, trends of non-thermal processing are required. The juice industries have used membrane technology for non-thermal pasteurization and concentration.

The demand is enhanced of functional drinks by adding bioactive ingredients to increase health benefits. For example, microfiltration (MF) is a promising technology because it requires low energy and produces high quality and natural fresh taste. In the same way, processes are simple to scale up and have low energy consumption processes (Laorko *et al*., 2013). Furthermore, the advantages of membrane filtration such as to remove suspended solids, colloids or microorganisms (in the size range of the submicron of the particle) (Bruijn and Bórquez, 2006). Microfiltration (MF) helps to maintain the quality of products. Colantuono *et al.* (2018) reported that mostly, the clarification or sterilization of fruit juices used microfiltration. Pagani *et al*. (2008) studied acerola juice using a ceramic microfiltration membrane presenting permeate flux of $73 Lh^{-1}m^{-2}$. The results showed that after the microfiltration has been no significant difference change in the physical properties of permeate and retentate juices. Laorko *et al*. (2013) reported that the viscosity and turbidity were reduced.

1.2.5.2 Cold sterilization and stabilization of fruit juice by using membrane filtration

Carnerio *et al.* (2002) studied the storage condition of pineapple juice at 8°C for 28 days using 0.3 µm microfiltration. The results showed that microorganisms, viscosity decreased and then did not significantly different in soluble solid content, pH, sugar and acidity in pineapple juice. Similarly, Youn *et al.* (2004) studied apple juice using 0.01 µm membrane filtration. They found that vitamin C, organic acid, and pH of fruit juice not significantly different and also improved color. Laorko *et al*. (2010) compared pore sizes of 0.1 and 0.2 µm for microfiltration and found that a membrane with a pore size of 0.2 µm can effectively preserve bioactive components such as vitamin C, total phenolic as well as antioxidant capacity of clarified pineapple juice. Bruijn *et al.* (2006) studied the shelf life using ultrafiltration on apple juice using MWCO of 15 and 50 kDa. Apple juice was storage at 16°C. They concluded that ultrafiltration in combination with pasteurization improved the efficiency of fruit juice preservation. Rai *et al*. (2008) studied the storage condition of mosambi juice using UF for 30 days. The UF used MWCO 50 kDa. Mosambi juice was stored at 2 conditions (room and refrigerated temperatures) and 2 packages including amber and

transparent vials. The results showed that Mosambi juice was stored at room temperature for 72 hours. On the other hand, amber vials retain the quality of vitamin C better than transparent vials. The decrease in vitamin C for 2 packages for 30 days, including amber and transparent vials was 18% and 20%, respectively.

1.2.5.3 Basic concepts of membrane filtration

The membrane process can separate the particles in the liquid by separating particles by particles larger than the pore size. Membrane filtration uses Darcy's law (Abdelrasoul *et al*., 2013).

$$J = \frac{\Delta P}{\mu R} \qquad (1)$$

Permeate flux (J) represented Permeate flux ($m^3/m^2.s$), ΔP represented transmembrane pressure (Pa), μ represented solution viscosity (Pa-s) and R represented hydrodynamic resistance (1/m)

$$\Delta P = \frac{(P_f + P_c)}{2} - P_p \qquad (2)$$

ΔP represented transmembrane pressure (Pa), P_f represented feed inlet stream (kPa); P_c represented concentrate stream pressure (kPa), and P_p represented permeate pressure (kPa).

The total membrane resistance can be explained as follows:

$$R_t = R_m + R_c \qquad (3)$$

R_m represented the flow resistance through the membrane, defined as the clean membrane resistance combined with the resistances caused by pore blockage, as in Equation

R_m represented the value of the clean and the resistance membrane. R_m represented the pore blocking and the flow resistance.

$$R_m = R_{m\ |c} + R \qquad (4)$$

1.2.6 Bioactive ingredient for functional foods

1.2.6.1 Prebiotic

Prebiotics are non-digestible short-chain carbohydrates but fermentable in the colon. It can improve the growth or activity of native bacteria in the colon which can give health benefits to the host. Prebiotics improved health benefits by enhancing short-chain fatty acid (SCFA) production, reducing the number of pathogenic bacteria populations, and improving host immunity (Slavin, 2013). The candidate of prebiotics must tolerate gastric acid and be unhydrolyzed by a humoral enzyme in the upper gastrointestinal tract. Furthermore, the prebiotic can ferment intestinal microbiota in the colon. It has a good effect on health. It needs to be proven by *in vivo* and *in vitro* tests (Morais *et al*., 2015). Prebiotics are across through the small intestine and utilized by probiotics to produce SCFAs that are used as a source of energy for the host organism to improve the host's health (Al-Sheraji *et al*., 2013).

1.2.6.2 Isomaltooligosaccharide (IMO)

Another interesting prebiotic is isomaltooligosaccharides (IMOs). In Japan, IMO was the first produced, which has been very popular in the form of syrup and powder. The advantages of IMO are sweeteners and low calories and are used for adding fibers to food products. Isomaltooligosaccharides are moderately sweet carbohydrate that occurs naturally. The test of Isomaltooligosaccharides is a little bit sweet. It can be found in fermented foods not only in miso, soy sauce, or sake but also in honey (Playne and Crittenden, 2004). In manufacturing, most IMOs are produced from industrial starch hydrolysates, which starch hydrolysates contain maltose and maltodextrins. In production, it is required α-transglucosidase (Roper and Koch, 1988). Alternatively, pullulanase is used in combination with α-amylase and α-glucosidase. (Tomiyasu *et al*., 2001).

1.2.6.3 Definition and structure of isomaltooligosaccharides

Isomaltooligosaccharides (IMO) consist of three to six glucose molecules linked together with an indigestible glycosidic linkage (α-1,6 glycosidic bonds). The mixture is mainly composed of isomaltose a disaccharide that a similar

structure to maltose, but with an α-(1-6)-linkage instead of the α-(1-4)-linkage, Isomaltotriose is a trisaccharide of α-D- glucosyl- (1→6) α-D-glucosyl (1→6) α-D-glucose. Panose is a trisaccharide of α-D-glucopyranosyl (1→6) α-D-glucopyranosyl (1→4)-D-glucose. IMO is a product that is enzymatically digestion from starch, which present various product such as isomaltotetraose and panose (Kaneko *et al*., 1994). This enzymatic process involves first amylase enzymes for starch liquefaction and the enzyme transglucosidase to convert glucose α-1,4 glycosidic bonds into starch to indigestible glycosidic bonds of α-1,6 in isomaltooligosaccharides chemical structure. Moreover, IMO generates some impurity of free glucose, maltose, and maltotriose. These impurities of such molecules are fermentable sugars that can be removed by yeast fermentation to produce pure forms of IMO with different degrees of glucose polymerizations including panose, isomaltose and some other higher oligosaccharides (Ibrahim, 2018) (Figure 4).

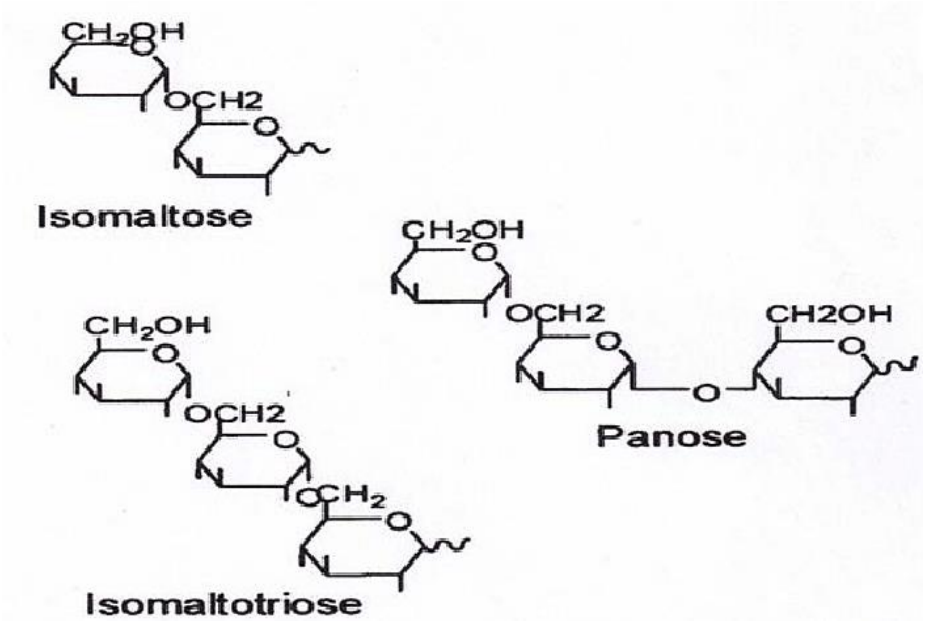

Figure 4. Chemical structure of isomaltooligosaccharides (IMO)

Source: Ibrahim (2018)

1.2.6.4 Isomaltooligosaccharides properties

IMO promotes the growth of vital microorganisms mainly bifidobacterium in the alimentary tracts. IMO quantitatively increases lactobacillus and

enhances the survival rate of bifidobacteria in acidic conditions or bile acid solutions. In addition, IMO decreases the Enterobacteria population. IMO almost entirely passes thought the mucosa epithelium of the small intestine and can act as a substrate for the growth of probiotic microorganisms for produced such as SCFAs. IMOs improve the activity of the immune system by enhancing pathogens resistance and improving the metabolism of lipids. In addition, the functions of the liver and kidneys are driven by the IMO. IMO is prebiotic since it can be digested by an enzyme in the gastrointestinal tract. IMOs is the only one that provided high degrees of polymerization such as isomaltotetraose and larger oligomers are considered indigestible. However, they have been illustrated to have bifidogenic properties in the gastrointestinal tract of a human. IMOs are normally recognized as safe (GRAS) by FDA in the United States of America and are approved by other worldwide regulatory agencies with a maximum daily intake of 30.0 grams per day. Overconsumption of IMO may cause gastrointestinal symptoms mainly flatulence, bloating, soft stool and diarrhea (Ibrahim, 2018).

1.2.6.5 Applications of IMO in food products

Prebiotics such as isomaltooligosaccharide (IMO) is a food ingredient. Generally, IMO is added to foods or drinks. IMO features have sweetness levels of around 60 % of sucrose. At present, IMO is important in daily life for example in foods, beverages, sauces, jams, processed fruits, sweeteners or toppings (Gerschenson, 2017).

1.2.6.6 Health benefits of IMO

(1) Bifidogenic effects

Kaneko *et al*. (1994) studied in vitro culture. They tested the commercial products Isomalto-900, panose, isomaltose and isomaltotriose. The results showed that the group of *Bifidobacterium* spp., such as *B. adolescentis*, *B. longum*, and *B. breve*. They can metabolize sugars also raffinose. In addition, the other groups of bacteria, such as Bacteroides and *Clostridium ramosum* can metabolize isomaltooligosaccharides (IMOs). The property of IMO is high PI values when compared with other prebiotics.

Nakakuki (1993) studied in human (*in vivo*). The results showed that after taking IMO (13.5 g/day) for two weeks for a healthy mature man and 18 elderly people, the numbers of Bifidobacteria increased when checked in feces (108.3 to 109.4 Bifidobacteria/g). As a result, *Clostridium perfringens* and *Enterobacteriaceae* were decreased.

Vinelli *et al.* (2022) studied PI by aggregation bacteria in gram-positive and gram-negative consisting of eubacteria and *E. coli*, respectively. The final products of fermentation were SCFAs and absorption of the substrate. The results showed IMO supplementation promoted *B. longum* growth.

(2) The advantages of bowel function

Goffin *et al.* (2011) studied the effects of IMO including other oligosaccharides on lipid composition and blood glucose for diabetic pigs. IMO did not affect the increased growth performance of pigs. IMO reduced the risk of diarrhea in piglets. The effects of IMO on intestinal function and metabolism were studied. First, seven elderly males are constipated and consumed a low-fiber diet for 30 days. They consumed the supplement with 10 grams of IMO daily. The result showed that the patient had no flatulence or diarrhea. Moreover, the wet mass of feces increased to 70% and 55% of the dry mass of feces (Chen *et al*., 2001).

(3) Effects on immunity

Mizubuchi *et al.* (2005) studied the effects of a diet supplemented with 20% IMO in mice. The result showed the levels of IgA increased. IMOs promotes the immune system of mice by increasing the activity of T cell receptor in the small intestine. Moreover, lactobacillus and immunoglobulin type A higher in mice's feces samples treated with 20 percent of IMO for 4 weeks compared with control treatment ($p<0.05$).

(4) Anti-cariogenic properties

Additionally, IMOs improve cell mechanisms to against cancer cells. The monomer of IMOs has low acidity compared with glucose and sucrose performed in vitro experiments using *Streptococcus mutant* (Moynihan, 1998). Goffin

et al. (2011) reported that a mixture of rich-IMOs and panose can produce acid similar to glucose and sucrose. Whereas the *S. mutans* and *S. sobrinus* did not react with the mixture. Therefore, glucan did not produce by *S. murans* and *S. sobrinus* indicating that the bacterium significantly inhibits the synthesis of sucrose.

1.2.7 Micronutrients

1.2.7.1 Calcium and vitamin D supplementation

For health, calcium and phosphate are key raw materials for the laying down of bone. Calcium is an important mineral for the body's functions such as the nervous system, muscles, and blood coagulation. Calcium is important, for example, to maintain a constant calcium concentration. Moreover, the benefits of calcium are related to bone and bone mineral density.

Fat-soluble vitamin (Vitamin D) and complex organic molecule. Vitamin D is obtained from cholesterol. Normally, humans are exposed to ultraviolet light from the sun. It affects the stimulation of the subcutaneous fat. The important role of vitamin D is to regulate and maintain blood calcium levels. Therefore, the stimulus must be in homeostasis. The main functions of vitamin D are to increase the absorption of calcium in the duodenum and stimulate osteoblastic bone resorption. In addition, phosphate is absorbed in the intestines by activated vitamin D. Both hypercalcemia and hyperphosphatemia are prevented by regulatory systems exist. Thus, these diseases may be effective in soft tissue calcification (Reid and Bolland, 2020).

1.2.7.2 Effects of calcium and vitamin D supplements

Mostly, the main function of vitamin D is to maintain the calcium balance in the body. Acquisition of vitamin D in humans can get through sunlight and diet. First, vitamin D is converted to 25-hydroxyvitamin D (25OHD) in the liver after that hydroxylated to active metabolite 1,25 dihydroxyvitamin D [1,25(OH)2D, or calcitriol] in the kidney. The decrease in calcium helps to stimulate parathyroid hormone (PTH). The major factors linked to blood calcium regulation are parathyroid hormone (PTH) and calcitriol. Calcitriol is a form of vitamin that is absorbed by

calcium from food or supplements. The hormone involved in calcium absorption is fibroblast growth factor 23 (FGF23). The FGF23 hormone effects calcium production by osteocytes.

Calcium absorption occurs in the passive process of transport and then in the active transport system. Generally, 1,25(OH)2D motivates and absorbs calcium. Many factors may affect calcium absorption and 1,25(OH)2D production by the kidney. For the elder, menopause is cause osteoporosis (lack of calcium) and decreases 1,25(OH)2D levels. The production of 1,25(OH)2D in the kidney negatively correlated with the age of people. Moreover, the kidney appertained the decline in renal function. (Silva and Bilezikian, 2015).

1.2.7.3 Mechanism of calcium and vitamin D_3

Vitamin D is usually of hormones more than vitamins. Normally UV is the main factor for producing vitamin D in the skin. 1,25-dihydroxyvitamin D [1,25(OH)2D] is important for the body. It can increase calcium absorption in the intestine. Thus, the main active metabolites and hormones are 1,25-dihydroxy vitamin D [1,25(OH)2D] and parathyroid hormone (PTH) help to maintain calcium homeostasis between bones and blood. Finally, the importance of 5-hydroxyvitamin D [25(OH)D] is circulating metabolites (Figure. 5).

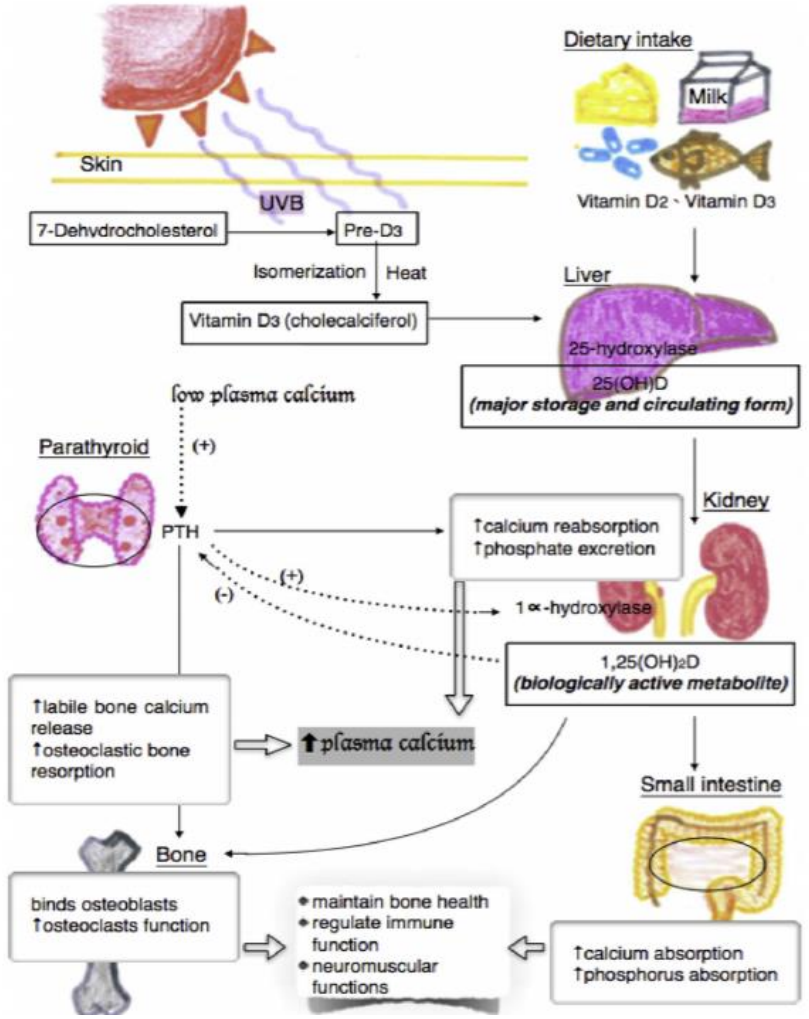

Figure. 5 Depicts the vitamin D synthesis mechanism, where D (25(OH)D) represents 25-hydroxyvitamin D, (1,25(OH)2D) represents 1,25-dihydroxyvitamin D, and PTH represents parathyroid hormone.

