Nutritional Compositions, Polyphenolic Profiles, Modulation of Gut Microbiota, Glycemic Index and Some Biological Properties of Pigmented Rice Varieties and Adlay Influenced by Soaking and Germination Conditions

Iyiola Oluwakemi Owolabi

A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Functional Food and Nutrition

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Academic Year	2018

ABSTRACT

Pigmented rice varieties are gaining interest due to their superior nutritional and phenolic properties compared to white rice varieties. With the rising risk of diabetes, cereals with higher protein contents should be consumed with rice in order to reduce the risk of diabetes. Adlay is one of the underutilized crops with higher protein contents and other functional properties. The germination process is one of the easy and innovative techniques for improving functional properties in seeds. Therefore, the aim of this study was to evaluate the effects of soaking and germination on the physicochemical properties, polyphenolic profiles and antioxidant activities of pigmented rice varieties and adlay. Two pigmented rice varieties and adlay seeds were soaked (24 h) and germinated for 12, 24, 36, and 48 h. The physicochemical properties, polyphenolic profiles, and the antioxidant activities were evaluated. Purple rice (PR) demonstrated the highest value of polyphenolic content, with 9 compounds detected for antioxidant activities. There were 6 phenolic compounds detected for red rice (RR). The adlay seeds had the least concentrations of phenolic compounds, with 6 compounds and higher nutritional properties identified. The following pheolic compounds were synthesized after soaking and germination, namely; gallic acid in purple rice; gallic acid, catechin and caffeic acid in red rice; vanillin in adlay, respectively. Among the red and purple rice samples, 24 h soaking gave the best results for phenolic and antioxidant properties, with 24 h germination in adlay seeds. Germination at 48 h yielded higher nutritional values in all the samples. The present study demonstrates how the process of soaking is a cheap and less time-consuming process of improving bioactive compounds and antioxidant activities in pigmented rice varieties and adlay seeds.

Bioactive components and anti-oxidative properties of purple rice after 24 h soaking and 36 h germination periods were investigated. The *in vitro* starch digestibility and glycemic index (GI) of soaked and germinated purple rice samples were determined. In addition, their prebiotic properties on the gut microbiota and their metabolites were investigated via batch culture fecal fermentation. The anti-inflammatory properties of the methanolic extracts of the purple rice were also evaluated on macrophage cells, stimulated with lipopolysaccharide. These conditions significantly increased ($P < 0.05$) the bioactive components and anti-oxidative properties. Total anthocyanin contents, total carotenoid contents and gamma-aminobutyric acid (GABA) were increased more than twofold in the soaked sample. All the extracts led to cell proliferation and inhibited nitric oxide (NO) production. Twenty-four hour soaking of purple rice exhibited highest bioactive compounds, anti-oxidative properties and showed greater NO inhibitory effects with IC_{50} of 234.00 ± 0.01 μg mL^{-1}. In addition to established information on germination, soaking greatly improved the bioactive components and anti-inflammatory properties of the purple rice variety.

Whole grain rice is known to contain fiber, phytochemicals and nutrients that could improve human health. The results from the study revealed that soaking and germination conditions significantly reduced ($P < 0.05$) the GI (72.64 ± 0.75 to 61.52 ± 3.11), but increased the total starch contents (58.1 ± 0.06 to 63.9 ± 0.09%). Lowest values for the GI were recorded in the soaked purple rice sample. All the samples evaluated in this study affected the gut microbiota in fecal batch culture. Interestingly, the soaked purple rice selectively increased the amount of bifidobacteria (log 9.62 to 10.21) and lactobacilli (log 8.55 to 9.30) groups and at 48 h fermentation and significantly inhibited ($P < 0.05$) the growth of clostridia and bacteroides groups. This correlates with the increasing production and highest concentration of acetate (35.27 ± 0.09 mM) in this purple rice after 48 h fermentation. It also exhibited a prebiotic index (PI) score of 0.79, thus indicating its potential gut microbiota modulation. The phenolic metabolites identified in the fecal fermented medium include 3-(4-hydroxyphenyl) propionic acid, pyrocatechol, *trans*-cinnamic acid and salicylic acid. Therefore, the soaked purple rice could be a potential functional hypoglycemic food products in food and nutraceuticals industry for gut microbiota modulation and reducing risks of colon cancer.

In conclusion, the best of our research knowledge obtained from this study would be about the process of ‘soaking’ which is an easy, innovative and economic technique of increasing bioactive components of rice with improved anti-inflammatory properties, lower glycemic index and gut microbiota modulation potential that could confer more health benefits to humans.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cereal grains are known to constitute one of the global main sources of food, thereby contributing up to 300 million tons of food grains produced annually. From this data, whole grains form a significant group as they are composed of exceptional nutritional and bioactive characteristics which have been attributed to the fractions, bran and germ that contain distinctive health-enhancing bioactive ingredients (Björck *et al.,* 2012; He *et al.,* 2010). These whole grains therefore exhibits a more composite and worthwhile nutritional characteristics than refined grains (Gong *et al.,* 2018). These factors have increased the interest of researchers in carrying out more studies about whole grain and their effects on human health, as well as the bioactive components which are promising ingredients for functional foods products. According to the report of World Health Organization for 2012–2016, the intake of whole grain cereals help reduce the prevalence of non-communicable diseases including type 2 diabetes, cardiovascular disease (CVD), obesity and hypertension. Thus, different epidemiological evaluations, specifically comprehensive prospective studies including millions of volunteers over years of monitoring, have emphasized the inverse relationship between the consumption of whole grain, including the bran component and the decreased risk of metabolic syndromes and chronic diseases (Cho *et al.,* 2013; Hongyu Wu *et al.,* 2015).

Free radicals are known as electron molecule that are not paired and could be produced through diversified biological and biochemical processes (Halliwell, 2001). When the amounts of these free radicals are at increased levels, they are capable of destroying and greatly altering the composition of the cell which may lead to stress (Halliwell, 2001; Sharma

et al., 2012). This has been studied on the pathogenesis of different diseases such as inflammation, cancer, neurological disorders, atherosclerosis, hypertension, diabetes, asthma and aging (Birben *et al.*, 2012). Increased levels of free radicals could be normalized by various antioxidant compounds including native phytochemicals, phenolic compounds, flavonoids, vitamin C, vitamin E, superoxide dismutase (SOD) which are present in whole cereal grains (Sen and Chakraborty, 2011).

Several studies have reported and confirmed the importance of the gut microbiota in host health including the physical, gut health, mental health and immunity. The gut bacteria may have many benefits as they help not only to improve the absorption of nutrients and energy, but also they are essential in health status (Power *et al.*, 2014)**.** Various global interventions have adopted lifestyle changes such as diet and physical activity as therapy for most chronic diseases These diets contain secondary metabolites called phenolic compounds and are found abundantly in a wide variety of foods, such as fruits, vegetables, herbs, whole seeds and cereals, and some beverages, such as coffee, tea, cocoa and wine (Vinson *et al.*, 2001). Some physicochemical factors affect the bioavailability of these metabolites which includes digestibility by gastrointestinal enzymes, and absorption into the enterocytes (Eckel *et al.*, 2010)**.**

Soaked and germinated cereals and their products have garnered a great deal of worldwide attention, especially in Asian countries. The process of soaking and germination is an economical technology that changes the composition of the cereals chemically in a radical way as the biochemical actions trigger the synthesis of some essential compounds. Rice (*Oryza sativa* L.), a main source of carbohydrate, is a staple food of ASEAN people and now spread worldwide. Scientific data has proven that several kinds of food consumed including germinated grains, especially rice exhibits high contents of GABA. *Coix lachryma*-Jobi L.,

commonly named Job's tears/adlay is also one of those cereal grains enhanced by soaking and germination conditions. Job's tear has its indigene from China, but has also been widely cultivated in many other Asian countries including Philippine, Burma, Sri Lanka and Thailand (Bender, 2006). When compared to rice seeds, adlay seeds contain higher contents of protein and fat but lower mineral contents.

Thus, in this experiment, soaked and germinated whole grain is aimed to be prepared in order to enhance the bioactive components with impact on the glycemic index, anti-inflammatory properties, gut microbiota and its metabolites evaluated.

1.2 Review of Literature

1.2.1 Whole grain cereals and their major fractions

Among the whole grain cereals, wheat, rye, rice, oats or barley represents a major global source of food for humans since from history. These grains have similar structures which are separated into three unique segments known as the outer bran (rich in fiber), the germ (micronutrient-rich) and endosperm known as the starchy central 'body' (Van der Kamp *et al.*, 2014). After grain processing, the kernel needs to retain the same proportion of bran, germ and endosperm just as the original grain before it could be pronounced as "whole grain" (Slavin *et al.,* 2013). The bran is the outer skin made up of several layers containing fiber, vitamins, minerals, and bioactive components. Among these constituents, phenolic acids have been of interest as they are classified as bioactive phytochemicals that confer vital health impact on humans (Ozcan *et al.*, 2014). The germ, also regarded as the embryo contains essential vitamins (B & E), fatty acids, selenium, and antioxidants. Likewise the endosperm which has the largest size of the kernel, is mostly composed of starchy carbohydrates such as glucose (Frølich *et al.*, 2013). However, the amount of nutritional composition as well as the bioactive compounds present in cereal grains is greatly affected by

the grains species, the type of cultivar used, and the germination conditions (Frølich *et al.*, 2013).

1.2.2 Bioactive compounds of whole grains

The prominent bioactive costituents in whole cereal grains includes the polyphenols such as ferulic acid and cinnamic acids; dietary fibers such as beta-glucan; lignans, phytic acid, inositols and betaine (Fardet, 2010; Gani *et al.*, 2012; Slavin *et al.*, 2013). The polyphenols are synthesized under glycosides groups that are connected to separate sugar moieties, with other groups connected to organic acids, amines, lipids, carbohydrates and other phenols, although the significant phenolic compounds are phenolic acids.

1.2.3 Categories of plant phenolics

The precursors of phenolic acid synthesis are phenylalanine and/or tyrosine via the shikimate pathway (Figure 1). The fundamental step involved in the biosynthesis of phenolic acids is the linkage of the hydroxyl groups to the phenyl ring (Tzin and Galili, 2010). Based on the heterogeneous of phenolic acids, ranging from single aromatic ring structure with low molecular weight to polymeric compounds with higher molecular weight, they can therefore be mainly grouped into simple and complex phenolics as shown in Figure 2.

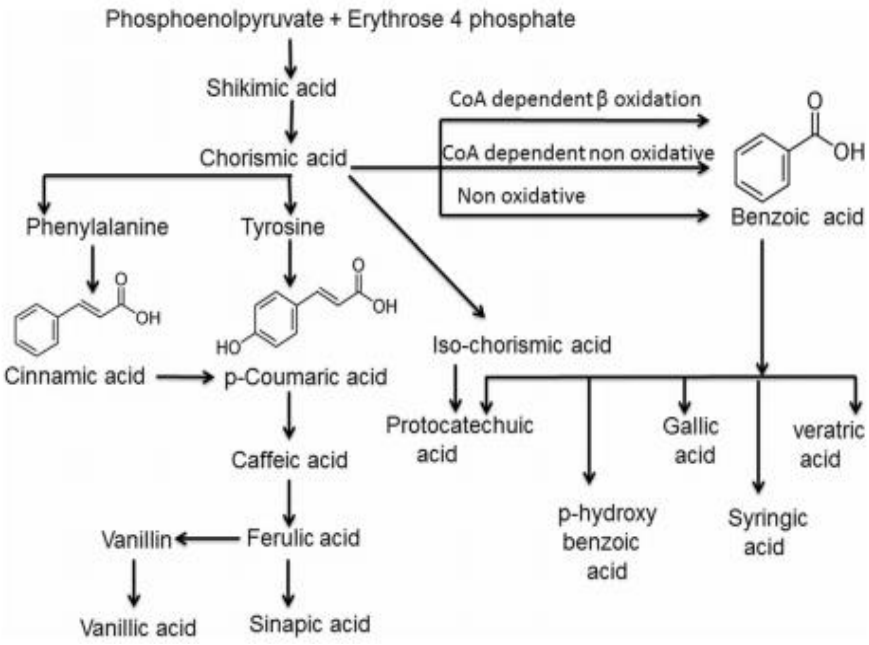


Figure 1: Shikimate pathway showing the biosynthesis of plant phenolic acids.

Source: Tzin and Galili (2010)

1.2.3.1 Simple phenolics

Cinnamic acids and benzoic acids have been studied as the simplest phenolic acids found in plants (Figure 2) with the 6- and 9- carbon framework Giada (2013). These phenolic compounds have a carboxylic group connected to the benzene ring with one or more methoxyl groups or hydroxyl groups linked to it (Yang *et al.*, 2001). Gallic acid for example has three hydroxy (-OH) groups linked at 3rd (Meta), 4th (Para) and 5th (Meta) carbon, while syringic acid contains two methoxy (-OCH_3) groups at the 3rd and 5th (Meta), also one -OH group at the 4th carbon (Para). In addition, the cinnamic acids are studied to contain unsaturated propionic acid side chain linked to the benzene ring (De *et al.*, 2011). Also, caffeic acid has two -OH groups at the 3rd and 4th carbon, whereas ferulic acid contains a single -OCH_3 and one -OH groups linked at the 3rd and 4th carbon atoms, respectively.

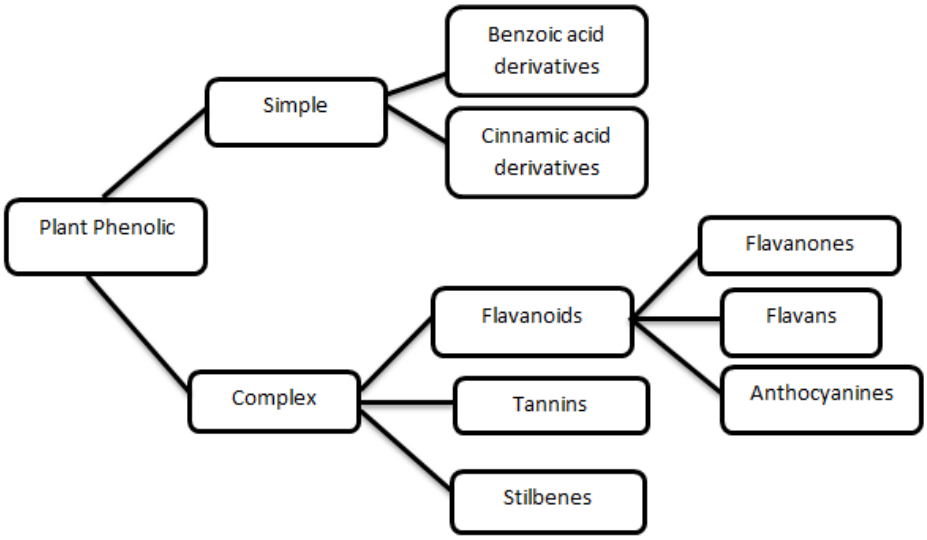


Figure 2: Categories of the plant phenolics according to their structure. Mainly, polyphenols could group into simple and complex phenolics. For simple phenolic acids, they can as well be categorized into benzoic and cinnamic acid derivatives, whereas the complex phenolics are grouped as flavonoids, tannins and stilbenes.

Source: Giada (2013)

1.2.3.2 Complex phenolics

The complex phenolics are phenolic components with higher molecular weights. Complex phenolics are mostly present in cell vacuoles (Cooper *et al.*, 2015). The prominent examples for this category of phenolics are tannins and flavonoids which can be found in fruits and vegetables. Flavonoids contain 2 phenolic rings, having an oxygenated heterocyclic pyran ring attached to it (Kumar and Pandey, 2013). Based on the oxygenated position of the pyran ring, flavonoids can be further classified into: anthocyanins, flavones, flavanols etc. Higher complexity of phenolics are attained owing to the acetylation or glycosylation of these molecules beyond their primary replacements with hydroxyl or methoxyl groups (Cheynier, 2005).

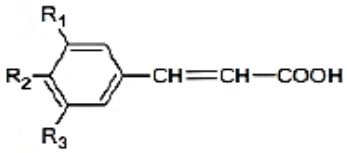
1.2.4 Distribution of plant phenolics

Phenolic acids in plants are mostly found in different parts of plants such as roots, leaves, fruits and vegetables (Achakzai *et al.,* 2009). Caffeic acid is known as the most abundant type of phenolic acids present in fruits, while ferulic acids are present in esterified forms in the cell walls of seed coat, bran and fruits (Dai and Mumper, 2010). Phenolic acids exist in higher levels at significantly varying amounts in the leaves and stems of plants among different species (Achakzai *et al.*, 2009). For example, polyphenols are present in cell vacuoles, tissues present in the leaf, epidermis, flowers and fruits (Pandey and Rizvi, 2009). Also, the barks, wood and fruit pods are abundant in tannins, whereas flowers have more flavonoids (Ververidis *et al.*, 2007).

1.2.5 Phenolic acids

Phenolic acids are part of the major bioactive compounds found in cereal grains and have antioxidant effects on human (Kim *et al.,* 2006; Li *et al.*, 2008). They are of considerable significance as they constitute about 30% of total polyphenols in Mediterranean diets (Laddomada *et al.*, 2015). These compounds are a particular group of polyphenols which are normally associated in the process of defense against biotic and abiotic stresses (Dicko *et al.,* 2005). Phenylalanine acts as the substrate for the biosynthesis of phenolic acids through the phenyl propanoid pathway (Heleno *et al.*, 2015). Some biotic and abiotic conditions including ambient factors and agronomic strategies, can affect the biosynthetic pathway; thereby varying the phenolic acid contents (Rawat *et al.*, 2013). In addition, the influences of genetics need to also be considered as the genetic-environmental interrelation may cause a significant variation in the phenolic contents among different cereal species as well as cultivars of the same species (Fernandez-Orozco *et al.*, 2010; Laddomada *et al.*, 2017).

The phenolic acids occur in free, conjugated or insoluble bound forms (Andersson *et al.,* 2014), while approximately 95% of phenolic compounds (PC) found in grains are ester- or ether-linked to polysaccharides of cell walls and cross-link them intra- and/or intermolecular in order to form networks. These are also regarded as dietary fiber-phenolic components (DF-PC) (Vitaglione *et al.,* 2008). Broadly, phenolic acids in whole grains are classified into hydroxybenzoic acids and hydroxycinnamic acids according to their C1–C6 and C3–C6 structures, respectively (Kim *et al.,* 2006). The hydroxybenzoic acids group includes vannilic, syringic, *p*-hydroxybenzoic and gallic acids, whereas, the hydroxycinnamic acids group comprises of *p*-coumaric, caffeic, sinapic, ferulic, and exist in esters and glycosides forms (Călinoiu and Vodnar, 2018). Of all the hydroxycycinnamic acids, ferulic acid is the main and most abundant. Other groups including benzoic acid derivatives exist in smaller quantities. The corresponding hydoxycinnamic and hydroxybenzoic acids derivatives structure are presented in the Figure 3.

Cinnamic Acid Derivatives	Substitutions		
	R1	R2	R3
Cinnamic acid	H	H	H
p-Coumaric acid	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	CH_3O	OH	H
Sinapic acid	CH_3O	OH	CH_3O

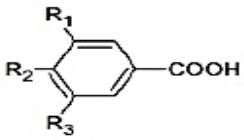
Benzoic Acid Derivatives	Substitutions		
	R1	R2	R3
Benzoic acid	H	H	H
p-Hydroxybenzoic acid	H	OH	H
Protocatechuic acid	H	OH	OH
Vanillic acid	CH_3O	OH	H
Syringic acid	CH_3O	OH	CH_3O
Gallic acid	OH	OH	OH

Figure 3: Hydroxycinnamic acids and hydroxybenzoic acids derivatives structures.

Source: Călinoiu and Vodnar (2018)

The bran is an important factor that determines the health benefits of whole grains. Based on previous studies, the total phenolic contents is basically 15 to 18-fold higher in the bran those observed in the endosperm, which only has 17% from the total phenolic contents in the grain (Fardet, 2010). The bran coats include the aleurone layer, the intermediate (hyaline) layer, the inner and outer pericarp.

Phenolic acids have been shown to have antioxidant activity. The *o*-diphenols (Figure 4) including caffeic acid, hydroxytyrosol, and oleuropein are known to demonstrate a stronger antioxidant activity when compared to phenolic acids that are sterically hindered to a lesser extent for example, tyrosol. Furthermore, phenolic acids such as caffeic, ferulic, sinapic and *p*-coumaric acids that are hydroxyl derivatives of hydoxycinnamic acids, have

more antioxidant potentials than the hydroxyl derivatives of benzoic acids such vanillic acid, syringic acid, *p*-hydroxybenzoic, and 3,4-dihydroxy benzoic acids (Parvathy, 2015).

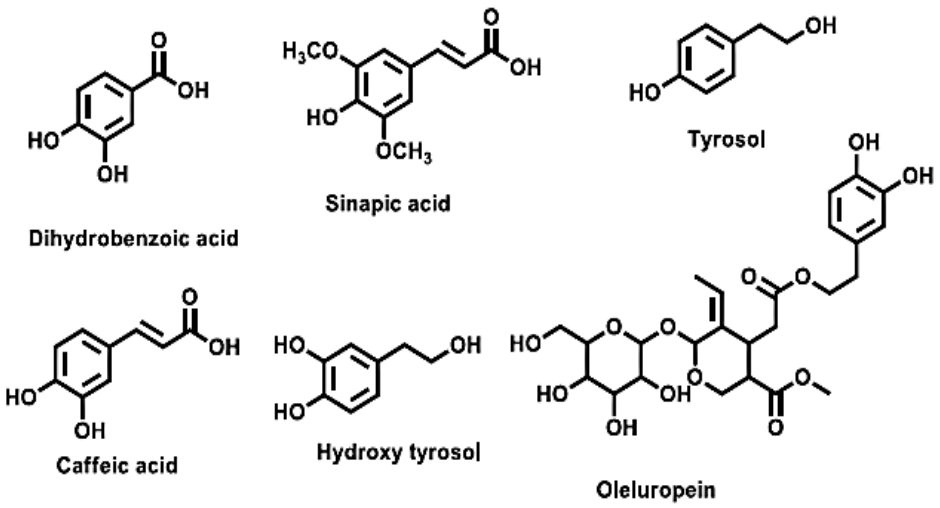


Figure 4: Structure of a few phenolic acids commonly occurring in plants.

Source: Parvathy (2015)

Antioxidants from polyphenols function in suppressing lipid oxidation by scavenging the peroxyl radical. This may be achieved by two processes as shown below:

$$LOO^{*} + ArOH \longrightarrow LOOH + ArO^{*}$$

$$LO^{*} + ArO^{*} \longrightarrow LOOAr$$

From the first step, the lipid peroxy radical (LOO^{*}) extracts a proton of hydrogen from the antioxidant (ArOH) in order to produce the aroxyl radical (ArO^{*}) and hydroperoxides (LOOH), respectively. In the second step, both the peroxy and aroxyl radicals react radical-radical coupling, thereby yielding a non-radical product.

1.2.5.1 Biosynthesis of phenolic compounds

Phenolics are formed by three different biosynthetic pathways (Figure 5): (i) the shikimate/chorismate or succcinylbenzoate pathway which produces the phenyl propanoid derivatives (C6-C3); (ii) the acetate/malonate or polyketide pathway, which produces the phenyl propanoids, including the large group of flavonoids (C6-C3-C6) and some quinones; and (iii) the acetate/ mevalonate pathway which produces the aromatic terpenes, mostly monoterpenes, by dehydrogenation reaction (Knaggs, 2001). The aromatic amino acid phenylalanine, synthesized in the shikimic acid pathway, is the common precursor of phenol containing amino acids and phenolic compounds. Different biosynthetic routes have been proposed (i) direct biosynthesis from an intermediate of shikimate pathway (ii) biosynthesis via phenylalanine, cinnamic acid, *p*-coumaric acid, caffeic acid and 3,4,5-trihydroxy cinnamic acid, or (iii) biosynthesis via caffeic acid and protocatechuic acid (Vermerris and Nicholson, 2007).

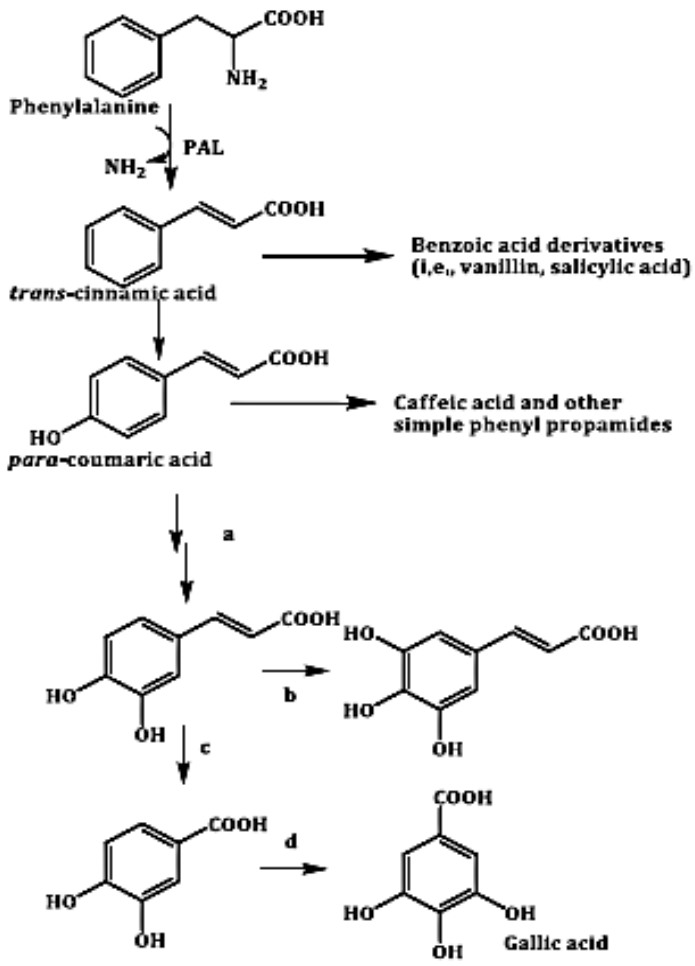


Figure 5: Biosynthesis of phenolic acids (a) enzymes involved in the general phenylpropanoid pathway, (b) proposed for caffeic acid 5-hydroxylase, (c) oxidizing enzymes that reduce propane side chain from C3 to C1, (d) proposed protocatechuic acid 5-hydroxylase.

Source: Vermerris and Nicholson (2007)

1.2.6 Flavonoids

Flavonoids can act as antioxidants by acting as hydrogen donors or by acting as chelating metals. Flavonoids can occur as glucosides (containing glucose as side chains) or as aglycons (without glucose side chains). Flavonoids are a group of plant phenols characterized

by the carbon skeleton C6-C3-C6. The major framework of these components involves two aromatic rings connected by a three- carbon aliphatic chain that generally has been condensed to yield a pyran or less frequently, a furan ring (Cuppett *et al.*, 1997). The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled A, B and C in Figure 6 below.

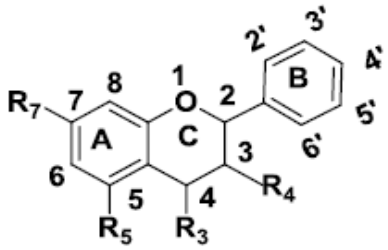


Figure 6: Structure of basic skeleton of a flavonoid.

Source: Cuppett *et al*. (1997)

Among many classes of flavonoids, those of particular interest are flavones, flavanones, isoflavones, flavonols, flavanols, flavan-3-ols and anthocyanidins. Das and Pereira (1990), evaluated the antioxidant capacity of flavonoids and attributed this property to their structures. They reported that the efficacy of flavonoids with the polyhydroxylated substitutions may be influenced by the position of the hydroxyl replacement on the B ring. A reduced antioxidant capacity is associated with a hydroxyl replacement in the *ortho* position in the B ring; nonetheless, the hydroxyl substitution in the *ortho* location aided by a supplementary hydroxyl group in the *para* location increased the activity. Flavonoids can also help to control the extent of lipid oxidation by chelating metal (copper) ions. Hudson and Lewis (Hudson and Lewis, 1983) studied the antioxidant activity of a series of polyhydroxy flavonoids and related their activity to their structure. Researchers predicted about an

association between the 4-carbonyl and the 3- or 5- hydroxyl categories, which function to chelate the copper ion. The flavones form ligands when reacted with the cupric ion, while the flavanones form complex after reaction with copper ions via their enediol or enolic groups.

1.2.7 Free radicals, reactive oxygen species and oxidative stress

A free radical is any chemical species that possesses one or more unpaired electrons. Free radicals have short life time and are highly reactive. Free radicals are highly reactive species that have potential to oxidize biological molecules including proteins, lipids, nucleic acids etc. (Pham Huy *et al.*, 2008).

In aerobic organisms, humans, oxygen is reduced to water at the end of the respiratory chain in mitochondria. Mitochondria are the "power houses" in our cells that provide the energy needed to maintain normal body function and metabolism. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process (Table 1). At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures.

Oxidative stress plays a major part in the development of chronic and degenerative diseases and conditions such as cancer, arthritis, autoimmune disorders, cardiovascular, neurodegenerative diseases and ageing process. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods, supplements (Frei, 1994; Pham Huy *et al.*, 2008).

There are two sources of ROS: exogenous and endogenous. Exogenous ROS can be produced from pollutants, tobacco, smoke, drugs, xenobiotics or radiation. Endogenous ROS are produced intracellularly through multiple mechanisms and depending on the cell and

tissue types. Under normal metabolic conditions, each cell in our body is exposed to about thousand numbers of superoxides each day. Once formed, superoxide is converted to other ROS (Frei, 1994).

Table 1: Different free radicals and their half-life periods

Radical	Name	Half life
HO^*	Hydroxyl radical	10^{-9} sec
LO^*	Lipid alkoxyl radical	10^{-6} sec
LOO^*	Lipid peroxide radical	7 sec
L^*	Lipid carbon centered radical	10^{-8} sec
H_2O_2	Hydrogen peroxide	Minutes
O_2^{-*}	Superoxide anion radical	10^{-5} sec
$^1O_2$	Singlet oxygen	10^{-6} sec
	Semiquinone	Days
NO^*	Nitric oxide radical	~1 sec

Source : Frei (1994); Pham Huy *et al.* (2008)

1.2.8 Antioxidants

Antioxidants are substances that when present in foods or in the body at low concentrations compared to that of an oxidizable substrate markedly delay or prevent oxidation of that substrate (Halliwell *et al.*, 1995). Antioxidants are known to act at different levels in the oxidative sequence involving lipid molecules. They may act by decreasing oxygen concentration, intercepting singlet oxygen, preventing first chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ion catalysts, decomposing primary products to non-radical compounds and chain-breaking to prevent

continued hydrogen abstraction from substrates. The extent to which oxidation of lipids occurs also depends on the chemical structure of fatty acids involved as well as other factors related to the storage of foods and reaction conditions (Shahidi, 1997).

Natural antioxidants from dietary sources include phenolic and polyphenolic compounds, chelators, antioxidant vitamins and enzymes, as well as carotenoids and carnosine. The mechanism by which these antioxidants are involved in the control of food autooxidation and rancidity prevention may be different. However, their presence in the live plants may be for the sake of protecting tissues from injurious damage. The process of autooxidation and development of rancidity in foods involves a free radical chain mechanism proceeding via initiation, propagation and termination steps (Shahidi and Naczk, 1995). While radicals are produced in the "initiation" step, they react with unsaturated fatty acids by abstracting a hydrogen atom from their molecules in the "propagation" step (Figure 7). The reactions in the propagation lead to a chain reaction until a "termination" reaction occurs.

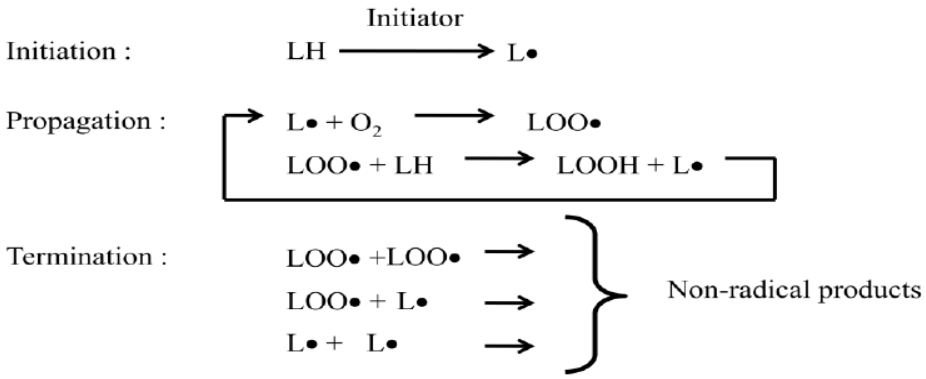


Figure 7: Free radical chain mechanism

Source: Shahidi and Naczk (1995)

1.2.9 Biological activities of polyphenols in the human body

The bioavailability of whole grain polyphenols greatly affects their corresponding biological activities (Manach *et al.*, 2005; Marín *et al.*, 2015) as the bound forms of polyphenols represent about 80% of the total content (Fernandez-Orozco *et al.*, 2010). The polyphenols in whole grains may present immediate biological activities including antioxidant, anticancer, anti-inflammatory, and antimicrobial properties (Shahidi and Yeo, 2018).

1.2.9.1 Antioxidant activity

The antioxidant capacity of phenolic compounds present in cereal grains have been the most interesting aspect studied. Based on previous report of Rice-Evans *et al.* (1996), all phenolic acids exhibit possible antioxidant activities as a result of the aromatic phenolic ring present in these compounds. Some *in vitro* studies have reported various mechanism of action of phenolic acids such as reducing agents, free-radical scavengers and quenchers of single oxygen formation (Sevgi *et al.*, 2015; Verma *et al.*, 2008). Their respective antioxidant capacities can be described by the donation of electron or by the transfer of hydrogen atom to free redicals (Sevgi *et al.*, 2015). Specifically, the potential of hydroxybenzoic acids to initiate various endogenous antioxidant pathways caused an increase in the amount of antioxidant enzymes, consequently reducing oxidative stress and its implicit end-result, namely; endothelial dysfunction and Inflammation processes (Juurlink *et al.*, 2014).

Generally, the phenolic compounds absorbed in the body are metabolized and conjugated (Fardet, 2010). Some specific enzymatic actions such as gastrointestinal esterase may liberate bond ferulic acid and diferulic acids from bran (Andreasen *et al.*, 2001; Liu, 2007). From earlier reports, phenolic acids from dietary fiber could be transformed in the colon into phenylpropionic, phenylacetic acid as well as benzoic acid (BA) metabolites

(Saura-Calixto, 2011; Vitaglione *et al.*, 2008). Their specific antioxidant activities and their absorption into the blood plasma have been reported from previous studies (Giacco *et al.*, 2014; Price *et al.*, 2012). Phenolics in cereal bran are adequately assimilated and may help to significantly promote the antioxidant properties. However, the report that the insoluble components can present an exceptional antioxidant activity (Serpen *et al.*, 2007) provides future insights as regards the production and consumption of dietary fiber-phenolic compounds, functioning as health-related ingredients. Based on the fate of bond phenolics in the gastrointestinal tract, some may prevent the passage of intestinal soluble radicals, which could be an important mechanism for most pathologies (Vitaglione *et al.*, 2008).

1.2.9.2 Anti-Inflammatory property

The anti-inflammatory properties exhibited by phenolic compounds in humans are aided by a remarkable decrease in pro-inflammatory cytokines, while ferulic acid demonstrates a crucial impact on inflammatory messengers in in various *in vitro* and in *vivo* models inflammation studies (Anson *et al.*, 2012).

In a recent *in vitro* study (Laddomada *et al.*, 2015), the exceptional anti-inflammatory property of the phenolic acids observed in whole-meal flour of durum and wheat has been recorded. To be precise, the anti-inflammatory property was shown in colon cells in humans (HT-29 human colon cells achieved by evaluating the amounts of interleukin 8 (IL-8) and the transforming growth component β1 (TGF-β1)), leading to a vital benefit for the intestinal health. In a different human study carried out on a randomized monitored group of obese human subjects which emphasized corresponding anti-inflammatory properties of phenolic acids from whole-wheat grain diets. To be precise, a decrease in the plasma tumor necrosis factor-α (TNF-α) after a period of 8 weeks and an improved interleukin (IL)-10 was observed only after a period of four weeks with the whole grain meal compared with processed grain

meal. From this study, the whole-wheat based diet contained higher amounts of ferulic and dihydroferulic acid in faeces, in contrast with the processed wheat-based diets (Vitaglione *et al.*, 2014).

Bioprocessed bran has been related with an higher level of anti-inflammatory properties in an *ex vivo* lipopolysaccharide (LPS)-induced inflammatory study, implying an exceptional increase in the phenolic acids bioaccessibility, besides their metabolites, which exhibit notable immunomodulatory properties *ex vivo* (Bast *et al.*, 2010).

1.2.10 Gut microbiota and their metabolites

The colonization of Microorganisms starts from the mouth to the rectum and exist all through the area of the gastro intestinal tract (GI) in humans. The microbiota composition and the degree of colonization, howbeit, vary significantly between anatomical sites which may be subjected to the rates of transit, host secretions, conditions of the environment, availability of substrate and the arrangement of the gut wall. Therefore, small amount of microorganisms which are resistant to oxygen exposure, pH conditions and to an extent, the prompt transit rates observed in these regions, are relatively harbored in the stomach and proximal small intestine. On the contrary, the large intestine conditions normally support the formation of an exceptionally heavy microbiota that is predominated by obligate anaerobic bacteria. These microorganisms obtain their energy mostly from the colonic fermentation of non-digested dietary constituents, and from host secretions, particularly mucin, yielding the SCFA acetate, propionate, and butyrate as main outcomes as well as the H_2, CO_2, CH_4, and H_2S gases produced simultaneously. Data on the microbial composition of the colon are mostly achieved from the study of fecal samples.

The gut microorganism community is known to be primary responsible for various vital biological roles such as production of short chain fatty acid (SCFAs) and vitamin,

synthesis of amino acid (AAs), transformations of bile acids, hydrolysis along with the non-digestible substrates fermentation (Putignani *et al.,* 2015). The health benefits of the gut microorganism community involve the following: (i) establishment of immune-cell balance, (ii) homeostasis of the epithelial cells, (iii) regulation of the nerve cells, (iv) angiogenesis help the digestion of food as well as modulation of fat metabolism (Holmes *et al.,* 2011). Through the production of organic metabolites, gut microbiota regulates pathway signaling associated with the intestinal mucosa homeostasis. When a healthy relationship between the gut microbiota and the GI tract is interrupted, both intestinal diseases and extra intestinal disorder may emerge (Putignani *et al*., 2015), including inflammatory bowel disease (IBD), obesity, allergy, cancer, diabetes, metabolic dysfunction, neuropathology and cardiovascular dyslipidemia (Holmes *et al.,* 2011).

According to reports on molecular analyses, most of the bacteria are members of 2 phyla, namely; Firmicutes and Bacteroidetes (Eckburg *et al.,* 2005). The Bacteroidetes phylum that is Gram-negative involves the genera Prevotella and Bacteroides; these bacteria have the ability to utilize a very broad diversity of substrates and are known to be the main propionate producers (Kaoutari *et al.*, 2013; Reichardt *et al.,* 2014). Firmicutes include several species identified as the dominant producers of butyrate and specialist degraders of indigestible polysaccharides (Ze *et al.*, 2012). Actinobacteria (including *Bifidobacterium* spp.), Proteobacteria (including *Escherichia coli*), and Verrucomicrobia (including *Akkermansia mucinophila*) are typically present in smaller numbers in the healthy gut microbiota, but these organisms have considerable potential to influence health outcomes. Only the most abundant species of human intestinal organisms accounting for 30% are currently represented by cultured isolates (Walker *et al.*, 2010), while most of the remaining organisms are probably capable of being cultured (Lagier *et al.*, 2012).