Source: Chapron *et al*. (2004)

1.2.8 Probiotic

There are more than 1000 species of microorganisms in the human body. Bedani *et al*. (2015) reported the advantages of gut microbiota for humans, such as the production or synthesis of useful vitamins. Moreover, the advantages of probiotics can help in the development of intestinal tissue, the immune system, reduce carcinogenesis, energy source for the host. Probiotics can inhibit pathogenic microorganisms that adversely affect the body.

Generally, probiotics known as lactobacilli and bifidobacterial should be safe for human consumption due to their mechanism. The important probiotics mechanisms are resistance to colonization, produce SCFAs (short-chain fatty acids), competitive when compare with pathogenic microorganisms and can control of the gastrointestinal transit in the body (Olveira and González-Molero, 2016). Anadon *et al*. (2016) reported that probiotics are live microorganisms such as LAB (lactic acid bacteria), and yeast. Probiotics effect to health benefits. Therefore, it can improve the health of the host when ingested in enough amounts.

1.2.9 Functional juice

A few years ago, there was an increasing focus on food and beverage's critical role in preventing and treating disease. Therefore, the production and consumption of healthy food are very important. Nowadays, beverages and healthy food are more in demand in the market. Healthy fruit juices are highly popular because they are easy to drink. There are many beneficial nutrients such as minerals, vitamins, prebiotics, probiotics, etc. Therefore, consumers are more aware of nutrition and health. Production of juices fortified with prebiotics and vitamins. Therefore, it is the demand for health trends. Functional drinks are increasingly popular all over the world. as shown in Table 3.

Table 3. Examples of commercially available fruit beverages

Brand	Producer	Active compounds
Vita Biosa	Biola Inc., Canada	Antioxidants and Probiotics
Goodbelly	NextFoods, U.S.A.	Probiotics
Gefilus	Valio Ltd., Finland	L-ascorbic acid, vitamin D and probiotics
Proviva	Skane Dairy, Sweden	Probiotics
Tropicana Farmstand	Tropicana, U.S.A	Vitamins A, L-ascorbic acid potassium
Tomato Juice Plus	Langer Juice Co., Inc., U.S.A	Vitamins and minerals
Daily Greens	Bolthouse Farms, U.S.A	Vitamins A, vitamin C, manganese, iron, and zinc

Source: Gurakan *et al.* (2010); Soccol *et al.* (2012)

1.2.9.1 Functional food claim

Functional food claim means recommending. Functional food claims include foods that provide and do not provide energy, such as carbohydrates, proteins, fats, vitamins, and minerals. Except for the following: identification in the ingredient list and substances are mandatory on nutrition labels.

Functional food claim is an indication of the level of nutrients added and contained in food or beverages.

(Examples: “source of calcium”; “high in fiber and low in fat”.)

(1) Comparing or claiming the level of two or more nutrients by using the words "reduced," "less than," "fewer," "increased," and "more than."

(2) Non-additional claims are ingredients or nutrients that consumers expect to find in food. No added ingredients.

The functional claim is to identify or show the relationship between food and health. as shown in Table 4. The daily recommended nutrient intake is shown in Table 5.

Table 4. Conditions for nutrient content claims

Component	Claim	Conditions (not more than)
Vitamins and Minerals Dietary Fiber Protein	Rich in, High, Excellent source of	≥20% of Thai RDI
	Increased, More, Added, Fortified, Enriched	≥10% of Thai RDI

Source: Food Innovation and Regulation Network (FIRN)

Table 5. (Thai Recommended Daily Intakes-Thai RDI)

Nutrient	Thai RDI	Unit
Vitamin D	5	µg
Vitamin E	10	Mg
Vitamin K	80	µg
Calcium	800	Mg
Phosphorus	800	Mg
Iron	15	Mg
Iodine	150	µg
Magnesium	350	Mg
Zinc	15	Mg
Copper	2	Mg
Potassium	3,500	Mg
Sodium	2,000	Mg
Manganese	3.5	Mg
Selenium	70	µg

Source: Notification of the Ministry of Public Health (No. 182) 1998

1.2.9.2 Non-thermally pasteurized functional mulberry juice

For the reason, functional drinks are popular. Consumers are more concerned with the benefits of food. Therefore, healthy foods and beverages supplemented with vitamins or minerals are acceptable. The main reason is that it can reduce the time spent eating fruits and vegetables. Thus, the fruit juice market is in high demand. Currently, the fruit juice trend is filled with vitamins, minerals, bioactive compounds, prebiotics and phytochemicals, such as β-carotene (beta-carotene), lutein, vitamin C and vitamin E (Putnik *et al*., 2020).

Research questions

1. Does microfiltration able to produce functional mulberry juice with minimum deterioration of heat-sensitive bioactive components?
2. Does IMO and bioactive substances in juice increase the prebiotic property of functional mulberry juice?

Hypothesis

1. Microfiltration can preserve heat-sensitive bioactive components in functional mulberry juice.
2. Supplementation with IMO and bioactive substances in juice improve the prebiotic property of functional mulberry juice.

1.3 Objectives

1. To produce non-thermally pasteurized functional-mulberry juices fortified with vitamins, minerals, and prebiotic using microfiltration

2. To study phytochemical, physical and chemical properties of non-thermally pasteurized functional mulberry juices processed by microfiltration

3. To evaluate the prebiotic properties of the developed functional mulberry juices

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CHAPTER 2

PRODUCTION OF NON-THERMALLY PASTERIZED FUNCTIONAL MULBERRY JUICE BY MICROFILTRATION AND ITS PHYTOCHEMICAL AND PHYSICAL PROPERTIES

2.1 Abstract

Microfiltration (MF), a promising non-thermal processing technique has been employed for fruit juice processing. This study aimed to produce functional mulberry juice using MF and investigate its quality changes including antioxidant capacity (2,2-diphenyl-1-picrylhydrazyl: DPPH, (Ferric reducing antioxidant power: FRAP), vitamin C, total phenolic content and physical-chemical properties (color, pH, viscosity, total soluble solid) of clarified (non-thermally pasteurized) and concentrated mulberry juices with different formulations of isomaltooligosaccharide, calcium lactate and vitamin D_3. The results indicated that MF did not have significant effects on the pH, reducing sugar and acidity of clarified and concentrate juices. It could preserve vitamin C and total phenolic content providing juice with high antioxidant capacity.

2.2 Introduction

Mulberry fruit (*Morus nigra*) is a fruit that is beneficial to the body. It is popular to eat around the world. Mulberry fruit contains beneficial nutrients and vitamins such as vitamin C, anthocyanins, flavonoids, minerals, and essential fatty acids (Bae and Suh, 2007). Mulberries have the advantage of aiding in hyperglycemic and anti-inflammatory conditions (Choi *et al*., 2016). Tchabo *et al*. (2018) reported that mulberry has many benefits for humans because of the presence of various bioactive compounds. Bioactive compounds such as organic acids, polyphenols, and particularly anthocyanin are major components of interest in producing healthy drinks or functional drinks. Sripakdee *et al*. (2017) reported that anthocyanin is important for health. Anthocyanin is present in the fruit. Mulberries are high in anthocyanins when ripe. Moreover, the main benefit of anthocyanin is its antioxidant properties. In mulberry,

there are many antioxidants such as anthocyanins (e.g., cyanidin and pelargonidin glycosides), flavonoids, and phenolic acids considered natural antioxidants in the body (Peng *et al*., 2011; Mikulic-Petkovsek *et al.*, 2012). Generally, anthocyanin is a pigment with a purple or red color that can be found in most fruits and vegetables. Kamiloglu *et al*. (2013) studied in mulberry analyzed the anthocyanin content in mulberry fruit. The results showed that mulberry contains the highest amounts of cyanidin-3-O-glucoside (C3G) and cyanidin-3-O-rutinoside (C3R) (>98%). Moreover, they also found many more anthocyanins.

Extend product shelf life in fruit juices or other products is mostly by using heat to extend shelf life, especially the concentration of fruit juice. Although heat processing is popular however the disadvantages of this technology are deterioration of nutritional value, color and taste. In particular, vitamin C and anthocyanins can be degraded (Toribio *et al*., 1986; Kato *et al*., 2003). In the same way, Lin *et al.* (2002) reported that thermal processes such as pasteurization or high-temperature evaporation affect degradation of vitamins in fruit juice.

In addition, thermal processing involves a high amount of energy to heat. The thermal process impact heat-sensitive compounds such as vitamin C and organoleptic attributes (Flores *et al.*, 2015). In general, heat treatment should be avoided for non-heat-tolerant nutrients such as vitamin C. The temperature above 50°C negatively affects the bioactive compounds, vitamins, and minerals in fruit juice (Shaw *et al*., 2001; Vaillant *et al*., 2005). Similarly, Vieira *et al.* (2016) studied the effect of heat on ascorbic acid and anthocyanin, which are important substances in mulberry, and consumers are aware of this. Therefore, heat treatment at temperatures of higher than 60 °C negatively affect the quality of the product (Sarkis *et al*., 2013).

Nowadays, functional drinks are very popular. As consumers become more health-conscious, technologies that help preserve nutrition are becoming viable alternatives, such as membrane technology, especially microfiltration (MF). Recently, membrane technology has been widely used in the beverage industry, such as the drinking water industry and the fruit juice industry. The key reason for the use of membrane technology is that both clear and concentrated parts can be produced. The

membrane is a technology that is easy to use, convenient, and energy-saving. (Nunes *et al*., 2001).

At present, membrane technology in various configulations and synthetic membranes are used for filtration, consisting of hollow fibers, spiral wounded, or tubular. Generally, the main function of the membrane is to clarify and concentrate the juice (Conidi *et al*., 2020). Microfiltration It's an easy-to-use technology. It is convenient and able to get a product with physical characteristics, chemistry, and nutrients that remain close to fresh. In addition, the membrane filtration principle will filter particles and large solids. The part that can pass through the membrane must have particles smaller than the membrane pore size. The membrane can filter out large solids such as suspended solids, pectin, starch, and some bacteria. Ribeiro *et al*. (2018) studied the storage condition of umbu juice at 6°C for 3 months using 0.2 µm ceramic tubular membranes and TMP 3.5 bar. They concluded that microfiltration maintained the quality of the juice. Phenolic compounds were not significantly different after 3 months of storage. Moreover, the microbiological evaluation found that salmonella, coliforms, mold, and yeast were not increased. Mahnot *et al.* (2019) studied the storage condition of coconut water using 0.45 µm microfiltration at refrigerated temperatures for 190 days. The results showed that sensory evaluation is acceptable in 30 days at refrigerated temperatures for non-MF while MF is acceptable for 90 days at refrigerated temperatures. On the other hand the effect of citric acid and vitamin C is accepted within 115 days. In the fruit juice industry before starting the process. They are treated by enzymatic treatment. The enzymatic treatment has many advantages, such as making the fruit clear and reducing the cloudiness of the juice. Pectinase is generally used to make the juice clear. Carvalho and Silva (2010) studied pineapple juice with 100 ppm of commercial pectinase from Novozymes for 30 minutes and the temperature at 40 °C. They used a tubular polyethersulfone membrane of 0.3 µm and then clarified by microfiltration. The results showed 57.7 L/m^2 /hours (1.5 bar) of permeate fluxes and the viscosity decreased when treated with pectinase. Oliveira *et al*. (2012) compared two membranes, including tubular ceramic and hollow fiber membranes. Transmembrane pressure (TMP) of 0.5 and 1.0 bar, respectively. They used microfiltration for clarified passion fruit juice and then treated it with 150 ppm of

enzymatic treatment. The result showed no significant difference in physical and chemical analyses. Marchese *et al*. (2011) studied the effects of a 0.2 µm microfiltration system on orange juice. MF can separate all total solid particles using filtration by allowing the liquid to flow through. They studied the effects of using membrane technology to compare physicochemical properties before and after microfiltration. Cisse *et al.* (2005) studied the transformation of juice compounds and vitamin C in juice using microfiltration. The result showed that microfiltration was able to maintain the properties of juice vitamin C. The purpose of microfiltration step was to clarify and pasteurize juice. In this study, mulberry juice was filtered using 0.2 µm polysulfone hollow fiber membrane and phytochemical, physical and chemical properties of the delivered products were also studied.

2.3 Materials and methods

2.3.1 Raw material and chemical agent enzyme

1. Ripe mulberry fruits of the variety (*Morus nigra* L.). Mulberry fruits collected from Phichit Sub-district, Na Mom District, Songkhla Province Hatyai, Thailand.

Figure 6. Mulberry fruits (*Morus nigra* L., Moraceae)

2. 63.84% isomaltooligosaccharide (IMOs) concentration was obtained from the previous study from the Faculty of Agro-Industry Prince of Songkla University in Hatyai, Songkhla, Thailand.

3. Food-grade (calcium lactate powder was supplied by Bulksupplements company and vitamin D3 was supplied by ADINOP company.

4. Juice extractor machine stainless steel used WFA3000 Juicer machine (United States). Juicer machine is used to extract mulberry juice and separated the waste.

Figure 7. Juice extractor machine stainless steel

5. Microfiltration

For the microfiltration used, the experimenters chose a hollow fiber membrane and a membrane pore size of 0.2 microns (Amersham, Biosciences, UK). The code number and model number are 56-4102-48 and CFP-2E-5A, respectively. This membrane length is 30 cm long and 1 mm in diameter shown in Figure 8. The material used for this membrane is polysulfone. Components of the microfiltration include a pump, the jacket-feed tank (8 liters), transducers, and water circulating cooling (MBS 3000, Danfoss, Denmark). The laminar flow cabinet consists of HEPA (High efficiency particulate air filter) with 0.1375 m^2 filtration area and 0.3 µm pore size. The air that exits the HEPA filter is clean and unidirectional air stream. The schematic of the membrane experimental set-up is shown in Figure 9.

Figure 8. Hollow fiber module

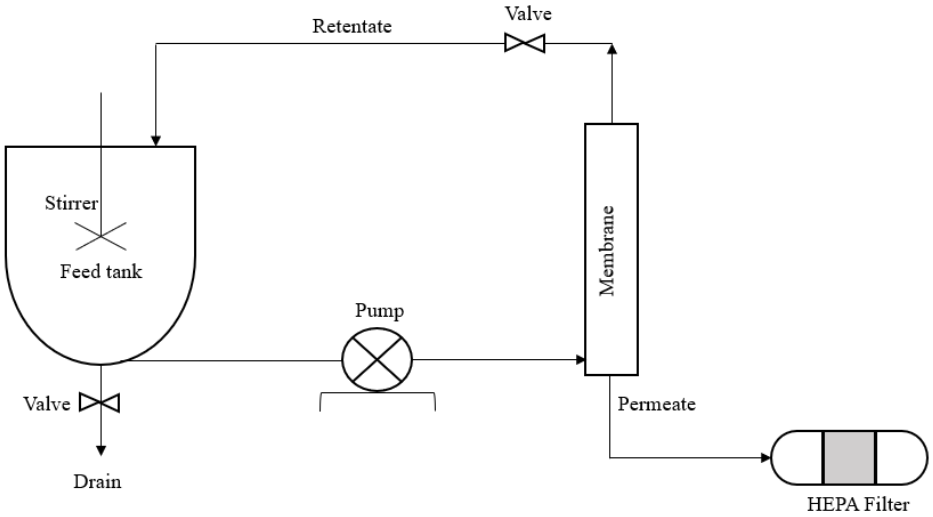

Figure 9. Schematic of the membrane experimental set-up

Chemicals

1. Alkaline salt (sodium carbonate; Na_2CO_3) is food grade.

2. Solutions: Sodium carbonate (Na_2CO_3), potassium persulfate($K_2S_2O_8$), sodium Acetate($CH_3COONa \cdot 3H_2O$), lead(II) acetate ($Pb(CH_3COO)_2$), potassium oxalate($C_2K_2O_4$), gallic acid, Trolox, DPPH, Folin-Ciocalteau, Fehling's solution (A and B) and D-glucose standard solution.

3. Sodium hydroxide (NaOH), hydrochloric acid (HCl), ethanol (C_2H_5OH), acetic acid ($C_2H_4O_2$), potassium hydroxide solution (KOH), CH_3COONa -KCl buffer, Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$).