According to Cardona *et al.* (2013), the gut microorganism community of human is affected by several conditions, from which diet is a very vital one (Figure 8). Also, researches have shown that the intestinal microbes are markedly affected by numerous factors such as host genetics, mode of delivery, lifestyle (urbanization and global mobility), medical interventions (use of antibiotics, vaccinations and hygiene) and health status (Burokas *et al.*, 2015). Furthermore, diet has repeatedly shown to be one of the most important factors affecting gut microbiota establishment and composition. Food ingredients, which are non-digestible by enzymes in humans (for example, fiber) help to provide substrates needed for the microbial fermentation in the intestines. As gut bacteria are exclusive fermenting various substrates, multiple diets can produce various growth-enhancing and growth-suppressing factors for particular phylotypes (Flint *et al.*, 2012). Also, the outcome from the metabolism of bacteria especially SCFA and vitamins are important for the health benefits of humans. As shown in Figure 9, a healthy or impaired gut microorganism community influences the gut as well as the host metabolic health via the modulation of gut physiology and lipopolysaccharide (LPS) infiltration, calorie intake, fat accumulation, and insulin action (Boulangé *et al.* 2016). An important mode of action of interest is the microbial balance of bile acids that influences the function of liver by committed receptors of bile acids including TGR5 and farnesoid X receptor. The rate of gluconeogenesis as well as glycolysis could be influenced by the indicators from the receptors of bile acid, therefore, altering the management of blood glucose (Giacco *et al.*, 2011). Nonetheless, there are few reports that connect the consumption of grains to the change in the circulation of bile acids to the liver and intestines and the subsequent generated profiles of bile acids.

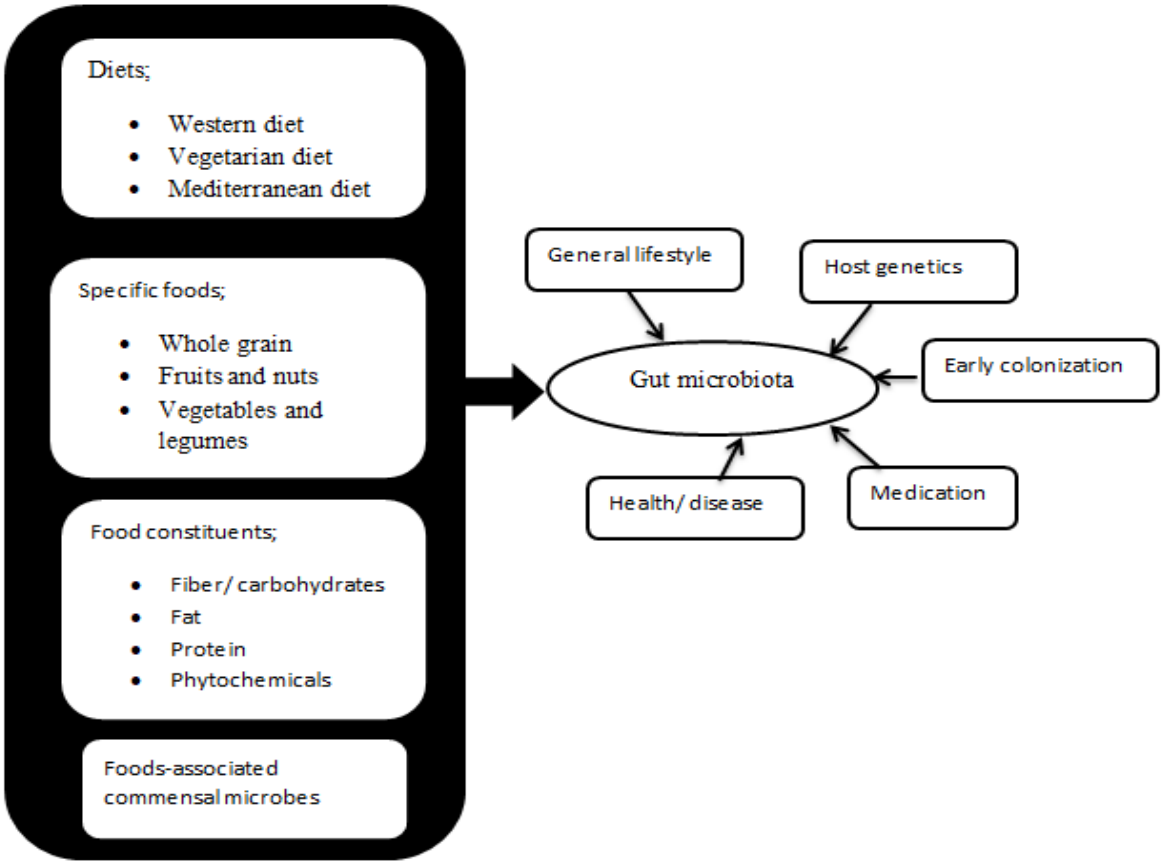


Figure 8: Dietary factors affecting the gut microbiota composition in humans

Source: Cardona *et al.* (2013).

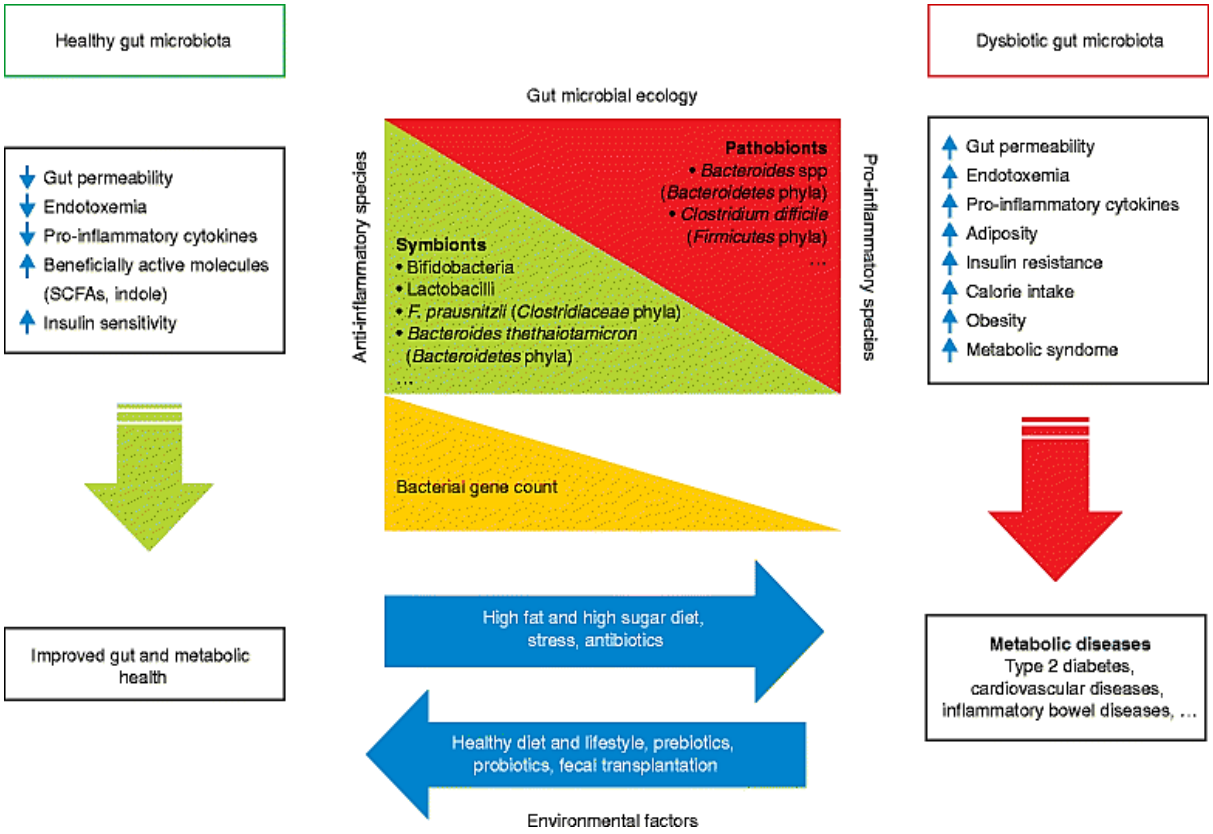


Figure 9: Effects of a healthy gut microbiota and dysbiosis on the gut and metabolic health of the host. A healthy microbiota comprises a balanced representation of symbionts (bacteria with health-promoting functions) and pathobionts (bacteria that potentially induce pathology). A shift toward dysbiosis results from a decrease in symbionts and/or an increase in pathobionts and is likely to be triggered by environmental factors (such as diet, stress, antibiotics, and infections). Low bacterial gene counts have also been associated with altered gut microbial functions and dysbiosis and have been linked to increased fat accumulation, lipopolysaccharide-induced inflammation, insulin resistance, obesity, and the metabolic syndrome. Individuals with these characteristics are more likely to develop metabolic diseases (such as diabetes, cardiovascular diseases, and inflammatory bowel diseases). LBP - Lipopolysaccharide Binding Protein, SCFA – Short - Chain Fatty Acid.

Source: Boulangé *et al.* (2016).

Whole cereal grains are made up of fibers are not readily digested by humans (such as arabinoxylans or β-glucan) and could only be fermented by the native bacteria (Hamaker and Tuncil, 2014). When dietary fibers are fermented, they are capable of producing some byproducts which are readily assimilated and utilized for various metabolic functions in human body (Den Besten *et al.*, 2013). The produced byproducts comprise of SCFAs, with acetate being the most abundant, followed by propionate and butyrate. Substantial amounts of butyric acids produce locally are used by the human colon, while the liver and peripheral tissues utilize propionic acids. On the other hand, acetic acids are capable of passing through the blood brain barrier and used as a source of energy by the glial cells (Everard and Cani, 2014; Frost *et al.*, 2014). Generally, SCFAs are believed to contribute to an estimate of 10% of all the energy requirements in human body (Everard and Cani, 2014).

1.2.10.1 Butyric acid

Firmicutes including *Faecalibacterium prausnitzii*, Roseburia, *Eubacterium rectale* and various *Clostridium* spp. are known to be major producers of butyric acid which has been studied for its role as a fundamental source of energy for the colon cells (Joyce and Gahan, 2014). Furthermore, butyric acid is also believed to preserve the integrity of the epithelial cells in the gut system due its ability to promote the transcription, which in turn improves the produced protein integrals and the development of tight connections within the colonocytes. When the connections are tighter, penetration of bacteria is minimized. Butyric acid aids the reduction of bacteria permeability in the GI tract by improving the stimulation of the peroxisomal proliferator-activated receptor (Cooper *et al*., 2015).

1.2.10.2 Propionic acid

Propionic acid is known to be mainly produced by some bacteria in the Bacteroidetes group, responsible for carbohydrates fermentation (Joyce and Gahan, 2014). Various studies

on rodents and humans revealed the relationship between propionic acid and G protein-coupled free fatty acid receptor (FFAR), otherwise referred to as G protein-coupled receptor (GPCR) 43, observed on an enteroendocrine L cells found in the colon. When these L cells are stimulated, they serve as an intermediary in the release of GLP-1. Stimulation of FFAR3 which is also regarded as GPR41 may help activate the peptide YY (PYY) liberation, with some studies suggesting that this pathway may as well be utilized to trigger the liberation of GLP-1 (Everard and Cani, 2014). The stimulation of FFAR2 on adipocyte cells could initiate leptin production. Higher amounts of leptin, GLP-1 and PYY are known as indicators of satiety which is believed to lower the food consumption in humans and rodents (Chambers *et al.*, 2015). Moreover, GLP-1 functions as inhibitors of production of gastric acids, slow down the emptying of gastric as well as reducing intestinal motility; PYY helps to suppress the stomach movement and emptying and promotes both water and electrolyte re-assimilation in the large intestine, thereby resulting into prolonged stay of food in the intestines; also, leptin reacts on the arcuate nucleus of the hypothalamus for the control of appetite (Shah and Vella, 2014).

1.2.10.3 Acetic acid

Bifidobacterium and *Ruminococcus bromii* are known producers of acetic acid through carbohydrates fermentation (Tagliabue and Elli, 2013). All the SCFAs have been reported to reduce drastic energy intake with no rise in GLP-1 or PYY accumulation humans as well as in rodent studies (Everard and Cani, 2014; Frost *et al.*, 2014). Various indications have suggested the possibility of this impact to be moderated by a rise in the concentration of acetic acid resulting from the breakdown of carbohydrates in the large intestine. In a study on rodents, acetic acid generated from the colon was observed to infiltrate into the blood stream, pass through the barrier between the blood and the brain and finally absorbed by the

hypothalamus in the brain. It was observed that the food intake of the animals after administering acetic acid (between 1-2 h) were significantly lower than that of the control groups due to the impact of improved concentration of acetic acid with no substantial differences in the levels of PYY and GLP-1. The authors believed that this may be attributed to the increased concentrations of acetic acid in the hypothalamus, thus improving the oxidative lactic acid synthesis from the pyruvic acid recovering pathways and improving the GABAergic neurotransmission in the hypothalamus. The synthesis of ATP (Adenosine triphosphate) could be enhanced by increasing the oxidative metabolism, thereby, lowering the proportion of AMP (adenosine monophosphate) to ATP. Consequently, this may reduce the actions of AMP kinase with improved malonyl-CoA levels and inducing the pro-opiomelanocortin (POMC) neurons, which in turn additionally increases the GABAergic neurotransmission. The improved stimulation of pro-opiomelanocortin (POMC) neurons and GABAergic neurotransmission will help to lower acute appetite, revealing the potential of acetic acid in boosting satiety (Frost *et al.*, 2014). A reduction of food consumption was reported with acetic acid in the study of obesity in animal studies via the mechanism of central nervous system (Frost *et al.*, 2014).

1.2.11 Phenolic compounds metabolism by the gut microbiota

Polyphenols found naturally are normally in complex molecules and glycosylated forms, which are less soluble and bioaccessible (Marín *et al.*, 2015). Once the phenolic compounds are consumed in diets, the insoluble components are transformed (Figure 10) by some microbial communities and enzymes in the GI system in order to yield phenolic compounds with improved bioaccessibility and additional pharmacological activities (Marín *et al.*, 2015). To be precise, some current research have shown that ellagic acid which is a phenolic compound that can obtained from berries was modified to urolithin by the microbial

community in the gut. The urolithin produced strongly suppressed the the heme peroxidases including lactoperoxidase and myeloperoxidase, leading to a reduction in cellular damage caused by inflammation (Saha *et al.*, 2016). Furthermore, urolithin helps to decrease the generation of superoxide radicals which may be quite impossible by ellagic acid. Urolithin, when administered to rats with a body weight of 40 mg/kg, significantly suppressed PMA-triggered edema and activity of MPO when compared to activities of ellagic acid (Saha *et al.*, 2016). A study carried out by Marín *et al.* (2015) revealed the synthesis of some phenolic compounds including *p*-coumaric and ferulic acids from ester linked arabinoxylans through the hydrolytic property of the gut microbial community. In addition, studies have as well reported that ferulic acid dimers were transformed into 3-(4 hydroxyphenyl)- propionic acid and vanillin with improved anti-inflammatory activity (Marín *et al.*, 2015). In summary, biodegradation of polyphenols by the gut microbial community provides various vital roles and also affects the biochemical activities, thereby leading to varying response to phenolic compound administration within humans (Vyas and Ranganathan, 2012).

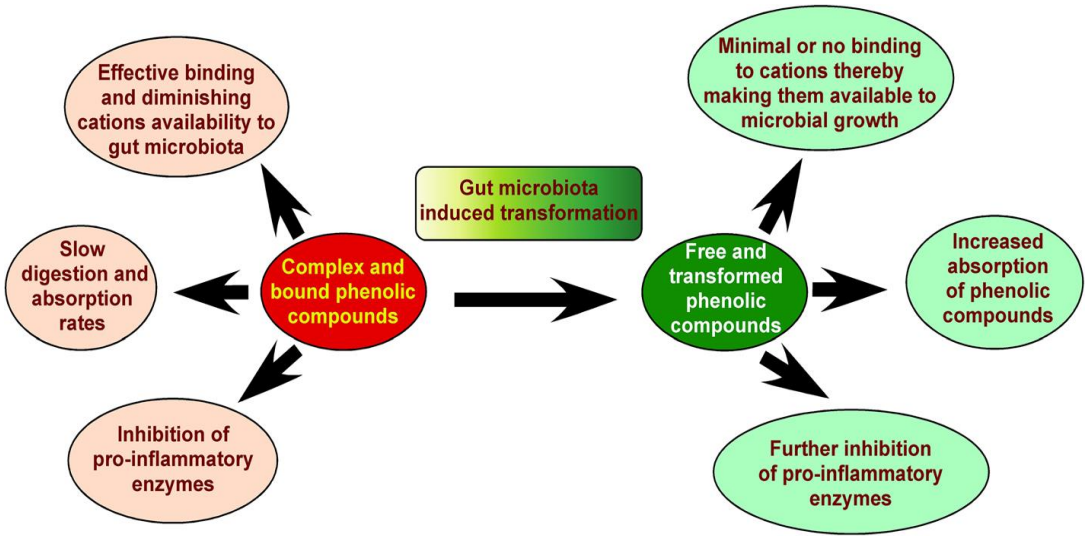


Figure 10: Gut microbial community influences the biodegradation of polyphenols and improves the health benefits.

Source: Marín *et al*. (2015).

The gut microbial community helps to regulate polyphenols via several mechanisms such as hydrolysis, reduction, ring-cleavage decarboxylation and demethylation. The metabolism of these phenolic compounds by the gut microbiota is important for their absorption as their biological properties are being regulated. (Hanhineva *et al.*, 2010). In plants, most polyphenols are found as glycosylated forms; however, their transformations like polymerization and esterification also exist. After ingesting phenolic compounds, they admitted as xenobiotics by humans with lower bioacessibility when compared to micro and macronutrients. Moreover, based on the level of polymerization and complexity of structure, these phenolic compounds may be freely assimilated in the upper gut (in other words, phenolic compounds with low molecular weights involving both the mono and dimeric structures) (Appeldoorn *et al.*, 2009). They may as well get to the large intestine untransformed (for example, polymeric and oligomeric phenolic compounds including hydrolysable and condensed tannins with values of molecular weights extending to about 40, 000 Da) (Monagas *et al.*, 2010). It has been estimated that only 5–10% of the total phenolic compound intake is absorbed in the small intestine. The remaining phenols (90–95% of total phenolic intake) may accumulate in the large intestinal lumen up to the millimolar range where, together with conjugates excreted into the intestinal lumen through the bile, they are subjected to the enzymatic activities of the gut microbial community (Kutschera *et al.*, 2011; Rothwell *et al.*, 2012). The gut microbial community is thus important for intense degradation of the main phenolic structures yielding different phenolic metabolites of small molecular weights that are easily assimilated in the body. This may be the key factor for the health benefits obtained from the consumption of diets abundant in polyphenols instead of the initial phenolic compounds found in the diet. Consequently, apart from the inter-individual variation in daily intake of polyphenols, inter-individual differences in the composition of the

gut microbiota may lead to differences in bioavailability and bio-efficacy of polyphenols and their metabolites (Gross *et al.*, 2010). When evaluating the connection between the gut microbiota and polyphenols, it becomes more complicated. Recently, different studies have revealed that the phenolic compounds (substrates) fed in to the gut microbiota via various forms of dietary consumption as well as the organic metabolites generated may consequently regulate and alter the formation of the gut microbiota by some selective prebiotic and antimicrobial properties resistant to some pathogenic bacteria in the gut (Queipo-Ortuño *et al.*, 2012). The formation of bioactive polyphenol-derived metabolites and the modulation of colonic microbiota may both contribute to host health benefits, although the mechanisms have not been delineated. The health properties attributed to beneficial bacteria for human hosts include protection against gastrointestinal disorders and pathogens, nutrient processing, reduction of serum cholesterol, reinforcement of intestinal epithelial cell-tight junctions and increased mucus secretion and modulation of the intestinal immune response through cytokine stimulus (Vitali *et al.*, 2010).

Figure 11 diagrammatically shows the destination of the phenolic compounds in diets consumed by humans. Concisely, about 5-10% of the overall polyphenols ingested especially with mono and dimeric structures are immediately assimilated in the upper gut normally following decojugation reactions including deglycosylation (Manach *et al.*, 2005). Following the assimilation of these phenolic compounds in the upper gut system, the ones with lower molecular weights may undergo intense oxidation, reduction and hydrolysis in phase I and especially conjugation in phase I biodegradation in the enterocytes and subsequently in the hepatocytes, leading to the production of various hydrophilic conjugated metabolites (such as glucuronide, methyl and sulfate derivatives) which are easily released in to the circulatory system for subsequent transportation to tissues and organs and later elimination in the urine.

Reaching the colon, the gut microbiota is believed to react enzymatically with about 90-95% of the overall polyphenol ingested which are unabsorbed in the upper gut, thereby yielding organic metabolites with various biological effects (Bowey *et al.*, 2003). Thus, it was admitted that the gut microbial community has an important influence on the bioaccessibility of phenolic compounds as well as their pharmacological prospective (Cardona *et al*., 2013). Nonetheless, the variation existing between separate gut microbial communities and the likely variation resulting from the day-day consumption of polyphenols may significantly affect their potency as well as their bioaccessibilty. Hitherto, various groups of bacteria are known to influence the transformations of polyphenols, which tends to be more peculiar rather than being ubiquitous (Cardona *et al*., 2013). Therefore, even the phenolic compounds in diets can in turn affect the gut microbiota composition.

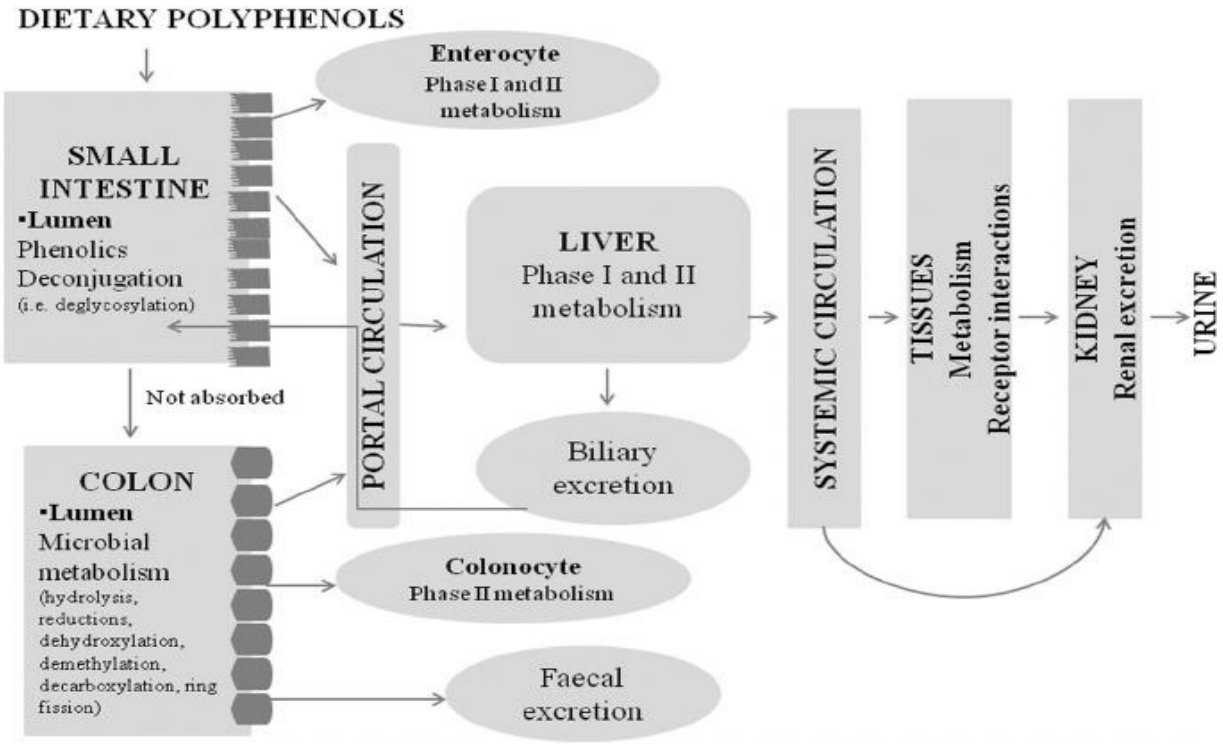


Figure 11: Routes for dietary polyphenols and their metabolites in humans. Within the host, dietary polyphenols and their microbial metabolites successively undergo intestinal and liver

phase I and II metabolism, biliary secretion, absorption in the systemic circulation, interaction with organs and excretion in the urine.

Source: Manach *et al.* (2005a).

Various metabolites are produced from the microbial transformation of dietary polyphenols and as well non-digested carbohydrates and amino acids (Lord and Bralley, 2008). Examples are the metabolites from colonic degradation of common polyphenols as presented in Table 2 (Jandhyala *et al.*, 2015). Chlorogenic acids are a class of phenolic compounds consisting of the hydroxycinnamates (such as ferulic, *p*-coumaric and caffeic acids) which may interact with quinic acid to yield different conjugated structures and acids including *p*-coumaroylquine acids. Chlorogenic acids are mostly present in plums, peaches and some seeds such as coffee. However, several microbial metabolites have been identified. Table 3 shows that the main microbial metabolites of hydroxycinnamates are 3-hydroxyphenylpropionic acid and benzoic acid, generated by the action of *E. coli*, *B. lactis*, and *Lactobacillus gasseri* (Marín *et al*., 2015). The first compound is produced by de-esterification, decrease in double bond, as well as dehydroxylation. In addition, β-oxidation reduces the side-chain and produces benzoic acid in small degree. These two metabolites are also produced from chlorogenic acid (Gonthier *et al.*, 2006). The most common metabolites from ferulic acid produced by colonic microbiota are vanillin and 3-(4-hydroxyphenyl) propionic acid (Couteau *et al.*, 2001). Recently, reports have shown that the gut microbiotas such as *Bifidobacterium* and *Lactobacillus* strains are capable of transforming chlorogenic acid in to quinic and caffeic acids (Marín *et al.*, 2015; Raimondi *et al.*, 2015), whereas caffeic acid can be subsequently transformed into 3-hydroxylphenylacetic, dihydroxyphenylpropionic acid and *p*-coumaric acids (also known as 3-hydroxycinnamic acid) (Konishi and Kobayashi, 2004). For the 3,4- dihydroxyphenylacetic acid, it could be

produced from rutin (Jaganath *et al.*, 2009). Contrarily, the metabolites derived from rutin were not found in a bacteria-free mice, which means products with ring-fission are produced mainly by the gut microbiota (Parkar *et al.*, 2013). The dietary polyphenol detection is performed using Liquid Chromatography Mass Spectrometry (LC-MS), Gas Chromatography Mass Spectrometry (GC-MS), and ^{1}H-NMR (proton nuclear magnetic resonance) spectroscopy platforms. Table 4 shows an elaborate list of the dietary polyphenols and the gut microbiota involved in its transformation. Appeldoorn *et al.* (2009), conducted a study on the transformation of procyanidin dimers by the human colon microbial community. Grape seed extracts were fermented for 0, 1, 2, 4, 6, 8 and 24 h. They identified several phenolic metabolites produced, including phenylvaleric acids, phenylvalerolactones and 3´,4´-dihydroxyphenyl-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol. Furthermore, Clostridium histolyticum population was observed to reduce after the fermentation period compared to the control samples.

Table 2: Types of dietary polyphenols present in various foods and the types of gut micro-organisms those are responsible for the degradation

Phenolic compounds	**Classes involved**	**Foods containing polyphenols**	**Gut bacteria**
Flavanols	Kaempferol, Quercetin, Myricetin	Onions, capers, apples, broccoli, grapes and plums	*Bacteroides distasonis*, *Bacteroides uniformis*, *Enterococcus caseliflavus* and *Eubacterium ramulus*
Flavones	Hesperetin, Naringenin	Citrus fruits and tomatoes	*Clostridium* spp., *E. ramulus*
Flavan-3-ols	Catechin, Epicatechin, Gallocatechin	Green tea, cocoa, kola, banana, pomegranate	*Bifidobacterium infantis* and *Clostridium coccides*
Anthocyanidins	Cyanidin, Pelagonidin, Malvidin	Bilberries and all red, blue and purple fruits (especially berries)	*Lactobacillus plantarum, L. casei, L. acidophilus* and *Bifidobacterium longum*
Isoflavones	Daidzein, Geinstein, Formononentin,	Soy, beans, lentils, chickpea (Fabaceae family)	*Lactobacillus* and *Bifidobacterium*
Flavones	Luteolin, Apigenin	Cereals, parsley, thyme, celery and citrus fruits	*Clostridium orbiscinden, Entrococcus avium*
Tannins	Gallo tannins, Ellagitannins	Raspberries, cranberries, strawberries, walnuts, grapes and pomegranate	*Butyrivibrio* spp.
Lignins	Secoisolariciesinnol, metaresinol, pinoresinol, larciresinol, isolarciresinol, syringiresinol	Flax seeds, cereals, strawberries, and apricots	Species of *Bacteroides*, *Clostridium*, *Peptostreptococcus* and *Eubacterium*
Chlorogenic acids	Caffeic acid, ferulic acid	Peach, plums and coffee	*E. coli*, *Bifidobacterium* spp. and *L. gasseri*

Source: Jandhyala *et al.* (2015)

Table 3: Main metabolites derived from non-flavonoids and identified bacteria involved in their transformation

Precursors	**Main identified metabolites**	**Bacteria**
Ellagitannins	Urolithins	*Butyrivibrio* spp
Lignans	Enterodiol Enterolactone	*Bacteroides distasonis, Bacteroides fragilis, Bacteroides ovatus, Clostridium cocleatum, Butyribacterium methylotrophicum, Eubacterium callanderi, Eubacterium limosum, Peptostreptococcus productus, Clostridium scindens, Eggerthella lenta*
Hydroxycinnamates	3-hydroxyphenylpropionic acid, 3-(4-hyroxyphenyl)propionic acid Vanillin	*Escherichia coli, Bifidobacterium lactis, Lactobacillus gasseri*

Source: Marín *et al.* (2015)

Table 4: Main metabolites derived from flavonoids and identified gut bacteria involved in their transformation.

Precursors		Main metabolites identified	Bacteria	References
Flavonols	Kaempferol	2-(4-Hydroxyphenyl)propionic acid 2-(3,4-Dihydroxyphenyl)acetic acid	*Clostridium orbiscidens* *C. orbiscidens, Eubacterium oxidoreducens*	Schneider *et al.* (2000)
	Quercetin	2-(3-Hydroxyphenyl)acetic acid 3-(3,4-Dihydroxyphenyl)propionic acid 3-(3-Hydroxyphenyl)propionic acid	*Eubacterium ramulus* *Enterococcus casseliflavus*	Rechner *et al.* (2004); Schneider *et al.* (2000)
	Myricetin	2-(3,5-Dihydroxyphenyl)acetic acid 2-(3-Hydroxyphenyl)acetic acid	*C. orbiscidens, E. oxidoreducens*	Schneider *et al.* (2000)
Flavanones	Naringenin	3-(4-Hydroxyphenyl)propionic acid	*Clostridium* strains *E. ramulus*	Manach *et al.* (2005); Rechner *et al.* (2004)
Flavan-3-ols	Catechin	3-(3-Hydroxyphenyl)propionic acid 5-(3´,4´-Dihydroxyphenyl)-γ-valerolactone	*Clostridium coccoides, Bifidobacterium* Spp.	Yamakoshi *et al.* (2001)
	Epicatechin	5-(3,4-Dihydroxyphenyl)valeric acid 3-(3,4-Dihydroxyphenyl)propionic acid		
	Epigallocatechin	5-(3´,4´-Dihydroxyphenyl)-γ-valerolactone 5-(3´,5´-Dihydroxyphenyl)-γ-valerolactone		
Anthocyanins	Cyanidin Peonidin Pelargonidin Malvidin	3,4-Dihydroxybenzoic acid 3-Methoxy4-hydroxybenzoic acid 4-Hydroxybenzoic acid 3,4-Dimethoxybenzoic acid	*Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12	Miladinović *et al.* (2014)

Table 4: Main metabolites derived from flavonoids and identified gut bacteria involved in their transformation (continued).

Precursors		Main metabolites identified	Bacteria	References
Isoflavones	Daidzein	(S)-Equol	*Bacteroides ovatus, Streptococcus intermedius, Ruminococcus productus, Eggerthella sp.Julong* 732, *Enterococcus*	Decroos *et al.* (2005)
		O-Demethylangolensin	*Clostridium spp.* HGHA136	Hur *et al.* (2002)
	Genistein	6´-Hydroxy-O-desmethylangolensin		Coldham *et al.* (2002)
	Formononetin	Daidzein		Heinonen *et al.* (2004)
	Biochanin A	Genistein		Heinonen *et al.* (2004)
Flavones	Luteolin, apigenin	3-(3,4-Dihydroxyphenyl)-propionic acid, 3-(4-hydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid, and 4-hydroxycinnamic acid, phloretin	*C. orbiscindens, Enterococcus avium*	Hanske *et al.* (2009)

1.2.12 Dietary phenols and carbohydrate metabolism

Carbohydrates obtained from diets consist of two most significant components, namely; starch and sucrose. Digestion, assimilation as well as the metabolism of starch and sucrose could be affected by the presence of polyphenols and their metabolites found in the diet. Almost all carbohydrates are absorbed in the upper gut system as monosaccharides, followed by subsequent assimilation in the circulatory system (Cardona *et al.*, 2013). The elevated glucose concentration in blood promotes secretion of insulin from the β-cells of the islets of Langerhans in the pancreas, and insulin mediates the uptake of glucose in peripheral tissues including muscle, adipose tissue and kidney, promotes storage of glucose in liver as glycogen, and inhibits lipolysis in adipose tissue. Another essential hormone in maintaining the glucose homeostasis is glucagon that is secreted from the pancreatic α-cells once the blood glucose level begins to fall below normal. Glucagon promotes liver glucose production by inducing glycogenolysis and gluconeogenesis to ensure adequate circulating glucose to fuel the body functions. Maintenance of glucose homeostasis is of utmost importance to human physiology, being under strict hormonal control. Failure of this control can result in the metabolic syndrome, a multi-symptom disorder of energy homeostasis encompassing obesity, hyperglycemia, impaired glucose tolerance, hypertension and dyslipidemia (Walle, 2004). The most characteristic abnormality in the metabolic syndrome is insulin resistance, which results from interactions between genetic and environmental factors, including diet and sedentary lifestyle (Monagas *et al.*, 2010). Products from whole cereal grains have been greatly studied due to their increased fiber components as well as their abundant phenolic reserves which could proffer some positive impacts on the glucose regulation (Larrosa *et al.*, 2010). The intake of plant food products, such as whole grains, berries, fruits and vegetables are not only known to be excellent sources of dietary fiber, but also good sources of variable

phenolic compounds. These compounds may influence glucose metabolism by several mechanisms, such as inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Hanhineva *et al.*, 2010) as shown in Figure 12.

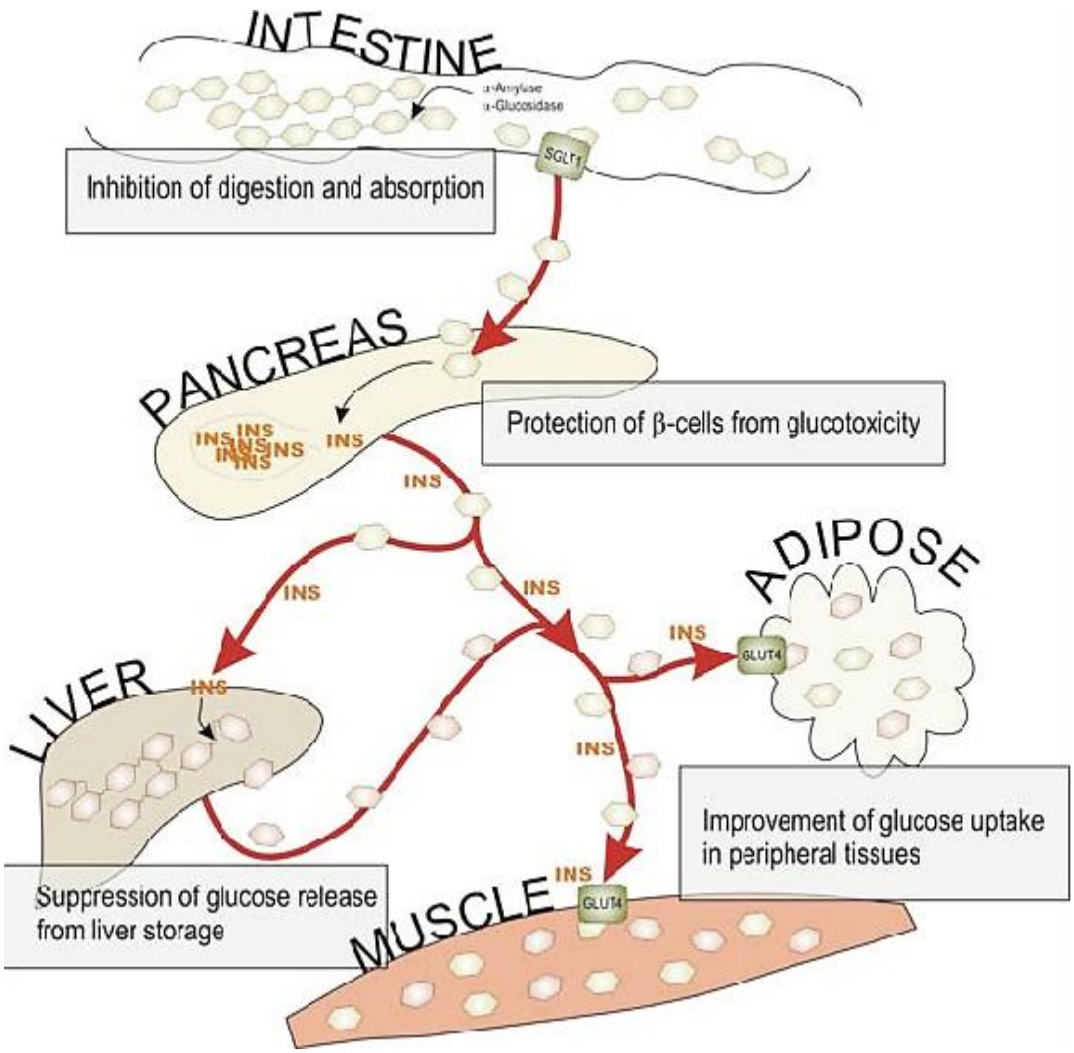


Figure 12: Potential sites of action of dietary polyphenols on carbohydrate metabolism and glucose homeostasis.

Source: Hanhineva *et al.* (2010)

The breakdown of carbohydrates and the absorption of glucose are important factors for an improved regulation of glycemic index after consuming diets high in carbohydrates. Fundamental enzymes that influence the breakdown of carbohydrates in diets are α-amylase and α-glucosidase. The glucose released is then assimilated in all parts of the enterocytes in the intestines through some certain transporters. The suppression of the glucose carrier and the enzymes responsible for digestion decreases the level of glucose released and the assimilation in the small intestine, thus, inhibiting the increase in glycemic index following the consumption of carbohydrate rich-diets (Hanhineva *et al.* (2010).

Various polyphenols have been studied to suppress the *in vitro* actions of α-amylase and α-glucosidase. These phenolic compounds encompass flavonoids (e.g. catechins, anthocyanins, flavanones, isoflavones, flavonols and flavones), ellagitannins and the simple phenolics. Furthermore, studies have revealed the suppressive properties (*in vitro*) of some phenolic compounds isolated from food substances such as vegetables (eggplant, corn, peas and pumpkin), berries (blue and black berries, raspberries and strawberries), red wine, black and green tea, pigmented grains including black rice. From the studies, substrates such as sucrose, *p*-nitrophenyl-α-D-glucopyranoside or maltose were utilized in the evaluation of the actions of α-glucosidase activity Hanhineva *et al.* (2010).

1.2.13 Germination

The process of germination, also known as sprouting is a conventional, non-heat and economical technique of improving grain quality as the absorption of nutrients is increases. This thereby helps to lower the amounts and actions of anti-nutrients, increases the available amino acids and carbohydrates and consequently enhances the functional properties. (Eyzaguirre *et al.*, 2006). The germination technique includes some chemical modification in the grains including starch, fat and protein degradation by the activities of amylolytic,

lipolytic as well as proteolytic enzymes, independently. During the soaking and germination period of cereal grains under room temperature, both the enzymes existing in the grain and the newly produced ones start to change the components of the seeds (Katina *et al.*, 2007). Therefore some macromolecules (complex) are degraded in to smaller molecules which are easier to digest and assimilated into the body

The germination technique can be confirmed by the penetration of the surrounding structures of the embryo by the radicle. This is also called visible germination. The process of germination starts with the uptake of water by a mature dry seed (Figure 13) characterized with rapid initial uptake (Phase 1). This is followed by a plateau phase (phase 2) in which the embryonic axes elongates and finally enters into complete germination, also known as phase 3 (Bewley, 1997).

Several factors may decrease the nutritional components of grains including the suppressive actions of enzymes as well as other anti-nutrients which may inhibit the quality and digestion of protein, reduction of the bioaccessibility of micronutrients by the activity of some metal chelating elements for example, zinc and iron (Mouquet-Rivier *et al.*, 2008). Nevertheless, some grain processing techniques may be used to eradicate or inhibit the actions of these factors such as soaking, germination, fermentation, dehusking as heat treatments (e.g. canning and boiling, infrared heating). The process of germination may also trigger or lead to the synthesis of some bioactive components of the grain. When grains are germinated, some important compounds are synthesized through biological activities, necessary for the seedling development. Consequently, this technique results in to the increase in the levels of peptides, simple sugars and amino acids in the grains germinated. These include germinated barley (Chung *et al.*, 2009), germinated wheat (Moongngarm and Saetung, 2010) and germinated rice (Saman *et al.*, 2008). Additionally, various enzymes are

stimulated to produce some compounds including GABA as well as vitamins. Example of such germinated grains include germinated rice (Kim *et al*., 2015; Komatsuzaki *et al.,* 2007; Thuwapanichayanan *et al*., 2015), barley (Chung *et al*., 2009) and soybean (Xu & Hu, 2014). In other words, germination is an evident process to improving bioactive compounds, nutritional and health benefits (Imam *et al.*, 2012; Imam and Ismail, 2015; F. Wu *et al.*, 2013). Due to perception, limited option and education, people consume white rice (WR) even it contains high glycemic index (GI). Therefore, consumption of GI material for a long time can lead to disorders like obesity, glucose intolerance, type 2 diabetes, and cardiovascular diseases (Barclay *et al.*, 2008). Studies have shown that the gut microbial community among volunteers consuming white bread was altered throughout the period of twelve weeks of human intervention studies in Finnish adults aged 60 ± 6 years. It was observed that the *Bacteroidetes* group was reduced, while the growth of *Collinsella*, *Clostridium* cluster IV and *Atopobium* spp. was promoted. As known that brown rice (BR), when compared to WR contains more bioactives with potential health benefits. Panlasigui *et al.* (2006) reported the lower GI as a result of BR consumption by healthy (12.1% lower) and diabetic subjects (35.6% lower) compared to WR has ability to prevent type 2 diabetes and control glycemia respectively. In addition, studies on metabolic outcomes relevant to type 2 diabetes (*in vitro* data, animal experiments, and clinical studies) due to germinated brown rice (GBR), have reported changes in bioactive constituents during the process of germination of BR. Shallan *et al.* (2010) reported that feeding with GBR for 5 weeks triggered a significant reduction in levels of glycaemia, total cholesterol, and low-density lipoprotein (LDL) cholesterol, respectively (up to 23%, 31%, and 45% in levels of glycaemia), in diabetic albino rats. High-density lipoprotein (HDL) cholesterol was also reported to have increased by 45%.

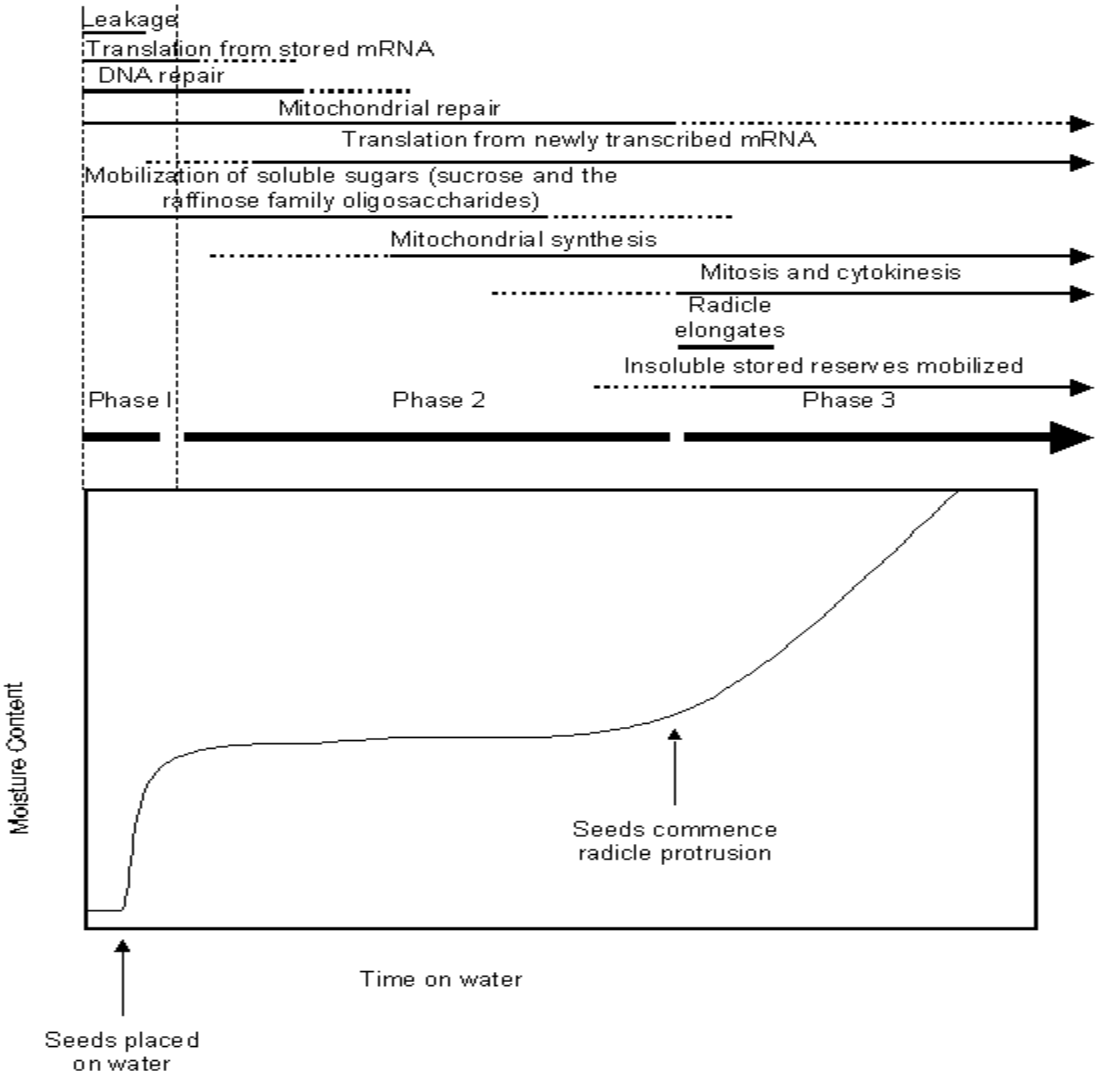


Figure 13: Chart showing the activity within a seed during germination.

Source: Bewley (1997)

1.2.13.1 Factors influencing germination

Seeds require both internal and external conditions for germination. Some factors are described below;

Seed viability

For successful germination, different seeds have unique features which often depend on the viability and life span of the seed. The period for which a seed remains viable is

dependent on the type of seed and also the storage conditions. Generally, the seed's viability is best retained under conditions in which there is low temperature and high carbon dioxide concentration.

Water

Water is very essential in germination process because mature seeds are usually dry and hence, the need for water uptake for growth to resume. The uptake of water by the seeds is called "imbibition" and leads to the swelling and breaking of the seed coat. The degree to which imbibition takes place is dependent on three factors; the seed's composition, the seed coat's permeability to water and the availability of water in either liquid or gaseous form in the environment. Most plants store food reserve in form of starch, protein, or oil within the seeds. This food reserve serves as nourishment to the growing embryo. So when there is imbibition of water by the seed, hydrolytic enzymes are activated which help to break down the stored food resources into metabolically useful chemicals.

Oxygen

The process of germination is related to living cells and requires consumption of energy by these cells. The energy-requirement of living cells is often sustained by oxidation whether in the presence or absence of oxygen (i.e. respiration or fermentation). These entails exchange of gases, an output of carbon dioxide in both cases and also the uptake of oxygen in the respiration process. As a result of this, seed germination is significantly affected by the composition of the ambient atmosphere.

Temperature

Seeds have different temperature ranges within which they germinate. Germination is inhibited at very low or very high temperatures as temperature affects cellular metabolic and

growth rate. Some seeds would germinate at temperature a bit higher than room temperature (16°-24°C), while some germinate in cool (-2 to 4°C) or warm (24-32°C) temperature.

Light/Darkness

Light or darkness can be environmental elicitors for germination. Most cultivated plants usually germinate well in both dark and in light. For example, changes in the content of starch, protein and RNA of rice (var IR8) were more progressive in the dark than in the light.

1.2.13.2 Impact of germination on carbohydrate

The process of germination triggers some biological activities in carbohydrates that decreases the values of energy in contrast to the energy values in both the raw and roasted grains, as reported in studies on germinated wheat flour (Kavitha *et al.*, 2014; Rumiyati *et al.*, 2012). Germination also stimulates the actions of enzymes in germinated grains, resulting into the fermentation of carbohydrates into simple sugars. Studies carried out on various kinds of cereal grains showed that some enzymes responsible for hydrolysis are stimulated during germination process. Thus, leading to the hydrolysis of starch as well as non-starch polysaccharides with increasing amounts of simple sugars, alongside the liberation of bound phenolics that have covalent bonds with cell walls (Hung *et al.*, 2012). The collective actions of α- and β-amylases, α-glucosidase and hydrolytic enzymes influence the degradation of starch. Notwithstanding, the level and form of enzymatic degradation of starch are based on their structures. Chu *et al.* (2014) reported that hydrolysis of starch varies among 2 genotypes of barley with different branching of amylopectin. The amount of starch was rapidly decreased during the germination process in genotypes with shorter chains of amylopectin, possibly due to the resistance of long-chain amylopectin to enzymes responsible for

hydrolysis. The simple sugars and low-molecular weight carbohydrates produced have higher digestibility and improved nutrient absorption, thus could be suitable for weaning diets.

Findings by Ito *et al.* (2005) to investigate the effects of GBR on glycemic and insulinemic responses, they used 19 (12 male, 7 females, age 23–41 years, body mass index 15.4–28.8 kg/m^2) and 13 (5 males, 8 females, age 25–32, body mass index 15.4–25.6 kg/m^2) normal human subjects, respectively, without any history of abnormal blood glucose readings within the previous year. In the study, germinated brown rice (GBR) containing 50 g available carbohydrate portion produced better glycemic and insulin responses than corresponding amounts of brown rice (BR) and white rice (WR). Within 120 min of ingestion of test diets among 19 subjects (WR, BR, or GBR), GBR produced the lowest glycemic index (56.9 ± 2.9) when compared to BR (61.5 ± 4.7) or WR (79.5 ± 6.6). Also, when increasing concentrations of GBR were used in WR diet among 13 subjects, an exclusive GBR diet produced the lowest glycemic index (54.4 ± 5.1) compared to 2/3 GBR in WR diet (63.7 ± 5.3), 1/3 GBR in WR diet (67.4 ± 2.9), or an exclusive WR diet 74.6 ± 6.2). Also, the hydrolysis index (HI) as well as the estimated glycemic index (eGI) were significantly reduced with germination as shown in Table 5, leading to breads with medium to low eGI (Cornejo *et al.*, 2015).

Table 5: Kinetic parameters of the *in vitro* starch digestibility and estimated glycemic index of gluten-free bread from raw (BR) pre-germinated (Pre-GBR) and germinated brown rice (GBR) at different times.

Treatment	C_∞ (g/100g)	k (min^{-1})	H_{90} (g/100g)	HI	eGI
BR	96.81 ± 1.58a	0.006 ± 0.001c	36.66 ± 1.56b	60.21 ± 3.89a	60.10 ± 3.25a
Pre-GBR	81.23 ± 4.56b	0.007 ± 0.001c	44.84 ± 1.05a	56.63 ± 1.93a	57.01 ± 1.66a
12 h GBR	39.29 ± 4.84c	0.025 ± 0.005b	32.65 ± 3.68b	47.04 ± 5.53b	48.74 ± 4.77b
24 h GBR	40.88 ± 5.46c	0.022 ± 0.001b	32.86 ± 6.03b	46.42 ± 5.61b	48.22 ± 4.84b
48 h GBR	25.27 ± 1.63d	0.041 ± 0.006a	26.15 ± 0.68c	34.30 ± 0.91c	37.76 ± 0.79c

C_∞: equilibrium concentration of starch hydrolyzed after 180 min, k: kinetic constant, H_{90}: starch hydrolysis at 90 min. Values with different letters in the same column are significantly different ($P < 0.05$).

Source: Cornejo *et al*. (2015)

1.2.13.3 GABA (Gamma-Aminobutyric Acid)

Gamma-aminobutyric acid (GABA), an amino acid containing 4 carbons is synthesized from the removal of carboxyl groups from L-glutamate by the action of enzyme glutamate decarboxylase as shown in Figure 14 (Komatsuzaki *et al.*, 2007). GABA is ubiquitous and has been studied as a suppressive neurotransmitter in association with the central nervous system (Wang and Kriegstein, 2009). Several health benefits on humans could be derived from GABA, namely; anti-oxidative, anti-hypertensive, anti-diuretic and anti-depressant properties (Chung *et al.*, 2009). Improved contents of GABA have been reported in germinated brown rice which is capable of suppressing the growth of cancer cells (Cho and Lim, 2016). Different types of foods including germinated cereal grains contain higher GABA contents. Additionally, various enzymes are stimulated to produce some compounds including GABA as well as vitamins. Example of such germinated grains include

germinated rice (Kim *et al.*, 2015; Komatsuzaki *et al.*, 2007; Thuwapanichayanan *et al.*, 2015), barley (Chung *et al.*, 2009) and soybean (Xu and Hu, 2014).

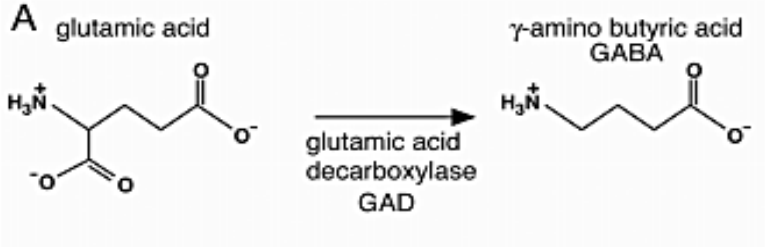


Figure 14: Structure of glutamic acid and GABA.

Source: Komatsuzaki *et al*. (2007)

1.2.14 Pigmented rice

Rice is mainly the species *Oryza sativa* L. and there are estimated to be about 100,000 rice varieties; only a small proportion is actually widely cultivated. They vary in grain weight, size and shape, degree of dormancy, longevity, and seedling vigor, and some have red to purple-black pigments. Rice is known as one of the major cereals in the world. Globally, about 95% of the total rice grains are produced in Asia and more than half of people in the world consume rice as basic diet. It is known as basic food for half of the population globally including Thailand. The paddy rice produced in Thailand was 31.65 million tons in 2008/9, making the country the world's largest rice exporter when sales reached 13.09 million tons (Teangpook and Paosangtong, 2011). Although the consumption of white rice has lasted for several years, other rice varieties which contain some color pigments (black, purple and red) are currently gaining attention due to their excellent source of polyphenols embedded in them. In other words, pigmented rice varieties have significant impact on human health as a result of their antioxidant activities that help to lower the levels

of free radicals which damage cells (Samyor *et al.*, 2017). Studies have shown that pigmented rice exhibit several biological properties such as anti-carcinogenic, antioxidant, anti-inflammatory as well as anti-allergic activities (Wang *et al.*, 2007).

Anthocyanins which naturally occur as reddish to purple in color are known as water-soluble flavonoids and are regarded as the major functional compound in pigmented rice (Delgado-Vargas *et al*., 2000). However, cyaniding-3-glucoside was studied as the predominant anthocyanin in red and black rice which accounts for 67% and 88% of the overall anthocyanin in the rice varieties, respectively (Deng *et al.*, 2013).

The structure of a matured rice grain consists of the hull, pericarp, seed coat, nucellus, embryo, aleurone layer and endosperm as shown in Figure 15. Rice produced in Thailand is affordable fit for processing. Pigmented rice is particularly essential because of their health benefits embedded in them due to the fact that the bran, nucellus, seed coat, pericarp and the aleurone layer exhibits numerous bioactive components including γ-oryzanols and vitamin E. Beside these bioactive components, rice also contains some other phytochemicals influences the colors of testa and pericarp in pigmented rice kernels. These bioactive components are believed to be capable of preventing several diseases related to oxidative stress including cancer (Leardkamolkarn *et al.*, 2011). Due to numerous health advantages exhibited, pigmented is currently being consumed in place of white rice as a basic diet.

1.2.14.1 Nutritional constituents

Table 6 shows the nutritional compositions of some pigmented rice varieties (brown, purple and black) obtained from India (Reddy *et al.*, 2017). As recorded, the process of polishing resulted in reduction in the values of moisture, ash, crude protein and fat contents, respectively in the rice verities. In contrast, the values of carbohydrate were increased. The values of the moisture contents in the raw rice varieties were significantly ($P < 0.05$) higher

when compared to the polished sample. In the same vein, the ash contents in the raw samples were recorded to be about 3 to 4 times (0.83% to 1.79%) more than that found in the polished samples (0.31% to 0.57%). The protein contents in the raw samples ranges from 5.57% to 8.75% and from 3.09% to 5.03% in the polished samples. They also reported loss of fat contents throughout the rice varieties due to polishing. In the overall, purple rice, especially the raw sample exhibited better values for nutritional compositions evaluated in the study.

Key bioactive compounds in germinated rough rice, for example, GABA, dietary fiber, ferulic acid, tocotrienols, magnesium, potassium, zinc, γ-oryzanol, and prolyl endopeptidase inhibitor, increase significantly in brown rice after germination (Komatsuzaki *et al.*, 2007). When these bioactive compounds were benchmarked against those of milled ungerminated rice, they were 10 times greater in GABA, nearly four times greater in dietary fiber, vitamin E, niacin, and lysine, and three times greater in thiamine, pyridoxine, and magnesium. GABA has several physiological properties such as neurotransmission and induction of hypotension and diuretic and tranquilizing functionalities (Okada *et al.*, 2000). GBR extracts contain GABA which can inhibit cancer cell from spreading (Oh and Oh, 2004).

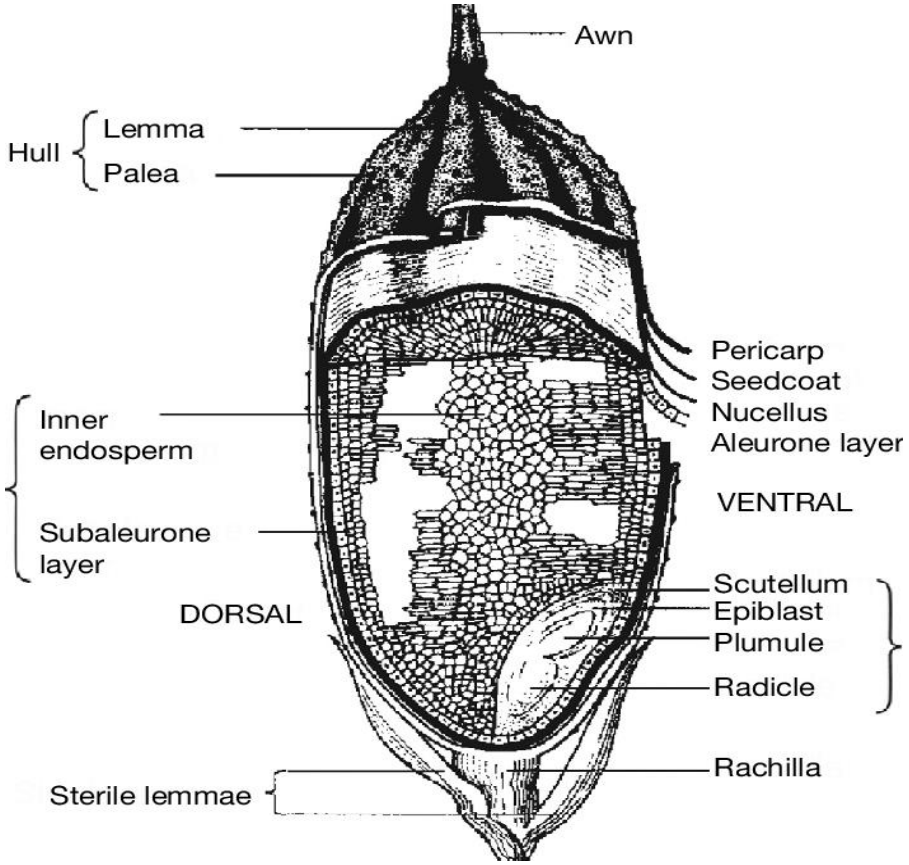


Figure 15: A cross-section of rice seed.

Source: Wrigley *et al.* (2015)

Table 6: Comparison of nutritional compositions in raw and polished pigmented rice varieties.

Parameter	Brown rice		Purple rice		Black rice	
	Raw	**Polished**	**Raw**	**Polished**	**Raw**	**Polished**
Moisture content (%)	11.40±0.30^{b}	8.72±0.60^{c}	12.05±0.20^{a}	8.54±0.22^{cd}	11.12±0.70^{b}	8.17±0.10^{d}
Ash (%)	0.83±0.10^{c}	0.33±0.03^{e}	1.79±0.20^{a}	0.57±0.09^{d}	1.38±0.20^{b}	0.31±0.03^{e}
Protein (%)	8.75±1.20^{a}	8.48±0.17^{a}	7.77±0.05^{b}	7.45±0.04^{c}	5.57±0.10^{d}	5.29±0.07^{e}
Fat (%)	3.33±0.20^{b}	0.41±0.07^{d}	3.73±0.20^{a}	0.34±0.05^{d}	3.05±0.10^{c}	0.21±0.03^{e}
Carbohydrate (%)	74.67±1.40^{d}	82.13±0.61^{b}	74.38±1.50^{d}	83.27±0.30^{b}	78.24±2.60^{c}	85.57±0.47^{a}

Values (Mean ± SD, n = 3) with the same lowercase letters in a row did not differ significantly ($P < 0.05$) by the Duncan's multiple range method.

Source: Reddy *et al.* (2017)

1.2.15 Job's tear

Coix lacryma-jobi L. is a distant relative of maize in the Maydae tribe of the grass family, Poaceae or Graminaeae. It is commonly named Job's tear, adlay, mayuen, Chinese pearl barley and hatomugi. Job's tear seeds are mainly produced in East and South-East Asia, including China, Japan, the Philippines, Burma, and Thailand. The seeds of Job's tear has a hard dark brown to gray colored shell/ hull and is pear or oval shaped with 5 mm diameter, has a milky white to black outer surface after the dehulling process as shown in Figure 16: a and b (Chhabra and Gupta, 2015). Job's tear has long been used in traditional Chinese medicine as a nourishing cereal. As presented in Table 7 (Corke *et al*. 2016), job's tear contains higher values of nutritional compositions compared to wheat, rice and maize. Hence, it is an excellent grain in alleviating the incidence of malnutrition. It is normally added in

soups and broths in the form of flour or whole grain. In Japan and Thailand, a non-dairy drink from Job's tears is available in the market as an alternative healthy food. Both animal and human clinical trials demonstrated that consumption of dehulled flour and seed of Job's tear can improve lipid metabolism, thereby decreasing the risk of heart diseases (Capule and Trinidad, 2016). In addition, it could reduce liver fat accumulation and protect from tumor stimulating compounds (C. C. Kuo *et al.*, 2012). Some bioactive compounds in Job's tear, especially coixenolide, inhibited tumors, prevented cancer and protected against viral infection (Manosroi *et al.*, 2016).

Figure 16: (a). Job's tears seeds with seed coat (b). Job's tears seeds without seed coat

Source: Chhabra and Gupta (2015)

Adlay has been consumed in orient countries as a nutritional food, as well as a traditional Chinese medicine which is traditionally used for wart treatment, rheumatism, neuralgia, promoting digestion, diuretic and inflammatory treatment (Wang *et al.*, 2012). Several physiological functions of adlay and its biological active components in different part of adlay, including hull, bran, testa and endosperm, have been investigated recently. Chen *et al.* (2011) found out that flavonoids in adlay bran partly contribute to its anti-inflammatory

effect. They investigated the antiulcer activity of dehulled adlay, and demonstrated that caffeic acid was one of the compounds indicative of a gastroprotective agent

1.2.15.1 Chemical components

Job's tear seeds are made up to various proteins and fatty acids (mainly prolamins) that affects both the biochemical and physiological activities in humans (S. j. Wu *et al.*, 2013). Furthermore, the seeds as well exhibits quite amount of benzoxazinones that proffer some anti-inflammatory properties. The segments of the seeds that are unsaponifiable exhibits several compounds with numerous health benefits such as phytosterols and squalene (Chhabra and Gupta, 2015). About six bioactive components of job's tear which manifest some anti-mutagenic activities were discovered by spectroscopic procedure, namely; vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, trans-coniferylaldehyde, coixol and sinapaldehyde. Cytoprotective activities of trans-coniferylaldehyde against DNA double-strand breaks triggered by tert-butyl hydroperoxide in cell cultures were observed. The chemo protective property of job's tear stems from trans-coniferylaldehyde triggered via the stimulation of some kinase indicators such as ERK1/2, JNK, MEK1/2, MSK1/2 and p38 (Chen *et al.*, 2011). Table 7 shows nutritional composition of job's tear compared with rice, wheat and maize.

Table 7: Typical composition of job's tear seed compared with other grains on a 14% moisture basis

Composition	Job's tear	Rice	Wheat	Maize
Energy (cal)	1500	1460	1480	1510
Protein (%)	14.2	7.8	9.9	8.4
Fat	3.6	1.3	1.8	4.3
Starch (%)	67.2	76.6	74.6	70.2
P (mg)	299	203	268	NR
Mg (mg)	126	NR	NR	NR
Ca (mg)	49.0	9	38	34
Fe (mg)	2.90	NR	4.2	NR
Zn (mg)	1.89	NR	NR	NR
Mn (mg)	1.89	NR	NR	NR

Per 100g for fat and mineral contents; NR – Not reported

Source: Corke *et al.* (2016)

1.2.15.2 Antioxidant activity

The methanolic extract of Job's tear's hull demonstrated an increased antioxidant property when compared to the remaining part of the seed (C.-C. Kuo *et al.*, 2012). Studies on the total phenolic contents (TPC) of both black and white husk flour from whole and milled seeds revealed that the TPC for the black husk was higher (approximately 8.055 mg GAE/g) compared to the white variety (approximately 7.19 mg GAE/g) in both whole and milled seeds. Based on GAE/g, TPC of the seeds was found to be higher than cereals like black rice (3.13 mg/gm), brown rice (0.54 mg/gm), barley (0.50 mg/gm). The DPPH scavenging activity did not show much of a difference for the black and white cultivar of Job's tears and hence comparable. However, the whole grain extract (~5.94%) exhibited lower activity in comparison to degermed flour (~7.04%). The reducing power assay for the

two cultivars again showed similar results, but greater compared to cereals like brown rice, white rice, mung bean (Jiraporn Chaisiricharoenkul *et al.*, 2011).

Phenolic acids such as protocatechuic acid, chlorogenic acid, p-coumaric acid, vanillic acid, ferulic acid and caffeic acid have been found in adlay seed in free and bound forms. P-coumaric acid and ferulic acid were reported to contribute largely to the *in vitro* xanthine oxidase inhibitory activity (Zhao *et al.*, 2014).

1.2.15.3 Nutritional advantage

The protein contents in job's tear between 15.4-18%, the crude fat content 6.2%, crude fiber content 0.8%, ash content 1.9%, carbohydrate content 65.3%, moisture content 11.2% with energy value of 380 calories per 100 g. The mineral component comprises of phosphorus – 435 mg, iron – 5 mg and calcium – 25 mg. Sodium, magnesium, zinc and potassium were not identified. The vitamin component involves vitamin B2 (riboflavin) – 0.19 mg, vitamin B1 (thiamine) – 0.28 mg and niacin – 4.3 mg (Sharma and Rawal, 2012).

1.2.16 Whole cereal grains as functional foods

Currently, studies on the health significance of whole grain cereals, especially regarding their bioactive components have emphasized the possibility of these grains as functional food products and their ability to alleviate numerous prevalent diseases (Cristina *et al.*, 2017; Fardet, 2010; Rawat *et al.*, 2013). Taking into consideration the yearly intake of cereal grains evaluated for 332 kg/person, their functional possibilities have unfolded throughout research (Organization, 2003).

Most of the functional properties of whole cereal grains include polyphenols, vitamins, mineral and dietary fiber which are predominantly found in the bran. Thus, the incorporation of bran in products from cereals could be regarded as a major strategy in the development of function food products from cereals (Laddomada *et al.*, 2015). Lately,

various researchers have reported that the functional prospects of food products from cereal grains is linked to their carbohydrate components such as arabinoxylan, β-glucan and inulin, and well as to their bioactive components including polyphenols.

The integration of these bioactive components into food products could be achieved instantaneously in its available form; notwithstanding, the nano- and macro-encapsulation procedures exhibited the most functional approach in ensuring the bioacessibility of these compounds, which in turn helps in preventing the inference of food processing and digestion (Ruiz and Segura-Campos, 2017).

Thus, it is imperative to elicitate the beneficial compounds in these whole grain cereals in order to produce cereals with higher bioactive compounds as well as higher anti-oxidative, anti-inflammatory, hypoglycemic properties and improved gut microbiota modulation.

1.3 Research objectives

1. To study nutritional compositions, polyphenolic profiles and antioxidant properties of pigmented rice varieties and adlay seeds under soaking and germination conditions
2. To evaluate bioactive components, antioxidant, cytotoxicity and the anti-inflammatory properties of soaked and germinated purple rice.
3. To determine the *in vitro* digestibility and estimated glycemic index.
4. To study *in vitro* gut microbiota modulation and their metabolites of polyphenolic compounds from the rice sample.

The overview of the experimental flow is presented in Figure 17.

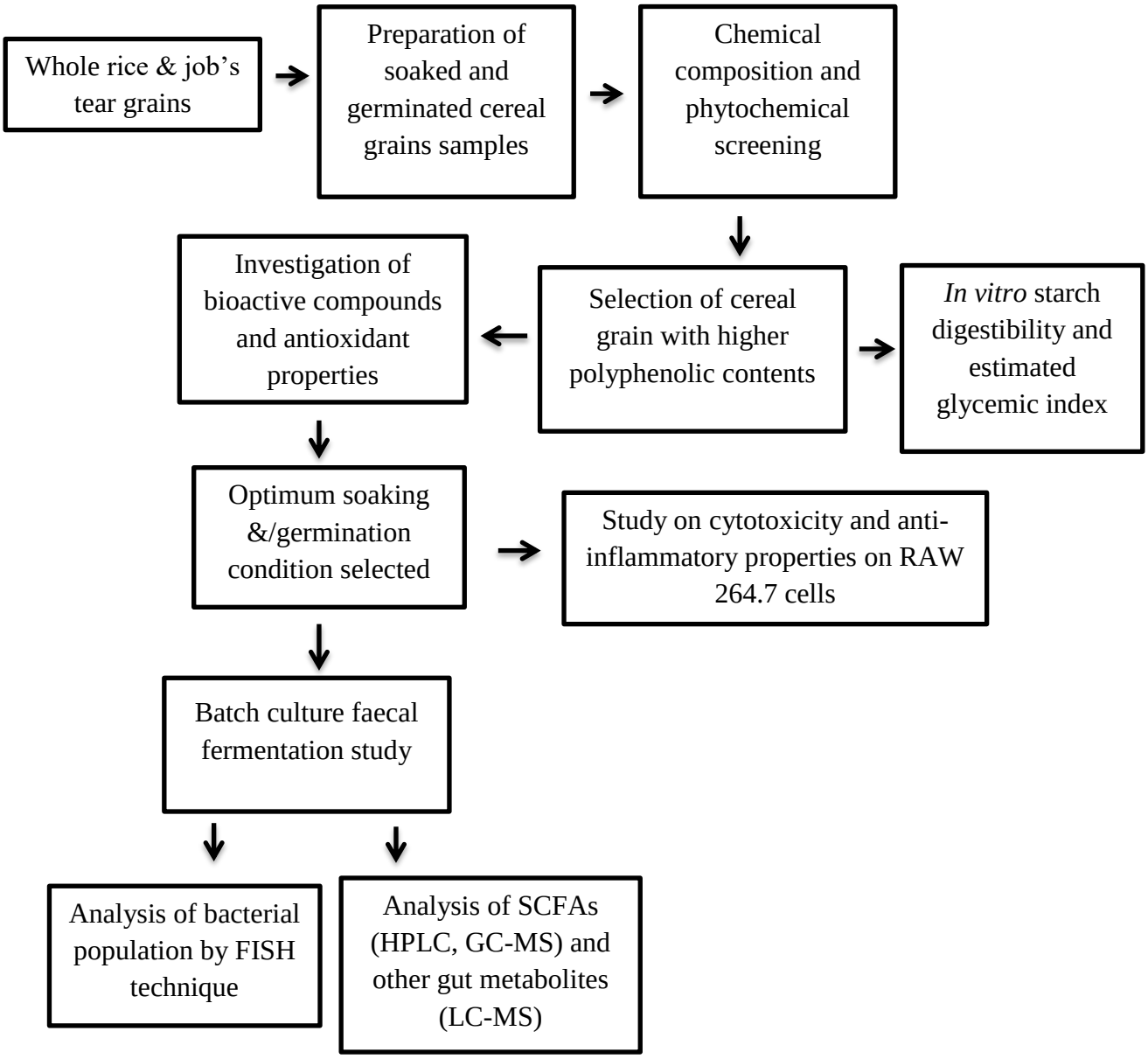


Figure 17: Experimental flow chart

1.4 References

Achakzai, A. K. K., Achakzai, P., Masood, A., Kayani, S. A. and Tareen, R. B. (2009). Response of plant parts and age on the distribution of secondary metabolites on plants found in Quetta. Pakistan Journal of Botany. 41(5): 2129-2135.

Andersson, A. A. M., Dimberg, L., Åman, P. and Landberg, R. (2014). Recent findings on certain bioactive components in whole grain wheat and rye. Journal of Cereal Science. 59(3): 294-311.

Andreasen, M. F., Kroon, P. A., Williamson, G. and Garcia-Conesa, M. T. (2001). Intestinal release and uptake of phenolic antioxidant diferulic acids. Free Radical Biology and Medicine. 31(3): 304-314.

Anson, N. M., Hemery, Y. M., Bast, A. and Haenen, G. R. M. M. (2012). Optimizing the bioactive potential of wheat bran by processing. Food and Function. 3(4): 362-375.

Appeldoorn, M. M., Vincken, J. P., Aura, A. M., Hollman, P. C. and Gruppen, H. (2009). Procyanidin dimers are metabolized by human microbiota with 2-(3, 4-dihydroxyphenyl) acetic acid and 5-(3, 4-dihydroxyphenyl)-γ-valerolactone as the major metabolites. Journal of Agricultural Food Chemistry. 57(3): 1084-1092.

Appeldoorn, M. M., Vincken, J. P., Gruppen, H. and Hollman, P. C. (2009). Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. The Journal of Nutrition. 139(8): 1469-1473.

Barclay, A. W., Petocz, P., McMillan Price, J., Flood, V. M., Prvan, T., Mitchell, P. and Brand-Miller, J. C. (2008). Glycemic index, glycemic load, and chronic disease risk—a meta-analysis of observational studies. The American Journal of Clinical Nutrition. 87(3): 627-637.

Bast, A., Haenen, G. R. M. M., Mateo Anson, N., Havenaar, R., Aura, A. M., Selinheimo, E., Mattila, I., Poutanen, K. and van den Berg, R. (2010). Bioprocessing of wheat bran in whole wheat bread increases the bioavailability of phenolic acids in men and exerts antiinflammatory effects *ex vivo*. The Journal of Nutrition. 141(1): 137-143.

Bender, D. A. (2006). *Benders' dictionary of nutrition and food technology*. Cambridge, England: Woodhead Publishing Limited.

Bewley, J. D. (1997). Seed germination and dormancy. The Plant cell. 9(7): 1055-1066.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S. and Kalayci, O. (2012). Oxidative stress and antioxidant defense. The World Allergy Organization journal. 5(1): 9-19.

Björck, I., Östman, E., Kristensen, M., Mateo Anson, N., Price, R. K., Haenen, G. R.., Havenaar, R., Bach Knudsen, K. E., Frid, A., Mykkänen, H., Welch, R. W. and Riccardi, G. (2012). Cereal grains for nutrition and health benefits: overview of results from *in vitro,* animal and human studies in the HEALTHGRAIN project. Trends in Food Science and Technology. 25(2): 87-100.

Boulangé, C. L., Neves, A. L., Chilloux, J., Nicholson, J. K. and Dumas, M. E. (2016). Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Medicine. 8(1): 42.

Bowey, E., Adlercreutz, H. and Rowland, I. (2003). Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. Food and Chemical Toxicology. 41(5): 631-636.

Burokas, A., Moloney, R. D., Dinan, T. G. and Cryan, J. F. (2015). Chapter One - microbiota regulation of the mammalian gut–brain axis. In S. Sariaslani and G. M. Gadd (Eds.), *Advances in Applied Microbiology* (Vol. 91, pp. 1-62). Cork, Ireland: Academic Press.

Călinoiu, L. and Vodnar, D. (2018). Whole grains and phenolic acids: a review on bioactivity, functionality, health benefits and bioavailability. Nutrients. 10(11): 1615-1646.

Capule, A. and Trinidad, T. (2016). Isolation and characterization of native and modified starch from adlay (*Coix lacryma-jobi* L.). International Food Research Journal. 23(3): 1199-1206.

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J. and Queipo-Ortuño, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. The Journal of Nutritional Biochemistry. 24(8): 1415-1422.

Chaisiricharoenkul, J., Tongta, S. and Intarapichet, K. O. (2011). Structure and chemical and physicochemical properties of Job's tear (*Coix lacryma-jobi* L.) kernels and flours. Suranaree Journal of Science and Technology. 18: 109-122.

Chambers, E. S., Viardot, A., Psichas, A., Morrison, D. J., Murphy, K. G., Zac-Varghese, S. E. K., MacDougall, K., Preston, T., Tedford, C., Finlayson, G. S., Blundell, J. E., Bell, J. D., Thomas, E. L., Mt-Isa, S., Ashby, D., Gibson, G. R., Kolida, S., Dhillo, W. S., Bloom, S. R., Morley, W., Clegg, S. and Frost, G. (2015). Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. Gut. 64(11): 1744–1754.

Chen, H. J., Chung, C. P., Chiang, W. and Lin, Y. L. (2011). Anti-inflammatory effects and chemical study of a flavonoid-enriched fraction from adlay bran. Food Chemistry. 126(4): 1741-1748.

Cheynier, V. (2005). Polyphenols in foods are more complex than often thought. The American Journal of Clinical Nutrition. 81(1): 223S-229S.

Chhabra, D. and Gupta, R. K. (2015). Formulation and phytochemical evaluation of nutritional product containing Job's tears (*Coix lachryma-jobi* L.). Journal of Pharmacognosy Phytochemistry. 4(3): 291-298.

Cho, D. H. and Lim, S. T. (2016). Germinated brown rice and its bio-functional compounds. Food Chemistry. 196: 259-271.

Cho, S. S., Qi, L., Fahey, G. C. and Klurfeld, D. M. (2013). Consumption of cereal fiber, mixtures of whole grains and bran, and whole grains and risk reduction in type 2 diabetes, obesity, and cardiovascular disease. The American Journal of Clinical Nutrition. 98(2): 594-619.

Chu, S., Hasjim, J., Hickey, L. T., Fox, G. and Gilbert, R. G. (2014). Structural changes of starch molecules in barley grains during germination. Cereal Chemistry. 91(5): 431-437.

Chung, H. J., Jang, S. H., Cho, H. Y. and Lim, S. T. (2009). Effects of steeping and anaerobic treatment on GABA (γ-aminobutyric acid) content in germinated waxy hull-less barley. LWT - Food Science and Technology. 42(10): 1712-1716.