4. Commercial pectinase (Pectinex® Ultra SP-L).

2.3.2 Preparation of mulberry juice

20 kg of ripe mulberries were washed with tap water and soaked and then drained. After that mulberry fruits were squeezed by a juice extractor machine stainless steel juicer heavy duty. The item model number is WF-A3000 (Weifeng, China). The percent yield of mulberry juice was 81.75% and got 3.65 kg of waste. The 16.35 L of fresh mulberry juice was stored at -20°C before use. The total soluble solids and pH of the mulberry juice were 7.00 ºBrix and 3.31-3.35, respectively. Maintaining the quality of fresh mulberry juice before entering microfiltration, the fresh mulberry juice was kept at 4ºC. It should be used up within 3 days. Then, the mulberry juice was treated by commercial pectinase 0.1% (V/V) of commercial pectinase (MB(EN)) at room temperature (25±3°C) for 60 min (Carneiro *et al*., 2002) is shown in Figure 10. The purpose is to enhance filtration performance, fruit juice is usually treated before filtration with the enzyme. Enzyme breakdown of the pectin in the juice, has changed the solubility and precipitation properties of pectin, thus reduce the pore blocking of the membrane surface and reduce the viscosity (Domingues *et al.*, 2014).

2.3.3 Formulation of functional mulberry juice

Benefits of mulberry fruit for medical treatment. Mulberry fruit has many bioactive compounds, such as flavonols, phenolic acids and other vitamins and minerals. Moreover, mulberry fruit has 2 major anthocyanins include, cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R).

Health benefits of mulberries, such as anti-diabetic, anti-inflammatory and anti-aging, anti-cancer and then antioxidant. Furthermore, decrease the risk of diabetes and obesity. Mulberry fruit is one of the important antioxidants. Mulberry fruit has 3000 mg/kg FW of anthocyanins. In addition, mulberry consists of phenolic compounds and stimulates macrophages with alkaloids.

Mulberry fruits are a high level of anthocyanin content than blueberry and blackberry. On the other hand, the disadvantage of mulberry fruits is their very short shelf-life and softness. The important vitamins in mulberry are vitamin C and vitamin

K, which are present in high amounts. The main functions of vitamin C include boosting the immune system, collagen production, and antioxidants.

The benefits of vitamin K are supporting bone tissue and essential component for blood clotting in the body. Although mulberries are many vitamins and minerals, lack vitamin D and calcium is not enough in the human body per day. Normally, vitamin D is important for health but gets hard from foods. Consequently, fortification of juices or added calcium for children who do not drink milk.

Normally, vitamin D is more important for females than males because the estrogen hormone rapidly decreases in old women (menopause). So, elderly people should consume foods with a high vitamin D content. Normally, the problem of decreased bone mass is an effect in males over 70. The population obtains both calcium and vitamin D to reduce fractures. Thus, the EAR and RDA values of vitamin D are not different from that of adults aged 19-50 years.

For this reason, elders should be consumed vitamin D of 400 IU (EAR) and 600 IU (RDA). Women who have aged 51-70 years old are being to lose bone mass faster than men, which begins at menopause (around 50 years of age). Increasing the risk of osteoporosis and its complications. However, the bone mass in both men and women is lost when they when reaching the age of 70. Moreover, the absorption of calcium in the small intestine is decreased, since the process involved in membrane transporter, is reduced. Thus, the rate of calcium absorption is vitally necessary. To allow calcium to move through the epithelium membrane of the small intestine throughout a process that does not require energy called passive transport to occur more for education as is available in Thailand. Calcium replacement was administered in postmenopausal. In the experiment, they compared women over 60 years obtained from food and supplements for 2 years and the group without supplements (control). The result showed total calcium doses of 813 mg/day (treated with supplement) and 297 mg/day (control). Bone density or bone mineral density (BMD) of 1.90 and 0.23 percent, respectively.

There is no data on calcium supplementation in males or calcium to prevent fractures in both men and women. From all the data, it can be assumed that the daily calcium intake in adults over 50 years is equal to 1,000 mg for both men and women.

Therefore, 25-hydroxyvitamin D [25(OH)D] and calcium lactate fortified in functional mulberry juice with 1.5, 5 µg and 80, 180 mg for elders. The products were claimed for two levels as shown in **Table 6**.

Table 6. Body weight median average vitamin D requirements and reference values for vitamin D requirements in the Thai population of different ages

	Ages	Adequate Intake (AI) International Unit (IU)	Estimated Average Requirement (EAR) International Unit (IU)	Recommended Dietary Allowance (RDA) International Unit (IU)
Vitamin D	51-70 y	-	400	600
Calcium	≥ 51 y	-	800	1000

Source: Bureau of Nutrition (2020)

Table 7. Table of conditions for nutrient content claims

Component	**Claim**	**Conditions (not more than)**
Vitamins and Minerals Dietary Fiber Protein	Rich in, High, Excellent source of	≥20% of Thai RDI
	Increased, More, Added, Fortified, Enriched	≥10% of Thai RDI

Source: Food Innovation and Regulation Network (FIRN)

Table 8. Formula treatments of Mulberry juice

Formulas	**Mulberry juice (ml)**	**IMO (ml)**	**Calcium (mg)**	**Vitamin D (µg)**
1	3,000	-	-	-
2 (added)	3,000	(2%) 60	80	1.5
3 (high)	3,000	(8%) 240	180	5

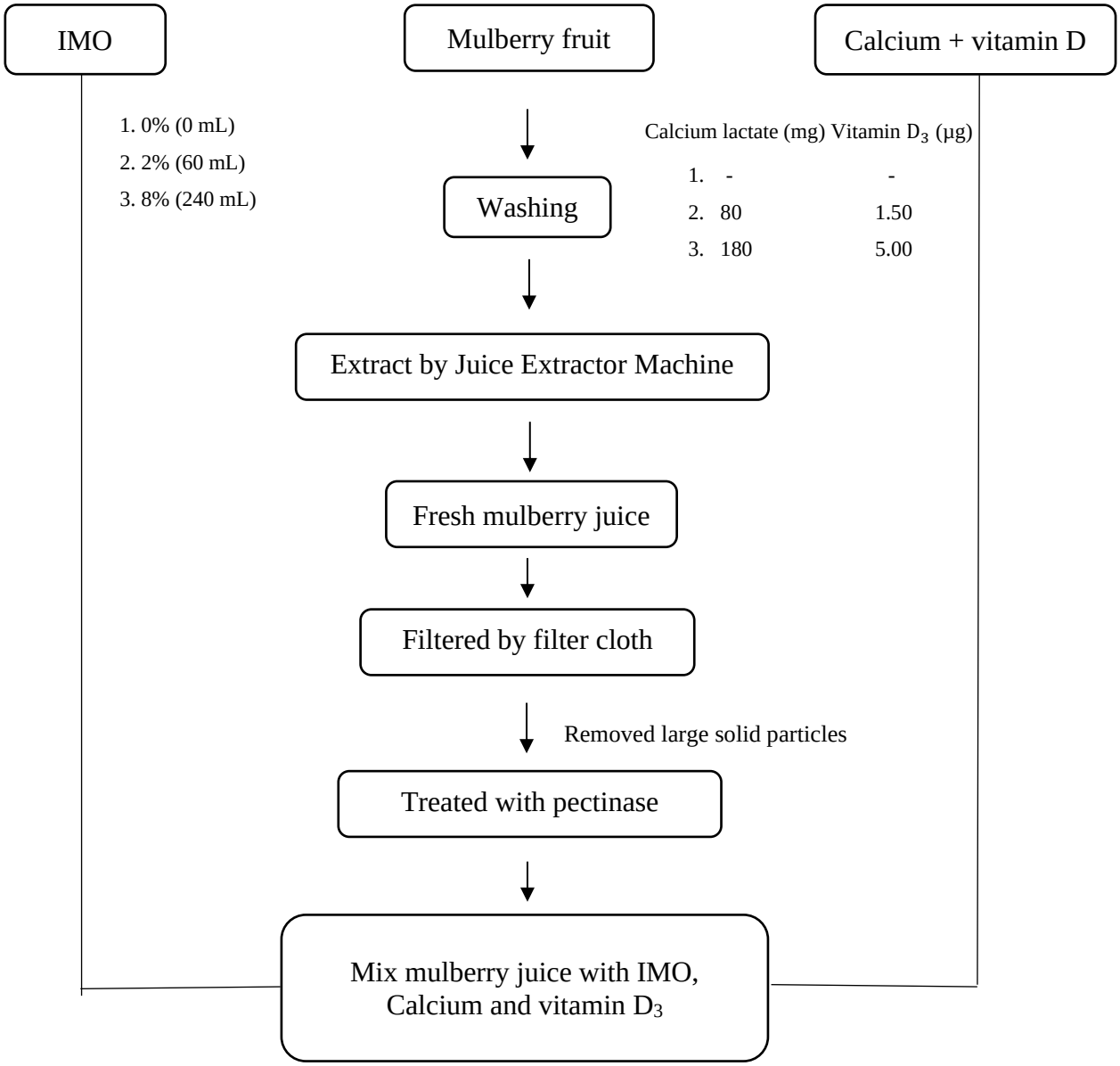

Figure 10. Mulberry juice preparation

2.3.4 Non-thermally pasteurized juice by microfiltration

The experiment chose a hollow fiber membrane and a membrane pore size of 0.2 microns (Amersham, Biosciences, UK). Components of the microfiltration include a pump, the jacket-feed tank (8 liters), transducers, and water circulating cooling (MBS 3000, Danfoss, Denmark). The MF system has two products: permeate and retentate. The temperature of the jacket-feed tank is controlled using a

water-circulating cooling bath. Before the membrane process, flash with clean water to remove stains. The second is to circulate with 0.5 N NaOH at 50°C for 1 hour and then flash with clean water. The next step, circulating with NaOCl was used to sterilize the system at 50°C for 1 hour. Finally, the membrane was rinsed with clean water before starting the filtration process (Laorko *et al*., 2010). The system was carried out by the retentate return to the feed tank. After that, the clarified mulberry juice was filled into sterile bottles. Note than, glass bottles were sterilized using a hot air oven at 180 °C for at least 3 hours before being used. Microfiltration was operated 20 °C, TMP was regulated at 0.8 bar and CFV of 1.0 m/s. In the filling process, mulberry juice is placed in a glass bottle in a laminar flow cabinet. It must be sprayed with 70% alcohol spray and exposed to UV light (UV-c 254 nm and the intensity at 76 µm/cm^2overnight before use. The laminar flow cabinet consists of HEPA (High efficiency particulate air filter) with 0.1375 m^2 filtration area and 0.3 µm pore size. The air that exits the HEPA filter is clean and unidirectional air stream.

After filtration of mulberry juice, the membrane was cleaned by membrane manufacturer methods to remove fouling and clean the membrane.

2.3.5 Mulberry juice analysis

In the mulberry sample, MB(F)), mulberry juice obtained by microfiltration consisting of 0% IMO mulberry juice (MBI0), 2% IMO mulberry juice (MBI2), and 8% IMO mulberry juice (MBI8) were all analyzed. The experiment analyzed the physical, chemical, and phytochemical properties of all samples for comparison before and after entering the MF. Importantly, analysis time should be taken within one month because long-term storage can affect the phytochemicals and nutrients in mulberry, such as L-ascorbic acid in the juice, which decreased during the first month of storage at 4 ºC.

The pH is measured using a pH meter. (Hanna, USA).

The total soluble solid (TSS) were measured using a hand refractometer (ATAGO, Japan).

Viscosity was analyzed by a U-Tube viscometer (Kapillarviskosimeter 50904, Schott, Germany) while the sample temperature was about 20 ºC.

Color analysis

The samples were analyzed for color values using a colorimeter. The analysis principle is based on the CIE Lab scale (L*, a*, b*) and Hunter Lab, which are popular systems for measuring color in samples. It consists of the hue, the valve, and the chroma. In the fundamentals of color reading, the L* represented brightness ranges from 0-100 (-L* is black and +L* is white). a* represented red to green (-a* is the color green and +a* is red) and b* represented blue to yellow (-b* is blue and +b* is yellow) using Eqs (Diamante, 2010):

$$\Delta E = (\Delta L^{*^2} + \Delta a^{*^2} + \Delta b^{*^2})^{1/2}$$

ΔE represented a value used to measure the difference between the initial color value and the final color value of the sample.

$$\text{Chroma} = (a^{*^2} + b^{*^2})^{1/2}$$

a* and b* represented the color values of the juice.

Total phenolics analysis

The total phenolic was carried out using a protocol of Sripakdee *et al.*, (2015). For this experiment, phenolic content was analyzed using the main chemical reagent, Folin–Ciocâlteu reagent (FCR). The phenolic solution was diluted and transferred into a test tube. The diluted phenolic compound was mixed with 2.5 mL of 0% (v/v) FCR and mixed for 3 min. Added 0.5 mL of saturated sodium carbonate (7%, w/v). The sample was approved to equilibrate for 2 hours. Subsequently, the sample was determined by the concentration by UV-Vis spectrophotometer (GENESYS 10S UV-VIS) at 765 nm. The reported phenolic content is expressed in mg GAE/100 ml. The phenolic content obtained must be compared with the standard gallic acid solution.

Antioxidants analysis

- Antioxidant capacity (DPPH method)

The sample was diluted with ethanol to be 0, 0.1, 0.2, 0.4 and 0.8 mg/ml. The sample was transferred into a 3 mL pf DPPH solution and mixed. The sample was

placed in a dark condition for 0 and 30 min. The inhibition percent of DPPH was calculated according to the following equation. For the determination of antioxidant contents, 0.0017 grams of 2,2-Diphenyl-1- picrylhydrazyl was dissolved with 5 mL of methanol in a volumetric flask. The concentration of the substance is 0.862 mM. Then, prepare the extracted samples dissolved in methanol 250 µL sample and dissolved in 750 µL methanol (total volume of 1 mL) and prepare 4 Eppendorf tubes. And prepare 62.5 µL sample extraction dilutes with 250 µL DPPH solution. Then pipette into the 96-well plate. After that, it was measured at a wavelength of 517 nm using a microplate reader.

$$DPPH^{+} = \frac{(\mathrm{Abs}_{t=0} - \mathrm{Abs}_{t=30}) \times 100}{\mathrm{Abs}_{t=0}}$$

- Antioxidant capacity (DPPH method)

First, the standard graph was prepared using 50-900 µM of Trolox solution (prepared from 1000 µM concentrated solution diluted with methanol). The FRAP solution was added to the 285 µL sample tube and the FRAP solution was then placed in the dark condition for 30 minutes. The samples were carried out at wavelength 593 nm (Miller and Rice-Evans, 1997).

L-ascorbic analysis

L-ascorbic 0.5 g was dissolved in 1% metaphosphoric acid and adjusted by a 100 ml volumetric flask. The solution was transferred in five flasks, which have 5 different volumes in each flask (0, 1, 2, 3, 4 and 5 ml). Subsequently, 1% metaphosphoric acid was used to adjust the volume up to 100ml in the all of flask and added 9 ml of 2,6-dichlorophenolindophenol. Sample was measured by spectrophotometer at wavelength 515 nm within 30 s. Finally, results are reported in mg of L-ascorbic per mL of juice obtained from a standard curve (Sripakdee *et al*., 2015).

Total soluble solid analysis

The three formulas of mulberry juice were analyzed for total soluble solids (TSS) by an instrumented hand refractometer (Atago, Japan) and then read the total amount of total dissolved solids in Brix (AOAC, 2000).

Analysis of calcium

Functional mulberry juices from microfiltration were collected to analyze calcium by Central Laboratory (Thailand) Co., Ltd. Functional mulberry juices (MBI2 and MBI8) were determined using Inductively coupled plasma atomic emission spectroscopy (ICP-AES) by AOAC (2019) 984.27.

Analysis of cholecalciferol (vitamin D_3)

Functional mulberry juices from microfiltration were collected to analyze calcium by Central Laboratory (Thailand) Co., Ltd. MBI2 and MBI8 were determined by chromatography method (López-Cervantes *et al*., 2006).

Analysis of total anthocyanin

In this experiment, the three formulas of mulberry juice were analyzed using the pH differential method. The instrument used for the analysis was a spectrophotometer (GENESYS 10S UV-VIS). Concisely, the experiment used two buffer solutions: 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5). After that, 1 ml of the extracted sample was taken and then adjusted to a volume of 10 ml. After that, the absorbance was determined at a wavelength of 510 nm, and 700 nm. In this experiment, distilled water was used as a blank. The results must be reported as (mg cyanidin-3-glucoside/100 mL). Then, take three formulas of mulberry extract samples, each 0.25 mL and added 2.25 mL of buffer solution at pH 1 and 4.5 before measuring absorbance values at 510 and 700 nm (Rapisarda *et al*., 2000).

Calculation of total anthocyanin contents (Laokuldilok and Kanha, 2015):

$$\text{Total anthocyanin contents} = \frac{A \times MW \times DF \times 1000}{€ \times 1}$$

A = (A510-A700) pH 1.0 - (A510-A700) pH 4.5

MW represented the molecular weight of cyanidin-3-glucoside (C3G) is 449.2 g/mol

DF represented dilution factor

€ represented molar absorptivity equal 26,900 l/mol x cm

Reducing sugars

AR grade glucose was weighed about 4.75 g. After that, the glucose volume was adjusted to 500 ml and the pH indicator used was the phenolphthalein indicator. Sample was neutralized by HCl orpH NaOH. The final step is to adjust to 100 ml using a volumetric flask. Sugar content determination can be performed by titration. Compared with Fehling's solution, the equation:

Calculations:

$$\text{Titration} = V1 = x \text{ ml}$$

$$\text{Factor for Fehling's solution (grams of invert sugar)} = \frac{\text{Titration (ml)} \times 2.5}{1000}$$

$$= 0.0025 \times V1 = x \text{ grams}$$

- Determination of reducing sugars

Preparation of sample

The sample was weighed 10 ml and homogenized sample. The 500 ml of sample was transferred to a volumetric flask. 100 ml of water was added and neutralized the sample by adding NaOH solution. The indicator used to indicate the end point is phenolphthalein. 10 ml of neutral lead acetate solution was added and mixed and incubated at room temperature for 10 min. In the next step, add a small amount of potassium oxalate solution and mix the solution. It is then filtered with filter paper No. 1 (Whatman® filter papers). The filtrate was transferred to a 50 ml burette having an off-set tip.

Preliminary titration

Fehling A and B solutions were transferred into a 250 ml conical flask. The experiment used glass beads to prevent excessive boiling. After that, boil the solution. In the experiment, the substance Methylene blue, which is an indicator, drops a few drops and observes until it reaches a brick-red endpoint. Finally, when the experiment is complete, record the titration values for use in calculating the sugar content.

Final titration

In the quantitative titration reducing sugar, Fehling A and B solutions were mixed into an Erlenmeyer flask containing glass beads. The next step is to use a hot plate to heat the Fehling A and B solutions. The titration is performed when 1 mL of the sample is added and titrated to a brick-red color. The endpoint is reached within 1 minute. Finally, when the experiment is complete, record the titration values for use in calculating the sugar content.

Calculations:

$$\text{Reducing sugars (\%)} = \frac{0.25 \times V1 \times V2}{V3 \times W} = x\ \%$$

Results

Reducing sugars (as invert sugar) = % by wt.

Total sugar

Pipette an aliquot of 50 ml of the clarified filtrate to a 100 ml volumetric flask. After that, add the concentrated HCl and leave overnight before adding the 0.1N NaOH concentrate to neutralize the pH. In the experiment, they used phenolphthalein as an indicator. In the titration, the solution is poured into a 50 mL burette and titrated, doing the same for reducing sugar.

Calculations :

Based on the factor for Fehling's solution:

$$V4\ mL = 0.0025 \times V1\ g$$

Therefore, $$\%\ \text{Total reducing sugars} = \frac{0.0025\ x\ V1x\ 2\ x\ V2\ x\ 100}{V4\ x\ W}$$

The SPSS program, analysis of variance (ANOVA), and mean difference test using Duncan's New Multiple Range Test (DMRT) were used to analyze statistical data.

2.4 Results and discussion

2.4.1 Effect of enzymatic treatment on physical and chemical properties of mulberry juice

Table 9 shows the physical and chemical properties of mulberry juice. Two samples were analyzed: fresh mulberry MB(F) and mulberry treated with pectinase MB(EN). There are significant differences in viscosity and color values. On the other hand, for the two samples, the pH, total soluble solid (TSS), total sugar, reducing sugar, and citric acid were not significantly different. This experiment showed that the addition of pectinase was able to reduce the viscosity and facilitate the further microfiltration process. The brightness of MB(EN), indicated by the L* value was lighter when treated with pectinase. Vaillant *et al*. (2005) reported that being treated by pectinase enzymes in fruit juices contributes to clearer juices. Pectinase is an enzyme commonly used to catalyze the transformation of pectin compounds into plant cell walls. Moreover, the advantages of pectinase are high yield, easier extraction, and easier filtering by microfiltration. For membrane processes, the use of enzymes increased the permeate flux (Girard and Fukumoto, 2000). So, pretreatment using the pectinase enzyme is acceptable and used before entering the membrane process.

Table 9. Physical and chemical properties of fresh mulberry juice (MB(F)) and enzymatic treated mulberry juice (MB(EN)).

Properties	MB(F)	MB(EN)
Total soluble solid (ºBrix)	7±0.00^{ns}	6.87±0.03^{ns}
pH	3.33±0.02^{ns}	3.25±0.06^{ns}
Citric acid (w/w%)	1.39±0.02^{ns}	1.39±0.03^{ns}
Total sugar (w/w%)	7.78±0.34^{ns}	7.54±0.21^{ns}
Reducing sugar (w/w%)	5.94±0.15^{ns}	6.12±0.12^{ns}
Viscosity (mPa s)	1.78±0.01^{a}	1.65±0.02^{b}
Color		
L*	37.01±1.94^{b}	38.66±1.05^{a}
a*	8.45±0.13^{b}	10.32±0.09^{a}
b*	14.33±0.04^{a}	11.47±0.11^{b}
ΔE (colour difference)	-	3.79±0.48
Chroma	16.64±1.65^{b}	15.43±1.15^{a}

Same letters in the same row present no statistic difference according to Duncan's test at $P<0.05$

2.4.2 Effect of microfiltration on L-ascorbic acid and anthocyanin of functional mulberry juice

Figure 11 shows anthocyanin content in fresh mulberry, clarified and concentrate functional mulberry juice. The result showed that microfiltration with a pore size of 0.2 µm was used to determine anthocyanin content in mulberry juice, with the highest anthocyanin value being 127.07 ± 2.28 mg/g. These results did not significantly different from the anthocyanin content in MBI 0, MBI 2 and MBI 8. There was no significant difference in fresh mulberry, clarified and concentrate functional mulberry juices. Sripakdee *et al.* (2015) reported that ripe mulberry contains high levels of health benefits such as anthocyanin, vitamin C, and flavonoids.

Figure 12 shows L-ascorbic acid in fresh mulberry, clarified and concentrate functional mulberry juice. The highest values of L-ascorbic acid (14.19±0.06 mg/ml) in clarified juice (MBI0) were obtained. These results did not significantly different from L-ascorbic acid in MBI 0, MBI 2 and MBI 8 when compared before and after microfiltration. There was no significant difference in fresh mulberry, clarified and concentrate functional mulberry juices. Normally, L-ascorbic acid in mulberry juice is sensitive to the conditions, for example, factors that affect vitamins and phytochemicals include high temperatures and UV light. (Santhirasegaram *et al.,* 2015). The experiment has reported that the content of L-ascorbic acid using microfiltration helps to reduce the loss of vitamin C in the process.

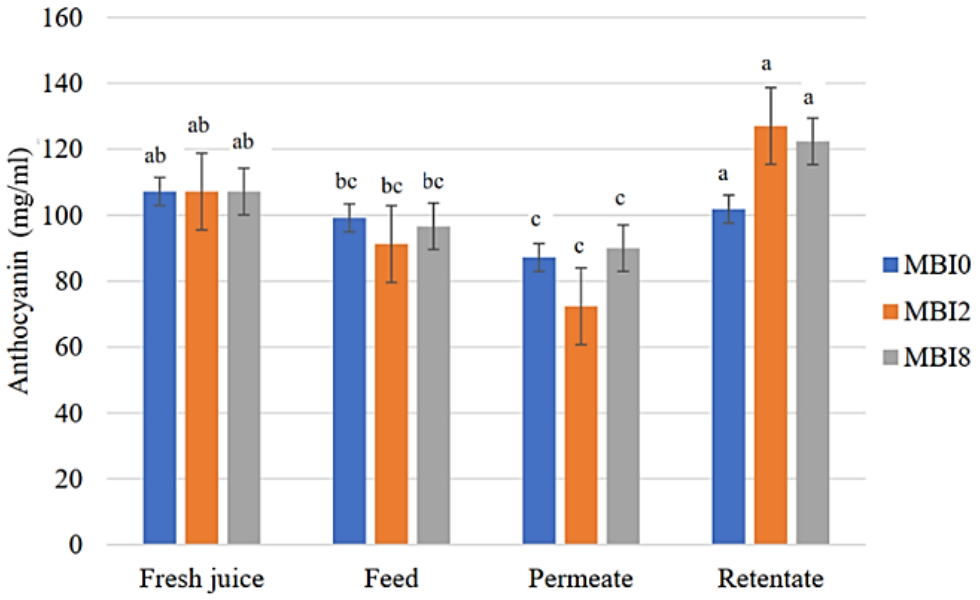

Figure 11. Anthocyanin content in fresh mulberry, clarified and concentrate functional mulberry juice with membrane pore size 0.2 µm (a-c represented mean values of mulberry juice are significantly different according to Duncan's multiple range test at $p<0.05$)

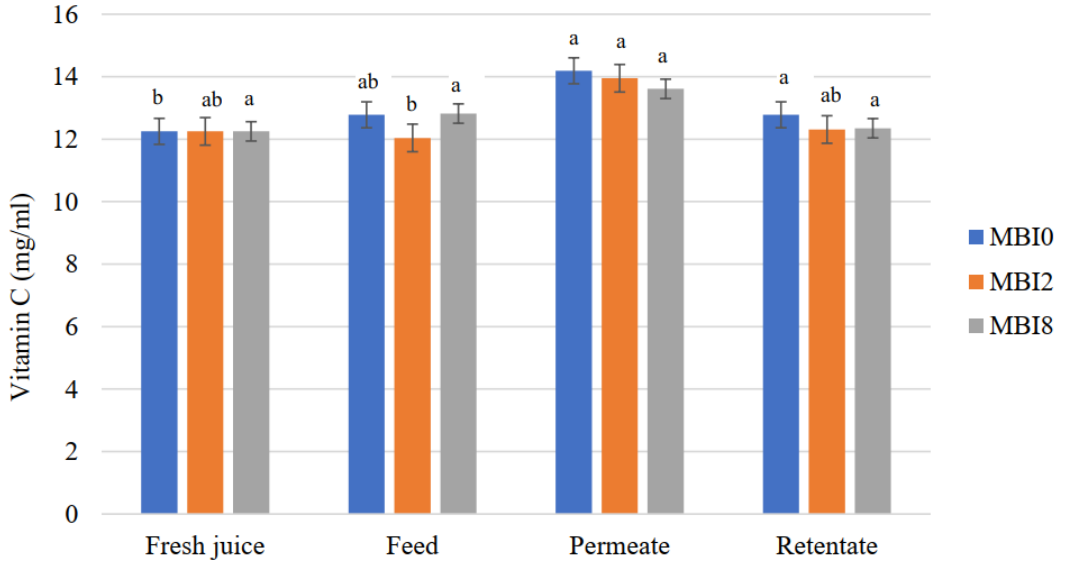

Figure 12. L-ascorbic acid in fresh mulberry, clarified and concentrate functional mulberry juice with membrane pore size 0.2 µm (a and b represented mean values of mulberry juice are significantly different according to Duncan's multiple range test at $p<0.05$)

2.4.3 Total phenolic content and antioxidant capacity

Table 10 showed the antioxidant (DPPH radical scavenging and FRAP reducing antioxidant) and phenolic content in the total mulberry juice before and after microfiltration. In this experiment, the highest total phenolic value was 373.67±14.40 mg GAE/100ml, DPPH radical scavenging (2514.41±14.88 mg TE/100g) and FRAP reducing antioxidant (1880.18±35.56 mg TE/100g) was obtained by microfiltration. The results showed no statistically significant difference in DPPH radical scavenging and FRAP reducing antioxidant before and after membrane filtration. Moreover, the total phenolic content was no significant difference before and after using membrane filtration. Laorko *et al*. (2013) reported that the storage time and temperature increase affected the total phenolic content and antioxidant capacity (DPPH) in clarified pineapple juice. They found that the storage condition at 4°C was suitable for extending shelf life. Aguilar-Rosas *et al.* (2007) studied the effect of pasteurization (HTST) of apple juice. The process controlled at 90 °C for 30 seconds. The results showed that phenolic compounds decreased about 32% when compared with control.

Table 10. Total phenolic content and antioxidant capacity in fresh mulberry, clarified and concentrate functional mulberry juice

Properties		Total phenolic content (mg GAE/100ml)	DPPH free radical scavenging (mg TE/100g)	FRAP reducing antioxidant (mg TE/100g)
Feed	MBI0	359.95±14.24^{a}	2604.56±12.55^{a}	1697.38±21.93^{a}
	MBI2	362.23±8.36^{a}	2365.79±10.78^{ab}	1609.62±33.68^{a}
	MBI8	326.83±4.64^{ab}	2314.21±16.75^{ab}	1574.46±19.74^{ab}
Permeate	MBI0	299.261±10.80^{b}	2514.41±14.88^{a}	1680.18±35.56^{a}
	MBI2	300.50±12.06^{b}	2138.54±10.04^{b}	1598.99±6.19^{ab}
	MBI8	288.76±11.76^{b}	2125.92±8.42^{b}	1526.94±41.3^{b}
Retentate	MBI0	315.32±7.50^{ab}	2252.87±9.46^{ab}	1625.18±20.48^{a}
	MBI2	373.67±14.40^{a}	2077.33±10.21^{b}	1554.86±5.32^{ab}
	MBI8	323.33±13.02^{ab}	2024.85±9.65^{b}	1519.19±15.28^{b}

Same letters in the same row present no statistic difference according to Duncan's test at $P<0.05$

2.4.4 Effect of microfiltration on cholecalciferol and calcium of functional mulberry juice

Table 11. showed the cholecalciferol and calcium in mulberry juices before and after microfiltration. In this experiment, fresh mulberry juice when analyzed for calcium by Inductively coupled plasma atomic emission spectroscopy (ICP-AES) by AOAC (2019) 984.27 was 29.262 mg/100mL. Fresh mulberry juice, was analyzed with vitamin D3 by chromatography method, but the result was not detected. After microfiltration, calcium lactate and vitamin D3 were added to MBI2 and MBI8, the results were 104.352, 202.564 mg/100 mL, and 1.30, 4.00 µg/100 mL, respectively. From the experimental results, vitamin D3 has decreased because this vitamin is susceptible to degradation by light. Therefore, it is one reason that vitamin D3 may be less than before the process. The IMO (non-pure) concentration was 63.84 (1mg/ml). Before MF, the IMO content was 38.30 and 153.22 for the formulas MBI2 and MBI8,

respectively. After the MF process, the value decreased slightly (no significant difference). Zhang *et al.* (2007) reported the effect of sterilization on oat-based beverages fortified with vitamin D3. The process was controlled at 140°C for 20 seconds. The results showed that heat treatment caused a 60 percent loss of vitamin D3 and a 47–74 percent loss of calcium and zinc. Dima *et al*. (2020) studied the effect of temperature on vitamin D3. The results showed no loss of vitamin D3 during one week when kept at refrigerated temperatures.

Table 11. Cholecalciferol and calcium in fresh mulberry and clarified functional mulberry juice

Properties	Fresh juice	Before MF		After MF (Permeate)	
		MBI2	MBI8	MBI2	MBI8
Calcium (mg/100 mL)	29.262	108.65	209.95	104.35	202.56
VitaminD3 (Cholecalciferol) (µg/100 mL)	Not detected	1.50	5.00	1.30	4.00

2.5 Conclusion

Cold pasteurization of functional mulberry juice was carried out by microfiltration (MF). The MF membrane (0.2 µm) was chosen to study the effect on physical and phytochemicals in mulberry juice. Microfiltration gave perfect clarification and cold pasteurization s in one step. The results found that the vitamin C content, antioxidant capacity and total phenol were no significant difference between MBI0, MBI2 and MBI8. Thus, microfiltration could be preserved temperature-sensitive vitamins such as vitamin C in mulberry juice. However, viscosity was significantly increased when added IMO. The cold pasteurization process could achieve the preservation of compounds in mulberry juice, including vitamin C, anthocyanin, phenolic compounds, and antioxidants (DPPH and FRAP). Claiming functional mulberry juices are considered claimable because of their calcium and vitamin D content within the quantity amount. For the MBI2 formula, which claims "increased or more" (≥10% of Thai RDI), calcium and vitamin D content are 104.35 mg/100 mL and

1.30 µg/100 mL. Finally, the MBI8 formula, which claims "rich in or high" (≥20% of Thai RDI) calcium and vitamin D content are 202.56 mg/100 mL and 4.00 µg/100 mL, respectively.

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CHAPTER 3
GUT FERMENTATION OF FUNCTIONAL MULBERRY JUICE

3.1 Abstract

The effect of isomaltooligosaccharide (IMO) on changes in human fecal bacterial populations, short-chain fatty acids (SCFAs) and phenolic compounds production were evaluated. The IMO effect was performed by fecal batch culture fermentation. Bacterial populations were analyzed using 16S RNA sequencing (Next generation sequencing, NGS). After fecal batch culture fermentation, bifidobacteria was increased by IMO supplementation. The *Bifidobacterium* spp. significantly ($p<0.05$) increased to 5.03% (MBI2) and 17.53% (MBI8) after 24 hours of fermentation. Isomaltooligosaccharide enhanced the production of propionic acid and butyric acid with the highest concentrations of 11.66 ± 1.69 mM (MBI2) and 13.68 ± 0.50 mM (MBI8) for propionic acid, 9.55 ± 1.01 mM (MBI2) and 10.79 ± 0.33 mM (MBI8) for butyric acid at 48h fermentation. Some anthocyanin metabolites were detected, such as 3,4-dihydroxybenzaldehyde, L-phenylalanine, and aminocaproic acid and 3-(2-hydroxyphenyl) propionic acid. Thus IMO supplementation in mulberry juice could evevate functional perperty on gut health by promoting the growth of *Bifidobacterium* spp., production of SCFA and phenolic metabolites derived from anthocynin.