Coldham, N., Darby, C., Hows, M., King, L., Zhang, A. Q. and Sauer, M. (2002). Comparative metabolism of genistin by human and rat gut microflora: detection and identification of the end-products of metabolism. Xenobiotica. 32(1): 45-62.

Cooper, D., Martin, R. and Keim, N. (2015). Does whole grain consumption alter gut microbiota and satiety? Healthcare. 3(2): 364-392.

Corke, H., Huang, Y. and Li, J. (2016). Coix: overview. Encyclopedia of Food Grains. 1: 184-189.

Cornejo, F., Caceres, P. J., Martínez-Villaluenga, C., Rosell, C. M. and Frias, J. (2015). Effects of germination on the nutritive value and bioactive compounds of brown rice breads. Food Chemistry. 173: 298-304.

Couteau, D., McCartney, A. L., Gibson, G. R., Williamson, G. and Faulds, C. B. (2001). Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. Journal of Applied Microbiology. 90(6): 873-881.

Cristina, C., Andreia, R., Maria Filomena, B. and Isabel, C. F. R. F. (2017). Phenolic compounds as nutraceuticals or functional food ingredients. Current Pharmaceutical Design. 23(19): 2787-2806.

Cuppett, S., Schnepf, M. and Hall, C. (1997). Natural antioxidants–Are they a reality. Natural Antioxidants, Chemistry, Health Effects Applications. 3: 12-24.

Dai, J. and Mumper, R. J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules. 15(10): 7313-7352.

Das, N. P. and Pereira, T. A. (1990). Effects of flavonoids on thermal autoxidation of palm oil: structure-activity relationships. Journal of the American Oil Chemists' Society. 67(4): 255-258.

De, P., Baltas, M. and Bedos-Belval, F. (2011). Cinnamic acid derivatives as anticancer agents-a review. Current Medicinal Chemistry. 18(11): 1672-1703.

Decroos, K., Vanhemmens, S., Cattoir, S., Boon, N. and Verstraete, W. (2005). Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. Archives of Microbiology. 183(1): 45-55.

Den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J. and Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of Lipd Research. 54(9): 2325-2340.

Deng, G. F., Xu, X. R., Zhang, Y., Li, D., Gan, R. Y. and Li, H. B. (2013). Phenolic compounds and bioactivities of pigmented rice. Critical Reviews in Food Science and Nutrition. 53(3): 296-306.

Dicko, M. H., Gruppen, H., Barro, C., Traore, A. S., van Berkel, W. J. H. and Voragen, A. G. J. (2005). Impact of phenolic compounds and related enzymes in sorghum varieties for resistance and susceptibility to biotic and abiotic stresses. Journal of Chemical Ecology. 31(11): 2671-2688.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. and Relman, D. A. (2005). Diversity of the Human Intestinal Microbial Flora. Science. 308(5728): 1635-1638.

Eckel, R. H., Alberti, K., Grundy, S. M. and Zimmet, P. Z. (2010). The metabolic syndrome. The Lancet. 375(9710): 181-183.

Everard, A. and Cani, P. D. (2014). Gut microbiota and GLP-1. Reviews in Endocrine and Metabolic Disorders. 15(3): 189-196.

Eyzaguirre, R. Z., Nienaltowska, K., de Jong, L. E. Q., Hasenack, B. B. E. and Nout, M. J. R. (2006). Effect of food processing of pearl millet (*Pennisetum glaucum*) IKMP-5 on the level of phenolics, phytate, iron and zinc. Journal of the Science of Food and Agriculture. 86(9): 1391-1398.

Fardet, A. (2010). New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre? Nutrition Research Reviews. 23(1): 65-134.

Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R. and Ward, J. L. (2010). Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. Journal of Agricultural and Food Chemistry. 58(17): 9341-9352.

Flint, H. J., Scott, K. P., Louis, P. and Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. Nature Reviews Gastroenterology and Hepatology. 9: 577-589.

Frei, B. (1994). Reactive oxygen species and antioxidant vitamins: Mechanisms of action. The American Journal of Medicine. 97(3): S5-S13.

Frølich, W., Åman, P. and Tetens, I. (2013). Whole grain foods and health–a Scandinavian perspective. Food Nutrition Research. 57(1): 18503-18510.

Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J. R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E. and Bell, J. D. (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nature Communications. 5: 3611-3622.

Gani, A., Wani, S., Masoodi, F. and Hameed, G. (2012). Whole-grain cereal bioactive compounds and their health benefits: a review. Journal of Food Processing and Technology. 3(3): 146-156.

Giacco, R., Costabile, G., Della Pepa, G., Anniballi, G., Griffo, E., Mangione, A., Cipriano, P., Viscovo, D., Clemente, G., Landberg, R., Pacini, G., Rivellese, A. A. and Riccardi, G. (2014). A whole-grain cereal-based diet lowers postprandial plasma insulin and triglyceride levels in individuals with metabolic syndrome. Nutrition, Metabolism and Cardiovascular Diseases. 24(8): 837-844.

Giada, M. D. L. R. (2013). Food phenolic compounds: main classes, sources and their antioxidant power. In *Oxidative Stress and Chronic Degenerative Diseases-A Role for Antioxidants* (pp. 87-112). Rijeka, Croatia: InTechOpen.

Gong, L., Cao, W., Chi, H., Wang, J., Zhang, H., Liu, J. and Sun, B. (2018). Whole cereal grains and potential health effects: Involvement of the gut microbiota. Food Research International. 103: 84-102.

Gonthier, M. P., Remesy, C., Scalbert, A., Cheynier, V., Souquet, J. M., Poutanen, K. and Aura, A. M. (2006). Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota *in vitro*. Biomedicine and Pharmacotherapy. 60(9): 536-540.

Gross, G., Jacobs, D. M., Peters, S., Possemiers, S., van Duynhoven, J., Vaughan, E. E. and van de Wiele, T. (2010). *In vitro* bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. Journal of Agricultural and Food Chemistry. 58(18): 10236-10246.

Halliwell, B. (2001). Free radicals and other reactive species in disease. Els: 1-9.

Halliwell, B., Aeschbach, R., Löliger, J. and Aruoma, O. (1995). The characterization of antioxidants. Food Chemical Toxicology. 33(7): 601-617.

Hamaker, B. R. and Tuncil, Y. E. (2014). A Perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. Journal of Molecular Biology. 426(23): 3838-3850.

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H. and Poutanen, K. (2010). Impact of dietary polyphenols on carbohydrate metabolism. International Journal of Molecular Sciences. 11(4): 1365-1402.

Hanske, L., Loh, G., Sczesny, S., Blaut, M. and Braune, A. (2009). The bioavailability of apigenin-7-glucoside is influenced by human intestinal microbiota in rats. The Journal of Nutrition. 139(6): 1095-1102.

He, M., van Dam, R. M., Rimm, E., Hu, F. B. and Qi, L. (2010). Whole-grain, cereal fiber, bran, and germ intake and the risks of all-cause and cardiovascular disease-specific mortality among women with type 2 diabetes mellitus. Circulation. 121(20): 2162-2168.

Heinonen, S.-M., Wähälä, K. and Adlercreutz, H. (2004). Identification of urinary metabolites of the red clover isoflavones formononetin and biochanin A in human subjects. Journal of Agricultural Food Chemistry. 52(22): 6802-6809.

Heleno, S. A., Martins, A., Queiroz, M. J. R. P. and Ferreira, I. C. (2015). Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. Food Chemistry. 173: 501-513.

Holmes, E., Li, J. V., Athanasiou, T., Ashrafian, H. and Nicholson, J. K. (2011). Understanding the role of gut microbiome–host metabolic signal disruption in health and disease. Trends in Microbiology. 19(7): 349-359.

Hudson, B. J. F. and Lewis, J. I. (1983). Polyhydroxy flavonoid antioxidants for edible oils. structural criteria for activity. Food Chemistry. 10(1): 47-55.

Hung, P. V., Maeda, T., Yamamoto, S. and Morita, N. (2012). Effects of germination on nutritional composition of waxy wheat. Journal of the Science of Food and Agriculture. 92(3): 667-672.

Hur, H. G., Beger, R. D., Heinze, T. M., Lay, J. O., Freeman, J. P., Dore, J. and Rafii, F. (2002). Isolation of an anaerobic intestinal bacterium capable of cleaving the C-ring of the isoflavonoid daidzein. Archives of Microbiology. 178(1): 8-12.

Imam, M. U., Azmi, N. H., Bhanger, M. I., Ismail, N. and Ismail, M. (2012). Antidiabetic properties of germinated brown rice: A systematic review Evidence-Based Complementary and Alternative Medicine. 2012: 1-12.

Imam, M. U. and Ismail, M. (2015). An overview on germinated brown rice and its nutrigenomic implications. Genomics, Proteomics metabolomics in Nutraceuticals Functional Foods: 504-517.

Ito, Y., Mizukuchi, A., Kise, M., Aoto, H., Yamamoto, S., Yoshihara, R. and Yokoyama, J. (2005). Postprandial blood glucose and insulin responses to pre-germinated brown rice in healthy subjects. The Journal of Medical Investigation. 52(3,4): 159-164.

Jaganath, I. B., Mullen, W., Lean, M. E. J., Edwards, C. A. and Crozier, A. (2009). *In vitro* catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. Free Radical Biology and Medicine. 47(8): 1180-1189.

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M. and Nageshwar Reddy, D. (2015). Role of the normal gut microbiota. World Journal of Gastroenterology. 21(29): 8787-8803.

Joyce, S. A. and Gahan, C. G. (2014). The gut microbiota and the metabolic health of the host. Current Opinion in Gastroenterology. 30(2): 120-127.

Juurlink, B. H. J., Azouz, H. J., Aldalati, A. M. Z., AlTinawi, B. M. H. and Ganguly, P. (2014). Hydroxybenzoic acid isomers and the cardiovascular system. Nutrition Journal. 13(1): 63-73.

Kaoutari, A. E., Armougom, F., Gordon, J. I., Raoult, D. and Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. Nature Reviews Microbiology. 11: 497-504.

Katina, K., Liukkonen, K. H., Kaukovirta-Norja, A., Adlercreutz, H., Heinonen, S. M., Lampi, A. M., Pihlava, J. M. and Poutanen, K. (2007). Fermentation-induced changes in the nutritional value of native or germinated rye. Journal of Cereal Science. 46(3): 348-355.

Kavitha, S., Parimalavalli, R. and Science, F. (2014). Effect of processing methods on proximate composition of cereal and legume flours. Journal of Human Nutrition and Food Science 2(4): 1051-1055.

Kim, K. H., Tsao, R., Yang, R. and Cui, S. W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chemistry. 95(3): 466-473.

Kim, M. Y., Lee, S. H., Jang, G. Y., Park, H. J., Li, M., Kim, S., Lee, Y. R., Noh, Y. H., Lee, J. and Jeong, H. S. (2015). Effects of high hydrostatic pressure treatment on the enhancement of functional components of germinated rough rice (*Oryza sativa* L.). Food Chemistry. 166: 86-92.

Knaggs, A. R. (2001). The biosynthesis of shikimate metabolites. Natural Product Reports. 18(3): 334-355.

Komatsuzaki, N., Tsukahara, K., Toyoshima, H., Suzuki, T., Shimizu, N. and Kimura, T. (2007). Effect of soaking and gaseous treatment on GABA content in germinated brown rice. Journal of Food Engineering. 78(2): 556-560.

Konishi, Y. and Kobayashi, S. (2004). Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal caco-2 cell monolayers. Journal of Agricultural and Food Chemistry. 52(9): 2518-2526.

Kumar, S. and Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview The Scientific World Journal. 2013: 1-16.

Kuo, C. C., Chen, H. H. and Chiang, W. (2012). Adlay (薏苡 yì yǐ; “soft-shelled job's tears”; the seeds of *Coix lachryma* L. var. ma-yuen Stapf) is a potential cancer chemopreventive agent toward multistage carcinogenesis processes. Journal of Traditional and Complementary Medicine. 2(4): 267-275.

Kutschera, M., Engst, W., Blaut, M. and Braune, A. (2011). Isolation of catechin-converting human intestinal bacteria. Journal of Applied Microbiology. 111(1): 165-175.

Laddomada, B., Caretto, S. and Mita, G. (2015). Wheat bran phenolic acids: bioavailability and stability in whole wheat-based foods. Molecules. 20(9): 15666-15685.

Laddomada, B., Durante, M., Mangini, G., D’Amico, L., Lenucci, M. S., Simeone, R., Piarulli, L., Mita, G. and Blanco, A. (2017). Genetic variation for phenolic acids concentration and composition in a tetraploid wheat *(Triticum turgidum* L.) collection. Genetic Resources and Crop Evolution. 64(3): 587-597.

Lagier, J. C., Million, M., Hugon, P., Armougom, F. and Raoult, D. (2012). Human gut microbiota: repertoire and variations. Frontiers in Cellular and Infection Microbiology. 2: 136-136.

Larrosa, M., García-Conesa, M. T., Espín, J. C. and Tomás-Barberán, F. A. (2010). Ellagitannins, ellagic acid and vascular health. Molecular Aspects of Medicine. 31(6): 513-539.

Leardkamolkarn, V., Thongthep, W., Suttiarporn, P., Kongkachuichai, R., Wongpornchai, S. and Wanavijitr, A. (2011). Chemopreventive properties of the bran extracted from a newly-developed Thai rice: The Riceberry. Food Chemistry. 125(3): 978-985.

Li, L., Shewry, P. R. and Ward, J. L. (2008). Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. Journal of Agricultural and Food Chemistry. 56(21): 9732-9739.

Liu, R. H. (2007). Whole grain phytochemicals and health. Journal of Cereal Science. 46(3): 207-219.

Lord, R. S. and Bralley, J. A. (2008). Clinical applications of urinary organic acids. Part 2. Dysbiosis markers. Alternative Medicine Review. 13(4): 292-307.

Manach, C., Williamson, G., Morand, C., Scalbert, A. and Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. The American Journal of Clinical Nutrition. 81(1): 230S-242S.

Manosroi, A., Sainakham, M., Chankhampan, C., Manosroi, W. and Manosroi, J. (2016). *In vitro* anti-cancer activities of Job's tears (*Coix lachryma-jobi* Linn.) extracts on human colon adenocarcinoma. Saudi Journal of Biological Sciences. 23(2): 248-256.

Marín, L., Miguélez, E. M., Villar, C. J. and Lombó, F. (2015). Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. BioMed research international. 2015: 905215-905215.

Miladinović, B., Kostić, M., Šavikin, K., Đorđević, B., Mihajilov-Krstev, T., Živanović, S. and Kitić, D. J. J. (2014). Chemical profile and antioxidative and antimicrobial activity of juices and extracts of 4 black currants varieties (*Ribes nigrum* L.). Journal of Food Science. 79(3): C301-C309.

Monagas, M., Urpi Sarda, M., Sánchez Patán, F., Llorach, R., Garrido, I., Gómez-Cordovés, C., Andres-Lacueva, C. and Bartolomé, B. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food and Function. 1(3): 233-253.

Moongngarm, A. and Saetung, N. (2010). Comparison of chemical compositions and bioactive compounds of germinated rough rice and brown rice. Food Chemistry. 122(3): 782-788.

Mouquet-Rivier, C., Icard-Vernière, C., Guyot, J.-P., Hassane Tou, E., Rochette, I. and Trèche, S. (2008). Consumption pattern, biochemical composition and nutritional value of fermented pearl millet gruels in Burkina Faso. International Journal of Food Sciences and Nutrition. 59(7-8): 716-729.

Oh, C. H. and Oh, S. H. (2004). Effects of germinated brown rice extracts with enhanced levels of GABA on cancer cell proliferation and apoptosis. Journal of Medicinal Food. 7(1): 19-23.

Okada, T., Sugishita, T., Murakami, T., Murai, H., Saikusa, T., Horino, T., Onoda, A., Kajimoto, O., Takahashi, R. and Takahashi, T. (2000). Effect of the defatted rice germ enriched with GABA for sleeplessness, depression, autonomic disorder by oral administration. Journal of the Japanese Society for Food Science Technology. 47(8): 596-603.

Organization, W. H. (2003). *Diet, nutrition, and the prevention of chronic diseases: report of a joint WHO/FAO expert consultation* (Vol. 916). Geneva,Switzerland: World Health Organization.

Ozcan, T., Akpinar Bayizit, A., Yilmaz Ersan, L. and Delikanli, B. (2014). Phenolics in human health. International Journal of Chemical Engineering Applications. 5(5): 393-396.

Pandey, K. B. and Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity. 2(5): 270-278.

Panlasigui, G. T., Leonora, N. and Thompson, L. U. (2006). Blood glucose lowering effects of brown rice in normal and diabetic subjects. International Journal of Food Sciences and Nutrition. 57(3-4): 151-158.

Parkar, S. G., Trower, T. M. and Stevenson, D. E. (2013). Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. Anaerobe. 23: 12-19.

Parvathy, R. (2015). *Phytochemical investigation on" Njavara"(Oryza Sativa* L., var." Njavara"), *the medicinal rice of Kerala.* Organic Chemistry Section, Chemical Sciences and Technology Division, National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram.,

Pham Huy, Ai, L., He, H. and Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. International Journal of Biomedical Science. 4(2): 89-96.

Power, S. E., O'Toole, P. W., Stanton, C., Ross, R. P. and Fitzgerald, G. F. (2014). Intestinal microbiota, diet and health. British Journal of Nutrition. 111(3): 387-402.

Price, R. K., Wallace, J. M. W., Hamill, L. L., Keaveney, E. M., Strain, J. J., Parker, M. J. and Welch, R. W. (2012). Evaluation of the effect of wheat aleurone-rich foods on markers of antioxidant status, inflammation and endothelial function in apparently healthy men and women. British Journal of Nutrition. 108(9): 1644-1651.

Putignani, L., Del Chierico, F., Vernocchi, P., Cicala, M., Cucchiara, S., Dallapiccola, B. and Group, D. S. (2015). Gut microbiota dysbiosis as risk and premorbid factors of ibd and ibs along the childhood–adulthood transition. Inflammatory Bowel Diseases. 22(2): 487-504.

Queipo-Ortuño, M. I., Boto-Ordóñez, M., Murri, M., Gomez-Zumaquero, J. M., Clemente-Postigo, M., Estruch, R., Cardona Diaz, F., Andres-Lacueva, C. and Tinahones, F. J. (2012). Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. The American Journal of Clinical Nutrition. 95(6): 1323-1334.

Raimondi, S., Anighoro, A., Quartieri, A., Amaretti, A., Tomás-Barberán, F. A., Rastelli, G. and Rossi, M. (2015). Role of bifidobacteria in the hydrolysis of chlorogenic acid. MicrobiologyOpen. 4(1): 41-52.

Rawat, N., Laddomada, B. and Gill, B. S. (2013). Genomics of cereal-based functional foodS. In P. K. Gupta and R. K. Varshney (Eds.), *Cereal Genomics II* (pp. 247-274). Dordrecht: Springer Netherlands.

Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., Debnam, E. S., Srai, S. K. S., Moore, K. P. and Rice-Evans, C. A. (2004). Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. Free Radical Biology and Medicine. 36(2): 212-225.

Reddy, C. K., Kimi, L., Haripriya, S. and Kang, N. (2017). Effects of polishing on proximate composition, physico- chemical characteristics, mineral composition and antioxidant properties of pigmented rice. Rice Science. 24(5): 241-252.

Reichardt, N., Duncan, S. H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K. P., Flint, H. J. and Louis, P. (2014). Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. The Isme Journal. 8: 1323–1335

Rice-Evans, C. A., Miller, N. J. and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine. 20(7): 933-956.

Rothwell, J. A., Urpi-Sarda, M., Boto-Ordoñez, M., Knox, C., Llorach, R., Eisner, R., Cruz, J., Neveu, V., Wishart, D., Manach, C., Andres-Lacueva, C. and Scalbert, A. (2012). Phenol-Explorer 2.0: A major update of the Phenol-Explorer database integrating data

on polyphenol metabolism and pharmacokinetics in humans and experimental animals. The Journal of Biological Databases and Curation. 2012: bas031-bas031.

Ruiz, J. C. R. and Segura-Campos, M. R. (2017). *New polymers for encapsulation of nutraceutical compounds*. West Sussex, UK: Wiley Online Library.

Rumiyati, R., James, A. P. and Jayasena, V. (2012). Effect of germination on the nutritional and protein profile of Australian sweet lupin (*Lupinus angustifolius* L.). Food and Nutrition Sciences. 3(5): 621-626.

Saha, P., Yeoh, B. S., Singh, R., Chandrasekar, B., Vemula, P. K., Haribabu, B., Vijay-Kumar, M. and Jala, V. R. (2016). Gut microbiota conversion of dietary ellagic acid into bioactive phytoceutical urolithin a inhibits heme peroxidases. PLOS ONE. 11(6): e0156811-e0156832.

Saman, P., Vázquez, J. A. and Pandiella, S. S. (2008). Controlled germination to enhance the functional properties of rice. Process Biochemistry. 43(12): 1377-1382.

Samyor, D., Das, A. B. and Deka, S. C. (2017). Pigmented rice a potential source of bioactive compounds: a review. International Journal of Food Science Technology. 52(5): 1073-1081.

Saura-Calixto, F. (2011). Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. Journal of Agricultural and Food Chemistry. 59(1): 43-49.

Schneider, H., Simmering, R., Hartmann, L., Pforte, H. and Blaut, M. (2000). Degradation of quercetin-3-glucoside in gnotobiotic rats associated with human intestinal bacteria. Journal of Applied Microbiology. 89(6): 1027-1037.

Sen, S. and Chakraborty, R. (2011). The role of antioxidants in human health. Oxidative Stress: Diagnostics, Prevention, Therapy. 1083: 1-37.

Serpen, A., Capuano, E., Fogliano, V. and Gökmen, V. (2007). A new procedure to measure the antioxidant activity of insoluble food components. Journal of Agricultural and Food Chemistry. 55(19): 7676-7681.

Sevgi, K., Tepe, B. and Sarikurkcu, C. (2015). Antioxidant and DNA damage protection potentials of selected phenolic acids. Food and Chemical Toxicology. 77: 12-21.

Shah, M. and Vella, A. (2014). Effects of GLP-1 on appetite and weight. Reviews in Endocrine and Metabolic Disorders. 15(3): 181-187.

Shahidi, F. (1997). *Natural antioxidants: chemistry, health effects, and applications*. Champaign, Illinois: The American Oil Chemists Society.

Shahidi, F. and Naczk, M. (1995). *Food phenolics*. Lancaster: Technomic Pub. Co.

Shahidi, F. and Yeo, J. (2018). Bioactivities of phenolics by focusing on suppression of chronic diseases: A review. International Journal of Molecular Sciences. 19(6): 1573-1586.

Shallan, M. A., El-Beltagi, H., Mona, A., Amera, T. and Sohir, N. (2010). Effect of amylose content and pre-germinated brown rice on serum blood glucose and lipids in experimental animal. Australian Journal of Basic Applied Sciences. 4(2): 114-121.

Sharma, P., Jha, A. B., Dubey, R. S. and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany. 2012: 1-26.

Sharma, T. and Rawal, G. (2012). Role of ayurveda in tumorigenesis: A brief review. International Journal of Green Pharmacy. 6(2): 93-101.

Slavin, J., Tucker, M., Harriman, C. and Jonnalagadda, S. (2013). Whole grains: definition, dietary recommendations, and health benefits. Cereal Foods World 58(4): 191-198.

Tagliabue, A. and Elli, M. (2013). The role of gut microbiota in human obesity: Recent findings and future perspectives. Nutrition, Metabolism and Cardiovascular Diseases. 23(3): 160-168.

Teangpook, C. and Paosangtong, U. (2011). The production and shelf life of high-iron, pre-cooked rice porridge with ferrous sulphate and other high-iron materials. Maejo International Journal of Science Technology. 5(2): 279-291.

Thuwapanichayanan, R., Yoosabai, U., Jaisut, D., Soponronnarit, S. and Prachayawarakorn, S. (2015). Enhancement of γ-aminobutyric acid in germinated paddy by soaking in combination with anaerobic and fluidized bed heat treatment. Food and Bioproducts Processing. 95: 55-62.

Tzin, V. and Galili, G. (2010). The biosynthetic pathways for shikimate and aromatic amino acids in arabidopsis thaliana. The Arabidopsis Book. 8: e0132-e0132.

Van der Kamp, J. W., Poutanen, K., Seal, C. J. and Richardson, D. P. (2014). The HEALTHGRAIN definition of 'whole grain'. Food and Nutrition Research. 58(1): 22100-22108.

Verma, B., Hucl, P. and Chibbar, R. N. (2008). Phenolic Content and Antioxidant Properties of Bran in 51 Wheat Cultivars. Cereal Chemistry. 85(4): 544-549.

Vermerris, W. and Nicholson, R. (2007). *Phenolic compound biochemistry*. West Lafayette, IN, USA: Springer Science & Business Media.

Ververidis, F., Trantas, E., Douglas, C., Vollmer, G., Kretzschmar, G. and Panopoulos, N. (2007). Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. Biotechnology Journal: Healthcare Nutrition Technology. 2(10): 1214-1234.

Vinson, J. A., Su, X., Zubik, L. and Bose, P. (2001). Phenol antioxidant quantity and quality in foods: fruits. Journal of Agricultural and Food Chemistry. 49(11): 5315-5321.

Vitaglione, P., Mennella, I., Ferracane, R., Rivellese, A. A., Giacco, R., Ercolini, D., Gibbons, S. M., La Storia, A., Gilbert, J. A., Jonnalagadda, S., Thielecke, F., Gallo, M. A., Scalfi, L. and Fogliano, V. (2014). Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber. The American Journal of Clinical Nutrition. 101(2): 251-261.

Vitaglione, P., Napolitano, A. and Fogliano, V. (2008). Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. Trends in Food Science and Technology. 19(9): 451-463.

Vitali, B., Ndagijimana, M., Cruciani, F., Carnevali, P., Candela, M., Guerzoni, M. E. and Brigidi, P. (2010). Impact of a synbiotic food on the gut microbial ecology and metabolic profiles. BMC Microbiology. 10(1): 1-13.

Vyas, U. and Ranganathan, N. (2012). Probiotics, Prebiotics, and Synbiotics: Gut and Beyond. Gastroenterology Research and Practice. 2012: 1-16.

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, M. D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J. and Flint, H. J. (2010). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. The Isme Journal. 5: 220-230.

Walle, T. (2004). Absorption and metabolism of flavonoids. Free Radical Biology and Medicine. 36(7): 829-837.

Wang, D. D. and Kriegstein, A. R. (2009). Defining the role of GABA in cortical development. The Journal of physiology. 587(Pt 9): 1873-1879.

Wang, L., Sun, J., Yi, Q., Wang, X. and Ju, X. (2012). Protective effect of polyphenols extract of adlay (*Coix lachryma-jobi* L. var. ma-yuen Stapf) on hypercholesterolemia-induced oxidative stress in rats. Molecules. 17(8): 8886-8897.

Wang, Q., Han, P. H., Zhang, M. W., Xia, M., Zhu, H. L., Ma, J., Hou, M. J., Tang, Z. H. and Ling, W. H. (2007). Supplementation of black rice pigment fraction improves antioxidant and anti-inflammatory status in patients with coronary heart disease. Asia Pacific Journal of Cinical Nutrition. 16(S1): 295-301.

Wrigley, C. W., Corke, H., Seetharaman, K. and Faubion, J. (2015). *Encyclopedia of food grains*. UK: Academic Press.

Wu, F., Yang, N., Touré, A., Jin, Z. and Xu, X. (2013). Germinated brown rice and its role in human health. Critical Reviews in Food Science and Nutrition. 53(5): 451-463.

Wu, H., Flint, A. J., Qi, Q., van Dam, R. M., Sampson, L. A., Rimm, E. B., Holmes, M. D., Willett, W. C., Hu, F. B. and Sun, Q. (2015). Association between dietary whole grain intake and risk of mortality: two large prospective studies in us men and womenwhole grain intake and mortalitywhole grain intake and mortality. JAMA Internal Medicine. 175(3): 373-384.

Wu, S. j., Fang, J. Y., Ng, C. C., Wang, C. Y. and Shyu, Y. T. (2013). Anti-inflammatory activity of Lactobacillus-fermented adlay-soymilk in LPS-induced macrophages through suppression of NF-κB pathways. Food Research International. 52(1): 262-268.

Xu, J. G. and Hu, Q. P. (2014). Changes in γ-aminobutyric acid content and related enzyme activities in Jindou 25 soybean (*Glycine max* L.) seeds during germination. LWT - Food Science and Technology. 55(1): 341-346.

Yamakoshi, J., Tokutake, S., Kikuchi, M., Kubota, Y., Konishi, H. and Mitsuoka, T. (2001). Effect of proanthocyanidin-rich extract from grape seeds on human fecal flora and fecal odor. Microbial Ecology in Health Disease. 13(1): 25-31.

Yang, X. D., Xu, L. Z. and Yang, S. L. (2001). Xanthones from the stems of Securidaca inappendiculata. Phytochemistry. 58(8): 1245-1249.

Ze, X., Duncan, S. H., Louis, P. and Flint, H. J. (2012). *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. The Isme Journal. 6: 1535–1543.

Zhao, M., Zhu, D., Sun-Waterhouse, D., Su, G., Lin, L., Wang, X. and Dong, Y. (2014). *In vitro* and *in vivo* studies on adlay-derived seed extracts: phenolic profiles, antioxidant activities, serum uric acid suppression, and xanthine oxidase inhibitory effects. Journal of Agricultural and Food Chemistry. 62(31): 7771-7778.

CHAPTER 2

NUTRITIONAL COMPOSITIONS, POLYPHENOLIC PROFILES AND ANTIOXIDANT PROPERTIES OF PIGMENTED RICE VARIETIES AND ADLAY SEEDS ENHANCED BY SOAKING AND GERMINATION CONDITIONS

2.1 Abstract

Pigmented rice varieties are gaining interest due to their superior nutritional and phenolic properties compared to white rice varieties. With the rising risk of diabetes, cereals with higher protein contents should be consumed with rice. Adlay is one of the underutilized crops with higher protein contents and other functional properties. The germination process is one of the easy and innovative techniques for improving functional properties in seeds. In this study, two pigmented rice varieties and adlay seeds were soaked (24 h) and germinated for 12, 24, 36, and 48 h. The physicochemical properties, polyphenolic profiles, and the antioxidant activities of these samples were evaluated. Purple rice (PR) demonstrated the highest values for polyphenolic contents, with 9 compounds detected for antioxidant activities. There were 6 compounds detected for red rice (RR). The adlay seeds had the least concentrations of phenolic compounds, with 6 compounds and higher nutritional properties identified. New compounds were synthesized. Among the rice samples, 24 h soaking (S24) gave the best results for phenolic and antioxidant properties, with 24 h germination in adlay seeds. 48 h germination yielded better results for the nutritional values in all the samples. The present study demonstrates how the process of soaking is a cheap and less time-consuming process of improving bioactive compounds and antioxidant activities in pigmented rice varieties and adlay seeds.

2.2 Introduction

Germination is a cheap, easy, innovative and effective method of enhancing the nutritive composition in cereals rice compared to other techniques of improving quality of cereals (F. Wu *et al.*, 2013). Before the germination process is carried out, grains are soaked in water for a few days, softening the kernel and stimulating germination. Germination enhances some changes biochemically, triggering some inactive enzymes responsible for the breakdown of large molecular substances. This leads to the synthesis of some bioactive compounds and increasing the bioavailability of nutrients and absorption (Moongngarm and Saetung, 2010). In Asian countries, the germination of rice is becoming more popular to the various nutritional advantages. Germinated grains, especially rice, are known to be good sources of some compounds beneficial to human health. These include vitamin B, E, β-carotene, γ- oryzanol, anthocyanin, and Gamma-aminobutyric acid (GABA).

Rice (*Oryza sativa* L.) is predominantly consumed across Asian countries among other cereals. Rice accounts for 50% of the total cereal production worldwide (Friedman, 1996). Pigmented rice varieties with purple (which is also known as black rice, forbidden rice, king's rice, etc.), red, or brown pericarp are popular for their surpassing nutritive contents and higher antioxidant properties than the white cultivars (Kang *et al.*, 2013; Min *et al.*, 2011). These cultivars have higher amounts of anthocyanins, phenolic compounds, and other bioactive compounds (Deng *et al.*, 2013; Frank *et al.*, 2012). The people who consume pigmented rice varieties are at low risk for diabetes, heart diseases, cancer, etc. (Ma *et al.*, 2001). Similarly, the intake of germinated colored rice varieties is capable of controlling postprandial glucose contents in the blood while balancing the secretion of insulin in subjects with high blood glucose content (Patil and Khan, 2011).

Other cereals improved by the process of germination is adlay (*Coix lachryma-jobi* L.), which is also known as Chinese pearl barley or Job's tears. Adlay is distributed throughout China, Japan, and Thailand. This cereal is a food and a useful ingredient in medicine (Zhao *et al.*, 2014). In addition to having many of the same bioactive components present in rice, adlay also contains coixol, coixenolide, and bioactive peptides (Manosroi *et al.*, 2014). Adlay has been investigated for it physiological and pharmacological activities, including anti-inflammation, anti-cancer, and anti-analgesic activities (Lu *et al.*, 2013). Some modern studies have reported that adlay seeds have several health benefits which include the potential to prevent tumor formation (Chang *et al.*, 2003), the ability to reduce inflammation (Chen *et al.*, 2011), and supporting immune system regulation (Lin and Tsai, 2008). Adlay is also widely recognized as a food supplement which can help decrease serum cholesterol and triglycerides, in addition to reducing liver lipids by increasing lipid excretion (Huang *et al.*, 2005).

The rise in the consumption of ready-to-eat rice porridge in Asian countries including Thailand poses a great concern with respect to the consequential effects on the postprandial blood glucose contents in subjects concerned. Alternatively germinated pigmented rice varieties can be prepared together with adlay (also known as rice-job's tears "*congee*") as breakfast. The increased bioactive components from these grains with higher protein and fat contents from adlay may help in reducing the incidence of some metabolic disorders.

Purple rice (*Khao neaw dam*), red rice (*Cho khing*), and adlay (*Look-Dei*) are less known and consumed, especially adlay. Adlay is an underutilized crop in Thailand and Asia. Detailed information on their chemical, antioxidants properties, phenolic composition, and profile are limited. Therefore, the aim of this study was to enhance the polyphenolic contents

and antioxidant properties of these pigmented rice and adlay under soaking and different germination conditions.

2.3 Materials and methods

2.3.1 Sample preparation, soaking and germination

Rough rice samples ((*Oryza sativa* L. cv. Niaw Dam Peuak Dam and cv. Cho khing) and adlay (Look-Dei) (Figure 18) were obtained from Songkhla Provincial Agricultural Office, Southern Thailand (Songkhla Province). The grains were soaked in 2.5% sodium hypochlorite solution (1:5 w/v) at 1-5% concentration for 5 min and drained. The purpose is to destroy or remove surface fungi and bacteria without killing internal organs. This was followed by washing the disinfected seed with distilled water in order to remove excess sodium hypochlorite.

The grains were soaked in distilled water (seed : water ratio, 1.5 w/v) at ambient temperature (30 ± 5ºC) for 24 h with the water changed every 8 h so that mold growth does not take over. After 24 h, the grains were collected for further process. For the germination process, the soaked seeds were placed in germination trays covered with moist cheese cloth and allowed to germinate for 12, 24, 36, and 48 h at 37ºC. Relative humidity was around 66% in a closed system. At the end of the germination period, the seeds were oven dried at 80ºC for 5 h, followed by de-husking. The de-husked seeds were grounded into a fine powder by 40-mesh sieve and stored at -20ºC for further analysis. The samples with their corresponding conditions are presented in Table 8.

2.3.2 Chemical compositions

The moisture, protein, fat, carbohydrate, ash, and fiber contents of both germinated and un-germinated samples were analyzed following the method of AOAC (2000). After determination of nitrogen content using micro-Kjeldahl apparatus, protein contents were

calculated as N x 5.95 for rice and N x 5.7 for Job's tears. The crude fat was determined using soxhlet apparatus. The ash content was determined by incinerating the sample in a furnace set at 600° C. Finally, the carbohydrate contents were determined using the following formula:

100% - protein content – moisture content – ash content – crude fat content.

2.3.3 Extraction of phenolic compounds

Phenolic compounds in both germinated and un-germinated samples were extracted with 80% methanol by centrifugation (Hatachi CR22G III) at 10,000 x *g* for 20 min. The extraction was carried out three times with the supernatants pooled and evaporated to ~10 mL at 35ºC under reduced pressure to remove the solvent. The evaporated samples were lyophilized to dryness and stored at -20°C for further analysis.

2.3.3.1 Phytochemical screening

Freeze dried powder of germinated and un-germinated samples were tested for the presence of numerous phytochemical constituents following the methods described by Sofowora (1993) and Harborne (1973). All analyses were performed in triplicate.

2.3.3.2 Total extractable phenolic content (TPC)

The total extractable phenolic contents of the extracts were measured using a Folin-Ciocalteu method from Singleton and Rossi (1965). Twenty µL of the extract was added to 96-well micro-plate. Next, 100µL of Folin-Ciocalteu reagent (10% v/v) and 80µL of Na_2CO_3 (7.5% w/v) were added and mixed thoroughly. After incubation for 30 min in the dark at ambient temperature, the absorbance was measured at 765 nm using the micro-plate reader. The total phenolic content was expressed as mg Gallic Acid Equivalent (GAE)/g DM.

2.3.3.3 Total extractable flavonoid content (TFC)

For the assay of TFC, 15µL of extract was mixed with 125 µL DI water and 10 µL of 5% sodium nitrite. This mixture was incubated at room temperature for 6 min, after which 10 µL of 10% aluminum chloride was added and incubated for 5 min. Finally, 50 µL of 1M NaOH was added, followed by incubation at room temperature for 15 min. The absorbance of the well-mixed mixture was measured at 510 nm. Total flavonoid content was calibrated with a standard curve of Catechin and expressed as Catechin Equivalent (mg CE/g DM).

2.3.4 Antioxidant activities

2.3.4.1 DPPH radical scavenging activity

2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity was determined using the modified method of Brand-Williams *et al.* (1995). One hundred and fifty µL of the extract was added with 150µL of 0.2 mM DPPH in 95% ethanol. The mixture was incubated in the dark for 30 min and the absorbance was determined at 517 nm using the micro-plate reader. The activity was reported as mg Trolox equivalent (TE/g DM).

2.3.4.2 ABTS radical scavenging activity

2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay was determined following the method of Arnao *et al.* (2001). The stock solutions of 7.4 mM ABTS solution and 2.5 mM $K_2S_2O_8$ solution were prepared. The ABTS radical solution was generated by mixing both stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The radical solution was diluted to obtain an absorbance of 1.1 ± 0.02 at 734 nm before performing the assay. 15µL of an extract was mixed with 285 µL of ABTS radical solution and left at room temperature for 2 h in the dark. The absorbance was

measured at 734 nm using the micro-plate reader. The activity was reported as Trolox equivalent (TE)/g DM.

2.3.4.3 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was performed according to the method of Benzie and Strain (1996). FRAP solution was freshly prepared from the mixture of 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution at the ratio 10:1:1 (v/v/v), and then warmed at 37ºC for 30 min before use. Fifteen µL of an extract was added with 285 µL of FRAP solution and stand for 30 min in the dark. The absorbance was read at 593 nm using micro-plate reader. The activity was reported as mg Trolox equivalent (TE/g DM).

2.3.5 Quantification of phenolic profiles un-germinated, soaked and germinated sample extracts

Phenolic compounds in the germinated and un-germinated extracts were estimated by reversed phase HPLC. All samples were filtered through a 0.22µm pore size syringe-driven filter before injection. A 20 µL aliquot of a sample solution was separated using Agilent HPLC technology system equipped with a diode array detector on an Agilent Eclipse XDB-C18, 5µm diameter, and 4.6 mm x 250 mm analytical column. The mobile phase consisted of Methanol (A) and purified water (HPLC grade) with 1% Formic acid (FA) at a flow rate of 0.8 mL/min. Gradient elution was performed as the following: from 0 to 10 min, linear gradient from 1 to 10% solvent A; from 10 to 20 min, 25% solvent A; from 20 to 30 min, 60% solvent A; and from 30 to 40 min, 70% solvent A. The stop time was 5 min in order to allow proper conditioning of the column before next injection. Column temperature was set at 25°C. Phenolic compounds in the samples were evaluated at wavelengths of 254, 280, and

300 nm. Each compound was identified by its corresponding retention time and also by spiking with standards in the same conditions.