3.2 Introduction

At present, microbiome studies have gained a lot of popularity and attention. For example, the study of intestinal microflora in diabetics and obese people. The gut microbiota is beneficial to the body in many ways, such as helping in the absorption of vitamins and nutrients and protecting the body from pathogens in the human intestine (Cani, 2018).

Lima *et al.* (2019) studied the effect of daily consumption of orange juice on the levels of blood glucose, lipids, and gut microbiota metabolites. The benefits of bioactive compounds in fruit juices are many. Orange juice contains hesperidin and

naringin. When consumed in the body, these compounds help balance the intestinal microflora. The study used 10 women for evaluation. They tested all of the volunteers' blood serum and feces. They were then analyzed for the microorganisms *Lactobacillus* spp. and *Bifidobacterium* spp. Short-chain fatty acids (SCFAs) were analyzed, consisting of acetic, propionic, and butyric acids. According to the findings of the study, in orange juice, the cholesterol, blood sugar, and insulin values of the volunteers could be adjusted. The study used volunteer feces. As a result, orange juice contributes to the normalization of intestinal microflora Bifidobacterium spp. and lactobacillus spp. were increased. Moreover, it increases the production of short-chain fatty acids (SCFAs). The consumption of fruit juice has a positive effect on the health of consumers.

Generally, acetic acid is the major SCFA in the colon, and it has been shown to increase cholesterol synthesis (den Besten et al., 2013). Acetic acids are an energy source for tissues such as muscles, kidneys, heart and brain. Production of acetate is widely distributed among different groups of bacteria compared to propionic and butyric production (den Besten et al., 2013). The lower pH in the colon affects the gut microbiota composition. Bacteria related to the production of acetic acid become dominant in the colon when pH increases to 6.5. Almost 70% of acetate is taken up by the liver and used as a substrate for other essential molecules such as long-chain fatty acid, cholesterol, glutamate and glutamic acid (Li *et al.*, 2016).

Propionic acid is one of the primary short-chain fatty acids that are metabolites by gut microbiota from dietary carbohydrates fermentation. It is mainly utilized by the liver and was shown to reduce food intake (Lin *et al.*, 2012), protect against diet-induced obesity and insulin resistance and induce gut hormones (Richard *et al.*, 2016). Propionate produces by more specific groups of substrates and bacteria compares to acetate production. It can be used in food preservation and also in food production because of characteristics to inhibit the growth of microorganisms (Fluegge, 2017).

Butyric acid is mostly produced from resistant starch (RS) fermentation especially RS type 3 (Morrison and Preston, 2016) which supports the health and healing of cells in the small and large intestines. Butyric acids have anti-carcinogenic that are important for keeping colon cells healthy (Borycka-Kiciak *et al*., 2017). Daily

energy in the gastrointestinal mucosa is provided by butyric acids for almost 50% (Tuohy *et al.,* 2005).

Isomaltooligosaccharide (IMO) consists of usually 3 to 6 glucose molecules bound with an indigestible glycosidic linkage. IMO is enzymatically processed from starch as a mixture of α-(1→6) linked glucosides, such as isomaltose, panose and isomaltotetraose however higher degree of polymerization (DP) is also obtained (Kaneko *et al.,* 1995). Chanowan et al. (2020) studied in IMO compositions analyzed by HPAEC-PAD. The results showed that the content of IMO consisted of isomaltotriose (DP2), panose, isomaltotriose (DP3), isomaltotetraose (DP4), isomaltopentaose (DP5), isomaltohexose (DP6) and isomaltoheptaose (DP7). Plongbunjong *et al*. (2017) studied the effects of isomaltooligosaccharides (IMOs). As a result, IMOs consist of DP2, 3, 4 and 6, i.e., isomaltose, D-panose, isomaltotetraose, and isomaltohexaose, respectively. The production of isomaltooligosaccharides (IMO) uses starch as the main raw material converted by enzymes. Amylase is an important enzyme in the digestion of starch and glucose. It is then converted from glucose glycosidic bonds α (1→4) to glycosidic bonds of α (1→6) by the transglucosidase enzyme. The proportion of isomaltose disaccharides will be high. Panose (IMO DP3) is obtained as the end product in the process (Ibrahim, 2018).

In this study, IMO was added to mulberry juice and its prebiotic property was evaluated by fecal fermentation using a colon system in batch culture.

3.3 Materials and methods

3.3.1 Materials

All chemicals and reagents used for fecal batch culture fermentation were analytical grade purchased from Sigma-Aldrich. The isomaltooligosaccharide (IMO) at 63.84% concentration was obtained from the previous study (Chanowan *et al.*, 2020). Mulberry fruits were supplied by Ban Suan Kaset Nai Fun (Na Mom District, Songkhla, Thailand). The mulberry fruits were washed with water and extracted using a fruit juice

extractor. The extract was then filtered through a filter cloth to remove solids. The mulberry juice was obtained with a total soluble solid of 8°Brix. Functional mulberry juice was formulated by the addition of isomaltooligosaccharide (IMO) at specific concentrations (0, 2, and 8%). Hollow fiber microfiltration with membrane pore size 0.22 µm was employed to produce non-thermally pasteurized mulberry functional juice, as explained in detail by (Laorko *et al*., 2010). Subsequently, the functional mulberry juice added IMO was further freeze-dried to obtain the powders. The preparation of mulberry juices for fecal batch culture fermentation is shown in Figure 13.

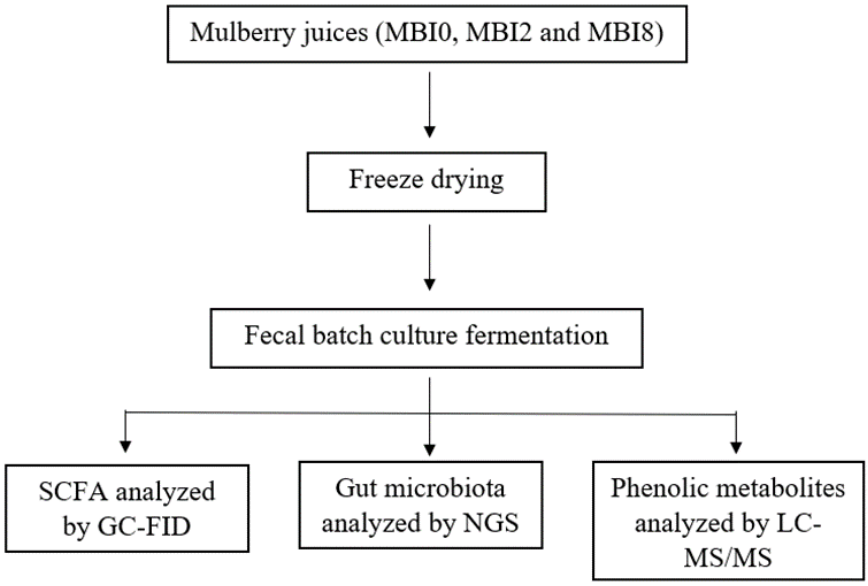

Figure 13. Preparation of mulberry juices for fecal batch culture fermentation

3.3.2 Preparation of fecal slurry

Samples of fresh feces were collected from four healthy donors to prepare the fecal slurry used as inoculum in the simulated colon system (batch culture). The inclusion and exclusion criteria for fecal donors were set. The donors had not consumed products containing prebiotics or probiotics, nor received antibiotic treatment for at least 3 months before donating the feces, and had no digestive system disease. The donors were healthy subjects between the ages of 25 and 45 years. Ethical clearance and consent were performed before collecting the feces. The fresh feces were

collected by the donors in the morning. Fresh feces of the donors were put into an anaerobic chamber, combined, and put in a stomacher's bag lined with filler. The 0.1 M phosphate-buffered saline (PBS; pH 7.0) was used to dilute the fresh feces at a ratio of 1:10 (w/v) or 10% concentration. After that, the fecal slurry was homogenized by the stomacher for 2 minutes at speed of 230 rpm before it was inoculated into the sterile vessels.

3.3.3 Batch culture of functional mulberry juice

The medium was prepared by mixing 0.8 g peptone water, 0.045 g NaCl, 0.9 g yeast extract, 0.018 g KH_2PO_4, 0.018 g K_2HPO_4, 0.045 g $CaCl_2 \cdot 6H_2O$, 0.045 g $MgSO_4 \cdot 7H_2O$, 0.9 g $NaHCO_3$, 0.225 g bile salts, 0.225 g L-cysteine·HCl, 0.0225 g hemin, 0.9 mL Tween 80, 4.5 µL vitamin K, and 0.45 mL 0.025% resazurin. The basal medium was sterilized at 121ºC for 15 min. The sterile vessel was filled with 90 mL of sterile basal medium and 1.0 g freeze-dried mulberry added IMO sample in a sterile basal medium, then continuously stirred using a magnetic bar in anaerobic conditions by flushing with nitrogen gas throughout fecal fermentation. The pH in the vessel was controlled at 6.8±0.1 by a pH controller and the temperature was controlled at 37ºC by circulating warm water into the double jacket of the vessel using a circulating water bath. These conditions were mimicked conditions in the human colon. Fecal slurry (10 mL) was filled into 90 mL of sterile basal media to obtain a final concentration of 1% (v/v). Fecal fermentation was performed for 48 h and samples were taken at 0, 6, 12, 24 and 48 h of fermentation. Samples were analyzed of gut microbiota by 16S RNA sequencing (next generation sequencing, NGS). Short-chain fatty acids (SCFAs) were analyzed by gas chromatography with a flame ionization detector (GC-FID). Phenolic metabolites were analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS). All samples were kept at -20ºC before analyses.

3.3.4 Short-chain fatty acids (SCFAs) analysis by GC-FID

Samples (1125 µL) were centrifuged at 13,000 x *g* at 4ºC for 5 min. The supernatant was filtered through a nylon membrane filter (0.22 µm). Aliquots of the filtered supernatant were mixed with acetone in a 1:1 ratio. Acetic, propionic, and butyric acid were used for the standard curves of each SCFA. Short-chain fatty acid production was analyzed by GC-FID. The GC column was HP-INNOWax of 30m x 0.32mm size with 0.25µm thick film. The initial oven temperature was set to 60°C and was increased to 100°C in 2 min. Then the temperature was increased to 250°C in 5 min (at 15°C/min). Helium (99.99%), hydrogen and oxygen were used as carrier gases in the system for GC-FID. The total run time was 25.67 min. The total volume injected was 2 µL. Standard curves of acetic, propionic, and butyric acid were used to determine the concentrations of SCFAs in the samples by comparing the peak height (mAU*s) for samples at respective retention times.

3.3.5 Enumeration of fecal bacteria by next generation sequencing (NGS)

The next generation sequencing (NGS) was used to determine the gut microbiota targeted regions (V3-V4) by amplicon sequencing of 16S RNA. Samples were extracted of microbial DNA to obtain a minimal concentration of DNA of about 5 ng/µL for further amplified by PCR. Primer F (1µM, 5 µL), 1µM primer R (5 µL), 5 ng/µl a DNA template (2.5 µL), and 2x KAPA HiFi HotStart Ready Mix (12.5 µL) were mixed. The tube was put in the Gene Amp following the user instructions of Gene Amp PCR 9700. The first step was an initial denaturation at 95°C for 3 min. Then the following was repeated for 25 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and a final 72°C for 5 minutes. Then, the sample was held at 4°C. The PCR product was tested by electrophoresis using Gel Documentation for DNA testing, according to the manufacturer's instructions. The amplicon obtained was approximately 550 bp. The details of PCR protocol were described below.

The 1st PCR clean-up used 20 AMPure XP beads per sample, 80% freshly prepared ethanol, and 10 mM Tris pH 8.5. The 2nd PCR step had an Index attached. After

reacting on ice with chemical addition, a 5 μL sample of extracted DNA was taken for the 2nd PCR (Index), and placed into a 0.2 mL PCR tube then mixed with Nextera XT Index Primer 1(N7xx) (5 μL), Nextera XT Index Primer 2 (S5xx) (5 μL), 2x KAPA HiFi HotStart Ready Mix (25 μL), and PCR grade water (10 μL) to obtain a final volume of 50 μL.

The operating procedures were followed using the Gene Amp PCR 9700 as follows. The first step was to perform an initial denaturation at 95°C for 3 min. Then the following was repeated for 8 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and a final 72°C for 5 min. Then, the sample was held at 4°C.

The 2nd PCR clean-up was done with AMPure XP beads one more time. After that, the Library Quantification and QC process included an electrophoresis test of PCR product using 2 μL samples with Gel Documentation. For DNA testing, an amplicon with approximately 630 bp was used in calculations, and the DNA concentration was diluted to 4 nM for next generation sequencing.

In library normalization and pooling the conversion of the DNA concentration unit to nM was calculated, and then DNA was diluted to 4 nM.

$$\frac{\text{Concentration in ng/μl}}{\text{660 mol x average library size}} \times 10^6$$

For library denaturation and MiSeq sample loading, PhiX was prepared to a concentration of 4 nM and denatured PhiX was diluted to 20 pM, then further to 4 pM (the same concentration as denatured DNA library) and DNA library was combined with the PhiX control.

3.3.6 Identification of phenolic metabolites by LC-MS/MS

The samples from fecal batch culture fermentation were taken at 0 and 24 hours for LC-MS/MS identification of the phenolic metabolites. Sample preparation was done as below.

1. MBI0 (0 h): Sample from fecal fermentation at 0 h of mulberry without the addition of IMO (27.55 mg) was dissolved with 0.5 mL water, and filtered via 0.22 nylon membrane before injection.

2. MBI0 (24 h): Sample from fecal fermentation at 24 h of mulberry without the addition of IMO (54.52 mg) was dissolved with 0.5 mL water, and filtered via 0.22 nylon membrane before injection.

3. MBI2 (24 h): Sample from fecal fermentation at 24 h of mulberry with an addition of 2% IMO (81.37 mg) was dissolved with 0.5 mL water, and filtered via 0.22 nylon membrane before injection.

4. MBI8 (48 h): Sample from fecal fermentation at 48 h of mulberry with an addition of 8% IMO (40.08 mg) was dissolved with 0.5 mL water, and filtered via 0.22 nylon membrane before injection.

The phenolic compounds in the mulberry sample were analyzed by using a liquid chromatograph-quadrupole time-of-flight mass spectrometer (LC-Q-TOF-MS), 1290 Infinity II LC-6545 Quadrupole-TOF, Agilent Technologies, USA. The LC column was Zorbax Eclipse Plus C18 Rapid Resolution HD of 150 mm length and 2.1 mm inner-diameter, particle size 1.8 μm. Mobile phase A was H_2O/MeOH (8:2) + 0.1% formic acid, and mobile phase B was 0.1% formic in acetonitrile. The column temperature was set at 40°C with a flow rate of 0.2 mL/min, and the injection amount was 5 μL. Phenolic compounds in mulberry juices were detected at 254 nm, 280 nm and 310 nm. A linear gradient program had 0–30 minutes from 95 to 45% (A), 30–40 minutes from 45 to 0% (A), 40–45 minutes from 0 to 95% (A), and 45–50 minutes 95% (A).

The device was operated at 40°C with a flow rate of 0.2 mL/min, and the injection amount was 5 μL. The mass spectrometer was set at 50-1200 m/z and for negative ion mode detection. The UV detector was set at the wavelengths 254 nm, 280 nm, and 310 nm. The gas temperature was set at 350°C, the gas flow was 8 L/min, and the nozzle voltage was 2000 V. Each sample was dissolved in water and filtered through

a 0.22 µm nylon membrane before injection. The phenolic compounds were identified according to ion molecular mass by using AutoMS2 software.

3.3.7 Phylogenetic analysis:

The bacterial DNA was compared to the NCBI database for 16S rRNA gene sequences. Then, the 16S rRNA gene sequences were aligned using ClustalW. The maximum likelihood tree (Kimura two-parameter model, 100 replications) was constructed using MEGA software version 11.

3.3.8 Statistical analysis

Statistical analysis using SPSS software version 21.0. The statistical significance level was set to $p<0.05$ and analyzed using analysis of variance (ANOVA) with Duncan's test.

3.4 Results and discussion

3.4.1 Prebiotic effect of mulberry juice

This study used next generation sequencing (NGS) by sequencing 16S rRNA gene to analyze the gut microbiome. It was found that some beneficial gut microbiota were detected such as *Bifidobacterium scardovii, Bifidobacterium stercoris, Megasphaera elsdenii, Bacteroides vulgatus* and *Lachnospira pectinoschiza*. The population of *Bifidobacteria* including *Bifidobacterium scardovii* and *Bifidobacterium stercoris* was significantly ($p<0.05$) increased in mulberry added IMO 2% and 8% compared to without addition of IMO 0% at 24 hours of fermentation (Figure 14-17). *Megasphaera elsdenii* are beneficial bacteria in the intestine that release ammonia from oligopeptides. The bacteria groups *Sporobacter termitidis, Clostridium leptum, Oscillospira guillermondii*, and *Lachnospira pectinoschiza* are anti-inflammatory and help against bowel disease (IBD). *Pseudobutyrivibrio xylanivorans* and *P. ruminis* are butyrate-producing bacteria that are beneficial in the human colon.