2.3.6 Statistical analysis

All data from the results of this study were statistically analyzed by one-way ANOVA using a completely randomized design. The mean values were analyzed by Turkey test for a multi-comparison of means and correlation analysis using Pearson correlation. Statistical analyses were carried out using the SPSS statistical software (SPSS, Inc., Chicago. IL).

2.4 Results and discussion

2.4.1 Water hydration characteristics of PR, RR, and adlay seeds

The hydration characteristics of the cereals evaluated are shown in Figure 19. Water absorption in the PR increased rapidly during the 24 h soaking up to four times and almost remained stable during the germination period. For the RR, the rate of water hydration increases about three times, drops after 24 h soaking, and becomes stable until 48 h germination where it increases slightly. The least moisture contents were observed in adlay seeds where the water absorption increases throughout the soaking and germination periods. The moisture content in the seeds is correlated with the percentage of germination and quality. The difference in the moisture contents in the seeds could be due to differences in seeds and varieties (Puangwerakul, 2007).

2.4.2 Changes in proximate composition during soaking and germination conditions

The proximate compositions of the two pigmented rice varieties and adlay are presented in Table 9. Germination process starts with the imbibition of water in the seeds. The moisture content increased across the samples. During the soaking germination periods, purple rice (PR) showed the highest moisture content at 48 h germination (44.64 g/100g). PR

is known as waxy rice with a soft kernel which may allow more rapid imbibition of water during soaking and germination. The values for the protein contents were similar for PR, while the values increased during germination in red rice (RR) and adlay with ranges between 3.52 -10.46 g/100g and 9.90 – 17.37 g/100g in RR and adlay respectively. The process of germination enables the activation of several enzymes that can synthesize some proteins via a series of chemical and biological reactions. Similarly, some proteins get hydrolyzed by protease enzymes. In other words, there is a process of dynamic regulation which influences the protein contents during germination (Zhang *et al.*, 2015).

The PR and adlay gave the highest protein contents at 48 h (11.39 and 17.37 g/100g) which could be an interesting source of protein in food products. The fat content increased significantly in adlay during germination (4.34 – 6.63 g/100g) but decreases at first and increases later up to 48 h in PR (3.02 g/100g). Meanwhile, the fat contents in RR were similar through all the germination periods. The fat components in rice seed are located around the aleurone layer of the rice bran. During the milling process, some portions of the fat could be lost as the bran is removed. Additionally, according to (Hettiarachchy, 2014) the fat components of the rice grain may contain some triacylglycerol that could be used up as a source of energy for the embryo during germination.

However, the fat contents of the purple rice variety were similar to the studies of (Fitzgerald, 2017) who reported fat contents of black rice (2.8 – 3.7%). For adlay, the protein and fat contents were higher than the values in the study carried out by (J. Chaisiricharoenkul *et al.*, 2011), who reported that protein contents in whole grain adlay range between 13.54 to 16.85% and between 4.86 to 5.35% for lipid. The crude fiber in the seeds also increased progressively. The presence of the bran layer in the rice varieties and the outer coat in adlay seeds contributes to the fiber contents (Ohtsubo *et al.*, 2005).

In all three samples, the carbohydrate contents were reduced during the germination periods, which may be a result of degradation in order to provide energy for the growth of the embryo. The high energy values obtained in adlay seeds were influenced by higher crude protein and fat contents. The values obtained are within the values obtained by Sompong *et al.* (2011) and (Oko *et al.*, 2012). The ash contents were also similar during soaking and germination period. These results demonstrate that the mineral contents in the seeds were intact and did not leach significantly during these periods.

Carbohydrates decreased considerably because as the seeds imbibed water during soaking, activities of some endogenous enzymes a-amylase are increased in the endosperm. These enzymes hydrolyze starch into soluble sugars to provide energy for cell division as the seeds germinates (Devi *et al.*, 2015). Carbohydrate degrading enzymes play a major role in the seed characteristics as the seed becomes softer, sweeter, with less cooking time. The degraded starch content improves the digestibility and easy assimilation of the grains but causes the flour paste to be less viscous. The decrease in the carbohydrate level is also believed to be of health benefits to diabetes mellitus patients.

2.4.3 Changes in the phenolic contents during soaking and germination conditions

The total phenolic and total flavonoid contents (TPC and TFC) of both germinated and un-germinated samples are illustrated in Figure 20 (A & B). In PR, TFC was observed to slightly decrease during germination compared to the control ($P < 0.05$). A similar trend was also observed in RR except for the higher TPC (7.93 mg GAE g^{-1}) during the 24 h soaking period compared with control (6.93 mg GAE g^{-1}). In contrast, germination increased the total phenolic contents in soaked and germinated adlay seeds. Additionally, for the TFC, values increased with soaking and germination in all three samples except for PR where significant

reductions were observed in TFC values at 24 h (3.09 mg CE g^{-1}) and 48 h (3.96 mg CE g^{-1}) germination periods compared with the control (4.17 mg CE g^{-1}). Secondary metabolites such as phenolic acids, flavonoids, condensed tannins, and others are crucial substances produced during germination. These substances are mainly synthesized via the shikimate pathway and also the phenylpropanoid biosynthesis pathway (Zhang *et al.*, 2015). During the soaking and germination process, L-phenylpropanoid is converted to cinnamic acid by phenylalamine ammonialyase (PAL). This leads to the synthesis of some phenolic acids including ferulic acid, caffeic acid, and *p*-coumaric.

Table 8: Sample and treatments description

Sample	Sample code	Treatments	Soaking time	Germination time
Purple rice	PR	Raw sample (BS)	0 h	0h
		S_{24}	24 h	0h
		S_{24} + 12 h germination	24 h	12 h
		S_{24} + 24 h germination	24 h	24 h
		S_{24} + 36 h germination	24 h	36 h
		S_{24} + 48 h germination	24 h	48 h
Red rice	RR	Raw sample (BS)	0 h	0h
		S_{24}	24 h	0h
		S_{24} + 12 h germination	24 h	12 h
		S_{24} + 24 h germination	24 h	24 h
		S_{24} + 36 h germination	24 h	36 h
		S_{24} + 48 h germination	24 h	48 h
Adlay	Adlay	Raw sample (BS)	0 h	0h
		S_{24}	24 h	0h
		S_{24} + 12 h germination	24 h	12 h
		S_{24} + 24 h germination	24 h	24 h
		S_{24} + 36 h germination	24 h	36 h
		S_{24} + 48 h germination	24 h	48 h

These phenolic compounds can also be transformed into flavonoids and lignins with some other compounds (Moongngarm and Saetung, 2010). Additionally, some processing conditions such as the frequent washing of rice varieties and soaking may cause some soluble phenols being leaching out (Laokuldilok *et al.*, 2011). These could relate to the decrease in TPC in some of the soaked and germinated samples in PR and RR, and consequently increase in TFC as reported in this study. A similar observation was reported by Bulatao and Romero (2014), who recorded a significant reduction (from 21 to 28%) in the TPC of black rice after

germination. Nonetheless, the values for TPC recorded in both PR and RR were far higher than those reported for black rice, black sticky rice, and red rice from Thailand (0.15 to 0.21 mg GAE g^{-1}) (Tanprasert *et al.*, 2008). The TPC values recorded in this study are also similar to those recorded by Xu *et al.* (2017), where they observed TPC in germinated rough adlay seeds from 1171.0 to 1850.4 μg GAE g^{-1}). Several studies observed changes in the phenolic contents and profiles during germination but virtually none during soaking period. Therefore, this study revealed that changes in the seed composition and phenolic contents composition do not only take place during germination but also during soaking condition. The results of this study support the results of the report of Vichapong *et al.* (2010), who revealed high values for phenolic compounds in pigmented rice varieties. The phenolic concentrations reported in this study in both rice varieties were higher than those reported by Sompong *et al.* (2011) where they recorded ranges between 7.4 to 10.5 mg g-1 for black rice varieties and 1.4 to 3.4 mg g^{-1} for red rice. In contrast to this study, ferullic acid and proto-catechuic acid were the predominant compounds detected in red rice. This variation in the phenolic composition could be due to different the rice varieties, germination locations, conditions, and time. The phenolic profile observed in adlay in this study is similar to the report of Xu *et al.* (2017), where they also identified caffeic acid vanillic acid, P-coumaric, and ferullic acid in germinated adlay seeds.

Decrease in the phenolic contents during germination could be due to *de novo* synthesis of phenolic compounds to meet the needs of early seedlings. During this time, it is hypothesized that key enzymes in the biosynthetic pathways are activated during germination process. For example, phenylalanine (PAL) which catalyzes the first committed step in phenylpropanoid synthesis, thus relevant for hydroxycinnamic acids, flavonoids, lignana and lignin is activated during the germination of grains including rice (Chen *et al.*, 2017; Cho and

Lim, 2018). This suggests synthesis of phenolic compounds at first but may be used up as the metabolic process continues to meet the early needs of the seeds. Also, partial enzymatic breakdown of insoluble cell wall polysaccharides substituted with ester-linked phenolic moieties, thereby altering the bioavailability of associated phenolic compounds. It is also proposed that as germination increases, the water contents gradually increase which may lead to a significant decrease in the soluble phenolic contents in the seeds.

Glucose is the original precursor for the synthesis of phenolic compounds and several other important molecular signaling pathways including the oxidative pentose phosphate pathway, glycolysis, acetate, malonate, shikimate and phenylpropanoid pathway which are involved in the synthesis and transformation of different phenolic compounds (Gan *et al.*, 2019). Glucose can be used up as a source of energy by the seed embryo, thereby altering the availability of the phenolic compounds.

2.4.4 The antioxidant activity of un-germinated, soaked and germinated extracts

The antioxidant capacity is closely interrelated to the phenolic contents of the evaluated samples (Elzaawely and Tawata, 2012). The antioxidant capacity is closely interrelated to the phenolic contents of the evaluated samples. Antioxidants are compounds with the potential of preventing lipid peroxidation that may trigger cancer and heart disease (Ohtsubo *et al.*, 2005). The results from the antioxidant activity of the samples from this study determined by DPPH, ABTS, and FRAP assays are shown in Figure 20.

A **B** **C**

Figure 18: Pictures of cereals used in the study

A – Purple rice; B - Red rice; C - Adlay.

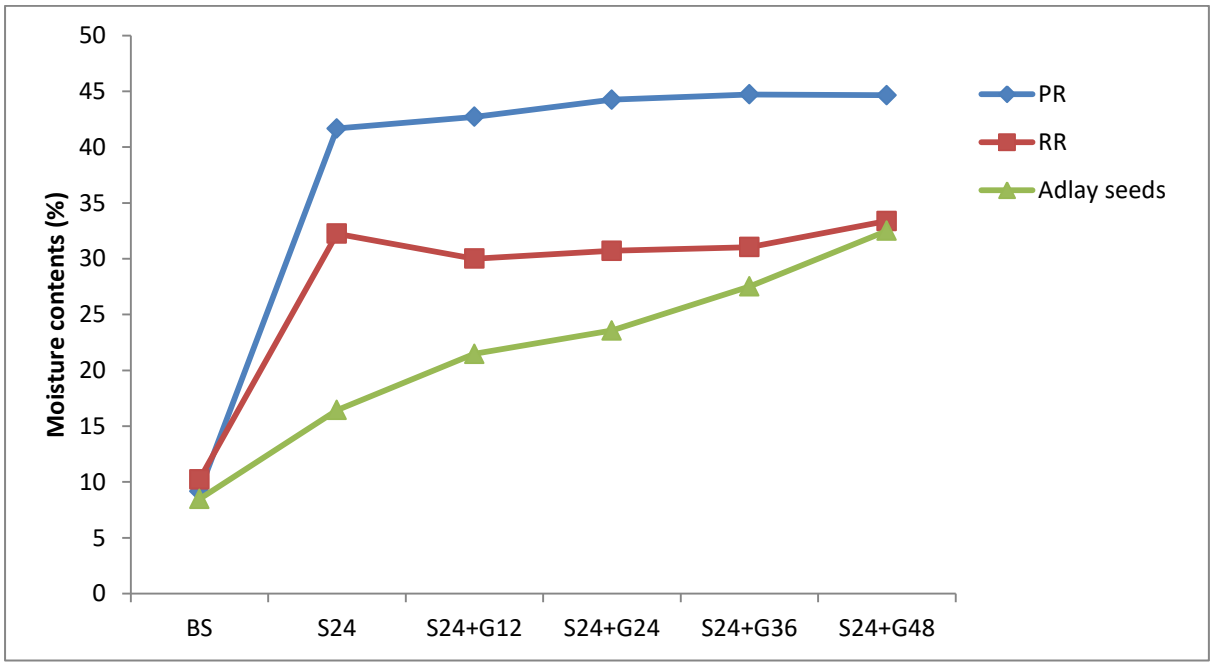


Figure 19: Rate of water hydration in PR, RR, and adlay seeds before soaking, after soaking and during germination at 12, 24, 36, and 48 h. BS - Before soaking; S24 - 24 h soaking; S24+G12 - 24 h soaking +12 h germination; S24+G24 – 24 h soaking + 24 h germination; S24+G36 - 24 h soaking + 36 h germination; S24+G48- 24 h soaking + 48 h germination. PR- Purple Rice; RR– Red Rice; db - dry basis.

Soaking and germination conditions led to an increase in the antioxidant activities in all the samples ($P < 0.05$). In this study, PR exhibited higher values for antioxidants with ranges between 6.77 ± 0.03 to 19.04 ± 0.06 mg TE g^{-1} in DPPH, 22.13 ± 0.11 to 47.45 ± 0.08 mg TE g^{-1} in ABTS and 16.18 ± 0.11 to 39.89 ± 0.04 mg TE g^{-1} in FRAP assays, which may highly contribute to the strongest antioxidant activity of the rice variety. Adlay seeds recorded the least antioxidant capacities in all the assays. Other studies have also revealed that germination positively affected the antioxidant properties of rice varieties and pigmented rice samples proved to have better antioxidant capacities than the white samples (Bulatao and Romero, 2014). Sompong *et al.* (2011), also reported that pigmented Thai rice varieties generally had better ferric reducing properties than other Chinese and Sri Lankan varieties evaluated. In their study, the black rice samples showed higher FRAP values with a mean of 5.6 mmol FE (II)/100g. However, in the study on Adlay seeds by Xu *et al.* (2017), total ABTS and FRAP values reduced during the first 24 h and increases until 60 h. These results are similar to the results obtained from this study, except for the reduction within the first 24 h which could be as a result of the difference in germination conditions and varieties.

2.4.5 Identification of phenolic profiles un-germinated, soaked and germinated extracts

The phenolic compounds detected in this study are presented in Table 10 and the phenolic standards and profiles in Figure 21. There were 9 compounds detected in PR, 6 in RR, and 6 in adlay. Gallic acid, proto-catechuic acid, catechin, caffeic acid, P-coumaric and ferullic acid were all detected in both PR and RR. Proto-catechuic acid, catechin, caffeic acid were the dominant compounds in PR and catechin in RR. Similar to the purple rice variety, caffeic acid, vanillin, P-coumaric and ferullic acid, rutin trihydrate and trans-cinnamic were also identified in adlay with rutin trihydrate at highest concentrations among the compounds.

Germination resulted in the synthesis of gallic acid in both rice varieties. Additionally, caffeic acid and catechin were synthesized during germination in the RR. Similarly, germination led to the production of vanillin in adlay seeds. From the results in this study, PR contained the highest concentrations for phenolic compounds (from 8.14 ± 0.07 to 11.86 ± 0.06 mg g^{-1}) with the highest values observed during soaking and 36 h germination condition. This was followed by RR (2.16 ± 0.18 to 8.02 ± 0.21 mg g^{-1}) where the soaking condition recorded the highest phenolic compounds. Adlay extracts contained the least phenolic compounds concentrations (1.10 ± 0.08 to 2.07 ± 0.11 mg g^{-1}) and the highest values were recorded at 24 h and 36 h germination period. These results attest to the values recorded in the PR where 2 h soaking and 36 h germination condition gave the highest values for TPC and TFC, the 24 h soaking condition in RR and 24 h germination in adlay in this study. During soaking and germination process, the activities of some amylolytic and proteolytic enzymes results into softening the structure of the kernel and thereby aiding the release of phenolic compounds. However, the oxidation of some phenolic substrates could also cause a reduction in some phenolic compounds as phenoloxidase and peroxidase enzymes resume activities (Rasane *et al.*, 2015). This may explain the increase and decrease in some of the compounds during soaking and germination conditions.

Flavonoids constitute a major component of polyphenols and they are believed to exhibit the potential to prevent some chronic diseases, such as cancer, heart disease and diabetes. The results from this study revealed that flavonoids comprise major part of the phenolic compounds identified. Xiang *et al.* (2017) in their study on germinated sweet corn identified five compounds including gallic acid, chlorogenic acid, syringic, hydroxycinnamic and ferullic acid. Zhang *et al.* (2015), also identified rutin together with four other phenolic compounds in buckwheat after germination. Studies of (Khang *et al.*, 2016) on six legume

extracts (adzuki beans, soybeans, black beans, mung beans, white cowpea and peanuts) detected thirteen phenolic compounds in the germinated legumes. These compounds also include protocatechuic, caffeic, cinnamic, ferullic, p-coumaric acids and vanillin identified in Thai purple rice extract in this study. Protocatechuic acid, syringic acid, caffeic acid and p-coumaric acid were also detected in black rice from Northern Thailand but only protocatechuic acid, syringic acid and chlorogenic acid were found in the brown rice variety (Pengkumsri *et al.*, 2015). All the identified compounds in this study were also detected in the Thai black rice evaluated Sumczynski *et al.* (2016).

2.4.6 Correlation between phenolics and antioxidant activity

The antioxidant activities were positively correlated with the total phenolic and flavonoid compounds before germination, during soaking and germination periods (Table 11). In PR, only the total flavonoid contents were correlated with the antioxidant activities ($P < 0.01$). In contrast, the TPC in the red variety were correlated with TPC and activities from ABTS assay ($P < 0.01$), while the TFC correlated with antioxidants activities from DPPH and FRAP ($P < 0.05$) and ABTS ($P < 0.01$). However, in adlay seeds, the TFC and TPC were all correlated with the activities from all assays ($P < 0.01$). A strong correlation was found between TFC and ABTS assay in the red rice ($P < 0.01$, $r= 0.978$) and adlay seeds ($P < 0.01$, $r = 0.97$). In other studies about rice varieties, red rice was recorded to have the greatest ABTS scavenging capacity (Sompong *et al.*, 2011). According to Awika *et al.* (2003), ABTS assay is proven to be a flexible method of estimating free radical scavenging activities. Additionally, the assay can also evaluate the antioxidant activities in hydrophilic as well as lipophilic compounds. High correlations between ORAC, ABTS, FRAP, and the phenolic contents ($r = 0.9979$, 0.9765, and 0.9607 respectively) and total flavonoid contents ($r =$

0.9798, 0.9868 and 0.9745, respectively) were extensively reported in adlay seeds (Xu *et al.*, 2017).

Table 9: Physicochemical properties of raw samples, soaked and germinated PR, RR and adlay at 0, 12, 24, 36, and 48 h

		(g/100g db)						
Samples	**Treatments**	**Moisture**	**Crude Protein**	**Total Fat**	**Ash**	**Crude Fiber**	**Total carbohydrate**	**Energy (Kcal/100g)**
PR	BS	9.16±0.08^{d}	10.28±0.82^{a}	2.23±0.40^{b}	0.99±0.01^{a}	0.78±0.13^{d}	77.33±0.46^{a}	370.51±0.09^{a}
	S_{24}	41.66±0.18^{c}	11.31±0.34^{a}	1.92±0.03^{b}	1.00±0.06^{a}	0.86±0.10^{c}	44.12±0.23^{b}	239.00±0.10^{b}
	S_{24}+G_{12}	42.71±0.39^{b}	10.42±0.60^{a}	3.60±0.36^{a}	1.00±0.00^{a}	0.88±0.16^{c}	42.27±1.16^{c}	243.16±0.02^{b}
	S_{24}+G_{24}	44.24±0.18a	10.28±0.54^{a}	1.97±0.75^{b}	0.99±0.01^{a}	0.94±0.06^{b}	42.52±0.46^{bc}	228.93±0.06^{b}
	S_{24}+G_{36}	44.72±0.50^{a}	10.78±0.92^{a}	2.38±0.28^{b}	0.99±0.01^{a}	0.98±0.11^{b}	41.12±0.14^{cd}	229.02±0.02^{b}
	S_{24}+G_{48}	44.64±0.62^{a}	11.39±0.48^{a}	3.02±0.28^{ab}	0.99±0.01^{a}	1.20±0.13^{a}	39.96±0.67^{d}	232.58±0.09^{b}
RR	BS	10.22±0.01^{d}	3.52±1.04^{c}	2.39±0.10^{a}	0.99±0.00^{a}	1.15±0.17^{d}	82.89±1.00^{a}	362.48±0.29^{a}
	S_{24}	32.24±0.58^{ab}	6.48±0.29^{b}	2.77±0.27^{a}	0.99±0.00^{a}	1.36±0.11^{cd}	57.52±0.90^{c}	302.19±0.18^{b}
	S_{24}+G_{12}	30.01±0.13^{bc}	7.56±0.93^{b}	2.69±0.18^{a}	0.99±0.00^{a}	1.38±0.26^{cd}	58.75±0.68^{bc}	298.92±0.03^{b}
	S_{24}+G_{24}	30.71±0.28^{bc}	6.62±0.52^{b}	2.80±1.19^{a}	0.99±0.00^{a}	1.6±0.02^{bc}	58.89±0.91^{bc}	280.75±0..14^{b}
	S_{24}+G_{36}	31.04±0.70^{bc}	4.39±0.81^{c}	2.49±0.17^{a}	1.00±0.02^{a}	2.01±0.11^{ab}	61.07±1.50^{b}	276.21±0.07^{b}
	S_{24}+G_{48}	33.37±1.12^{a}	10.46±0.49^{a}	2.62±0.41^{a}	0.99±0.00^{a}	2.02±0.13^{a}	52.56±1.50^{d}	267.55±0.09^{b}
Adlay	BS	8.48±0.04^{f}	9.90±0.12^{b}	4.34±0.11^{d}	1.97±0.01^{a}	2.25±0.17^{e}	76.31±0.03^{a}	383.90±0.12^{a}
	S_{24}	16.45±0.08^{e}	14.35±0.13^{b}	5.63±0.06^{c}	1.97±0.01^{a}	2.66±0.11^{d}	62.60±0.09^{b}	358.47±0.03^{b}
	S_{24}+G_{12}	21.48±0.14^{d}	17.40±0.12^{a}	5.76±0.04^{bc}	1.97±0.01^{a}	3.15±0.26^{c}	54.40±0.06^{c}	339.04±0.10^{c}
	S_{24}+G_{24}	23.57±0.08^{c}	16.78±0.15^{a}	5.94±0.04^{b}	1.98±0.01^{a}	3.26±0.02^{bc}	52.73±0.15^{d}	331.5±0.02^{c}
	S_{24}+G_{36}	27.51±0.16^{b}	15.69±0.15^{b}	6.19±0.06^{a}	1.98±0.01^{a}	3.31±0.11^{b}	49.63±0.10^{e}	316.99±0.05^{d}
	S_{24}+G_{48}	32.5±0.06^{a}	17.37±0.14^{a}	6.63±0.10^{a}	1.98±0.01^{a}	3.62±0.13^{a}	42.52±0.18^{f}	299.23±0.07^{e}

Notes: Values represent mean ± standard error. The experiments were carried out 3 times separately. In each row sample means not having the same letter are significantly different (Turkey's multiple comparison test ($P<0.05$)). BS-Before soaking; S_{24}- 24 h soaking; S_{24}+G_{12}- 24 h soaking +12 h germination; S_{24}+G_{24} – 24 h soaking + 24 h germination; S_{24}+G_{36}- 24 h soaking + 36 h germination; S_{24}+G_{48}- 24 h soaking + 48 h germination. PR- Purple Rice; RR– Red Rice. db- dry basis.

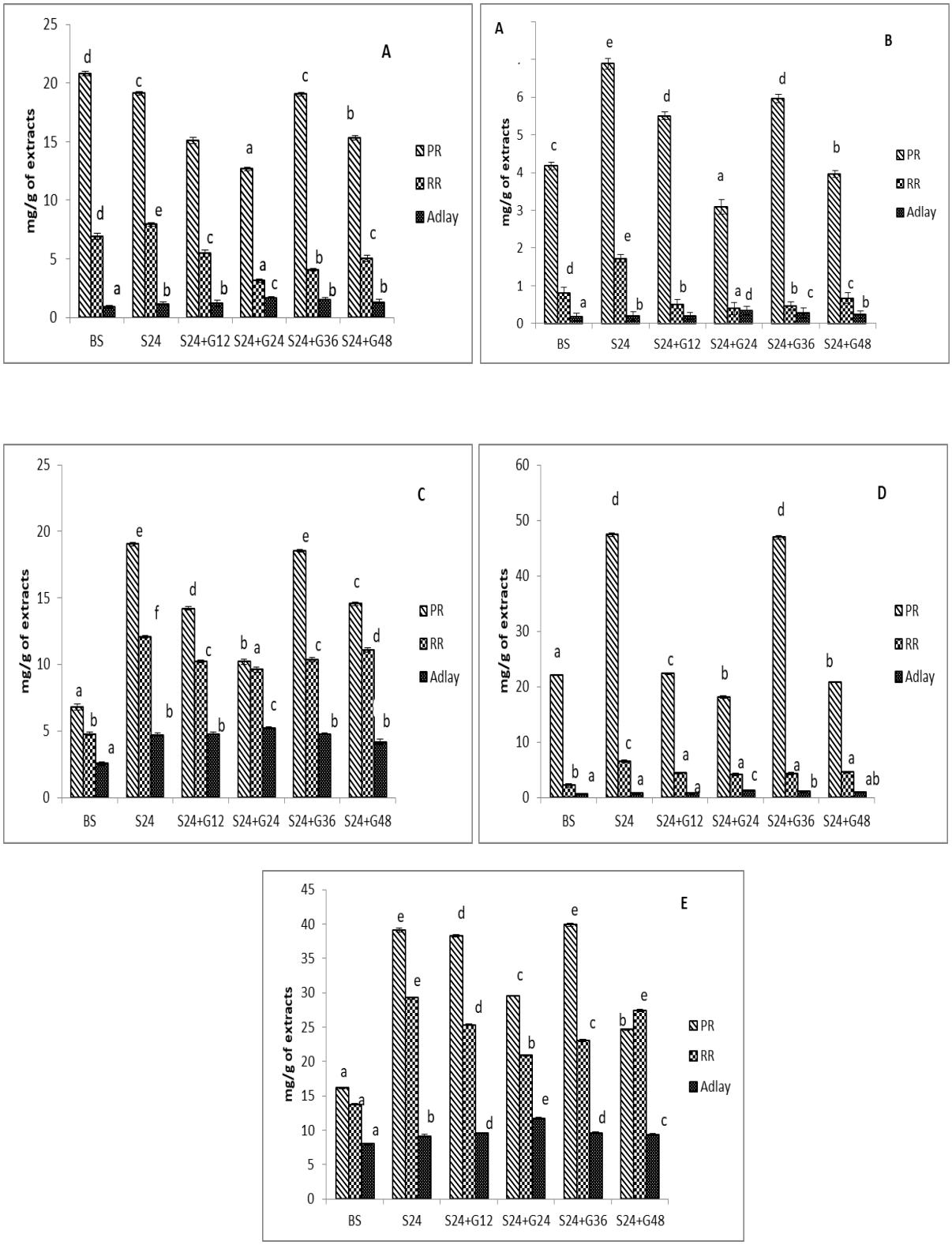


Figure 20: A- Total Pheolic Contents (TPC), B- Total Flavonoid Contents and antioxidant properties (C- DPPH; D- ABTS; E- FRAP) of PR, RR, and Adlay before soaking, at 24h soaking and germination periods.BS-Before soaking; S24- 24 h soaking; S24+G12- 24 h soaking +12 h germination; S24+G24 – 24 h soaking + 24 h germination; S24+G36- 24 h soaking + 36 h germination; S24+G48- 24 h soaking + 48 h germination. PR- Purple Rice. RR– Red Rice. TPC (mg GAE/g), TFC (mg CE/g), DPPH, ABTS, FRAP (mg TE/g).

Table 10: Polyphenolic components and concentrations (mg g-1 dry weight) of extracts during soaking and germination periods

Sample	Treatment	Compounds ($mg^{-1}g$)											
		Gallic acid	**Proto-catechuic acid**	**Catechin**	**Caffeic acid**	**Syringic acid**	**Vanillin**	***P*-coumaric acid**	**Ferullic acid**	**Rutin trihydrate**	**Quercetin dihydrate**	***Trans*-cinnamic acid**	**Total Phenolics detected**
PR	BS	ND	1.60±0.02^{a}	1.38±0.23^{a}	0.98±0.32^{a}	ND	0.34±0.02^{a}	0.31±0.02^{a}	1.03±0.02^{c}	0.29±0.22^{d}	ND	0.22±0.01^{a}	8.14±0.07
	S_{24}	0.09±0.02^{a}	1.61±0.22^{b}	2.48±0.02^{c}	1.37±0.12^{c}	ND	ND	0.73±0.02^{c}	0.38±0.02^{a}	3.02±0.22^{c}	ND	0.61±0.02^{b}	10.29±0.22
	$S_{24}+G_{12}$	0.08±0.01^{a}	1.65±0.12^{c}	2.29±0.11^{b}	1.32±0.02^{b}	ND	ND	0.47±0.02^{b}	1.30±0.02^{d}	1.61±0.21^{b}	ND	0.70±0.02^{c}	9.42±0.16
	$S_{24}+G_{24}$	0.59±0.03^{c}	1.84±0.02^{e}	2.72±0.01^{e}	1.45±0.03^{d}	ND	ND	0.74±0.02^{c}	0.54±0.02^{b}	ND	ND	0.83±0.03^{d}	8.71±0.09
	$S_{24}+G_{36}$	0.70±0.06^{d}	2.39±0.02^{f}	3.12±0.12^{f}	2.05±0.02^{f}	ND	ND	1.03±0.02^{e}	1.70±0.02^{f}	ND	ND	0.88±0.01^{e}	11.86±0.06
	$S_{24}+G_{48}$	0.33±0.02^{b}	1.71±0.02^{d}	2.71±0.02^{d}	1.33±0.13^{b}	ND	ND	0.91±0.02^{d}	1.52±0.02^{e}	ND	ND	0.98±0.02^{f}	9.49±0.08
RR	BS	ND	0.31±0.02^{d}	ND	ND	ND	ND	0.01±0.20^{a}	0.34±0.05^{c}	ND	ND	ND	2.89±014
	S_{24}	0.85±0.04^{d}	0.52±0.30^{e}	3.07±0.10^{c}	0.37±0.02^{c}	ND	ND	0.36±0.01^{c}	1.06±0.02^{e}	ND	ND	ND	8.02±0.21
	$S_{24}+G_{12}$	0.09±0.01^{a}	0.27±0.01^{c}	1.60±0.02^{b}	0.22±0.10^{b}	ND	ND	0.03±0.02^{ab}	0.36±0.01^{d}	ND	ND	ND	4.18±0.25
	$S_{24}+G_{24}$	0.14±0.04^{c}	0.16±0.05^{a}	1.45±0.06^{a}	0.15±0.11^{b}	ND	ND	0.02±0.04^{a}	0.26±0.03^{b}	ND	ND	ND	2.16±0.18
	$S_{24}+G_{36}$	0.11±0.02^{b}	0.27±0.03^{c}	2.74±0.06	0.14±0.02^{b}	ND	ND	0.042±0.01^{b}	0.20±0.03^{a}	ND	ND	ND	3.49±0.09
	$S_{24}+G_{48}$	0.11±0.01^{b}	0.31±0.02^{d}	3.69±0.01^{d}	0.07±0.07^{a}	ND	ND	ND	0.29±0.05^{b}	ND	ND	ND	4.48±0.10
Adlay	BS	ND	ND	ND	0.24±0.02^{c}	ND	ND	0.09±0.02^{d}	0.25±0.10^{b}	0.69±0.02^{d}	ND	0.11±0.04^{b}	1.38±0.19
	S_{24}	ND	ND	ND	0.13±0.10^{a}	ND	0.17±0.03^{a}	0.07±0.05^{c}	0.33±0.03^{c}	0.31±0.03^{a}	ND	0.10±0.02^{a}	1.10±0.08
	$S_{24}+G_{12}$	ND	ND	ND	0.18±0.05^{b}	ND	0.30±0.02^{b}	0.11±0.01^{e}	0.34±0.10^{c}	0.39±0.04^{b}	ND	0.15±0.18^{e}	1.46±0.10
	$S_{24}+G_{24}$	ND	ND	ND	0.40±0.10^{d}	ND	0.59±0.20^{c}	0.05±0.20^{b}	0.34±0.11^{c}	0.49±0.06^{c}	ND	0.18±0.22^{f}	2.05±0.21
	$S_{24}+G_{36}$	ND	ND	ND	0.18±0.02^{b}	ND	0.77±0.01^{d}	0.01±0.20^{a}	0.23±0.08^{b}	0.74±0.01^{e}	ND	0.14±0.11^{d}	2.07±0.11
	$S_{24}+G_{48}$	ND	ND	ND	0.30±0.03^{d}	ND	1.12±0.04^{e}	0.15±0.02^{f}	0.15±0.01^{a}	ND	ND	0.12±0.02^{c}	1.84±0.21

Notes: Values represent mean ± standard error. The experiments were carried out in triplicate. In each row, sample means not having the same letter are significantly different (Turkey's multiple comparison test ($P<0.05$)). BS-Before soaking; S24- 24 h soaking; S24+G12- 24 h soaking +12 h germination; S24+G24 – 24 h soaking + 24 h germination; S24+G36- 24 h soaking + 36 h germination; S24+G48- 24 h soaking + 48 h germination. PR- Purple Rice; RR– Red Rice.

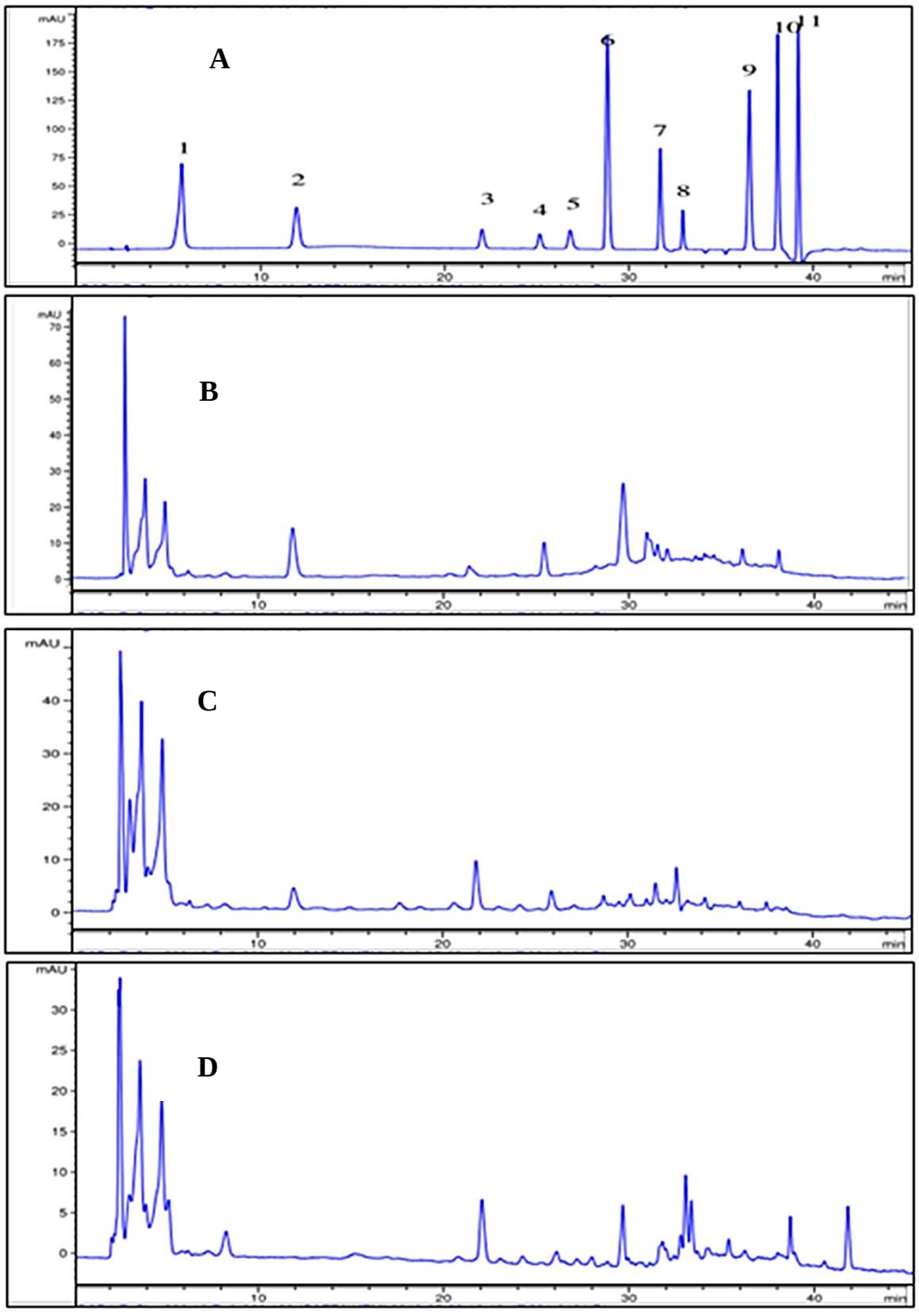


Figure 21: High Performance Liquid Chromatography (HPLC) chromatograms of phenolic compounds. A- Standards; 1: Gallic Acid; 2: Protocatechuic Acid; 3: Catechin; 4: Caffeic Acid; 5: Syringic Acid; 6: Vanillin; 7: P-Coumaric Acid; 8: Ferulic Acid; 9: Rutin Trihydrate; 10: Quercetin Dihydrate; 11: Trans-cinnamic Acid. B- Purple rice at 24 h soaking. C- Red rice at 24 h soaking. D- Adlay at 24 h germination.

Table 11: Pearson correlation between total phenolic, total flavonoid contents and antioxidants properties of un-germinated and germinated PR, RR and Adlay

	PR		**RR**		**Adlay**	
Antioxidant activity	**TPC**	**TFC**	**TPC**	**TFC**	**TPC**	**TFC**
DPPH	0.22	0.80**	0.28	0.59**	0.80**	0.64**
ABTS	0.20	0.88**	0.89**	0.98**	0.97**	0.97**
FRAP	0.13	0.85**	0.23	0.48*	0.84**	0.91**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

PR- Purple Rice; RR – Red Rice.

2.5 Conclusions

Prevention of occurrences of some prevalent diseases is more effective than curing. Therefore, consumption of functional foods high in polyphenols and antioxidants could be helpful in human health. From this study, soaking especially and some germination conditions led to an increase in the nutritional values, polyphenolic and antioxidant properties in evaluated samples. Adlay though contains polyphenolic contents at lower concentrations compared with the pigmented rice varieties, the higher protein and fat contents it contains could be exceptional functional foods when combined with the rice varieties. Interestingly, the PR is an excellent source of polyphenols and flavonoids improved by soaking and germination conditions. Various food products derived from these cereals may not only help to prevent some various diseases in humans but also improve the market for these indigenous pigmented rice varieties and adlay. Development of bioactive compounds from them could as well be an exquisite source of functional food products and nutraceuticals.

2.6 References

AOAC. (2000). Official method of analysis. association of official analytical chemists 17th ed. Washington D.C., USA.

Arnao, M. B., Cano, A. and Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chemistry. 73(2): 239-244.

Awika, J. M., Rooney, L. W., Wu, X., Prior, R. L. and Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. Journal of Agricultural and Food Chemistry. 51(23): 6657-6662.

Benzie, I. F. F. and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry. 239(1): 70-76.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology. 28(1): 25-30.

Bulatao, R. M. and Romero, M. V. (2014). Effects of germination on the proximate composition, antioxidant property and eating quality of brown rice (*Oryza sativa* L.). Philippine Agricultural Scientist. 97(1): 19-27.