The four samples tested show larger increases in bifidobacteria than in strains of non-beneficial bacteria. Figure 14-17 shows the populations of bifidobacteria. The percent of bifidobacteria significantly ($p<0.05$) increased as indicated in Figures

15 (5.03%) and 16 (17.53%) in 24 hours of fermentation, compared to Figures 14 and 15. Therefore, the addition of IMO in mulberry juice promoted the growth of bifidobacteria in the simulated colon system. Zhang *et al*., (2021) showed the effects of IMO levels on the gut microbiota. IMO is prebiotic and supports the growth of bacteria in the colon, such as bifidobacteria. Plongbunjong *et al*., (2017) produced isomaltooligosaccharides (IMOs) from Sangyod rice starch. In *in vitro* fermentation, the population of bifidobacteria and lactobacilli increased. This study showed a significant ($p<0.05$) increase in bifidobacteria. On the other hand, bacteroides that are pathogenic bacteria decreased significantly ($p<0.05$) when IMO was added. The species of *Megasphaera hominis* was detected at 24 hours of fermentation (Figure 15-17), and *Megasphaera* species is more common in women, especially in postmenopausal women with decreased lactobacilli.

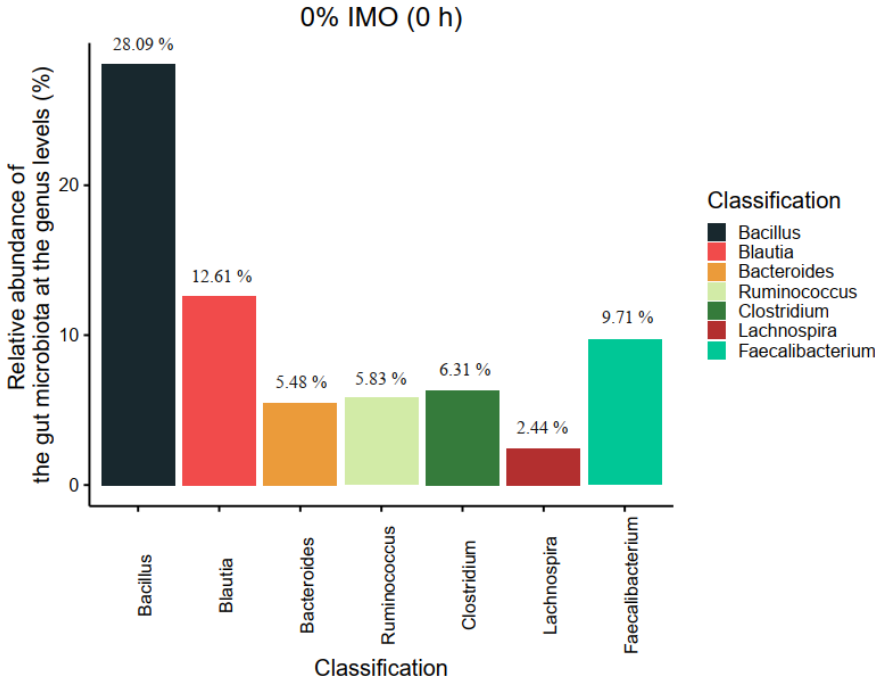

Figure 14. Percentages of mean relative abundances of the gut microbiome at the genus levels during fecal fermentation 0% IMO (0 hour)

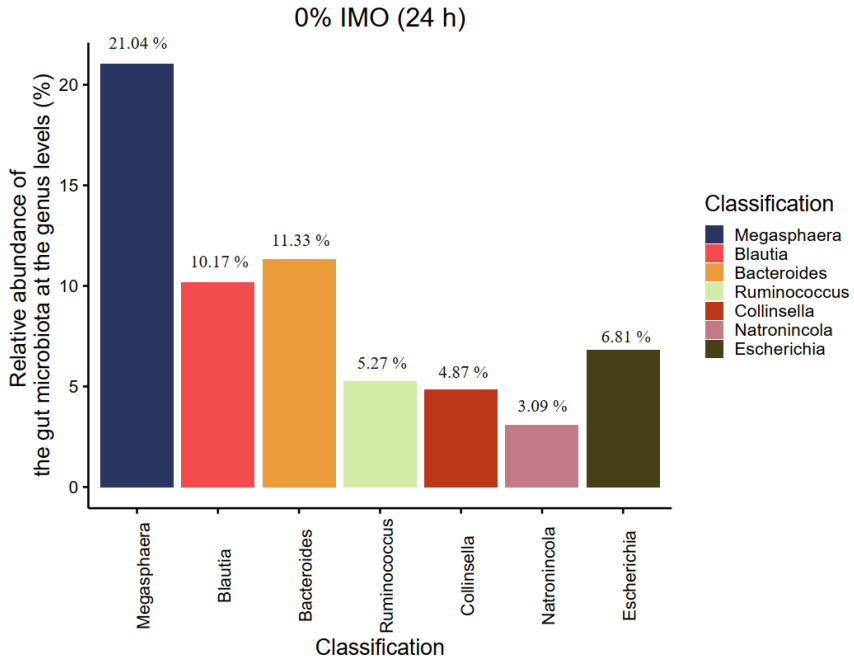

Figure 15. Percentages of mean relative abundances of the gut microbiome at the genus levels during fecal fermentation 0% IMO (24 hours)

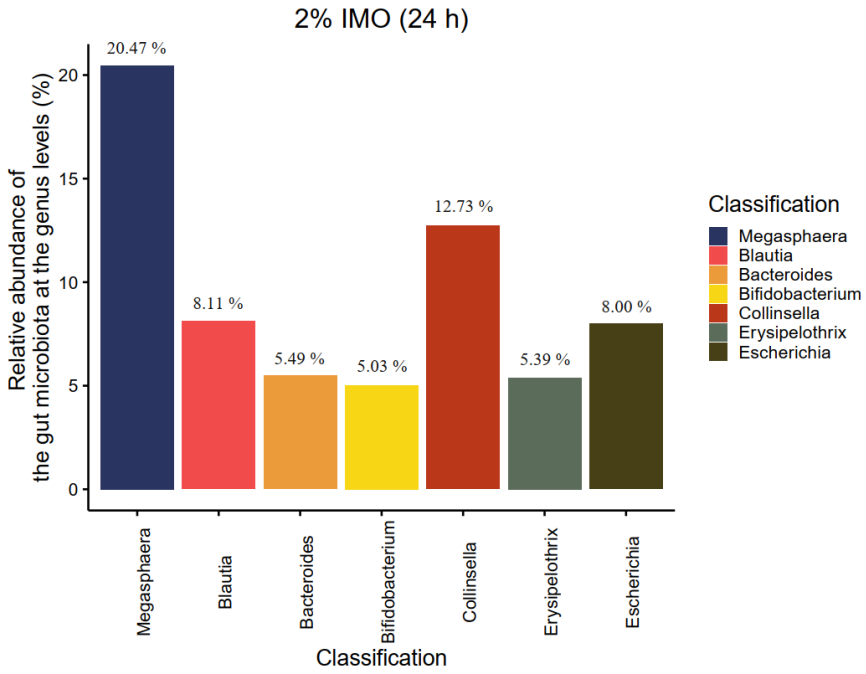

Figure 16. Percentages of mean relative abundances of the gut microbiome at the genus levels during fecal fermentation 2% IMO (24 hours)

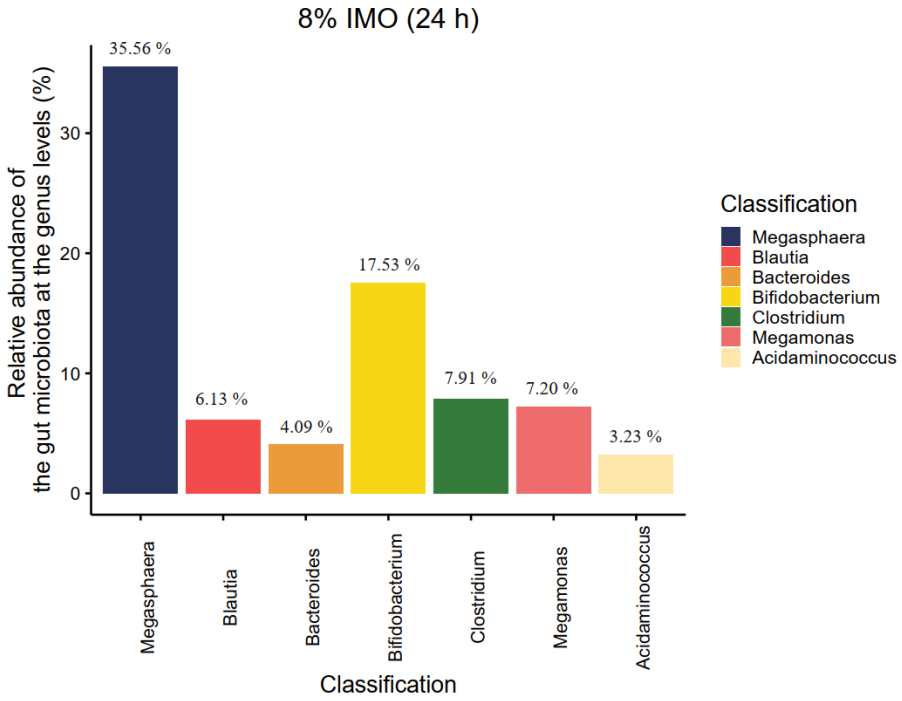

Figure 17. Percentages of mean relative abundances of the gut microbiome at the genus levels during fecal fermentation 8% IMO (24 hours)

3.4.2 Short-chain fatty acids (SCFAs) production

Short-chain fatty acids (SCFA) were found in the simulated colon from fecal fermentation. Mainly acetic acid, propionic acid, and butyric acid were produced by microbiota in the colon. Gas chromatography with a flame ionization detector (GC-FID) was used to analyze SCFA. The production of SCFA was found during fecal fermentation of mulberry juices added IMO (0% IMO, 2% IMO and 8% IMO) at 0, 6, 12, 24 and 48 hours as shown in Table 12. Interestingly, butyric acid and propionic acid were mostly the main SCFAs generated during fermentation meanwhile acetic acid was produced in less amount. Mulberry juice added 2% IMO (MBI2) had a high concentration of acetic acid, and mulberry juice added 8% IMO (MBI8) also had a high acetate concentration at 24 and 48 hours. The control sample (without the addition of IMO, 0% IMO), MBI2 and MBI8 fermented at 0, 6 or 12 hours) had no significant ($p>0.05$) acetate concentration.

Propionate increased during fermentation time increased. The increase was significant ($p<0.05$) at 24 hours for all samples (MBI0 4.00 mM, MBI2 5.06 mM, and

MBI8 7.03 mM). Wu *et al*. (2012) reported that propionic acid was significantly higher than acetic acid and butyric acid during fecal fermentation. The control showed a significant increase ($p<0.05$) at 24 hours of fecal fermentation (4.00 mM). The results indicate that propionic acid was generated by the colonic bacteria from IMO added and/or components originated in mulberry juice. Anggela *et al*. (2022) studied short-chain fatty acids (SCFAs) in porang oligo-glucomannan. The results showed a significant increase ($p<0.05$) in butyric acid from 3.58 to 6.68 mM within 48 hours. Plongbunjong *et al.* (2017) studied the effect of isomaltooligosaccharides (IMOs) on SCFA production. The result showed that IMO produced the highest butyric acid on day 11 of fecal fermentation.

On the other hand, MBI2 and MBI8 showed the highest concentrations of butyric acid at 9.55 ± 1.01 and 10.79 ± 0.33 mM, respectively at 48 hours, while the control had no increase in butyric acid. Butyric acid has anti-inflammatory activity, reducing colonic inflammation and the occurrence of colon cancer. So production of butyric acid was derived from IMO added in the mulberry juice, not from components that originated in mulberry juice.

Table 12: Short-chain fatty acid (SCFA) profiles in fecal batch culture fermentation of mulberry juice added IMO at different concentrations.

Sample	SCFA concentration (mM)			
	Acetic acid	Propionic acid	Butyric acid	Total SCFA
Control (0% IMO)				
0 h	1.35 ± 0.02^{a}	0.78 ± 0.13^{c}	1.19 ± 0.01^{b}	3.31 ± 1.06^{c}
6 h	1.45 ± 0.07^{a}	2.13 ± 1.25^{b}	2.21 ± 0.11^{b}	5.79 ± 1.42^{b}
12 h	1.45 ± 0.08^{a}	2.34 ± 0.09^{b}	2.37 ± 2.44^{b}	6.18 ± 0.25^{b}
24 h	2.01 ± 0.01^{a}	4.00 ± 0.07^{a}	3.44 ± 3.36^{b}	9.45 ± 0.15^{a}
48 h	2.14 ± 0.07^{a}	4.98 ± 0.28^{a}	4.68 ± 5.45^{a}	11.81 ± 1.14^{a}

MBI2 (2% IMO)				
0 h	1.56 ± 0.14^{c}	1.04 ± 0.13^{c}	2.63 ± 0.25^{c}	5.23 ± 0.53^{c}
6 h	1.46 ± 0.06^{c}	1.49 ± 0.07^{c}	1.84 ± 0.05^{c}	4.79 ± 0.18^{c}
12 h	4.01 ± 0.12^{b}	2.45 ± 0.12^{c}	2.39 ± 0.17^{c}	8.85 ± 0.41^{c}
24 h	5.87 ± 0.11^{b}	5.06 ± 0.12^{b}	4.24 ± 3.50^{b}	15.16 ± 3.73^{b}
48 h	9.7 ± 4.96^{a}	11.66 ± 1.69^{a}	9.55 ± 1.01^{a}	30.91 ± 7.66^{a}
MBI8 (8% IMO)				
0 h	3.39 ± 0.11^{bc}	7.45 ± 3.44^{b}	2.93 ± 0.14^{c}	13.77 ± 3.69^{b}
6 h	1.59 ± 0.02^{c}	1.24 ± 0.07^{c}	2.32 ± 0.04^{c}	5.15 ± 0.12^{c}
12 h	1.83 ± 0.04^{c}	0.84 ± 0.08^{c}	2.61 ± 0.12^{c}	5.28 ± 0.24^{c}
24 h	6.01 ± 0.11^{b}	7.03 ± 0.19^{b}	5.86 ± 0.17^{b}	18.89 ± 0.47^{b}
48 h	8.08 ± 0.16^{a}	13.68 ± 0.50^{a}	10.79 ± 0.33^{a}	32.56 ± 0.98^{a}

Values are given as mean ± SD of duplicate fermentation; Different superscripts within a column (in the rows for the same sample) indicate significant differences ($p<0.05$)

3.4.3 Gut microbiota population

This study, shows the classification of the gut microbiota analyzed by NGS, and a phylogenetic tree was constructed using MEGA11 with its maximum-likelihood method. The result shows three main bacterial groups were identified during fecal fermentation in batch culture (Figure 17).

The four samples (0% IMO (0 h), 0% IMO (24 h), 2% IMO (24 h) and 8% IMO (24 h)) show higher increases in bifidobacteria than in strains of non-beneficial bacteria. Figure 17 shows the populations of bifidobacteria. The result shows that beneficial for health such as *Bifidobacterium scardovii, Bifidobacterium stercoris* were increased in IMO by 2% and 8% when compared to 0% IMO, after 24 hours of fermentation significantly ($p<0.05$). Moreover, the bacteria groups *Sporobacter termitidis, Clostridium leptum, Oscillospira guillermondii*, and *Lachnospira pectinoschiza* are anti-inflammatory and help against bowel disease (IBD) *(Satokari et al*., 2014). *Pseudobutyrivibrio xylanivorans* and *P. ruminis* are butyrate-producing

bacteria that are beneficial in the human colon (Kopečný *et al*., 2003). Wichienchot and Keawyok (2021) developed a product containing IMO for kidney patients and identified gut microbiota using the NGS method. The beneficial microorganisms such as *Lactobacillus* spp. and *Bifidobacterium* spp. were increased by 53.74% and 29.35%, respectively, after 48 hours of fermentation. Plongbunjong *et al.* (2017) reported that IMO increased the number of beneficial bacteria, such as bifidobacteria and lactobacilli. Similarly, Azis *et al.* (2021) studied the effect of vitamin A supplementation on rice. The results showed that *Clostridium* spp. and *Enterobacter* spp. decreased but *Bifidobacterium* spp. and *Lactobacillus* spp. increased significantly ($p<0.05$).

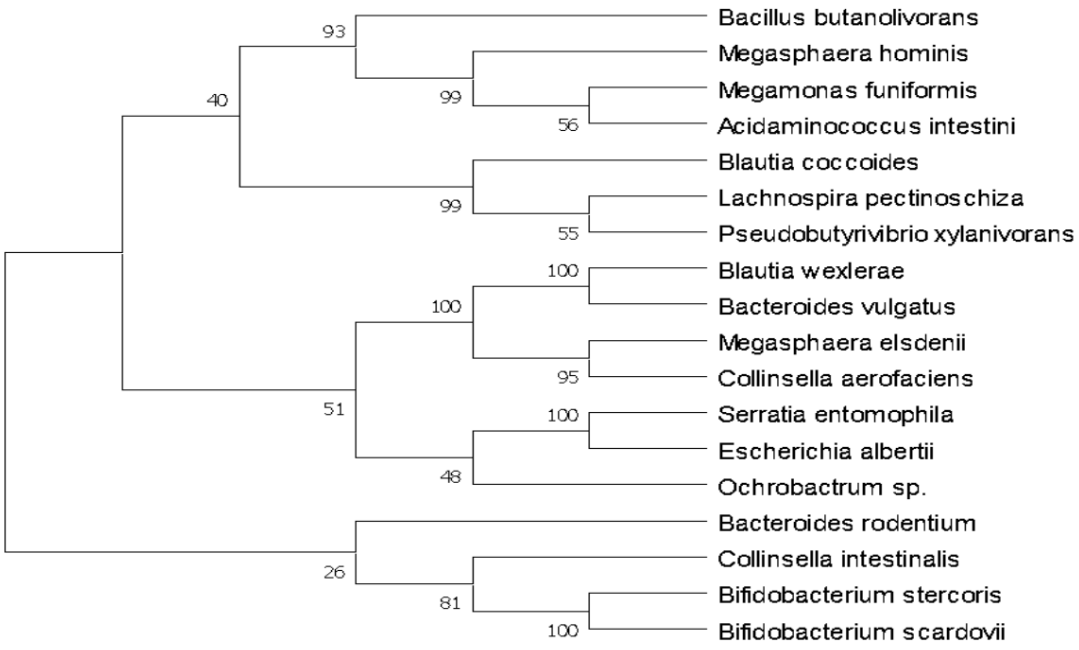

Figure 18. Phylogenetic analysis of the 16S rRNA sequence. The maximum likelihood tree (100 bootstraps) was constructed from the 16S rRNA sequence collected from the NCBI database.

3.4.4 Phenolic metabolites from fecal fermentation analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS)

Liquid chromatography-mass/mass spectrometry (LC–MS/MS) was used to analyze the phenolic metabolites during fecal fermentation of mulberry juice added IMO in batch culture. The results are presented in Table 13-16. The gut microbiota

metabolized compounds in mulberry juice and the prebiotic IMO added. The compounds in mulberry juice are phenolic compounds and other components such as flavonoids, such as rutin. Phenolic compounds in mulberry juice were detected at 254 nm, 280 nm, and 310nm, as they are usually detectable at these wavelengths (Zhang *et al.*, 2013). The results showed that the added prebiotic likely contributed higher concentrations of some phenolics than in the control. The most abundant phenolic compounds were 3-(2-hydroxyphenyl) propionic acid, 3,4-dihydroxybenzaldehyde, L-phenylalanine, aminocaproic acid, and cholic acid (Table 14-15). Bao *et al*. (2019) studied phenolic components in digested mulberry cultivars and it was found that during *in vitro* fermentation, the anthocyanins increased with fermentation time increased. Some metabolites of anthocyanin (such as 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid, etc.) were produced by bifidobacteria and lactobacilli. Cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside were the major phenolic compounds in mulberry analyzed by LC–MS/MS after *in vitro* fecal fermentation. Some colonic bacteria were increased such as *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Eubacterium hallii*, and *Clostridium barlettii*. Isomaltooligosaccharide added in mulberry juice promoted gut microbiota. Owolabi *et al*. (2020) studied *in vitro* fecal fermentation of purple rice. It was found that some phenolic metabolites, including 3-(4-hydroxyphenyl) propionic acid, phenylpropionic acid, and pyrocatechol, were produced.

Table 13. Identification of phenolic compounds in fecal fermentation of control (0% IMO at 0 hour) analyzed by liquid chromatography-mass/mass spectrometry (LC–MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.185	Citric acid	192.0272	192.027	$C_6H_8O_7$	-1.01	96.76	1745060
2	3.099	L-Phenylalanine	165.0791	165.079	$C_9H_{11}NO_2$	-0.88	92.77	648981
3	3.813	3,4-Dihydroxybenzoic acid	154.0264	154.0266	$C_7H_6O_4$	1.05	97.53	125963
4	4.151	Hydroxyphenyllactic acid	182.0577	182.0579	$C_9H_{10}O_4$	1.02	95.14	89641
5	4.79	3,4-Dihydroxybenzaldehyde	138.0313	138.0317	$C_7H_6O_3$	3.08	89.46	104315
6	8.449	3-(2-Hydroxyphenyl) propionic acid	166.0628	166.063	$C_9H_{10}O_3$	1.34	86.62	152197
7	8.737	D-(+)-3-Phenyllactic acid	166.0631	166.063	$C_9H_{10}O_3$	-0.68	89.47	366439
8	8.749	trans-Cinnamic acid	148.0522	148.0524	$C_9H_8O_2$	1.5	92.8	49403
9	14.838	Quercetin	302.0423	302.0427	$C_{15}H_{10}O_7$	1.31	94.21	45720
10	23.608	7-Ketodeoxycholic acid	406.2714	406.2719	$C_{24}H_{38}O_5$	1.24	49.19	229449
11	27.391	Cholic acid	408.2872	408.2876	$C_{24}H_{40}O_5$	0.8	96.41	452817
12	37.326	Allolithocholic acid	376.2976	376.2977	$C_{24}H_{40}O_3$	0.25	49.07	546142

Table 14. Identification of phenolic compounds produced by fecal fermentation of mulberry juice without addition of IMO (control, 0% IMO) at 24 hours analyzed by liquid chromatography-mass/mass spectrometry (LC–MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.761	3-(3,4-Dihydroxyphenyl) lactate	198.0527	198.0528	$C_9H_{10}O_5$	0.43	86.87	91635
2	3.375	3,4-Dihydroxybenzoic acid	154.0266	154.0266	$C_7H_6O_4$	-0.24	94.9	66030
3	3.989	Dihydroxyphenylacetic acid	168.0423	168.0423	$C_8H_8O_4$	-0.04	97.66	43434
4	4.853	3,4-Dihydroxybenzaldehyde	138.0315	138.0317	$C_7H_6O_3$	1.25	86.76	235655
5	5.141	Hydroxyphenyllactic acid	182.0578	182.0579	$C_9H_{10}O_4$	0.42	98.84	218504
6	5.292	Pyrocatechol	110.0367	110.0368	$C_6H_6O_2$	0.39	87.67	115429
7	6.169	4-Hydroxybenzaldehyde	122.0367	122.0368	$C_7H_6O_2$	1.03	83.52	39795
8	7.321	2-Hydroxyphenylacetic acid	152.0472	152.0473	$C_8H_8O_3$	0.93	87.43	28886
9	7.547	Isoleucyl-Phenylalanine	278.1629	278.163	$C_{15}H_{22}N_2O_3$	0.45	98.48	44601
10	7.722	Chenodeoxycholic acid sulfate	472.2516	472.2495	$C_{24}H_{40}O_7S$	-4.52	83.26	64931
11	8.048	Isocaproic acid	116.0834	116.0837	$C_6H_{12}O_2$	3.18	86.94	19926
12	21.729	Cholic acid	408.2875	408.2876	$C_{24}H_{40}O_5$	0.13	99.56	151425
13	37.339	Allolithocholic acid	376.2976	376.2977	$C_{24}H_{40}O_3$	0.28	98.41	409526

Table 15. Identification of phenolic compounds produced by fecal fermentation of mulberry juice without addition of IMO (control, 2% IMO) at 24 hours analyzed by liquid chromatography-mass/mass spectrometry (LC–MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.692	3-(3,4-Dihydroxyphenyl) lactate	198.0526	198.0528	$C_9H_{10}O_5$	1.29	85.56	49768
2	3.043	L-Phenylalanine	165.0787	165.079	$C_9H_{11}NO_2$	1.42	87.43	85107
3	4.083	Dihydroxyphenylacetic acid	168.0421	168.0423	$C_8H_8O_4$	0.84	98.67	47527
4	4.797	3,4-Dihydroxybenzaldehyde	138.0315	138.0317	$C_7H_6O_3$	1.44	87.22	181050
5	5.123	Hydroxyphenyllactic acid	182.0578	182.0579	$C_9H_{10}O_4$	0.79	99.21	69396
6	5.298	Pyrocatechol	110.0366	110.0368	$C_6H_6O_2$	1.82	87.37	39297
7	8.054	Isocaproic acid	116.0834	116.0837	$C_6H_{12}O_2$	2.77	86.03	21834
8	21.785	Cholic acid	408.2872	408.2876	$C_{24}H_{40}O_5$	0.93	98.76	32731
9	27.297	Ursodeoxycholic acid	392.2922	392.2927	$C_{24}H_{40}O_4$	1.15	97.89	25957

Table 16. Identification of phenolic compounds produced by fecal fermentation of mulberry juice added 8% IMO for 24 hours analyzed by liquid chromatography-mass/mass spectrometry (LC–MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.915	Pyrocatechol	109.0296	110.0368	$C_6H_6O_2$	-0.9	87.83	71933
2	2.94	3,4-Dihydroxybenzoic acid	153.0193	154.0266	$C_7H_6O_4$	-0.4	98.91	46386
3	3.065	L-Phenylalanine	165.0791	165.079	$C_9H_{11}NO_2$	-0.86	99.33	166048
4	4.757	3,4-Dihydroxybenzaldehyde	138.0316	138.0317	$C_7H_6O_3$	0.73	99.29	129761
5	5.045	Hydroxyphenyllactic acid	182.058	182.0579	$C_9H_{10}O_4$	-0.47	99.58	75475
6	5.521	Parahydroxyphenylacetic acid	152.048	152.0473	$C_8H_8O_3$	-4.21	89	20190
7	5.797	Aminocaproic acid	131.0947	131.0946	$C_6H_{13}NO_2$	-0.55	87.43	165237
8	7.701	Chenodeoxycholic acid sulfate	472.2516	472.2495	$C_{24}H_{40}O_7S$	-4.57	82.61	93019
9	8.051	Isocaproic acid	116.0836	116.0837	$C_6H_{12}O_2$	0.74	99.38	14485
10	8.402	3-(2-Hydroxyphenyl) propionic acid	166.0634	166.063	$C_9H_{10}O_3$	-2.69	85.77	1049504
11	8.603	D-(+)-3-Phenyllactic acid	166.0631	166.063	$C_9H_{10}O_3$	-0.5	89.21	39014
12	8.628	Lithocholic acid sulfate	456.2566	456.2546	$C_{24}H_{40}O_6S$	-4.52	84.21	41956
13	21.281	1-(3,4-Dihydroxyphenyl)-5-hydroxy-3-decanone	280.1679	280.1675	$C_{16}H_{24}O_4$	-1.72	83.39	41433
14	21.732	Cholic acid	408.2874	408.2876	$C_{24}H_{40}O_5$	0.33	99.55	124285

3.5 Conclusion

Mulberry juice added IMO enhanced the production of propionic acid and butyric acid during fecal fermentation compared to mulberry juice without addition of IMO. Isomaltooligosaccharide could promote the growth of bifidobacteria with bifidogenic effects were *Bifidobacterium scardovii* and *Bifidobacterium stercoris.* The effect of IMO on phenolic compounds is that IMO can produce more phenolic compounds such as hydroxyphenyllactic acid, 3,4-dihydroxybenzaldehyde, etc. Thus, IMO has the potential used as a prebiotic for functional mulberry juice production. Functional mulberry juice is suitable for the elderly. It contains many nutrients, such as calcium, vitamin D, and prebiotics.

3.6 References

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CHAPTER 4
CONCLUSION AND SUGGESTION

4.1 Conclusion

Functional mulberry juice was produced by membrane technology. Microfiltration used was a hollow fiber membrane module with a pore size of 0.2 µm. An enzymatic treatment of juice was used in this experiment with 0.1 percent pectinase. before entering the microfiltration to reduce viscosity and increase filtration efficiency. Functional mulberry juices were formulated using enzymatically treated mulberry juice as the main ingredient and mixed with IMO (0, 2, and 8 percent), calcium lactate (0, 80, and 180 mg), and vitamin D3 (0, 1.5, and 5 µg). In general, microfiltration could deliver high-quality products which are similar to fresh functional mulberry juice.

The results showed that important substances in mulberry (anthocyanin, phenolic compounds, antioxidants, and vitamin C) were not significantly different before and after MF. On the other hand, the viscosity and color values were significant differences after using the enzyme. For MBI2, vitamin D3 and calcium lactate were 1.3 µg/100 mL and 104.352 mg/100 mL. MBI8 contained vitamin D3 and calcium lactate: 4.00 µg/100 mL and 202.564 mg/100 mL. Isomaltooligosaccharide (IMO) was added to mulberry juice to study the properties of prebiotics. The functional mulberry juice could promote the growth of Bifidobacterium, including *Bifidobacterium scardovii* and *bifidobacterium stercoris* after 24 hours of fermentation by NGS. The results of the SCFA analysis in the MBI0, MBI2, and MBI8 samples showed that more butyric acid and propionic acid occurred during fermentation, but acetic acid was produced in less amount. The phenolic compounds were analyzed in the samples (MBI2 and MBI8) by the LC-MS/MS method. The most abundant phenolic compounds were 3-(2-hydroxyphenyl) propionic acid, 3,4-dihydroxybenzaldehyde, L-phenylalanine, aminocaproic acid, cholic acid and allolithocholic acid.

Therefore, the addition of IMO promotes the proliferation of beneficial bacteria in the body. The membrane technology can produce functional mulberry juice, it does not affect the valuable nutrient content of mulberry juice.

4.2 Suggestion

This study aimed to investigate the function of mulberry juice in elders. We test the mulberry juice with invitro fecal batch culture using the feces from the volunteer who have aged 25 to 45 years old. In a further study, we suggested that the experiment of the *in vitro* fecal batch culture model could use a feces sample obtained from volunteers ages sixty years old since the results must be more precise with the target sample. However, the results of this study can be explained as representative.

Appendices

APPENDIX A
STANDARD CURVE OF VITAMIN D3

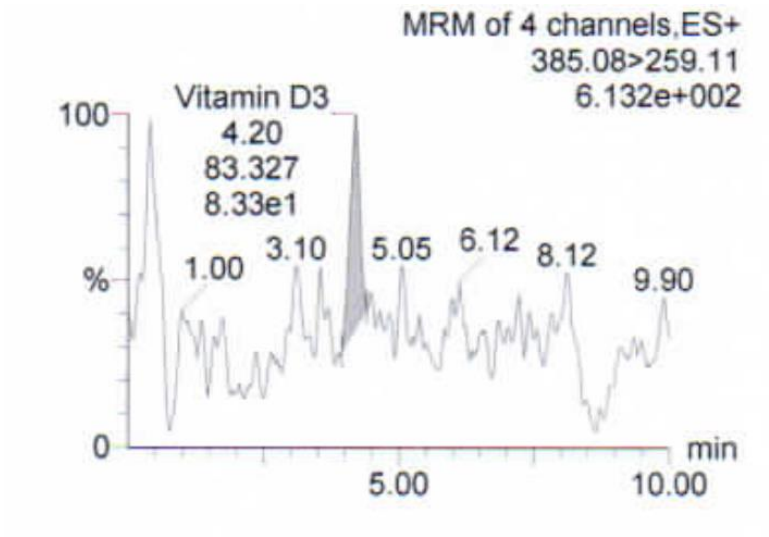
Compound name: Vitamin D3 (MBI2)

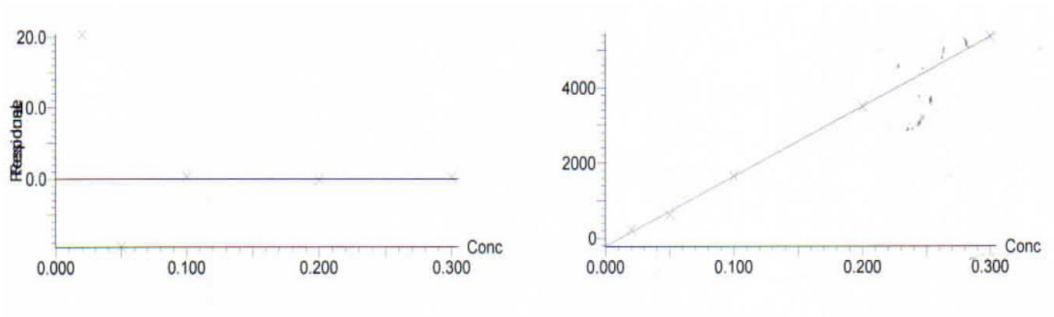
Correlation coefficient: r = 0.999625, r^2 = 0.999251