Chaisiricharoenkul, J., Tongta, S. and Intarapichet, K. O. (2011). Structure and chemical and physicochemical properties of Job's tear (*Coix lacryma-jobi* L.) kernels and flours. Journal of Science and Technology. 18(2): 109-122.

Chang, H. C., Huang, Y. C. and Hung, W. C. (2003). Antiproliferative and chemopreventive effects of adlay seed on lung cancer *in vitro* and *in vivo*. Journal of Agricultural and Food Chemistry. 51(12): 3656-3660.

Chen, H. J., Chung, C. P., Chiang, W. and Lin, Y. L. (2011). Anti-inflammatory effects and chemical study of a flavonoid-enriched fraction from adlay bran. Food Chemistry. 126(4): 1741-1748.

Chen, Z., Wang, P., Weng, Y., Ma, Y., Gu, Z. and Yang, R. (2017). Comparison of phenolic profiles, antioxidant capacity and relevant enzyme activity of different Chinese wheat varieties during germination. Food Bioscience. 20: 159-167.

Cho, D. H. and Lim, S. T. (2018). Changes in phenolic acid composition and associated enzyme activity in shoot and kernel fractions of brown rice during germination. Food Chemistry. 256: 163-170.

Deng, G. F., Xu, X. R., Zhang, Y., Li, D., Gan, R. Y. and Li, H. B. (2013). Phenolic compounds and bioactivities of pigmented rice. Critical Reviews in Food Science and Nutrition. 53(3): 296-306.

Devi, C. B., Kushwaha, A. and Kumar, A. (2015). Sprouting characteristics and associated changes in nutritional composition of cowpea (*Vigna unguiculata*). Journal of Food Science and Technology. 52(10): 6821-6827.

Elzaawely, A. A. and Tawata, S. (2012). Antioxidant activity of phenolic rich fraction obtained from *Convolvulus arvensis* L. leaves grown in Egypt. Asian Journal of Crop Science. 4: 32-40.

Fitzgerald, M. (2017). Chapter 12 - Rice: Grain-quality characteristics and management of quality requirements. In C. Wrigley, I. Batey, and D. Miskelly (Eds.), *Cereal Grains* (*Second Edition*) (pp. 291-315): Woodhead Publishing.

Frank, T., Reichardt, B., Shu, Q. and Engel, K. H. (2012). Metabolite profiling of colored rice (*Oryza sativa* L.) grains. Journal of Cereal Science. 55(2): 112-119.

Friedman, M. (1996). Nutritional value of proteins from different food sources. a review. Journal of Agricultural and Food Chemistry. 44(1): 6-29.

Gan, R. Y., Chan, C. L., Yang, Q. Q., Li, H. B., Zhang, D., Ge, Y. Y., Gunaratne, A., Ge, J. and Corke, H. (2019). 9 - Bioactive compounds and beneficial functions of sprouted grains. In

H. Feng, B. Nemzer, and J. W. DeVries (Eds.), *Sprouted Grains* (pp. 191-246): AACC International Press.

Harborne, J. (1973). Phytochemical methods chapman and Hall. Ltd. London. 4: 49-188.

Hettiarachchy, N. S. (2014). Effects of germination on nutrient composition of long grain rice and its protein physico-chemical and functional properties. Journal of Food and Nutrition. 1: 1-9.

Huang, B. W., Chiang, M. T., Yao, H. T. and Chiang, W. (2005). The effect of adlay oil on plasma lipids, insulin and leptin in rat. Phytomedicine. 12(6): 433-439.

Kang, M. Y., Rico, C. W., Bae, H. J. and Lee, S. C. (2013). Antioxidant capacity of newly developed pigmented rice cultivars in Korea. Cereal Chemistry. 90(5): 497-501.

Khang, T. D., Dung, N. T., Elzaawely, A. A. and Xuan, D. T. (2016). Phenolic profiles and antioxidant activity of germinated legumes. Foods. 5(2): 1-10.

Laokuldilok, T., Shoemaker, C. F., Jongkaewwattana, S. and Tulyathan, V. (2011). Antioxidants and antioxidant activity of several pigmented rice brans. Journal of Agricultural and Food Chemistry. 59(1): 193-199.

Lin, Y. and Tsai, C. E. (2008). A study of adlay on lowering serum and liver lipids in hamsters. Journal of Food Lipids. 15(2): 176-189.

Lu, X., Liu, W., Wu, J., Li, M., Wang, J., Wu, J. and Luo, C. (2013). A polysaccharide fraction of adlay seed (*Coixlachryma-jobi* L.) induces apoptosis in human non-small cell lung cancer A549 cells. Biochemical and Biophysical Research Communications. 430(2): 846-851.

Ma, J., Cheng, Q. X., Wang, T. and Ling, W. H. (2001). Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. The Journal of Nutrition. 131(5): 1421-1426.

Manosroi, J., Khositsuntiwong, N. and Manosroi, A. (2014). Biological activities of fructooligosaccharide (FOS)-containing *Coix lachryma-jobi* Linn. extract. Journal of Food Science and Technology. 51(2): 341-346.

Min, B., McClung, A. M. and Chen, M. H. (2011). Phytochemicals and antioxidant capacities in rice brans of different color. Journal of Food Science. 76(1): C117-C126.

Moongngarm, A. and Saetung, N. (2010). Comparison of chemical compositions and bioactive compounds of germinated rough rice and brown rice. Food Chemistry. 122(3): 782-788.

Ohtsubo, K. i., Suzuki, K., Yasui, Y. and Kasumi, T. (2005). Bio-functional components in the processed pre-germinated brown rice by a twin-screw extruder. Journal of Food Composition and Analysis. 18(4): 303-316.

Oko, A. O., Ubi, B. E., Efisue, A. A. and Dambaba, N. (2012). Comparative analysis of the chemical nutrient composition of selected local and newly introduced rice varieties grown in Ebonyi State of Nigeria. International Journal of Agriculture and Forestry. 2(2): 16-23.

Patil, S. B. and Khan, M. K. (2011). Germinated brown rice as a value added rice product: A review. Journal of Food Science and Technology. 48(6): 661-667.

Pengkumsri, N., Chaiyasut, C., Saenjum, C., Sirilun, S., Peerajan, S., Suwannalert, P., Sirisattha, S. and Sivamaruthi, B. S. (2015). Physicochemical and antioxidative properties of black, brown and red rice varieties of northern Thailand. Food Science and Technology. 35: 331-338.

Puangwerakul, Y. (2007). Malt characteristic of 40 rice varieties cultivated in Thailand. Kasetsart Journal (Natural Science). 41: 15-20.

Rasane, P., Jha, A., Sabikhi, L., Kumar, A. and Unnikrishnan, V. S. (2015). Nutritional advantages of oats and opportunities for its processing as value added foods - a review. Journal of Food Science and Technology. 52(2): 662-675.

Singleton, V. L. and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture. 16(3): 144-158.

Sofowora, A. (1993). Medicinal plants and traditional medicine in Africa. Ibadan. Nigeria: Spectrum Books Ltd.

Sompong, R., Siebenhandl-Ehn, S., Linsberger-Martin, G. and Berghofer, E. (2011). Physicochemical and antioxidative properties of red and black rice varieties from Thailand, China and Sri Lanka. Food Chemistry. 124(1): 132-140.

Sumczynski, D., Kotásková, E., Družbíková, H. and Mlček, J. (2016). Determination of contents and antioxidant activity of free and bound phenolics compounds and in vitro digestibility of commercial black and red rice (*Oryza sativa* L.) varieties. Food Chemistry. 211: 339-346.

Tanprasert, K., Uthairatanakij, A., Kanlayanarat, S., Htwe, N. N. and Srilaong, V. (2008). Characterization of bioavailable compounds in black and red pigmented rice cultivars in Thailand. In Asia Pacific Symposium on Assuring Quality and Safety of Agri-Foods 837: 65-72.

Vichapong, J., Sookserm, M., Srijesdaruk, V., Swatsitang, P. and Srijaranai, S. (2010). High performance liquid chromatographic analysis of phenolic compounds and their antioxidant activities in rice varieties. LWT - Food Science and Technology. 43(9): 1325-1330.

Wu, F., Yang, N., Touré, A., Jin, Z. and Xu, X. (2013). Germinated brown rice and its role in human health. Critical Reviews in Food Science and Nutrition. 53(5): 451-463.

Xiang, N., Guo, X., Liu, F., Li, Q., Hu, J. and Brennan, C. S. (2017). Effect of light- and dark-germination on the phenolic biosynthesis, phytochemical profiles, and antioxidant

activities in sweet corn (*Zea mays* L.) sprouts. International Journal of Molecular Sciences. 18(6): 1-13.

Xu, L., Wang, P., Ali, B., Yang, N., Chen, Y., Wu, F. and Xu, X. (2017). Changes of the phenolic compounds and antioxidant activities in germinated adlay seeds. Journal of the Science of Food and Agriculture. 97(12): 4227-4234.

Zhang, G., Xu, Z., Gao, Y., Huang, X., Zou, Y. and Yang, T. (2015). Effects of germination on the nutritional properties, phenolic profiles, and antioxidant activities of buckwheat. Journal of Food Science. 80(5): H1111-H1119.

Zhao, M., Zhu, D., Sun-Waterhouse, D., Su, G., Lin, L., Wang, X. and Dong, Y. (2014). *In vitro* and *in vivo* studies on adlay-derived seed extracts: phenolic profiles, antioxidant activities, serum uric acid suppression, and xanthine oxidase inhibitory effects. Journal of Agricultural and Food Chemistry. 62(31): 7771-7778.

CHAPTER 3

BIOACTIVE COMPONENTS, ANTI-OXIDATIVE AND ANTI-INFLAMMATORY PROPERTIES (ON RAW 264.7 MACROPHAGE CELLS) OF SOAKED AND GERMINATED PURPLE RICE EXTRACTS

3.1 Abstract

The bioactive components and anti-oxidative properties of Thai purple rice after 24 h soaking and 36 h germination periods were investigated. Anti-inflammatory properties of the rice extracts were also evaluated on macrophage cells, stimulated with Lipopolysaccharide. These conditions significantly increased the bioactive components and anti-oxidative properties. Total anthocyanin contents, total carotenoid contents and gamma-aminobutyric acid were increased more than twofold in the soaked sample. All the extracts led to cell proliferation and inhibited nitric oxide (NO) production. Twenty-four hour soaking exhibited highest bioactive compounds, anti-oxidative properties and showed greater NO inhibitory effects with IC_{50} of 234.00 ± 0.01 μg mL^{-1}. In addition to established information on germination, soaking greatly improved the bioactive components and anti-inflammatory properties of the rice variety.

3.2 Introduction

Of all the cereal foods in the world, rice is the third most important and the staple food consumed in several countries across Asia. About 85% of humans globally consumed the white rice variety until there was a rising interest in pigmented rice varieties (i.e. dark purple, red and black rice). This is because these colored varieties contain polyphenols in their bran layers (Petroni *et al.*, 2017). The black and purple varieties have been studied to contain mainly anthocyanins (Gamel *et al.*, 2019), while the red rice mainly contain pro-anthocyanidins (Samaranayake *et al.*, 2017). In Thailand, the red, brown and black rice varieties are extensively cultivated and can be found in all the provinces. Pigmented rice, specifically the black or dark purple varieties have been reported to contain several properties that are beneficial to human health such as antioxidant, anti-inflammatory, anti-obesity, antitumour, hypoglycaemic and anti-allergic (Chmiel *et al.*, 2018). In addition to these properties, they are also rich in fiber, minerals, ligans, c-oryzanol, vitamins B1, B2, B3 and B6, Vitamin E, phytic, polyphenols and selenium (Irakli and Katsantonis, 2017; Reddy *et al.*, 2017). Bioactive compounds in these rice varieties can also serve as natural hypoglycaemic food ingredients as an alternative to artificial medications which could cause many side effects (Boue *et al.*, 2016).

Thai Purple rice (Khao Niaw Dam Peuak Dam) is one of the most beneficial pigmented rice indigenous to Southern Thailand and known for its functional properties and health benefits. Southern Thailand is between Indian and Pacific Ocean and the rice grown in this part is quite different in yield and quality (Pathak *et al.*, 2017). The purple rice has been used as a traditional food, dessert and also for medicinal purposes (Yamuangmorn *et al.*, 2018). Several of the pigmented rice especially Khao Niaw Dam Pleuak Dam grown in the Southern Thailand still lack substantial information on the nutritional composition and health benefits. The process of soaking

and germination in improving the bioactive compounds and seed quality of this indigenous rice variety will be a novel approach area that would promote the consumption and its market.

Several pollutions, diet, stress majorly contribute to oxidative stress which in turn causes different health issues in human beings. In order to fight these complications, diets rich in antioxidants are required to prevent diseases caused by reactive oxygen species (ROS) (Oghbaei and Prakash, 2015). The immune system in response to injury or external bodies reacts by producing inflammation cytokines like nitric oxide (NO). Normally, NO plays a role in regulating vasodilation and neurological processes but its overproduction may led to damage of tissues with related chronic inflammation. Many antioxidants and anti-inflammatory substances from synthetic sources are in use but the risk of toxicity remains the concern of the consumers. Hence, optimum attention has been paid on developing substances from natural sources that are efficient in both anti-oxidative and anti-inflammatory properties.

Therefore, aim of this study was to investigate the bioactive components, anti-oxidative properties of soaked and germinated Thai purple rice extracts and their effects on lipopolysaccharide-activated RAW 264.7 macrophage cells.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Sodium hypochlorite, methanol, ethanol, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), potassium acetate (CH_3CO_2K), sodium acetate ($C_2H_3NaO_2$), potassium persulphate ($K_2S_2O_8$), petroleum ether, anhydrous sodium sulphate (Na_2SO_4), hydrochloric acid (HCl), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), fluorenylmethylloxycarbonyl chloride (FMOC), sodium tetraborate, trifluoroacetic acid, acetonitrile (ACN), acetone, gallic acid, protocatechuic acid, catechin, caffeic acid, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, rutin trihydrate, quercetin dihydrate, trans-cinnamic

acid, 2,2-diphenyl- 1-picryl hydrazyl (DPPH), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylicacid(trolox),2,20-azino bis(3ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), formic acid, HPLC grade acetonitrile (ACN), HPLC grade methanol, lipopolysaccharide (LPS) from Escherichia coli, L-nitro-arginine (L-NA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma Aldrich Co. (St. Louis, MO, USA). Reagents and media used for cell line such as trypan blue dye, trypsin-EDTA, fetal bovine serum (FBS), penicillin, streptomycin and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Gibco BRL, Life Technologies Inc. (Rockville, MD, USA) and Griess reagent from Merck (Darmstadt, Germany).

3.3.2 Sample preparation, soaking and germination

Rough rice sample (*Oryza sativa* L. cv. Khao Niaw Dam Peuak Dam) as shown in Figure 22 was obtained from Songkhla Provincial Agricultural Office, Southern Thailand (Songkhla Province). The grains were soaked in 2.5% sodium hypochlorite solution (1:5 w/v) at 1-5% concentration for 5 min and drained. The purpose is to destroy or remove surface fungi and bacteria without killing internal organs. This was followed by washing the disinfected seed with distilled water in order to remove excess sodium hypochlorite.

The grains were soaked in distilled water (seed : water ratio, 1.5 w/v) at ambient temperature (30 ± 5ºC) for 24 h with the water changed every 8 h so that mold growth does not take over. After 24 h, the grains were collected for further process. For the germination process, the soaked seeds were placed in germination trays covered with moist cheese cloth and allowed to germinate for 12, 24, 36, and 48 h at 37ºC. Relative humidity was around 66% in a closed system. At the end of the germination period, the seeds were oven dried at 80ºC for 5 h, followed by de-husking. The de-husked seeds were grounded into a fine powder by 40-mesh sieve and stored at -20ºC for further analysis.

3.3.3 Sample extraction

Phenolic compounds in both germinated and un-germinated samples were extracted with 80% methanol by continuous stirring for 30 mins, followed by centrifugation (Hitachi CR22G III, Hitachi Koki Co., Ltd, Japan) at 10 000 x *g* for 20 min at 4ºC. The extraction was carried out three times with the supernatants pooled and evaporated to ~10 mL at 35 °C under reduced pressure to remove the solvent. The evaporated samples were lyophilized to dryness and stored at -20 °C for further analysis.

3.3.4 Determination of total anthocyanins contents

The total anthocyanin content (TAC) of the rice extracts was determined according to the pH- differential method (H. Wu *et al.*, 2015). Briefly, 1 mL of the methanol extracts was transferred into 10 mL volumetric flasks to prepare two dilutions. In one flask, the volume was adjusted with potassium acetate (pH 1.0) and the other flask adjusted with sodium acetate (pH 4.5). These mixtures were incubated and allowed to react for 25 min. The absorbance was measured at 510 and 700 nm for each dilution in order to correct haze effects and the absorbance from the diluted sample mixtures was calculated from the Equation 1.

$$A = (A_{510} - A_{700})\ \text{pH 1.0} - (A_{510} - A_{700})\ \text{pH 4.5} \qquad (1)$$

The monomeric anthocyanin pigment was calculated as in Equation 2.

$$\text{Monomeric anthocyanin pigment (mg/L)} = \frac{(A \times MW \times DF \times 1000)}{\varepsilon \times 1} \qquad (2)$$

Where;

MW = molecular weight of cyaniding-3-glucoside (449.2)

DF = dilution factor

ε = molar absorptivity (26,900). This was converted into mg/100 g of the total anthocyanin contents.

3.3.5 Extraction and determination of total carotenoid contents

The total carotenoid contents (TCC) were extracted by adding 25 mL of acetone to 1 g of sample. This was homogenized, filtered with Whatman No. 1 filter paper. The supernatant was transferred into a separatory funnel and 25 mL of petroleum ether was added. This was allowed to stand for 10 min at room temperature and the top layer was washed with 25 mL distilled water. The process was repeated three times or until the sample gave a colorless solution. Fifteen gram of anhydrous sodium sulphate was added to the extract in a 50 mL volumetric flask and evaporated under vacuum at 50 °C using a rotary evaporator. The acetone was removed by slow addition of distilled water until there was no residual of the solvent left. Petroleum ether was added to dissolve the residue and the volume was made up to 10 mL. The absorbance was measured at 468 nm and the total carotenoid contents were determined as shown in Equation 3.

$$\text{Carotenoid (µg/g sample)} = \frac{\text{A468 x volume of extract x dilution}}{\text{0.2 x weight of sample used in grams}} \qquad (3)$$

Where 0.2 = A_{468} of 1 µg mL^{-1} standard Canthaxanthin

3.3.6 Quantification of gamma-Aminobutyric acid (GABA) content

The GABA analysis was carried out at Central Lab Thai, Hat-yai, Songkhla, Thailand. Briefly, the soaked and germinated rice seeds were finely grounded and 1 g per sample was extracted with 50 mL of ethanol and distilled water (50%: 50%). One milliliter of the extract was pipetted and 1 mL of fluorenylmethylloxycarbonyl chloride (FMOC) was added. Three milliliter of 0.1 M of Sodium tetra borate was added to the mixture and vortexed within 1 min. This was followed by filtering the mixtures using 0.45 lm and injected in HPLC (Agilent Technologies 1200 series, Hewlett- Packard-Strasse 8 76337 Waldbronn, Germany). The mobile phase consists of trifluoro acetic acid (TFE), acetonitrile (ACN) and methanol (MeOH). The gradient elution was performed with TFE 55%, ACN 25%, MeOH 20% (0–4 min); TFE 42%, ACN 28% MeOH 30% (4–6 mins); TFE 42%, ACN 28% MeOH 30% again for another 2 min; TFE 0%, ACN 75%

MeOH 25% (11–15 min); TFE 55%, ACN 25% MeOH 20% (13–15 min) and this was allowed to run in the same condition for 3 min. C18 (3.9 x 150 mm, 5 µNm) column (Agilent Technologies) was used at 1 mL min^{-1} flow rate with fluorescence detector with Ex 270 nm, In 315 nm (Agilent Technologies 1200 series, Hewlett- Packard-Strasse 8 76337 Waldbronn, Germany).

3.3.7 Antioxidant assays

3.3.7.1 Ferrous ion chelating activity (FCA)

The Ferrous ion chelating activity was measured applying the procedure of Ebrahimzadeh *et al.* (2009). A 277.5 µL of the sample extract was made to react with 7.5 µL of 2 mM $FeCl_2$ and 15 µL of 5 mM ferrozine for 10 minutes at ambient temperature. The activities were evaluated at an absorbance of 562 nm spectrophotometrically with the use of a micro-plate reader (BioTeK PowerWave XS, USA). A standard curve of EDTA at concentrations 20 - 40 µg/mL was constructed and the activities were expressed as µmol EDTA equivalent (EDTAE)/g sample extract.

3.3.7.2 Hydroxyl radical scavenging activity (HRSA)

The hydroxyl radical scavenging capacity was calculated based on protocol of . Concisely, 1 ml of sample extract was reacted with 1 ml of 1.865 mM 1, 10-phenanthroline solution and 2 ml sodium phosphate buffer pH 7.4, followed by stirring to achieve a homogeneous solution. 1 ml of 1.865 mM $FeSO_4 \cdot 7H_2O$ solution was added to the solution. The subsequent reaction was commenced by pipetting 1 ml of H_2O_2 (0.03% v/v) into the solution. After allowing it to stand for 60 minutes in a water bath at a temperature of 37ºC, the antioxidant activity of the sample was measured at a wavelength of 536 nm against reagent blank. Trolox standard curve of concentrations between 100 - 1000 µg/L was constructed and the antioxidant activity reported as mg Trolox equivalent (TE)/g sample extract.

3.3.7.3 Superoxide dismutase (SOD) - like activity

To assess the capability of the methanolic extracts to scavenge superoxide anions, superoxide dismutase assay was performed following the method of Marklund and Marklund (1974) with some modifications. The reaction mixture, consisting of 1 mL of 50 mM Tris-HCl/10 mM EDTA buffer (pH 8.5), 60 µL of 7.2 mM pyrogallol, and 60 µL of the methanolic extract, was incubated at 25℃ for 10 min. The mixture was added with 30 µL of 1 N HCl and the absorbance was measured at 420 nm. The SOD-like activity was calculated as shown in Equation 3.

$$\text{SOD-like activity (\%)} = [1 - (S_{abs} - B_{abs}) / C_{abs}] \times 100 \quad (3)$$

Where;

S_{abs} = absorbance of the sample solution

B_{abs} = absorbance of the reaction mixture containing buffer instead of pyrogallol

C_{abs} = absorbance of the reaction mixture containing buffer instead of the methanolic extract.

3.3.8 Cell culture model

The RAW 264.7 murine macrophage cells used were obtained from the American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium with 1.5% sodium bicarbonate, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin supplementation in order to maintain the cells at 37 °C, 5% CO2, in a completely humidified incubator. During the experiment, the cells were washed with phosphate buffer (PBS) of pH 7.2.

3.3.8.1 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay for cell viability

The cells were grown at 80–90% confluence and harvested using 0.25% trypsin-EDTA. The number of cells was estimated with the use of a hemocytometer trypan blue-based technique (Louis and Siegel, 2011). The cells with density of 1 x 10^6 cells mL^{-1} were seeded into 96-well

plates and were left to attach to the plates at 37 °C for 2 h. This was followed by allowing the cells to react with the mixture of medium and rice extracts at different concentrations (200–1000 μg mL^{-1}) and incubated for 24 h. After 24 h, the cell viability was determined by taking 100 μL of the supernatants with 10 μL of the MTT solution added. The MTT solution was removed after 2 h and the formazan crystals were dissolved by adding 100 μL of dimethyl sulphoxide (DMSO). The absorbance was measured at 570 nm with a microplate reader (BioTeK PowerWave XS, USA) and the cell viability percentage calculated using the Equation 4.

$$A = \frac{\text{(Absorbance of sample)}}{\text{(Absorbance of control)}} \times 100 \qquad (4)$$

3.3.8.2 Nitric oxide (NO) assay

The inhibition of NO production was determined as a method for screening the soaked and germinated extracts for anti-inflammatory properties. RAW 264.7 cells with a density of 1 x 10^6 cells mL^{-1} were seeded into a 96-well plates and left to adhere for 2 h. The cells were treated with 1 μg mL^{-1} lipopolysaccharide (LPS) from *E. coli* in order to stimulate the cells to produce NO. One hundred microliter of the rice extracts at different concentrations (200–1000 μg mL^{-1}) was added and incubated for 24 h and L-nitro arginine (L-NA) was used as the positive control. The nitrite accumulation in the culture supernatants was used to estimate the percentage of NO production inhibition using the Griess reagent. One hundred microliter of the supernatants was transferred to a 96-well plate with 100 μL Griess reagent added. The mixtures were read at 570 nm with a microplate reader (BioTeK PowerWave XS, USA). The percentage of NO production inhibition was calculated from the Equation 5.

$$\text{A} = \frac{[(\text{Control-Blank of control})-(\text{Sample-Blank of sample})]}{\text{(Control-Blank of control)}} \text{ x } 100 \qquad (5)$$

3.3.9 Statistical analysis

Data were statistically analyzed by one-way ANOVA using a complete randomized design. The mean values were analyzed by Tukey test for a multi-comparison of means and correlation analysis using Pearson correlation. Statistical analyses were carried out using the SPSS statistical software (SPSS, Inc., Chicago. IL).

Figure 22: Picture of rice sample (Khao Niaw Dam Peuak Dam)

3.4 Results and discussion

3.4.1 Changes in bioactive compounds of Thai purple rice extracts

The bioactive compounds and the antioxidant properties of soaked and germinated purple rice are presented in Table 12. TAC increased after 24 h soaking from 19.04 ± 0.05 to 44.48 ± 0.08 mg Cy3-glc E/100 g but decreased to 36.49 ± 0.03 mg Cy3-glc E/100 g after 36 h

germination. The GABA and the TCC were also increased compared to the control ranging between 34.41 and 87.14 mg kg^{-1}, 1.46 mg BE/100 g and 2.23 mg BE/100 g respectively.

Previous studies by Kushwaha (2016) also found TAC to increase in black rice after germination. The significant increase in the TAC could be explained by the fact that some inactive enzyme are usually activated in order to break down larger molecular substances during soaking and germination process, resulting to an increase in the available nutrients and bioactive components (Phattayakorn *et al.*, 2016). Studies by Chalorcharoenying *et al.* (2017) on different pigmented sprouted corn varieties also confirmed that corn genotypes with purple kernels were observed to contain the highest anthocyanin content (68.2 mg/g). The TCC observed in this study (1.46 ± 0.11, 2.23 ± 0.07 and 2.08 ± 0.09 mg BE/100 g) were higher than those reported by Petroni *et al.* (2017) who compared bioactive components of black/purple, red and white rice varieties. In their study, they observed that the black rice exhibited higher values for bioactive components with TCC 232.8 μg/100 g compared to red rice (20.59 μg/100 g) with no carotenoid detected in brown rice. Pengkumsri *et al.* (2015), also reported higher values for TAC (487.25 mg cyaniding equivalent per g) in black rice, while red and white rice varieties exhibited lower yields with no TAC detected.

The significant increase in the GABA contents after soaking and germination indicates partial decomposition of storage protein and translocation to the growing parts of the seeds. During this process, the enzyme glutamate decarboxylase would be activated in order to convert glutamic acid into GABA (Tiansawang *et al.*, 2016). Also, some studies have reported that GABA could be produced in response to environmental stress (Scholz *et al.*, 2017). Banchuen *et al.* (2010), who evaluated effects of germination on the GABA contents of black, red and brown rice from Southern Thailand reported higher GABA contents for black rice (18.70 mg/100 g), red rice (10.67 mg/100 g) and brown rice (10.66 mg/100 g). The GABA contents in the ten brown rice

varieties studied by Kaur *et al.* (2017) ranged between 145.6 ± 1.9 and 200.5 ± 2.6 mg kg^{-1} which are much lower than those reported in the purple rice from this study. The results on GABA in this study agree with the previous studies on GABA contents of brown rice varieties where a significant increase during soaking and germination Katsuno *et al.* (2015) was observed. Ding *et al.* (2016), also reported a significant increase in GABA contents of red rice after germination for with further increase with ultrasonication treatment. In the same vein, Wang *et al.* (2015) reported that the GABA contents of Chinese soybean increased 36.7-fold after germination compared to the non-germinated soybean. However, the values observed in this study were lower than those reported by Boonpattararuksa *et al.* (2016) after 24 h soaking and 36 h germination. This may be due to the difference in varieties, location and germination conditions. Higher GABA contents observed during soaking could be as a result of hypoxia due to limited amount of oxygen during soaking in water which increases GABA in plant tissues rapidly in response to hypoxia (Lin *et al.*, 2015). Amino acid metabolism is adaptive to hypoxic stress. This is made up of a coordinated modulation of the flux of organic N going through glutamic acid, the downregulation of alanine- and adenosine phosphate (ATP)- consuming pathways, leading to the accumulation of alanine and GABA (Limami *et al.*, 2014).

3.4.2 Effects on the anti-oxidative properties

The anti-oxidative properties of the raw, soaked and germinated samples are presented in Table 2. The results show that soaking and germination significantly ($P < 0.05$) increased the anti-oxidative properties of the purple rice extracts. In the FCA assay, the value increased from 16.77 ± 0.04 μmol EDTAE per g to 39.04 ± 0.09 μmol EDTAE per g. For the HRSA and SOD assays, the values increased from 52.13 ± 0.06 mg TE per g to 87.45 ± 0.05 mg TE per g and 40.18 ± 0.08 % to 89.09 ± 0.04 %, respectively. In all, S_{24} showed the highest antioxidant properties in these assays. The results from the anti-oxidant assays have also confirmed that the polyphenolic

compounds in the extracts could be responsible for most of the antioxidant activities of the rice extracts. The FCA assay measures the ability of compounds in the sample to compete with ferrozine for ferrous ion which is often used to evaluate the secondary anti-oxidant activity of a sample (Vladimir-Knežević *et al.*, 2011). The SOD – like assay measures the capacity of the evaluated sample to catalyze the conversion of superoxide radicals to hydrogen peroxides and provide a defense mechanism against oxidative damage (Nagami *et al.*, 2004). Also, HRSA assay measures the ability of the sample extract to scavenge hydroxyl radicals which is the most reactive among reactive oxygen species (Lee *et al.*, 2004). Therefore, the higher anti-oxidative activity of the purple rice extracts especially with the S_{24} observed in this study reveals that this sample has stronger primary and secondary anti-oxidant potential. These properties could be attributed to be as a result of the higher total phenolic contents, total flavonoid contents and anthocyanin which are considered as the most dominant compounds found in the rice. These results are similar to the studies of Dadi *et al.* (2018) on fifty antioxidant-rich foods.

It should be noted that S_{24} sample and S_{24} + 36 h germinated sample were selected and evaluated for further studies based on the higher bioactive compounds and anti-oxidant properties exhibited.

Table 12: Bioactive components and antioxidant properties of Thai purple rice extracts before soaking, at 24 h soaking and 36 h germination periods

Sample	TAC Cy3-glc E (mg/100g)	TCC (mg BE/100g)	GABA (mg/100g)	FCA (µmol EDTAE/g)	HRSA (mg TE/g)	SOD (%)
BS	19.04±0.05^{e}	1.46±0.11^{d}	34.41±0.09^{f}	16.77±0.04^{f}	52.13±0.06^{c}	40.18±0.08^{e}
S_{24}	44.48±0.08^{a}	2.23±0.07^{a}	87.14±0.03^{a}	39.04±0.09^{a}	87.45±0.05^{a}	89.09±0.04^{a}
S_{24} + G_{12} germ	36.18±0.06^{b}	2.06±0.10^{b}	79.54±0.04^{c}	26.77±0.10^{c}	73.45±0.02^{c}	67.09±0.06^{c}
S_{24} + G_{24} germ	34.45±0.03^{c}	1.89±0.05^{c}	84.04±0.03^{b}	18.77±0.06^{d}	69.45±0.08^{e}	56.09±0.03^{d}
S_{24} + G_{36} germ	36.49±0.03^{b}	2.08±0.09^{b}	64.91±0.07^{d}	28.55±0.09^{b}	81.97±0.09^{b}	79.89±0.05^{b}
S_{24} + G_{48} germ	23.77±0.04^{d}	2.04±0.07^{b}	44.55±0.02^{e}	26.77±0.08^{c}	70.45±0.03^{d}	69.09±0.06^{c}

EDTA - (Ethylenediaminetetracetic acid) equivalent (EDTAE/g); Cy3-glc E - (Cyanidin-3-O- glucoside equivalent); BE - (Beta carotene equivalent); TE (trolox equivalent).; BS - Before soaking; S_{24} - 24 h soaking; S_{24}+G_{12} - 24 h soaking + 12 h germination; S_{24}+G_{24} - 24 h soaking + 24 h germination; S_{24}+G_{36} - 24 h soaking + 36 h germination; S_{24}+G_{48} - 24 h soaking + 48 h germination. In each column, sample means not having the same letter in superscripts are significantly different ($P < 0.05$).

3.4.3 Assessment of cell viability and NO production inhibition in LPS-stimulated RAW 264.7 murine macrophage cell

It is important to check the cytotoxicity of the rice extracts on the macrophage cell in order to ascertain the safety of each the sample before its introduction as a functional food product into the market. Raw murine macrophage cell was selected as it has been studied to express the response of some compounds especially in the immune system, which is regarded as the initial step for defense in the human body (Cekici *et al.*, 2014). Figure 23 shows the percentage of cell viability before and after treating with the rice extracts. One hundred percent of the 264.7 RAW cells were still viable after inducing with LPS for a period of 24 h. Treatments with the rice extracts at all levels of concentrations (200 – 1000 µg mL^{-1}) all led to an increase in the RAW cell viability above 100%. However, the highest cell viabilities were observed with the treatment of

1000 μg mL^{-1} extract concentration with values of 142.22% at 24 h soaking, 124.37% at 24 h soaking combined with 36 h germination and 124.37% with the raw sample. The results confirmed the safety of all the extracts as they cause no damage or cell death even at higher concentrations. Moreover, S_{24} greatly led to the cell proliferation (113.58%) of the macrophage cell ($P < 0.05$) compared with the control even at a low concentration of (200 μg mL^{-1}). The presence of some polyphenolic compounds in the purple rice extracts mainly contributes to the cell viability and proliferation of the RAW 264.7 murine macrophage cells. (Limtrakul *et al.*, 2015; Limtrakul *et al.*, 2016) reported that red and black rice extracts even at a higher concentration of 200 μg mL^{-1} had no significant effect on the cell viability of the macrophage cell. Also, Petroni *et al.* (2017) observed no growth effects on the macrophage cells with red and black rice extracts (125 μM). There is almost no information on the effects of soaked and germinated pigmented rice extracts on the viability and NO inhibition of the 264.7 RAW macrophage cells. However, the results from this study proved that soaked and germinated purple rice extracts led to a significant ($P < 0.05$) increase in the cell viability even at a concentration of 200 μg mL^{-1}. The cell viability increased from 103.48% in the raw sample to 113.58% at S_{24} and 109.40% at S_{24} +G_{36} germination.

It is crucial to note that over production of NO results into the lipid cell membrane damage which in turn may lead to the generation of cancer. Therefore, substances capable of inhibiting the NO production would be helpful in preventing such occurrence. The NO inhibition by the rice extracts was determined by inducing the cells with LPS to produce NO and treating with different concentration of the extracts (200 – 1000 μg mL^{-1}). The percentage of NO inhibition increased in all sample extracts as the concentration increases ($P < 0.05$; Figure 24) with values ranging between 24.96% and 100.79% in the raw sample and 5.86 – 121.86% in 24 h soaking combined with 36 h germination. Highest values were recorded at 24 h soaking where the percentage NO inhibition increased from 30.87% to 129.30%. The results from this study agree with the works of

Vichit and Saewan (2016) who reported that germinated white, red and black rice extracts inhibited NO production in RAW 264.7 macrophage cells with highest values recorded in the pigmented varieties. They concluded that germination increased % NO inhibition nearly two times in black rice (from 31.47% to 64.96%), red rice (from 36.38% to 63.06%) and brown rice (from 24.18% to 45.31%).

Gamisoyo-san (GMSYS), an herbal extract was evaluated on RAW 264.7 macrophage cells Jin *et al.* (2016) and they reported no significant cytotoxicity in the cells up to 1000 μg mL^{-1} and markedly reduced NO production.

The IC_{50} of NO inhibition concentrations (μg mL^{-1}) is presented in Figure 25. In all the rice extracts, 24 h soaking gave the least IC_{50} NO inhibition concentration with value 243 ± 1.01 μg mL^{-1}. This was followed by 24 h soaking combined with 36 h germination (338 ± 1.10 μg mL^{-1}) and the raw sample gave 450 ± 2.01 μg mL^{-1}. It may be assumed that the phenolic compounds especially flavonoids at higher concentrations in the soaked sample were responsible for the strongest suppressive activity against NO production. Although all the values obtained were much higher than the positive control, L-NA (iNOS inhibitor) which gave an IC_{50} of 50.14 ± 2.10 μg mL^{-1}, the purple rice at all treatments could still be considered as functional food products as they promoted cell proliferation and inhibited NO production.

3.5 Conclusion

Soaking could be considered as an economic, less-time consuming and easy technique for improving polyphenolic compounds, other bioactive components and anti-oxidative properties in Thai purple rice varieties. The rice extracts can also be classified safe as it caused no toxicity on the macrophage cells. In addition, S_{24} led to the cell proliferation and greatly inhibited NO production compared to other samples. This study would however be the first to observe the outstanding cell proliferation and NO inhibition activities of Thai purple rice extracts especially at

24 h soaking condition. In conclusion, soaked and germinated Thai purple rice may be produced as functional foods and supplements such as functional drinks, confectionaries and as deserts. They could also be added to functional food products for lower risks of inflammatory diseases and oxidative stress occurrences.