Calibration curve: 18558.3* x + -213.955

Response type: External Std, Area

Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None





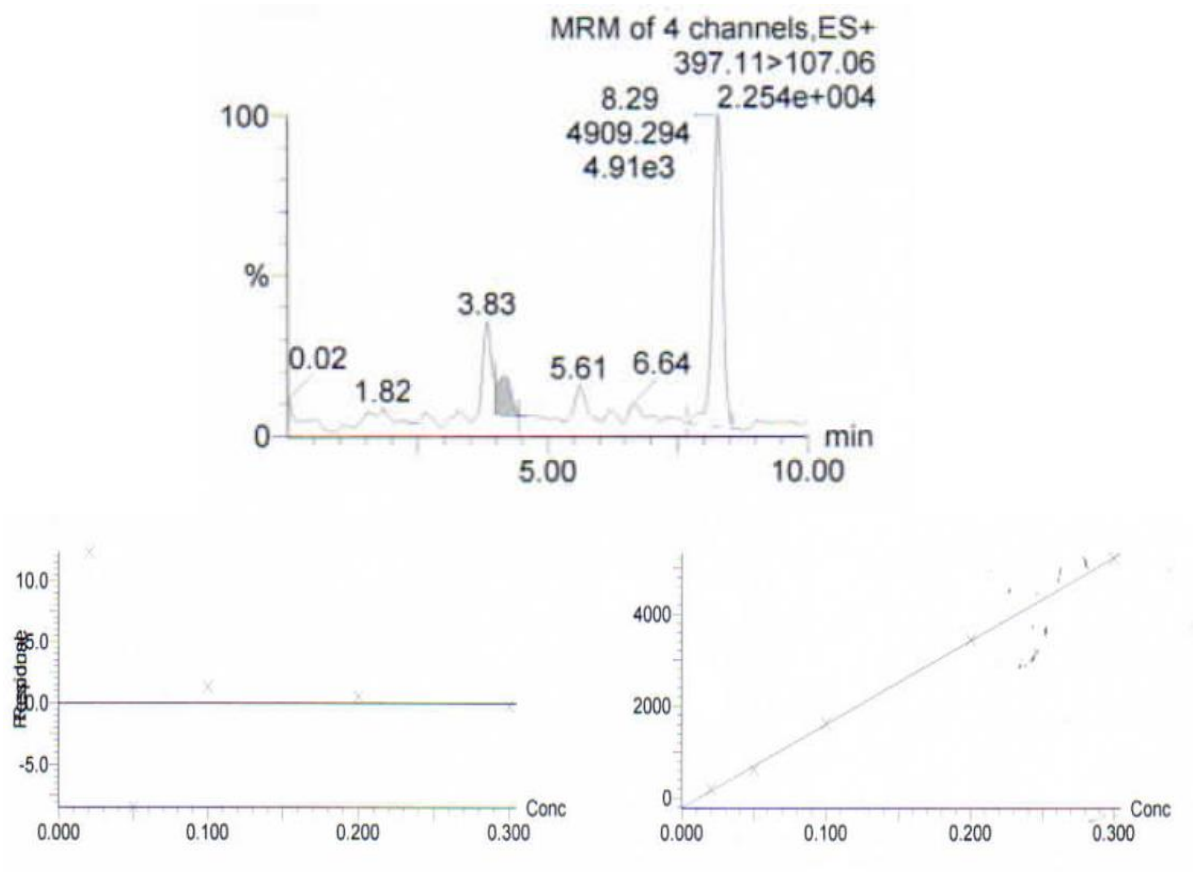
Compound name: Vitamin D3 (MBI8)

Correlation coefficient: r = 0.999744, r^2 = 0.999489

Calibration curve: 18175.7* x + -213.84

Response type: External Std, Area

Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



Name	Trace	RT	Area	IS Area	Response	Flags	Conc.
Vitamin D3 (MBI2)	385.08>107.20	4.17	24.126		24.126	MM	0.013
Vitamin D3 (MBI8)	397.11>105.06	4.14	518.031		518.031	MM	0.040

APPENDIX B

STANDARD CURVE OF IMO AND SUGAR CONTENT

Samples:

Glucose (10, 15, 20, 25 mg/ml)

Maltose (10, 15, 20, 25 mg/ml)

Isomaltotriose (0.5, 10, 15 mg/ml)

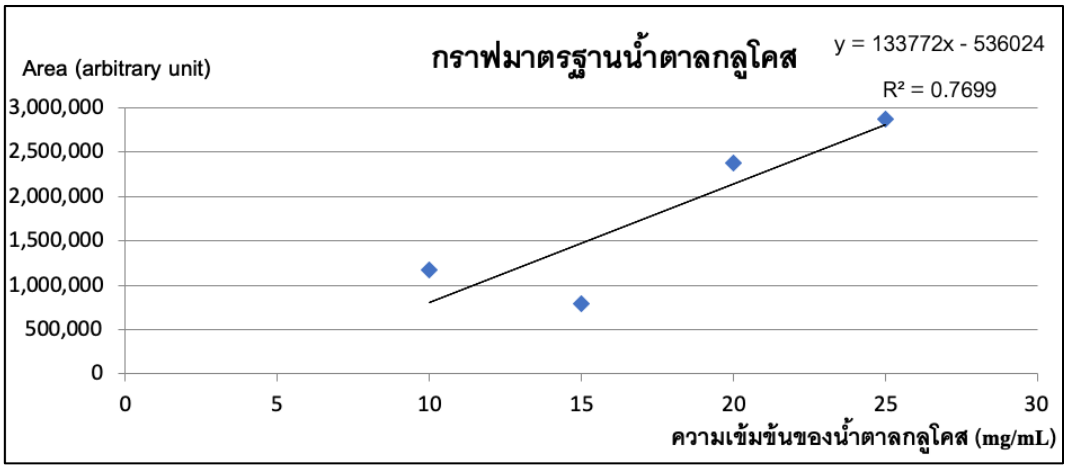
Isomaltopentose (0.125, 0.25, 1 mg/ml)

Panose (0.125, 0.25, 1 mg/ml)

IMO

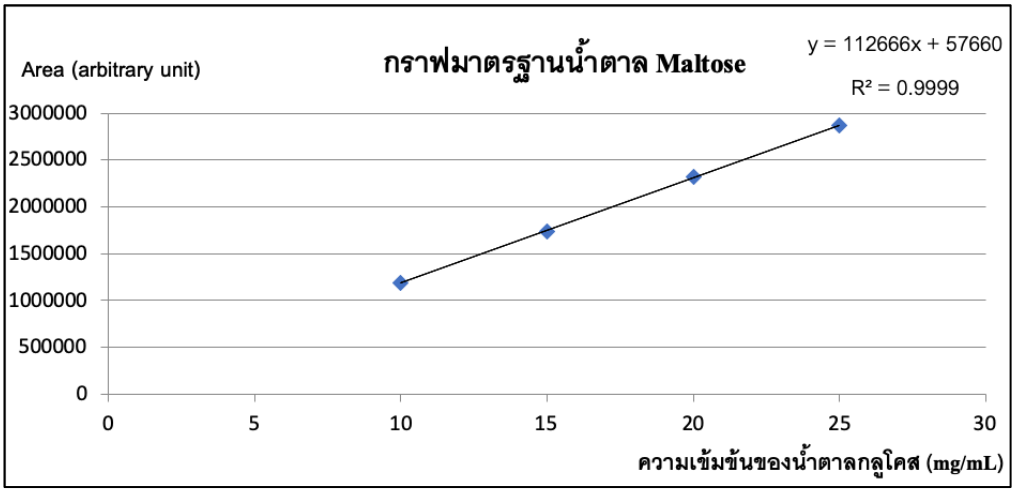
- **Standard curve of sugar and oligosaccharide**
- Standard curve of glucose

Concentration (mg/mL)	**Area (arbitrary unit)**	**Retention time (min)**
10	1,176,400	21.176
15	1,788,050	21.186
20	2,380,300	21.269
25	2,875,180	21.207



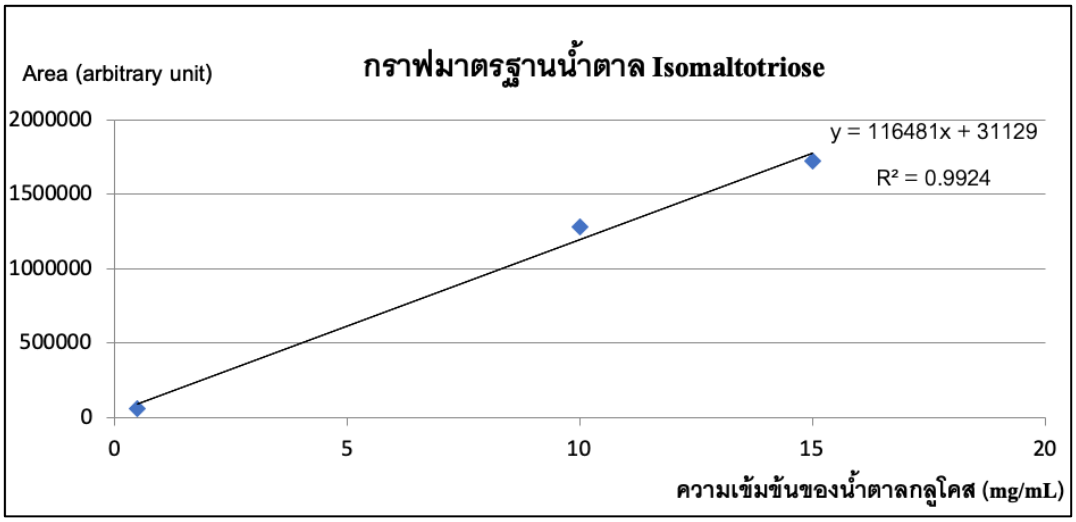
- Standard curve of maltose

Concentration (mg/mL)	Area (arbitrary unit)	Retention time (min)
10	1,187,910	17.406
15	1,738,620	17.404
20	2,318,270	17.414
25	2,872,460	17.431



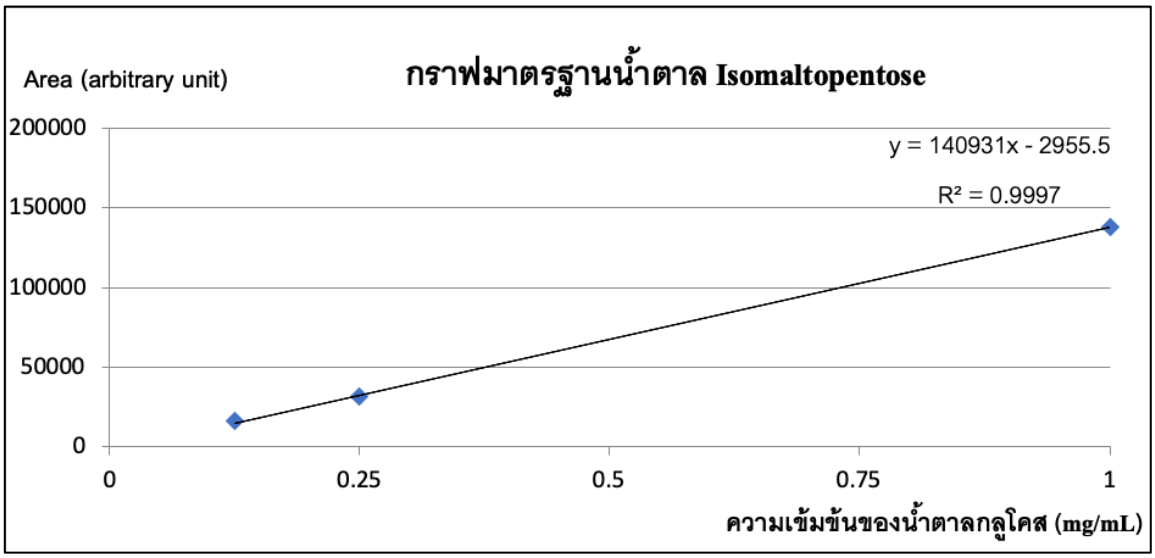
- Standard curve of isomaltotriose

Concentration (mg/mL)	Area (arbitrary unit)	Retention time (min)
0.5	59,877.1	15.173
10	1,281,470	15.186
15	1,722,310	15.186



- Standard curve of isomaltopentose

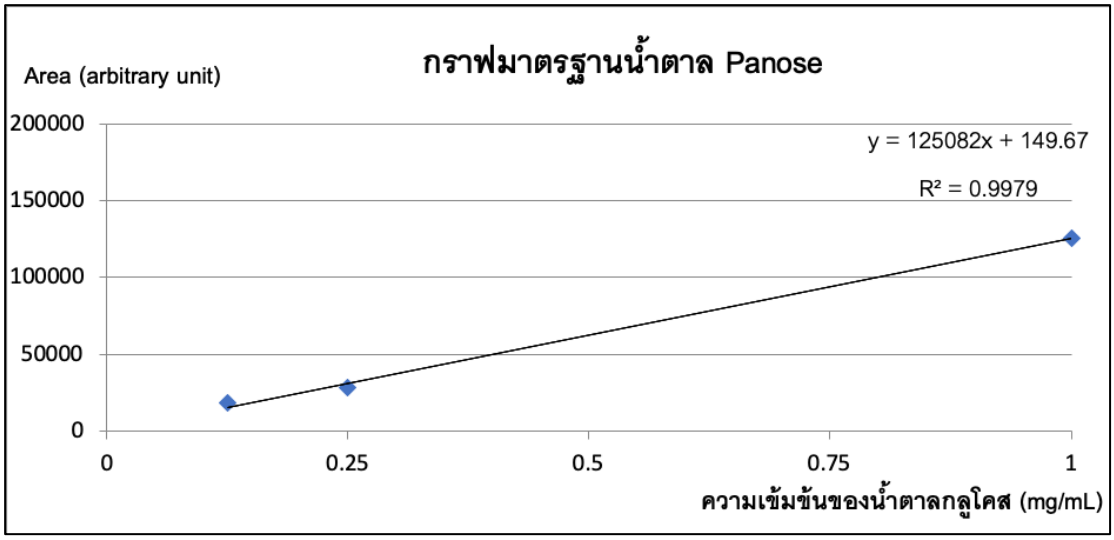
Concentration (mg/mL)	Area (arbitrary unit)	Retention time (min)
0.125	15,716.1	13.450
0.25	31,046	13.385
1	138,151	13.452



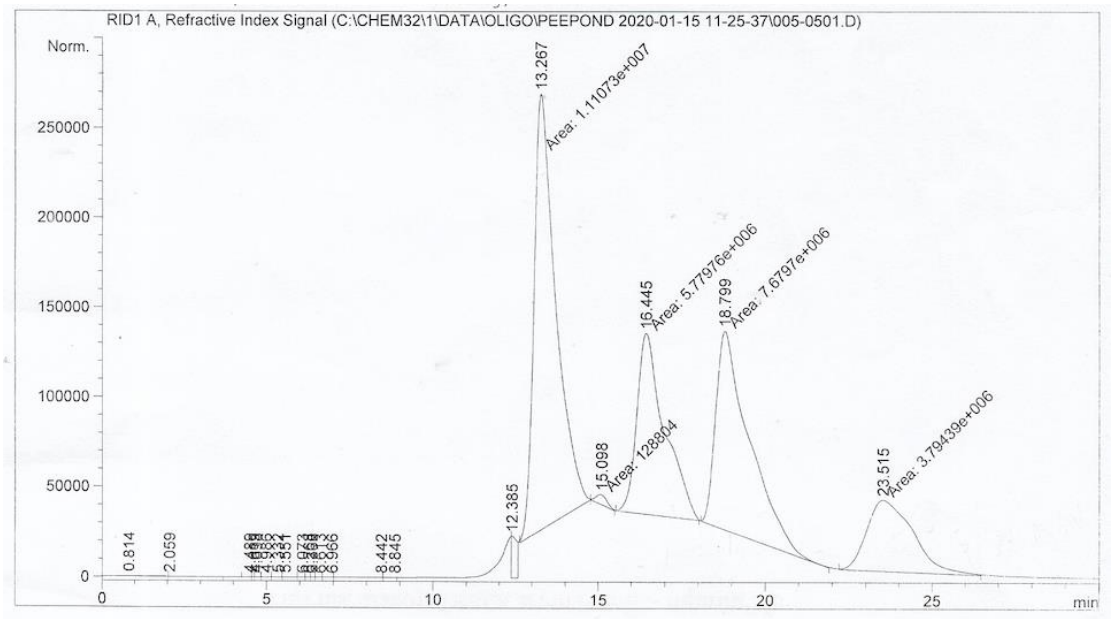
- Standard curve of panose

Concentration (mg/mL)	Area (arbitrary unit)	Retention time (min)
0.125	18,278.9	15.045
0.25	28,510.4	15.048

1	125,647	15.043



IMO



Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.814	BV	1.0091	1.87074e4	218.63322	0.0650
2	2.059	VV	0.1598	980.40247	84.04913	3.406e-3
3	4.489	VV	0.1189	37.64333	4.32091	1.308e-4
4	4.619	VV	0.0633	20.79410	4.47043	7.225e-5
5	4.698	VV	0.1218	55.07737	5.46820	1.914e-4
6	4.986	VV	0.1972	88.10873	5.45074	3.061e-4
7	5.332	VV	0.1823	99.18889	6.66530	3.446e-4
8	5.551	VV	0.1696	74.89478	5.42796	2.602e-4
9	6.073	VV	0.1255	20.17999	2.09181	7.011e-5
10	6.268	VV	0.0881	26.18770	3.64668	9.099e-5
11	6.368	VV	0.0718	17.49597	3.12304	6.079e-5
12	6.613	VV	0.1037	25.28118	3.10119	8.784e-5
13	6.966	VV	0.1591	108.11024	8.38062	3.756e-4
14	8.442	VV	0.6131	1759.70386	34.70592	6.114e-3
15	8.845	VV	0.2779	879.82031	37.81652	3.057e-3
16	12.385	BV	0.1692	2.69524e5	2.29835e4	0.9364
17	13.267	MM	0.7634	1.11073e7	2.42486e5	38.5906
18	15.098	MM	0.3859	1.28804e5	5563.18359	0.4475
19	16.445	MM	0.9573	5.77976e6	1.00631e5	20.0809
20	18.799	MM	1.1580	7.67970e6	1.10533e5	26.6819
21	23.515	MM	1.6102	3.79439e6	3.92743e4	13.1830
Totals :				2.87824e7	5.21898e5	

IMO	Glucose	Maltose	IDP2	IDP3	Panose	IDP4	IDP5	IDP6	IDP7	Total IMO (%)
Content	3.86	6.79	16.2	0.06	14.07	9.04	6.18	6.65	11.64	63.84

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List of Publications

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