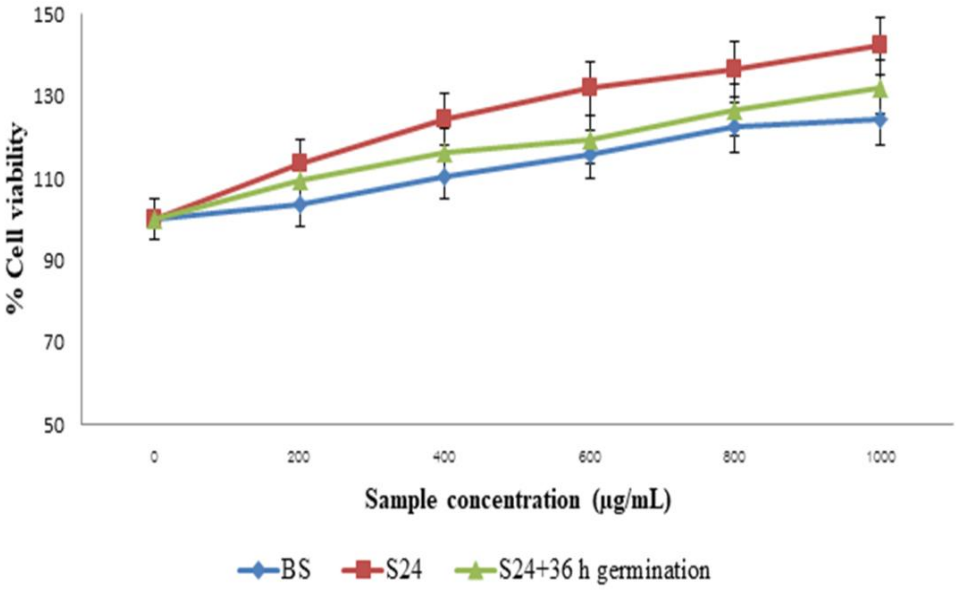


Figure 23: Percentage cell viability of 264.7 RAW murine macrophage cell after treating with Thai purple rice extracts at BS, S_{24} and S_{24}+36h germination

Before soaking (BS); soaked sample (S_{24}); 24 h soaking + 36 h germination (S_{24}+G_{36})

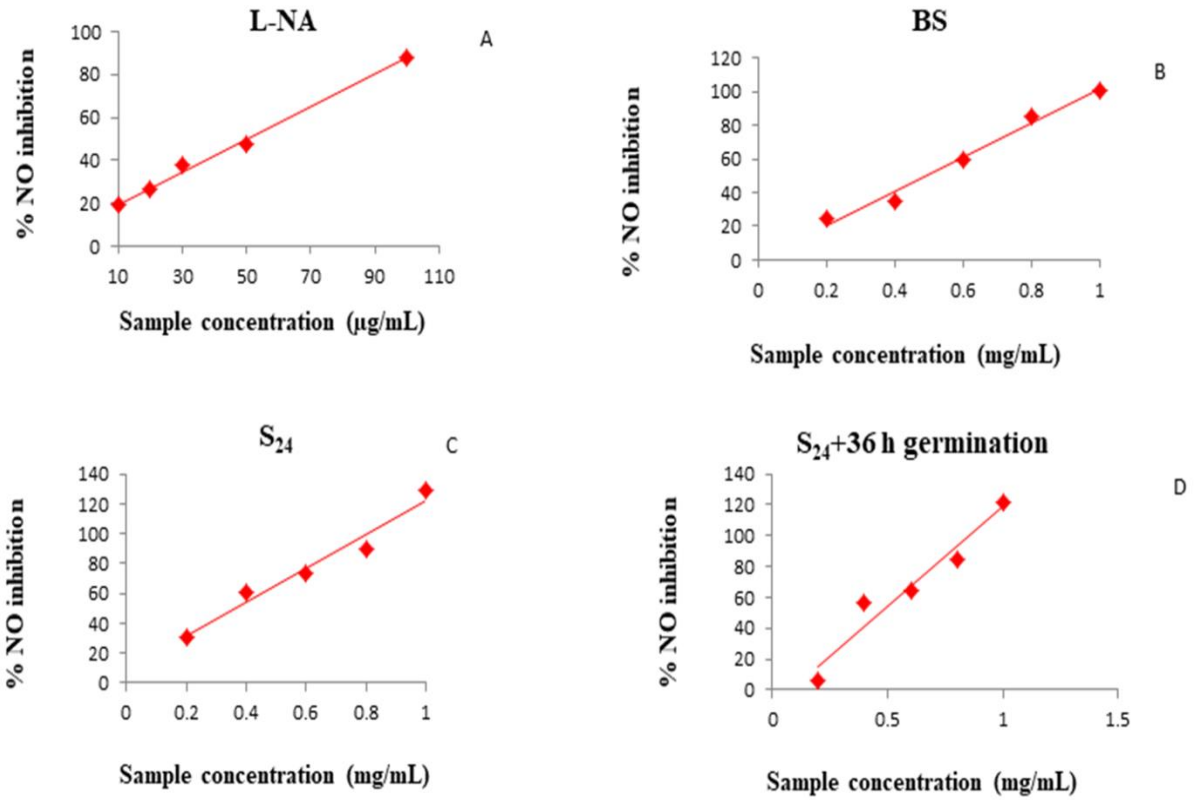


Figure 24: Percentage NO production inhibition of Thai purple rice extracts. A- L-nitro arginine (L-NA), B- Before soaking (BS), C- soaked sample (S_{24}), D- 24 h soaking + 36 h germination (S_{24}+G_{36})

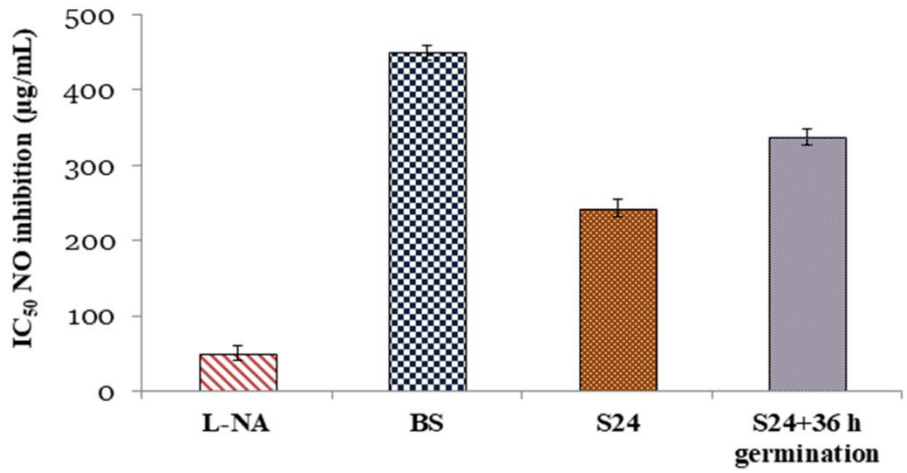


Figure 25: IC_{50} of NO production inhibition (µg/mL).

L-nitro arginine (L-NA); before soaking (BS); soaked sample (S_{24}); 24 h soaking + 36 h germination (S_{24}+G_{36})

3.6 References

Banchuen, J., Thammarutwasik, P., Ooraikul, B., Wuttijumnong, P. and Sirivongpaisal, P. (2010). Increasing the bio-active compounds contents by optimizing the germination conditions of Southern Thai Brown Rice. Songklanakarin Journal of Science and Technology. 32: 219-230.

Boonpattararuksa, J., Songchitsomboon, S., Akkaravessapong, P., Wongpiyachon, S., Wasusun, A. and Komindr, S. (2016). Effects of two pre-germinated brown rice of Thailand on postprandial blood glucose and insulin responses in type 2 diabetes. Food and Applied Bioscience Journal. 4: 138-150.

Boue, S. M., Daigle, K. W., Chen, M. H., Cao, H. and Heiman, M. L. (2016). Antidiabetic Potential of Purple and Red Rice (*Oryza sativa* L.) Bran Extracts. Journal of Agricultural and Food Chemistry. 64(26): 5345-5353.

Cekici, A., Kantarci, A., Hasturk, H. and Van Dyke, T. E. (2014). Inflammatory and immune pathways in the pathogenesis of periodontal disease. Periodontology 2000. 64(1): 57-80.

Chalorcharoenying, W., Lomthaisong, K., Suriharn, B. and Lertrat, K. (2017). Germination process increases phytochemicals in corn. International Food Research Journal. 24: 552–558.

Chmiel, T., Saputro, I. E., Kusznierewicz, B. and Bartoszek, A. (2018). The impact of cooking method on the phenolic composition, total antioxidant activity and starch digestibility of rice (*Oryza sativa* L.). Journal of Food Processing and Preservation. 42(1): e13383-e13395.

Dadi, D. W., Emire, S. A., Hagos, A. D. and Assamo, F. T. (2018). Influences of different drying methods and extraction solvents on total phenolic and flavonoids, and antioxidant capacity

of *Moringa stenopetala* leaves. Journal of Pharmacognosy and Phytochemistry. 7(1): 962-967.

Ding, J., Yang, T., Feng, H., Dong, M., Slavin, M., Xiong, S. and Zhao, S. (2016). Enhancing Contents of γ-Aminobutyric Acid (GABA) and Other Micronutrients in Dehulled Rice during Germination under Normoxic and Hypoxic Conditions. Journal of Agricultural and Food Chemistry. 64(5): 1094-1102.

Ebrahimzadeh, M. A., Nabavi, S. and Nabavi, S. (2009). Correlation between the in vitro iron chelating activity and poly phenol and flavonoid contents of some medicinal plants. Pakistan Journal of Biological Sciences. 12(12): 934.

Gamel, T. H., Wright, A. J., Tucker, A. J., Pickard, M., Rabalski, I., Podgorski, M., Di Ilio, N., O'Brien, C. and Abdel-Aal, E. S. M. (2019). Absorption and metabolites of anthocyanins and phenolic acids after consumption of purple wheat crackers and bars by healthy adults. Journal of Cereal Science. 86: 60-68.

Irakli, M. and Katsantonis, D. (2017). Rice bran: a promising natural antioxidant component in breadmaking. Journal of Nutraceuticals and Food Science. 2: 14-17.

Jin, M., Cai, Y., Li, J. and Zhao, H. (1996). 1, 10-Phenanthroline-Fe2+ oxidative assay of hydroxyl radical produced by H2O2/Fe2+. Progress in Biochemistry and Biophysics. 23(6): 553-555.

Jin, S. E., Kim, O. S., Yoo, S. R., Seo, C. S., Kim, Y., Shin, H. K. and Jeong, S. J. (2016). Anti-inflammatory effect and action mechanisms of traditional herbal formula Gamisoyo-san in RAW 264.7 macrophages. BMC Complementary and Alternative Medicine. 16(1): 219-221.

Katsuno, N., Sakamoto, C., Yabe, T., Yamauchi, R., Nishizu, T. and Kato, K. (2015). Methods for Enrichment of γ-Aminobutyric Acid in Sesame Seeds. Food Science and Technology Research. 21(6): 787-791.

Kaur, M., Asthir, B. and Mahajan, G. (2017). Variation in Antioxidants, Bioactive Compounds and Antioxidant Capacity in Germinated and Ungerminated Grains of Ten Rice Cultivars. Rice Science. 24(6): 349-359.

Kushwaha, U. K. S. (2016). *Black Rice: Research, history and development* (1st edn ed.). Switzerland: Springer International Publishing.

Lee, J., Koo, N. and Min, D. B. (2004). Reactive oxygen species, aging, and antioxidative nutraceuticals. Comprehensive Reviews in Food Science and Food Safety. 3(1): 21-33.

Limami, A. M., Diab, H. and Lothier, J. (2014). Nitrogen metabolism in plants under low oxygen stress. Planta. 239(3): 531-541.

Limtrakul, P., Yodkeeree, S., Pitchakarn, P. and Punfa, W. (2015). Suppression of inflammatory responses by black rice extract in RAW 264.7 macrophage cells via downregulation of NF-kB and AP-1 signaling pathways. Asian Pacific Journal of Cancer Prevention. 16: 277–4283.

Limtrakul, P., Yodkeeree, S., Pitchakarn, P. and Punfa, W. (2016). Anti-inflammatory effects of proanthocyanidin-rich red rice extract via suppression of MAPK, AP-1 and NF-κB pathways in Raw 264.7 macrophages. Nutrition Research Practice. 10(3): 251-258.

Lin, Y. T., Pao, C. C., Wu, S. T. and Chang, C. Y. (2015). Effect of different germination conditions on antioxidative properties and bioactive compounds of germinated brown rice. BioMed Research International. 2015: 1-10.

Louis, K. S. and Siegel, A. C. (2011). Cell viability analysis using trypan blue: manual and automated methods. In M. J. Stoddart (Ed.), *Mammalian Cell Viability: Methods and Protocols* (pp. 7-12). Totowa, NJ: Humana Press.

Marklund, S. and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry. 47(3): 469-474.

Nagami, H., Umakoshi, H., Shimanouchi, T. and Kuboi, R. (2004). Variable SOD-like activity of liposome modified with Mn(II)–porphyrin derivative complex. Biochemical Engineering Journal. 21(3): 221-227.

Oghbaei, M. and Prakash, J. (2015). Antioxidant components and their *in vitro* bioaccessibility in processed and stored chick pea and amaranth greens mix. Croatian Journal of Food Technology, Biotechnology and Nutrition. 10: 44-49.

Pathak, K., Rahman, S. W., Bhagawati, S. and Gogoi, B. (2017). Assessment of nutritive and antioxidant properties of some indigenous pigmented hill rice (*Oryza sativa* L.) cultivars of Assam. Indian Journal of Agricultural Research. 51(3): 214-220.

Pengkumsri, N., Chaiyasut, C., Saenjum, C., Sirilun, S., Peerajan, S., Suwannalert, P., Sirisattha, S. and Sivamaruthi, B. S. (2015). Physicochemical and antioxidative properties of black, brown and red rice varieties of northern Thailand. Food Science and Technology. 35: 331-338.

Petroni, K., Landoni, M., Tomay, F., Calvenzani, V., Simonelli, C. and Cormegna, M. (2017). Proximate composition, polyphenol content and anti-inflammatory properties of white and pigmented italian rice varieties. Universal Journal of Agricultural Research. 5: 312-321.

Phattayakorn, K., Pajanyor, P., Wongtecha, S., Prommakool, A. and Saveboworn, W. (2016). Effect of germination on total phenolic content and antioxidant properties of 'Hang' rice. International Food Research Journal. 26(1): 406-409.

Reddy, C. K., Kimi, L., Haripriya, S. and Kang, N. (2017). Effects of polishing on proximate composition, physico-chemical characteristics, mineral composition and antioxidant properties of pigmented rice. Rice Science. 24(5): 241-252.

Samaranayake, M., Yathursan, S., Abeysekera, W. and Herath, H. (2017). Nutritional and antioxidant properties of selected traditional rice (*Oryza sativa* L.) varieties of Sri Lanka. Sri Lankan Journal of Biology. 2(2): 25-35.

Scholz, S. S., Malabarba, J., Reichelt, M., Heyer, M., Ludewig, F. and Mithöfer, A. (2017). Evidence for GABA-Induced Systemic GABA Accumulation in Arabidopsis upon Wounding. Frontiers in Plant Science. 8(388): 1-9.

Tiansawang, K., Luangpituksa, P., Varanyanond, W. and Hansawasdi, C. (2016). GABA (Gamma-aminobutyric acid) production, antioxidant activity in some germinated dietary seeds and the effect of cooking on their GABA content. Food Science and Technology. 36: 313-321.

Vichit, W. and Saewan, N. (2016). Effect of germination on antioxidant, anti-inflammatory and keratinocyte proliferation of rice. International Food Research Journal. 26: 2006-2015.

Vladimir-Knežević, S., Blažeković, B., Bival Štefan, M., Alegro, A., Kőszegi, T. and Petrik, J. (2011). Antioxidant Activities and Polyphenolic Contents of Three Selected Micromeria Species from Croatia. Molecules. 16(2): 1454-1470.

Wang, F., Wang, H., Wang, D., Fang, F., Lai, J., Wu, T. and Tsao, R. (2015). Isoflavone, γ-aminobutyric acid contents and antioxidant activities are significantly increased during germination of three Chinese soybean cultivars. Journal of Functional Foods. 14: 596-604.

Wu, H., Johnson, M. C., Lu, C. H., Fritsche, K. L., Thomas, A. L., Cai, Z. and Greenlief, C. M. (2015). Determination of anthocyanins and total polyphenols in a variety of elderberry juices by uplc-ms/ms and other methods. Acta Horticulturae. 1061: 43-51.

Yamuangmorn, S., Dell, B. and Prom-u-thai, C. (2018). Effects of cooking on anthocyanin concentration and bioactive antioxidant capacity in glutinous and non-glutinous purple rice. Rice Science. 25(5): 270-278.

CHAPTER 4

IN VITRO STARCH DIGESTIBILITY, GUT MICROBIOTA MODULATION AND PHENOLIC METABOLITES OF SOAKED AND GERMINATED PURPLE RICE

4.1 Abstract

This study evaluated the *in vitro* starch digestibility and glycemic index (GI) of soaked and germinated purple rice. Additionally, their prebiotic properties on the gut microbiota and their metabolites were investigated using batch culture fecal fermentation. The results from the study revealed that soaking and germination conditions significantly reduced ($P < 0.05$) the GI (72.64 ± 0.75 to 61.52 ± 3.11%). Lowest value for the GI was recorded in the soaked rice. Raw purple rice, 24 h soaked purple rice, 24 h soaking combined with 36 h germination rice, resistant starch type II, and gallic acid evaluated in this study affected the gut microbiota. Interestingly, the fermented soaked rice selectively increased the amount of *Bifidobacterium* (log 9.62 to log 10.21) and *Lactobacillus* (log 8.55 to log 9.30) at 48 h fermentation and significantly inhibited ($P < 0.05$) the growth of *Clostridium/Enterobacter* and *Bacteroides* groups. This correlates with the increasing production and highest concentration of acetate (35.27 ± 0.09 mM) in this sample after 48 h fermentation. The prebiotic index (PI) score was 0.79, thus indicating its potential gut microbiota modulation. Among the gut microbiota identified, phenolic metabolites such as pyrocatechol, 3-(4-hydroxyphenyl) propionic acid, salicylic acid and *trans*-cinnamic acid were found. Therefore, the soaked khao niaw dam peuak dam could be a potential functional hypoglycemic ingredient in foods and nutraceuticals industry for management of gut health, diabetes and obesity.

4.2 Introduction

There has been a global rise in human death and it is believed to be related to non-communicable diseases (NCDs) such as diabetes mellitus, cardiovascular diseases, obesity, etc. In the developed and developing countries, an estimate of 630 million people are predicted to be living with diabetes by the year 2030 (WHO, 2016). In the same vein, the world obesity rate from the year 1980 to 2014 has increased two-fold. More than 1.9 billion of adult population were overweight in 2014, while 600 million were obese (WHO, 2016). It is estimated that by 2030, the digits could increase to 1.12 billion if the observed trend continues, reaching about 20% of the adult population worldwide (Kelly *et al.*, 2008). In 2015, 31% of all the deaths in the world were attributed to cardiovascular disease, which is the present leading cause of human death globally (WHO, 2016). The health statistics is quite alarming and calls for attention on diet-based nutritional intervention across the world.

The gut microbiota plays an important role in regulating energy homeostasis and energy intake. It also regarded as a "forgotten organ" due to its influence on the absorption and fermentation of food nutrients, thereby having a direct linkage with human health and metabolism (Hara and Shanahan, 2006). The interactions of human colon and non-digestible dietary compositions play vital roles in human health and disease (Singh *et al.*, 2017; Voreades *et al.*, 2014). Moreover, the gut microbiota also relates with the host immune system and provides signals which helps to promote the maturation and normal development of immune cells and immune functions (Chow *et al.*, 2010). Several factors affect the gut microbial populations such as dietary intake, mode of birth and age (Voreades *et al.*, 2014).

Recently, the consumption of whole grain rice and functional rice products that are capable of reaching the colon has gained so much attention. These include dietary fiber, prebiotics and other non-digestible dietary components such as polyphenols (Friedman, 2013;

Sharif *et al.*, 2014). These food components help to modulate the colon, support the production of short-chin fatty acids (SCFA), which have been proven to impart substantial positive colon health benefits (Macfarlane and Macfarlane, 2003). Imbalanced human gut microbiota is related to inflammatory bowel disease, colon cancer and gastroenteritis (Venter, 2007). Some carbohydrates are fermentable, escape digestion in the upper gut and activate the growth and activities of bacteria in the colon (Gibson *et al.*, 2004). These are known as prebiotics and help to improve the host gut health. Prebiotics serve as food materials for the colonic bacteria and are regarded as vital when compared to other dietary fibers because of their distinctive characteristics to be utilized selectively by beneficial colonic bacteria such as *Bifidobacterium* and *Lactobacillus* (Tuohy *et al.*, 2005).

Glycemic index (GI) has been used as a key clinical marker in the characterization of starchy food and in the assessment of dietary carbohydrate quality (Sacks *et al.*, 2014). Though the most generally-acceptable and reliable method of estimating GI is via *in vivo* assay, this however is expensive, highly influenced by physiology, metabolism, genetics and intra-individual variability of the subjects (Matthan *et al.*, 2016). Hence, a proxy approach was developed which measures the GI *in vitro* and has been generally acceptable (Woolnough *et al.*, 2008). This method employs the enzymatic hydrolysis, mimicking starch digestion in humans (Dona *et al.*, 2010).

The process of soaking and germination is also known to breakdown starch granules in grains by the carbohydrate-active enzymes. The amylolysis of starch components during the soaking and germination process is the main metabolic route for the utilization of starch in the endosperm (De Guzman *et al.*, 2017). This cheap and innovative process establishes the *de novo* synthesis of enzymes such as α-amylase and α-glucosidase in the aleurone and scutellum cells (Xu *et al.*, 2012). Only few studies that have reported the effects of soaked

and germinated rice on *in vitro* starch digestibility. From the results obtained in their studies, they confirmed that soaked and/ germinated whole grain rice may be a potential hypoglycemic food products but the mechanism behind this attribute is still inconclusive.

There are several varieties of pigmented rice especially khao niaw dam peuak dam cultivated in the Southern Thailand still lacking substantial information about their starch digestibility and effects on gut microbiota. The process of soaking and germination in improving the *in vitro* digestibility and functional quality of this indigenous rice variety will be a novel approach area that would promote the consumption and its market. Therefore, the aim of this study was to *in vitro* digestibility, gut microbiota and metabolites modulation of this indigenous soaked and germinated purple rice.

4.3. Materials and methods

4.3.1 Chemicals and reagents

Potassium hydroxide (KOH), sodium acetate, amyloglucosidase (*Aspergillus niger*), glucose standard, DNS reagent, phosphate buffer, α-amylase (Termamyl), sodium hydroxide, protease (Megazyme®), hydrochloric acid (HCl), potassium chloride (KCl), ethanol, acetone, α-amylase, bile extract, pancreatin, sodium chloride (NaCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), dipotassium phosphate (K_2HPO_4), haemin, resazurin, L-cysteine, paraformaldehyde, pepsin and DNA probes used for FISH technique were Bif164, Lab158, Bac303, Chris150 and Eub338 were all purchased from Sigma Co. (St. Louis, MO, USA). All other chemicals used in this study were analytical grade. Resistant starch type II (Hi-Maize®) was purchased from National Starch and Chemical Ltd, Bangkok, Thailand.

4.3.2 Sample preparation, soaking and germination

The paddy rice seeds (*Oryza sativa* L. cv. Khao Niaw Dam Peuak Dam) were obtained from Songkhla Provincial Agricultural Office in Southern Thailand (Songkhla

Province). The rice seeds were soaked in 2.5% sodium hypochlorite solution (1:5 w/v) at concentrations between 1 - 5% for 5 min with the water drained. The reason was to eliminate the fungi and bacteria on the surface of the grains. Subsequently, the seeds were washed with distilled water so as to ensure that residual sodium hypochlorite is removed from the seeds (Owolabi *et al.*, 2018).

The rice seeds were soaked in distilled water in the ratio of 1.5; w/v at room temperature (30 ± 5ºC) for 24 h with the water drained and replaced every 8 h in order to prevent the growth of mold. The rice seeds were eventually gathered for subsequent processing. For the germination process, the soaked rice seeds were spread in germination trays covered with damp cheese cloth and allowed to germinate for 12, 24, 36, and 48 h at 37ºC. Relative humidity was around 66% in an enclosed system. Finally, the rice seeds were dried in the oven at 80ºC for the period of 5 h, followed by de-husking. The resulting de-husked seeds were grounded into a fine powder by 40-mesh sieve and preserved at -20ºC for subsequent analysis.

4.3.3 Analysis of total starch (TS)

The total starch was determined by enzymatic reaction according to the modified method of Goñi *et al.* (1997). Thirty milligram of the ground and sieved purple rice flour was dispersed in 6 mL of 2 M KOH and intensely shaken for 30 min at room temperature in a water bath. The solubilized starch was then hydrolyzed by the addition of 3 mL of 0.4M Sodium acetate buffer pH (4.75) and 60 mL of amyloglucosidase from *Aspergillus niger* followed by at 60ºC for 45 min in a shaking water bath. The samples were centrifuged for 10 min at 3000 *g* and the glucose concentration in the supernatant was measured by dinitrosalicylic acid (DNS) method (Miller, 1959). The total starch was calculated as glucose concentration x 0.9.

4.3.4 Analysis of glucose

The glucose contents in the rice samples were measured by the 3,5-dinitrosalicylic acid (DNS) method using anhydrous glucose as the standard (Zhao *et al.*, 2008). The ground and sieved purple rice flour (2 g) was extracted with the addition of 50 mL followed by continuous stirring for 30 min and the resulting solution was centrifuged at 8228 × *g* for 10 min. Two mL of sample that was appropriately diluted, glucose standard, or blank (water) was reacted with 1.5 mL of DNS reagent solution in a water bath at 80ºC for 5 min. After the reaction, the mixture was cooled in an ice bath and distilled water was added to 10 mL. The absorbance was read at 540 nm against a blank solution using micro-plate reader (BioTeK PowerWave XS, USA).

4.3.5 Analysis of total dietary fiber (TDF)

The TDF was determined according to AOAC method 985.29 (Prosky *et al.*, 1985). Ground defatted rice sample (1 g) was suspended in 50 mL of 0.08 M phosphate buffer (pH 6.0) with 0.1 mL Termamyl and left to incubate for 30 min at 100°C in a shaking water bath. The extract was left to cool at room temperature and the pH was adjusted pH 7.5 by adding 10 mL of 0.275 M sodium hydroxide (10 mL). 100 microliters of protease (50 mg/mL ~ 350 tyrosine U/mL) was added and the sample was left in the shaking water bath at 60°C for 30 min. The pH was again adjusted to 4.5 with 10 mL of 0.325 M hydrochloric acid with 0.1 mL (~ 330 U/mL) amyloglucosidase added. The sample extract was incubated in the shaking water bath at 60°C for another 30 min. The soluble dietary fiber (SDF) was precipitated by the addition of 280 mL of 95% ethanol (preheated to 60 °C) and left at room temperature for 60 min. The insoluble dietary fiber (IDF) was recovered by filtration process. The residue was washed consecutively by adding three 20 mL portions of 78% ethanol, two 10 mL portions of 95% ethanol, and two 10 mL portions of acetone. The residue from sample was

analyzed for ash and protein. Ash was measured by incineration at 525°C in a muffle furnace for 5 h, while undigested protein was determined by the Kjeldahl method (N x 6.25). The blank determinations were also carried out. Total dietary fiber (TDF) was calculated as the weight of residue minus the weight of residual undigested protein and ash as in equation 1 below. All determinations were done in triplicate.

TDF = SDF + IDF (1)

4.3.6 *In vitro* starch digestion and glycemic index (GI)

The GI of the purple rice flour was carried out according to the method of Goñi *et al.* (1997). Fifty milligram of purple rice flour and 10 mL of HCl-KCl buffer (pH 1.5) were weighed and added to screw cap test tubes and homogenized using a homogenizer (40 s, 200 rpm). This was followed by adding 0.2 mL of pepsin solution containing 1 mg pepsin from porcine gastric mucosa was added to hydrolyze protein (Sigma Aldrich, St Louis, MO, USA, P6887) made in 10 mL of HCl-KCl buffer (pH 1.5) was added to each sample followed by incubation at 40°C for 60 min in a shaking water bath. The volume was raised to 25 mL with the addition of 25 mL tris-maleate buffer (pH 6.9). In order to start the hydrolysis, 5 mL of 2.6 IU α-amylase solution from porcine pancreas (Sigma Aldrich, St Louis, MO, USA, P7545) made in tris-maleate buffer was added to each sample. The flasks containing the samples were incubated in a shaking water bath at 37ºC with moderate agitation. Aliquots (0.1 mL) were taken from each flask every 30 min from 0-180 min and placed in boiling water for 5 min in order to inactivate the α-amylase. One milliliter of 0.4 M Sodium acetate buffer (pH 4.75) and 30 µL of amyloglucosidase from *Aspergillus niger* (Sigma A9913, Sigma-Aldrich Inc., US) were added to each aliquot to hydrolyze the digested starch into glucose and incubated at 60°C for 45 min in a shaking water bath. The released glucose content was determined by dinitrosalicylic acid (DNS) method (Miller, 1959). The glucose

was converted into starch by multiplying the released glucose weight by 0.9. The rate of starch digestion was expressed as a percentage of total starch hydrolyzed at different times (0, 30, 60, 90, 120 and 180 min). The total starch hydrolysis (%) of the rice starches at different periods was calculated according to equation 2.

$$\text{Total starch hydrolysis} = \frac{\text{Released glucose weight x 0.9}}{\text{Total starch weight}} \times 100 \qquad (2)$$

The *in vitro* starch digestion kinetics was determined using the non-linear model established by Goñi *et al.* (1997). The first-order equation is represented in equation 3 below.

$$C = C_\infty (1 - e^{-kt}) \qquad (3)$$

Where,

C = percentage starch hydrolyzed at time t (min),

C_∞ = equilibrium percentage of starch hydrolyzed after 180 min

k = kinetic constant

The parameters C_∞ and *k* were estimated for each sample based on data obtained from the *in vitro* starch digestion. The area under the hydrolysis curve (AUC) was calculated using the following equation 4.

$$AUC = C_\infty (t_\infty - t_0) - \left(\frac{C_\infty}{k}\right)[1 - \exp[-k(t_\infty - t_0)]] \qquad (4)$$

Where,

C_∞ = equilibrium percentage of starch hydrolyzed after 180 min

t_∞ = final time (180 min)

t_o = initial time (0 min)

k = kinetic constant

The hydrolysis index (HI) indicates the rate of starch digestion and the glycemic index (GI) represents the digestibility of the purple rice starch in relation to the digestibility of starch in a reference material, white bread. The HI, a good predictor of glycemic response,

was calculated by dividing the AUC of each treatment by the AUC of a reference (white bread). The GI was then estimated using the following equation 5 by (Goñi *et al.*, 1997).

$$GI = (0.549 \times HI) + 39.71 \quad (5)$$

4.3.7 Preparation of non-digestible soaked and germinated purple rice samples

Before the *in vitro* fecal fermentation, the rice samples were digested enzymatically, simulating the upper gut (mouth, stomach and the small intestine) digestion as shown in Appendix 2. This was achieved by independently homogenizing 30 g of raw rice; soaked rice sample; and germinated rice sample, with 500 mL of pepsin solution (0.462% pepsin, 49 mM NaCl, 12 mM KCl, 10 mM $CaCl_2$, 2.4 mM $MgCl_2$ and 2.5 mM K_2HPO_4). This followed the procedure of Jensen *et al.* (2009) with little adjustments. The pH of the samples was modified to 5.5, 3.8 and 1.5 with corresponding incubation duration of 120 min at 37°C, with continuous mixing at 220 rpm. This mimicked the human oral and gastric digestion. Subsequently, 300 mL of bile extract and pancreatin solution (1.25 g of bile extract, 0.2 g of pancreatin, and 50 mL of distilled water containing 0.1 M $NaHCO_3$) was included in the mixture, with the pH adjusted to 5.0, and afterwards to 6.5 for 30 and 240 min, respectively so as to mimic small intestine digestion. The reaction was stopped eventually by heating at 95°C for 10 min in order to terminate the enzyme reaction and cooled to room temperature in an ice bath. The non-digestible rice samples were collected with 1 KDa MWCO dialysis bag (Spectrum Laboratories, Inc., Rancho Dominguez,CA, USA) accompanied by constant removal of the distilled water permeate, until equilibrium point of solution was reached for up to 72 h. The corresponding samples were freeze dried and kept at -20°C for the subsequent batch culture fermentation study.

4.3.8 *In vitro* fecal fermentation

4.3.8.1 Preparation of fecal slurry

Fresh feces were collected from five healthy volunteers consisting of both male and female human who generally consume a regular meal, showed without digestive tract disease and had not ingested probiotics, prebiotics or antibiotics for the previous 3 months. Freshly passed feces received from the volunteers were placed directly in an anaerobic chamber, combined and homogenized with the addition of 0.1 M phosphate-buffered saline (PBS), pH 7.0 to 10% (w/v) of the fecal slurry. The slurry was then filtered through a 1 mm sieve and used directly as inoculum. This process is illustrated in Appendix 3.

4.3.8.2 *In vitro* pH-controlled batch culture fermentation

The batch culture fermentation was performed in water-jacketed vessels, filled with 90 mL of pre-sterilized basal growth medium. The medium consisted of 0.9 g of peptone water, 0.9 g of yeast extract, 0.045 g of NaCl, 0.018 g of K_2HPO_4, 0.018 g of KH_2PO_4, 0.0045 g of $MgSO_4.7H_2O$, 0.0045 g of $CaCl_2.6H_2O$, 0.9 g of $NaHCO_3$, 0.225 g of L-cysteine.HCl, 0.225 g of bile salts, 0.9 mL of Tween 80, 4.5 µL of vitamin K, 0.0225 g of haemin, and 0.45 µL of resazurin at pH7.0 and were inoculated with 10% (v/v) of fecal slurry (Rueangwatcharin and Wichienchot, 2015). Six different samples including the rough purple rice, soaked purple rice, germinated purple rice, resistant starch type II and gallic acid were aseptically fed into the to five vessels with a final concentration of 1% (w/v) as shown in Figure 26. The last fermenting vessel represents the control (with no carbon source). The vessels were maintained at 37°C with a circulating water bath. The contents in the vessels were continuously stirred magnetically with the pH controlled at 6.8. Nitrogen gas (oxygen-free) was passed constantly at 15 mL min^{-1} via the vessels in order to maintain an anaerobic condition. Aliquot samples (10 mL) were taken from each of the vessels at 0, 6, 12, 24 and 48

h of fermentation and processed for short chain fatty acids (SCFAs) and fecal bacteria analysis by HPLC/GC-MS and FISH technique, respectively.

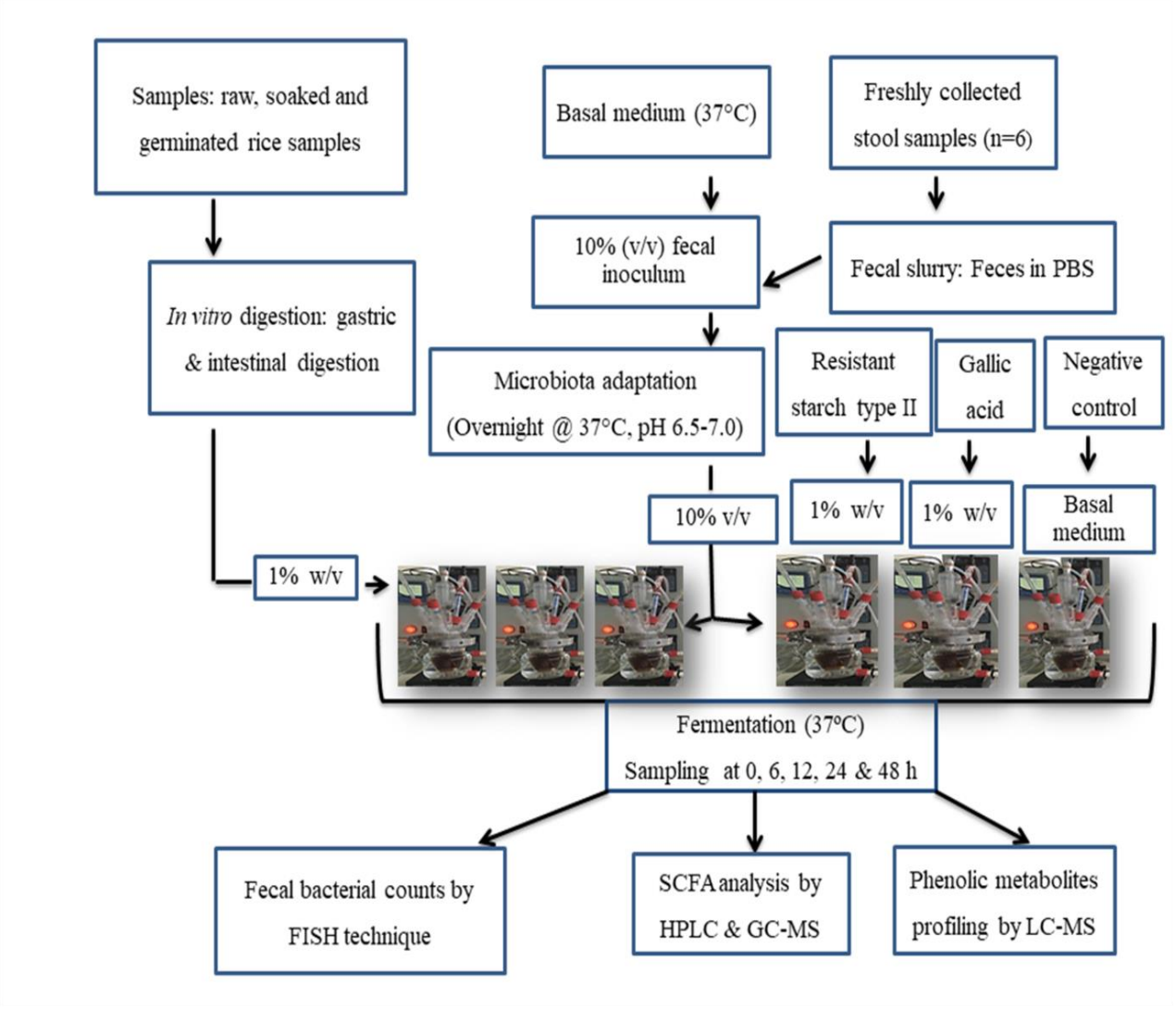


Figure 26: Summary of the gut fermentation experiment

4.3.8.3 Enumeration of fecal bacteria by fluorescent *in situ* hybridization (FISH)

The differences in the fecal bacterial compositions were evaluated using FISH technique with oligonucleotide probes, aimed at targeting specific diagnostic regions of 16S rRNA according to the method of Wichienchot *et al.* (2017). Five different commercial probes (Bif 164, Lab 158, Bac 303, Chis 150 and Eub 338) identified with the fluorescent dye Cy3 (Sigma-Aldrich, UK) were selected and used for the individual bacterial category as presented in Appendix 4. The samples (375 µL) taken from each vessel at different fermentation period were kept for 4 h at 4°C in 1125 µL 4% (w/v) paraformaldehyde. This was followed by centrifuging the fixed cells at 17000 x *g* for 5 min and washed twice in 1 mL filtered sterile PBS. The corresponding cells were re-suspended in 150 µL filtered PBS and stored in 150 µL ethanol (95%) at -20°C for further use. The samples were ideally diluted in appropriate volumes of PBS, and 20 µL of each diluted sample was added to each well of a six-well PTFE/poly-L-lysine coated slide (Tekdon Inc., Myakka City, USA). The samples were dried in a drying chamber at 46 – 50°C for 15 min. For the cells targeted with Lab 158 probe, the method was slightly modified in order to enhance the permeability cell. Briefly, 20 µL of diluted samples were treated with 20 µL of lysozyme at room temperature for about 15 min and washed immediately in cool water for 2 min. Afterwards, the slides were dehydrated using different alcohol concentrations (50%, 80% and 96% v/v ethanol) for 3 min in each solution and were kept in the drying chamber for 2 min for them evaporate excess ethanol before the addition of hybridization buffer to each well. The hybridization was performed for 4 h in a hybridization oven (Grant-Boekel, Cambridge, UK). After the hybridization, slides were washed again for15 min in a pre-warmed wash buffer (50 mL), and afterwards steeped in cold water for 3 s. These slides were then dried using a compressed air, followed by adding 5 µL of anti-fade mix to each well and mounting cover slips on the slides. Slides were

assessed by epifluorescence microscopy (Eclipse 400, Nikon, Surrey, UK) with the Fluor 100 lens. For each slide well, 15 fields with a maximum of 20 - 200 cells were enumerated (Wichienchot *et al.*, 2016; 2017).

The quantitative measurements of the microbial balance effect of non-digestible purple rice samples were derived with the following prebiotic index (PI) score modified from the method of Wichienchot *et al.* (2006) as shown in equation 6.

PI = (Bif/Total) + (Lac/Total) - (Bac/Total) - (Clos/Total) (6)

Where,

Bif, Lac, Bac and Clos = Bifidobacteria, Lactobacilli, Bacteroides, and Clostridia, respectively.

Total = Eubacteria at different time of fermentation compared to 0 h.

4.3.8.4 Analyses of the SCFAs by high-performance liquid chromatography and gas chromatography – mass spectrometry (HPLC & GC-MS)

Aliquot samples (1.5 mL) from various fermentation periods (0, 6, 12, 24 and 48 h) were centrifuged for 10 min at 13000 rpm, 4°C and filtered using a 0.22 μm membrane filter (Millipore, USA) into 2 mL vial bottles. The samples were stored at -20°C for the subsequent SCFAs analyses. These samples were evaluated for the SCFAs (acetic, propionic, butyric and isobutyric acids), using a HPLC equipment (Agilent model 1200 series, CA, USA) with a diode array detector on an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) from Bio-Rad (Richmond CA, USA). The elution was performed at a flow rate of 0.6 mL min^{-1} and the mobile phase consisted of 0.005 M H_2SO_4. The SCFAs in the samples were evaluated at a wavelength of 210 nm using diode array (DAD) detector. Each SCFA was identified by its corresponding retention time and also by spiking with standards in the same conditions.

The amount of each SCFA was evaluated according to the peak area with the standard curve for quantitative analysis (Wichienchot *et al.*, 2010).

For the analysis of SCFAs in the gallic acid, GC-MS was employed in order to give an accurate result due to the limitation of using HPLC. The conditions used for the GC-MS are briefly described. Gallic acid samples from the batch culture were filtered using 0.22 µm membrane filter and injected by split (1 µL) into an Agilent HP 6890 GC. Helium was used as the carrier gas at a flow rate of 1.5 mL/min and the separation was carried out on a capillary column (HP 19091N-113, USA) with 30 m in length, 320 µm diameter and thickness of 0.25 µm. The oven was kept at 60 ºC for 1 min, was ramped up to 100 ºC at a rate of 4 ºC/min and maintained for 2 min. Finally, it was ramped up to 230 ºC at 15 º C/min and for 5 min. The flow rates of hydrogen, air and nitrogen (the makeup gas) in the FID were 30, 300 and 25 mL/min, respectively. The temperature of the FID used was 250 ºC, while the total run time for each analysis was 25.67 min. The equilibrium time was 1 min, post time 1 min and the post temperature was 240 ºC. Standards of SCFAs were also analyzed in order to obtain the precise retention times for each of the SCFAs using the same conditions and the calibration curves constructed. This was later used for the identification of the SCFAs in the samples.

4.3.8.5 Identification of phenolic metabolites by liquid chromatography - mass spectrometry (LC-MS)

The fermentation retentates from the raw, soaked, and germinated purple rice samples at 48 h were selected for the LC-MS profiling and identification of the phenolic metabolites according to the method of Daud *et al.* (2017). A chromatographic separation Agilent HPLC (6200 series) in combination with a mass analyzer G6545A MS-Q-TOF LC/MS (Agilent Technologies, USA) made up of electrospray source system (ESI) was employed. Diode

Array Detector-DAD (G7117A), an auto sampler (G7129B), column compartment (G7116B), and binary pump 1260 (G7120A) were fitted with the chromatographic system. The column section kept at 40°C was employed for the chromatographic separation. An auto sampler injection volume of 5 µL, binary pump at 55 min with a post time of 60 s, flow rate of 0.2 mL/min, max flow gradient of 100 mL/min with a maximum pressure of 600 bar were applied. The mobile phase consisted of water (solvent A) - 88% and 100% acetonitrile (solvent B) - 12%. Diode array detector (DAD) set at a wavelength of 280 and 330 nm was employed in order to observe the eluent.

The method of acquisition for the mass spectrometer was set for dual ESI ion source within the range of 100–1000. The gas temperature was kept at 350°C and the gas flow at 8 L/min, nebulizer gas at 30 psi, capillary voltage at 4000 V, fragment voltage at 175 V, skimmer voltage at 65 V, and OCT 1 RF at 750 V. Scan compartment was performed for both positive (+) and negative (−) mode. The data management was carried out using Agilent Mass Hunter Workstation software. Also, the reference masses concerned were TFA anion (112.99 and TFA adduct (1033.98) in order to confirm the high resolution of mass spectrometry, which attest to the fact that the parent ion and source-induced dissociation fragment could proffer a precise mass information. Online database and TOF LC-MS data processor were employed in the recognition of the components. The LC-MS instrument regarded as “formula calculator” was employed in investigating the subfraction of ESI chromatogram and obtaining the mass value (m/z). On the chromatogram, the mass and molecular formula from the chosen peak were created from the system with the ESI scan employed in analyzing the mass to charge ratio (m/z) of the evaluated peaks.

4.3.9 Statistical analysis

Data from the results were analyzed statistically using one-way ANOVA. The mean values were analyzed by Tukey test for a multi-comparison of means. Statistical analyses were performed using the SPSS version 22.0 statistical software (SPSS, Inc., Chicago. IL, USA).

4.4 Results and discussion

4.4.1 Effects of soaking and germination on total starch (TS), glucose and total dietary fiber (TDF)

The results of changes in the total starch, glucose and dietary fiber during the soaking and germination periods are presented in Table 1. Soaking and germination significantly influenced ($P < 0.05$) the above evaluated parameters. The total starch contents in the rice variety at most conditions increased from 58.1 ± 0.06% to 63.9 ± 0.09% with maximum values recorded during the soaking period and at soaking combined with 36 h germination. In contrast a slight decrease in glucose in the rice samples was observed and the aforementioned conditions exhibited lowest values for glucose (3.05 ± 0.07 mg/mL and 3.47 ± 0.14 07 mg/mL) compared to the raw sample (4.54 ± 0.11 mg/mL). In cereals, starch is the main storage compound and functions as the major carbohydrate storage in most seeds (Shaik *et al.*, 2014). The increase in the TS contents as compared to the raw rice sample is similar to the earlier observations of Kandil *et al.* (2013). They found that the increase in the TS in soybean seeds during soaking and germination could be as a result of the newly formed starch which is produced via gluconeogenesis, utilizing precursors from the oil reserves in the seeds. Also, there could be a decrease in the α-amylase activity in the seed cotyledons during different soaking and germination conditions. Begcy *et al.* (2018) in their study reported elevated starch content in germinated rice seeds (from 54 g starch/100g to 70 g starch/100g)

which they believed could have accelerated the germination speed in the rice seeds. Also, precocious cellularization may help to initiate an early accumulation of starch in the rice seeds.

The slight decrease in the glucose could be explained by the studies of Sucheta *et al.* (2007) who observed a decline in the total soluble sugars in soybean seeds. They suggested that the carbohydrate contents in the seed cotyledons are the main components used up during soaking or germination. Furthermore, the sugars may also be utilized by the seed embryo in respiration, incorporated into the cell walls and translocated to the growing seed embryo. The loss of sugars during soaking especially could be from the diffusion of sugars after solubilization, while the increase during germination may result from the hydrolysis and mobilization of polysaccharides in the seeds, yielding more available sugars.

The TDF decreased slightly during the soaking period (9.06 ± 0.02 g/100g) compared to the raw sample (10.24 ± 0.06 g/100g). Afterwards, a drastic decline was observed during the germination period with the least value recorded at soaking combined with 24 h germination (3.13 ± 0.06 g/100g). This may be attributed to a decrease in the polysaccharide and mucilage contents which are broken down for utilization by the growing seed embryo (Hooda and Jood, 2003). It may also be due to increase in the activity of hydrolyzing enzymes such as cellulase, α-galatosidase which may hydrolyze the components of the dietary fiber, thereby converting the insoluble dietary fiber to the soluble component during the soaking and germination period (Jood *et al.*, 2012). In the same vein, the soaking process might have resulted into a decrease in the soluble fiber due to leaching out into the soaking water and also conversion of some insoluble fiber into soluble forms (Jood *et al.*, 2012). According to their study on sorghum seeds, they reported a decline in the dietary fiber after 12 h soaking and germination. A significant decline in total dietary fiber in jackfruit seed

flour was also recorded in the works of Zuwariah *et al.* (2018) where the TDF decreased from 25.43 ± 0.54 g/100g in the raw fruit to 24.48 ± 0.10 g/100g in the germinated fruit and 14.33 ± 0.04 g/100g in the thermal jackfruit seed flour (44% decrease). Total dietary fibers were decreased significantly ($P < 0.05$) in germinated kidney bean (from 54.27 ± 0.60 to 32.99 ±0.51 g/100g) and mung beans (from 26.79 ±0.48 to 24.60 ±0.12 g/100g) from as observed by Megat Rusydi *et al.* (2011), but conversely increased in the germinated rice varieties. The difference in the response of TDF to soaking and germination conditions could be due to differences in the rice varieties and processing conditions

4.4.2 *In vitro* starch digestion profile and glycemic index (GI)

The *in vitro* starch digestibility curves of the rice samples are shown in Figure 27; the GI and its parameters are presented in Table 13. Generally, soaking and germination conditions were observed to significantly influence ($P < 0.05$) the starch hydrolysis of the tested samples. All the samples had their starch hydrolysis lower than the reference sample (white bread). The soaked purple rice flour especially, followed by soaking combined with 36 h germination exhibited more resistance to hydrolysis (17.46 ± 0.23% and 20.58 ± 0.12%) when compared to the control (24.59 ± 0.22%) and other germination conditions after the digestion time (180 min). During the soaking period, some amounts of soluble substances may be leached out into water, making it less digestible with higher level of resistant starch and lower GI. This may be as a result of gelatinization and interactions between the starch molecules during soaking as observed by Kaur *et al.* (2016). Germination may proffer some resistance to the starch granules due to the annealing during this process which include soaking and drying (Cornejo *et al.*, 2015). In addition, the drying, high shear, freezing, thawing and cooling processes that the samples were exposed to could also result into an increase in the crystalline starch structures, which could in turn increase their resistance to

digestion. The gelatinization during cooking before testing the samples may improve the action of degrading enzymes and hence, increased starch digestibility. The results from this study is in agreement with the findings of (Xu *et al.*, 2012) who reported a reduction in the digestibility of germinated brown rice flour. They concluded that the decrease in the starch digestion may be attributed to the presence of increased crystalline starch structure after soaking and germination as hydrolytic enzymes first hydrolyses the amorphous layers which are easier to digest (Dura *et al.*, 2014). Additionally, the thermal temperature during the drying and other processing condition could cause some starch structural changes triggered by the heat-moisture treatment which may induce the hardness of the starch granules and consequently, less susceptibility to actions of hydrolytic enzymes (Chung *et al.*, 2012).

Results from the GI and its parameters reflected that there was significant reduction in the values of C_∞ and *k in* the soaked and germinated purple rice samples compared to the raw sample. This reflected less accessibility and more resistance of starch granules leading to reduced digestibility. The values for C_∞ and *k* observed in this study are within the values reported by (De La Hera *et al.*, 2014). The HI as well as the GI was significantly decreased with soaking and germination, resulting into lower GI. The amylose components in the rice variety may form complexes with lipid contents during drying and other heat related processes. Also, recrystallization of previously gelatinized rice starch could also be attributed to the lower glycemic index responses in the soaked and germinated samples.

Low values for glycemic index values are regarded as beneficial to human health which may serve as a potential tool in the prevention of type 2 diabetes as well as other diseases glycemic control exhibits a crucial role e.g., obesity and hyperlipidemia (Cornejo *et al.*, 2015). The observed GI values for the soaked and all the germinated samples were lower than the raw sample and fall within the intermediate or medium group, while the raw sample

is classified in the high glycemic index group. The soaked sample exhibited much lower values (61.52 ± 3.11%) when compared to the raw sample (72.64 ± 0.75%). This reveals that soaking could be a cheap and less time consuming process of producing purple rice samples with reduced lower sugar contents and moderate glycemic index values. The GI values in this study is within the range of values reported for a Thai rice variety (Sangyod Phatthalung) by Inpun (2014) who observed that the glycemic index ranges between 40-71% with the germinated samples exhibited lower values. The GI values of pre-germinated and germinated brown rice (12 h, 24 h and 48 h) in the study of Cornejo *et al.* (2015) ranged between 34-56% compared to the un-germinated sample (60%). This is quite lower than the values in this study and may be attributed to difference in the rice varieties, amylose and amylopectin contents, soaking and germination techniques.

Table 13: Total starch (TS), glucose, total dietary fiber (TDF) and glycemic index (GI) of raw, soaked and germinated purple rice

Sample	Glucose (mg/mL)	Total Starch (%)	Total Dietary Fiber (g/100g)	C_∞	*K*	Calculated HI (%)	Glycemic Index (GI)
BS	4.54±0.11[a]	58.1±0.06[c]	10.24±0.06[a]	24.59±1.07	0.03	56.34±0.88[a]	72.64±0.75[a]
S_{24}	3.05±0.07[d]	63.9±0.09[a]	9.06±0.02[b]	17.46±2.05	0.01	39.73±3.51[e]	61.52±3.11[e]
$S_{24}+G_{12}$	4.09±0.15[b]	61.7±0.10[b]	5.04±0.09[d]	18.44±1.09	0.02	45.48±2.23[d]	64.68±2.76[d]
$S_{24}+G_{24}$	4.47±0.09[a]	57.2±0.09[c]	3.13±0.06[f]	24.84±1.22	0.03	52.99±1.62[b]	68.80±1.27[b]
$S_{24}+G_{36}$	3.47±0.14[c]	63.6±0.11[a]	6.64±0.09[c]	20.58±2.06	0.02	40.90±1.33[e]	62.16±1.12[e]
$S_{24}+G_{48}$	4.13±0.10[a]	60.9±0.06[b]	4.22±0.04[e]	21.07±2.02	0.03	48.22±3.12[c]	66.18±3.77[c]

BS - Before soaking; S_{24} - 24 h soaking; $S_{24}+G_{12}$ - 24 h soaking + 12 h germination; $S_{24}+G_{24}$ - 24 h soaking + 24 h germination; $S_{24}+G_{36}$ - 24 h soaking + 36 h germination; $S_{24}+G_{48}$ - 24 h soaking + 48 h germination. In each column, sample means not having the same letter in superscripts are significantly different ($P < 0.05$).

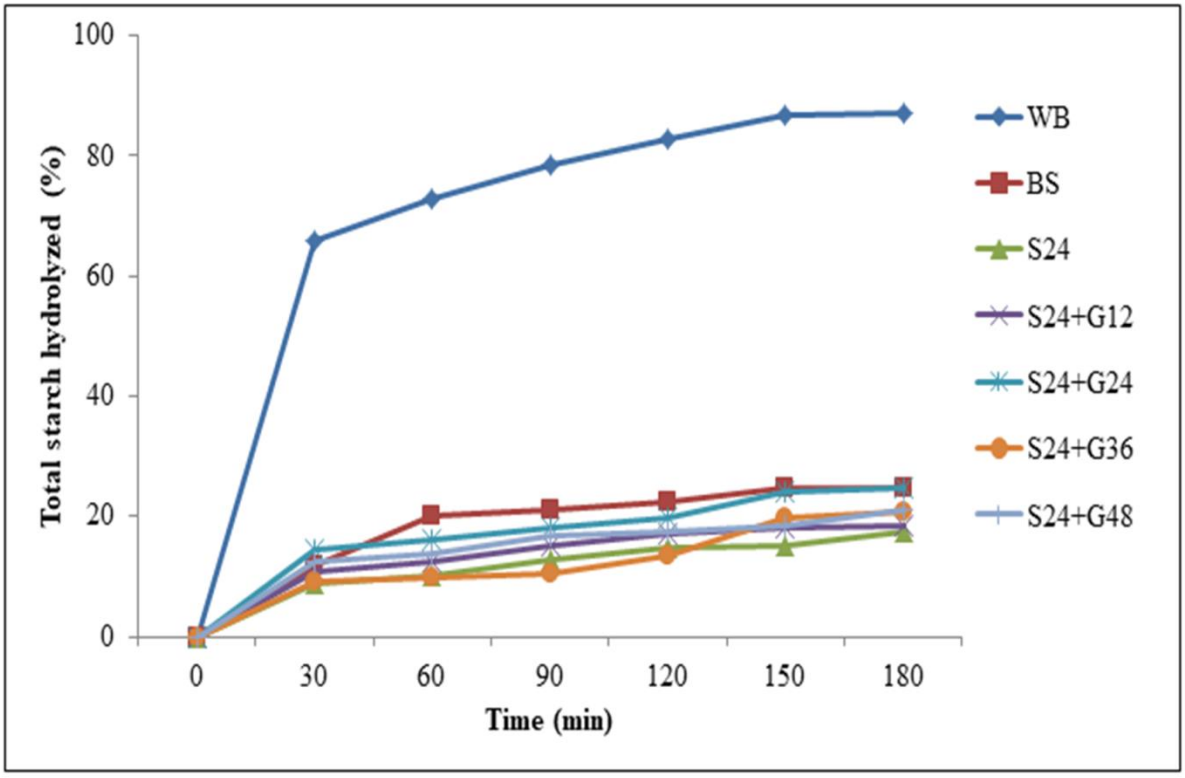


Figure 27: *In vitro* starch digestibility of raw, soaked and germinated purple rice flour

WB- White bread; BS - Before soaking; S24 - 24 h soaking; S_{24}+G_{12} - 24 h soaking + 12 h germination; S_{24}+G_{24} - 24 h soaking + 24 h germination; S_{24}+G_{36} - 24 h soaking + 36 h germination; S_{24}+G_{48} - 24 h soaking + 48 h germination

4.4.3 Gut microbiota modulation

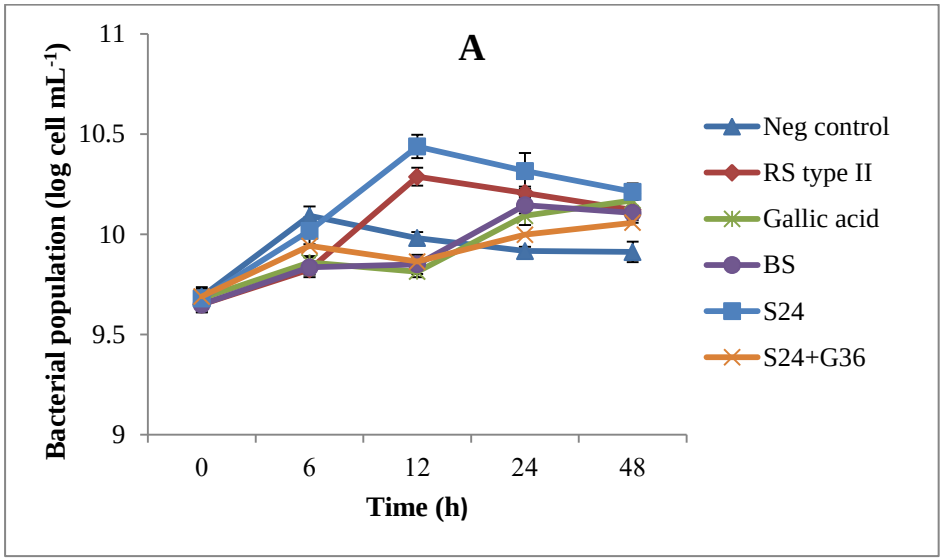
The consumption of whole grain cereals has been associated with the reduction in the prevalence of some metabolic disorders such as cardiovascular disease, diabetes and colon cancer (Kushi *et al.*, 2012). Metabolism of dietary prebiotics contained in these cereals via the gut microbiota produces SCFA and a modulation in the bacterial composition in the colon is linked to improved human colon health (Pham *et al.*, 2017).

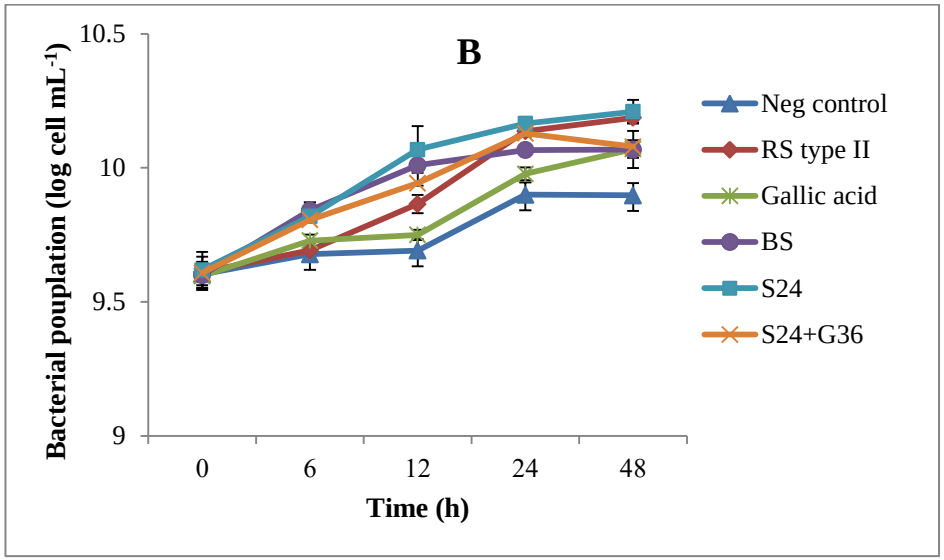
The soaked and germinated rice, after digestion under simulated upper gut conditions (mouth, stomach and small intestine); the residues were evaluated for their prebiotic properties in fecal batch culture. Changes in the bacterial populations after fecal fermentation at 0, 6, 12, 24 and 48 h are shown in Figure 28. The amount of bacterial populations as shown at 0 h were all similar in the fermented samples which makes it a baseline from which the populations change as fermentation time increases. Most of the bacterial populations significantly increased ($P < 0.05$) as fermentation time increases especially for the first 6 h. Eubacteria tends to decrease after 12 h in most of the samples. The decrease in the eubacteria (Figure 28a) could be attributed to the decrease in *Clostridium* and *Bacteroides* as observed after 12 and 24 h, respectively. *Bifidobacterium* and *Lactobacillus* groups recorded a significant increase ($P < 0.05$) except at 12 h with their peak values observed at 48 h. The human colon is made up of various bacterial groups which plays crucial role in human health and diseases (Gullón *et al.*, 2014). Fermentation of carbohydrate which is the major component in the fermented sample except for the gallic acid provides the gut microbiota the major energy needed to produce SCFAs. The fermented purple rice samples, especially the soaked sample significantly ($P < 0.05$) promoted the growth of *Bifidobacterium* (Figure 28b) which had the maximum values among the bacterial groups. The values increased from log 9.60 to log 10.07, log 9.62 to log 10.21 and log 9.61 to log 10.08 in the fermented raw, soaked

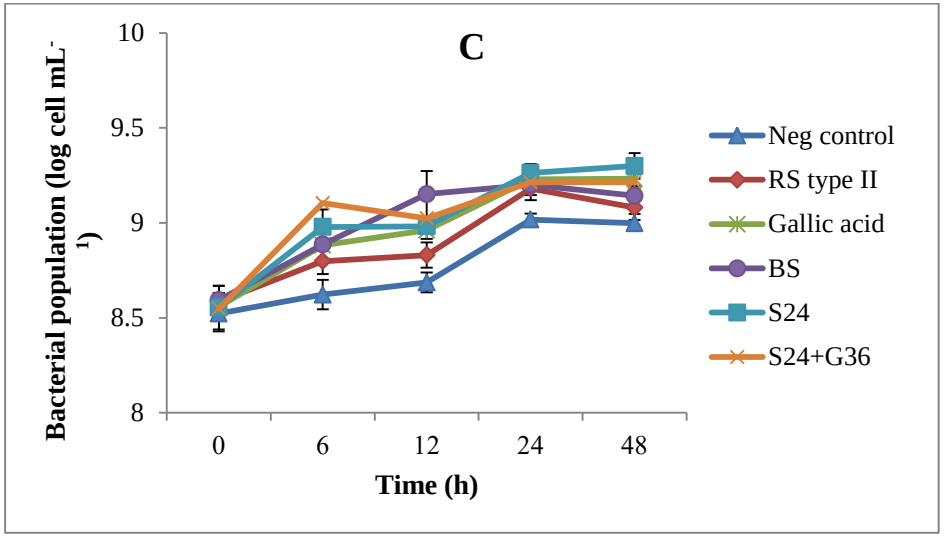
and germinated rice samples, respectively. The positive controls; RS type II and gallic acid had highest values for *Bifidobacterium* also at 48 h (log 10.18 and log 10.07, respectively). Among the rice samples, only the soaked rice sample exhibited higher values for this bacterial group than those observed in the positive controls. The *Bifidobacterium* groups are well known to be more abundant and highly beneficial to human colon health via suppression of growth of pathogens, production of vitamin B complex and stimulation of the immune system (Hidalgo *et al.,* 2012). Some recent works such as (Bergillos-Meca *et al.*, 2015; Sirisena *et al.*, 2018) have reported that certain compounds alongside carbohydrates such as anthocyanin, flavonoids, lipids as well as proteins could stimulate the growth of gut microbial population especially *Bifidobacterium* and *Lactobacillus*. The evaluated samples in this study were reported to contain bioactive compounds which include anthocyanin, phenolic acids, flavonoids, etc., in the previous study (Owolabi *et al.*, 2019). For *Lactobacillus* group (Figure 28c), most of the evaluated samples exhibited a significant increase ($P < 0.05$) across the fermentation period except at 12 h. The maximum value for this group was recorded with gallic acid (log 8.56 to log 9.23), soaked rice sample (log 8.55 to log 9.30) and germinated rice sample (log 8.55 to log 9.21) which did not differ significantly as compared with the RS type II (log 8.59 to log 9.08) and negative control (from log 8.52 to log 8.99). All these variations confirm that the gut microbiota carry out normal processes in a quite complicated and delicate manner. The increase in the bacterial populations also suggests that the pH-dependent fermentation systems were appropriately maintained. In all the substrates evaluated, the populations of *Clostridium* (Figure 28d) increased for the first 12 h and decreases afterwards except for the negative control, while the most of the substrates induced the growth of *Bacteroides* group significantly ($P < 0.05$) for the first 24 h in most of the substrates and slightly decline afterwards. Interestingly, the lowest values for the *Clostridium*

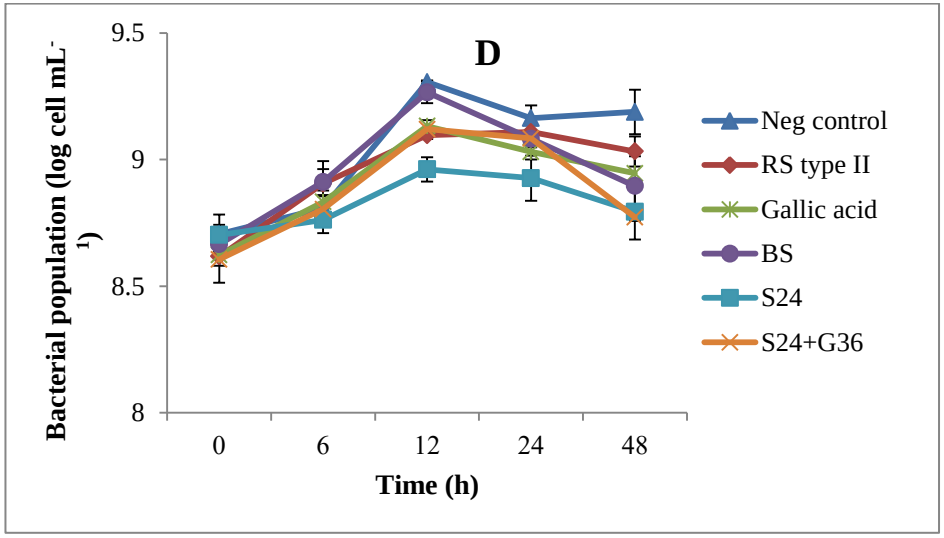
group were achieved with the soaked sample and the 36 h germinated sample (log 8.79 and log 8.77), while gallic acid and the soaked rice substrate exhibited lowest values for *Bacteroides* (log_{10} 8.58 and log_{10} 8.59). The increase in *Clostridium* and *Bacteroides* may not directly occur from the substrates but from the fermentation conditions. *Bifidobacterium* and *Lactobacillus* are studied not to only improve the lysozyme activities, but also promote the disruption of certain pathogenic bacteria (Sánchez-Moya *et al.*, 2017).

Nonetheless, the substrates evaluated in this study expressed varying values of the bacterial groups higher than the basal medium (negative control). *Bacteroides* is known to be one of the most abundant bacterial groups in the human colon. Besides its association with increases in the propionate production, it has also been studied to exhibit some protective potential against the influx of exogenous bacterial in the gut, thereby maintaining the human colon ecosystem (Pham *et al.*, 2017). Studies on oat flakes on the gut microbiota revealed that there was no significant increase in the *Bifidobacterium* but promoted the growth of *Lactobacillus* and *Bacteroides* (Pham *et al.*, 2017). Ayimbila and Keawsompong (2019), evaluated the colonic fermentation of riceberry rice hydrolysates and found that *Lactobacillus* and *Bacteroides* growth were enhanced while the pathogenic bacteria were neither enhanced nor suppressed. Similarly, soy protein hydrolysate promoted the growth of *Lactobacillus* and *Clostridium* but inhibited the growth of *Bacteroides* as reported by Ashaolu *et al.* (2019). Studies on batch culture fermentation of grape fiber promoted the growth of *Lactobacillus reuteri* and *Lactobacillus acidophilus*, while the growth of *Bifidobacterium, Lactobacillus, Bacteroides* were increased with decrease in the *Clostridium histolyticum* during frmentation of red wine (Dueñas *et al.*, 2015).


A
Bacterial population (log cell mL-1)
11
10.5
10
9.5
9
0
6
12
24
48
Time (h)
Neg control
RS type II
Gallic acid
BS
S24
S24+G36


B
Bacterial pouplation (log cell mL-1)
10.5
10
9.5
9
0
6
12
24
48
Time (h)
Neg control
RS type II
Gallic acid
BS
S24
S24+G36


C
Bacterial population (log cell mL-1)
10
9.5
9
8.5
8
0
6
12
24
48
Time (h)
Neg control
RS type II
Gallic acid
BS
S24
S24+G36


D
Bacterial population (log cell mL-1)
9.5
9
8.5
8
0
6
12
24
48
Time (h)
Neg control
RS type II
Gallic acid
BS
S24
S24+G36

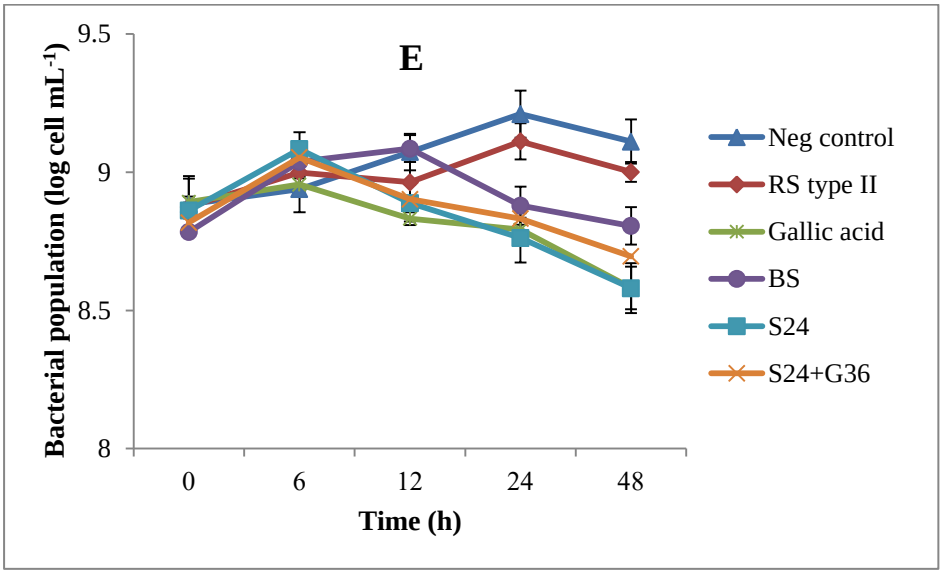


Figure 28: Bacterial populations during batch culture fermentation

RS II- Resistant starch type II; BS - Before soaking; S_{24} - 24 h soaking; S_{24}+G_{36} - 24 h soaking + 36 h germination. A - *Eubacterium*; B – *Bifidobacterium*; C – *Lactobacillus*; D – *Clostridium*; E – *Bacteroides*.

4.4.4 Prebiotic index (PI)

Prebiotic index is defined as the positive or negative relationship between the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* and pathogenic bacteria including *Clostridium* and *Bacteroides*, in relation to the total bacterial group (eubacteria) (Vardakou *et al.*, 2008). Evaluation of the PI by *in vitro* fermentation using human feces provides a cheap and fast technique for assessing the gut microbiota modulation potential of various substrates on a relative basis in laboratory scale (Rivière *et al.*, 2016). The PI scores ($p < 0.05$) for the non-digestible portions of soaked and germinated purple rice are shown in Figure 29. The highest value obtained was observed in the soaked rice sample (0.79) when compared with gallic acid (0.69), RS type II (0.58), germinated rice sample (0.52) and the raw rice sample (0.45) at 48 h. The highest PI score observed in this substrate at this fermentation time corresponds with the report on the bacterial population in this study.

However, all the samples evaluated within the fermentation period presented positive values for PI. These values are lower than those reported in the study on tuna products added inulin where the PI score ranged between 0.77 – 1.82 (Rueangwatcharin and Wichienchot, 2015). Nevertheless, the variability in the PI scores among the sample could result from varying compositions of non-digestible residue, starch structures, bioactive compound and resistant starch. The PI scores of soy protein and its hydrolysates reported by Ashaolu *et al.* (2019) ranged with 0.22 - 0.23 which were lower than the PI score from this study.

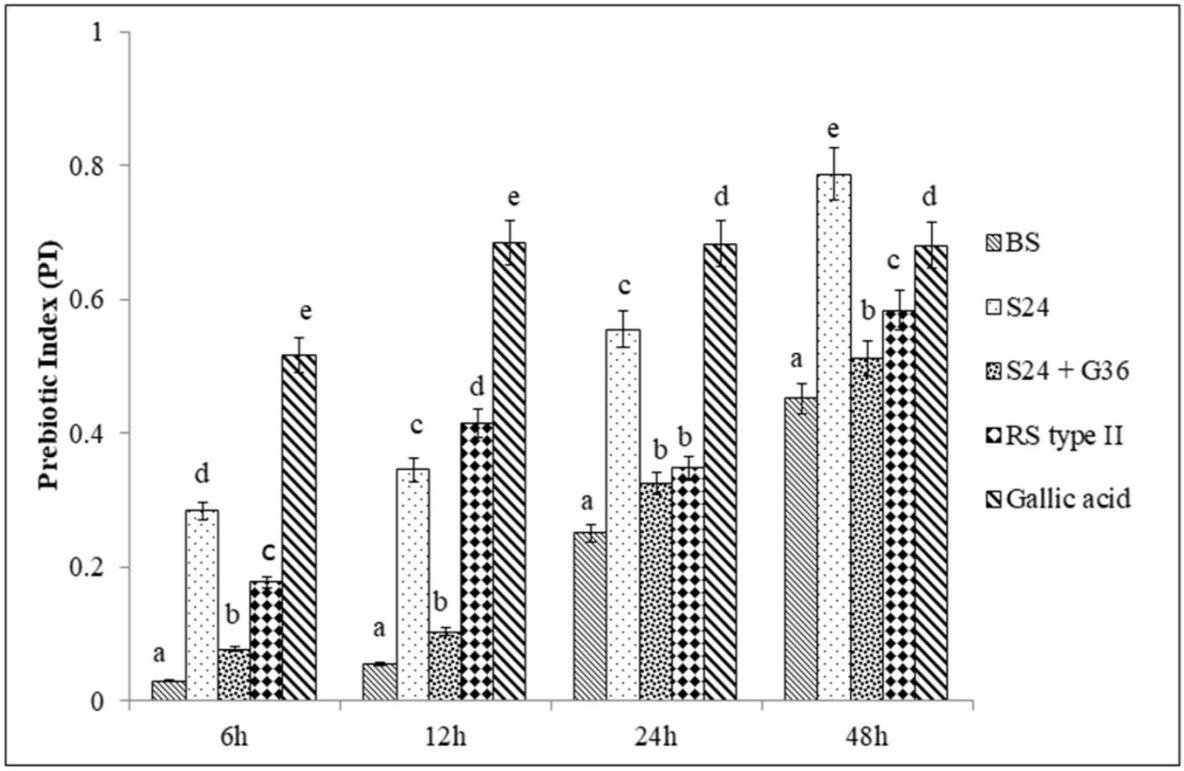


Figure 29: Prebiotic index of fermented samples during batch culture fermentation

RS II- Resistant starch type II; BS - Before soaking; S_{24} - 24 h soaking; S_{24}+G_{36} - 24 h soaking + 36 h germination. Sample means not having the same letter are significantly different ($P < 0.05$).

4.4.5 Production of short chain fatty acids (SCFAs)

The SCFAs concentrations such as acetic, propionic, butyric and isobutyric acids which were produced during the 48 h fermentation period are presented in Table 14. Fermentation of the soaked purple rice significantly ($P < 0.05$) promoted the production of SCFAs in higher amounts, with acetate exhibiting highest values (35.27 ± 0.09 mM), compared to the basal medium (13.15 ± 0. 06 mM), RS type II (12.65 ± 0.11 mM), gallic acid (19.85 ± 0.12 mM), raw rice sample (26.35 ± 0.06 mM) and germinated sample (29.90 ± 0.06 mM).

Acetic, propionic and butyric acids are known to be the most predominant SCFAs in the gastrointestinal system (≥95%), while formic, valeric and caproic acids, etc., consist of the remaining percentage (Den Besten *et al.*, 2013). From these SCFAs, acetate and propionate have been reported to be majorly produced by *Bacteroidetes*, while *Firmicutes* are the main producers of butyrate (Levy *et al.*, 2016). Several studies have discovered that acetate is often produced by some gut bacteria including *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Prevotella* spp., *Akkermansia muciniphila, Ruminococcus* spp., as well as *Streptococcus* spp. through Wood-Ljungdahl pathway and acetyl-CoA pathway (Fernández *et al.*, 2016). For propionate, it is mostly produced by *Bacteroides* spp., *Dialister* spp., *Phascolarctobacterium succinatutens, Megasphaera elsdenii, Veillonella* spp. *Coprococcus catus, Roseburia inulinivorans, Ruminococcus obeum, Salmonella* spp., through the succinate, acrylate and propanediol pathways (Koh *et al.*, 2016). *Clostridium leptum, Eubacterium hallii, Roseburia* spp. *Eubacterium rectale, Coprococcus eutactus, Anaerostipes caccae, Faecalibacterium prausnitzii, Eubacterium rectale,* and *Coprococcus catus* are known producers of butyrate through the butyryl-CoA:acetate CoA-transferase and the phosphotransbutyrylase/butyrate kinase pathways (Fernández *et al.*, 2016). The molar

proportion of acetate, propionate and butyrate in the colon is approximately 3:1:1 (Canfora *et al.*, 2015). The molar proportion and amounts of SCFAs in the colon are greatly affected by various factors such as quantity and strains of the gut bacteria, sample used, the host genotype and the transition time of the intestine (Canfora *et al.*, 2015).

Acetate is not only known as an important metabolite of *Bifidobacterium*, it is also useful for the normal muscle and tissue functioning, while the propionate and are butyrate as well play important roles in liver and colonic mucosa functioning, respectively (Furusawa *et al.*, 2013). Unlike the latter, acetate is less usually less metabolized and it is easily absorbed, decreases the pH in the colon, inhibits pathogenic bacteria and helps to increase calcium and magnesium bioavailability (Wong *et al.*, 2006). The increasing populations of *Bifidobacterium*, and *Lactobacillus* in this study, which are known as producers of acetate could have resulted in the increase in acetate production throughout the fermentation period. Propionate in most of the substrate recorded a decrease for the first 6 h, increases again at 12 h and becomes quite stable afterwards. Similarly, the amount of butyrate was observed to decline throughout the fermentation period in all the substrates and the lowest amount was observed at 48 h. In contrast, gallic acid increases significantly the amount of butyrate from 0 h to 48 h with the highest observed at 48 h (21.65 ± 0.10 mM). However, isobutyrate was found to increase throughout the fermentation time in all the substrates and had its highest concentration in the raw rice sample at 48 h (4.48 ± 0.04 mM). *Bacteroides* and *Clostridium* are known producers of propionate, and were observed to decrease during fermentation in this study, which may suggest the reason for a decline in the propionate concentrations. The type or amounts of substrates influenced the composition of the gut microbiota and thus, the type and concentrations of SCFAs produced. Data from the propionate and butyrate concentrations revealed that in the initial stage of fermentation, the colonic bacteria only

differentiated among the substrates according to the readily fermentable carbohydrates. As the fermentation time increases, the bacterial did not differentiate among the substrate, leading to higher initial productions but lower or similar concentrations as fermentation time increases (Teixeira *et al.*, 2017). The results from this study is similar to the study of Fernando *et al.* (2011) who studied the effect of rice fiber on probiotic fermentation. They reported that the SCFAs were produced as acetate, propionate and butyrate according to their abundance. They suggested that rice fiber has more tendencies to produce acetate than propionate and butyrate, respectively. Due to numerous health benefits of these SCFAs, rice fiber could be added by food manufactures in food products as an additive in order to improve the human colon health. In contrast, SCFAs and BCFAs concentrations increased throughout the fermentation period as reported by Ashaolu *et al.* (2019). Differences in the substrates fermented, other chemical compositions such as protein may as well influence the concentrations of SCFAs produced.

Table 14: SCFA concentrations (mM) during pH-controlled batch fermentation of non-digestible raw, soaked and germinated purple rice.

		Fermentation Time (h)				
Sample	**SCFA (mM)**	**0**	**6**	**12**	**24**	**48**
Negative control	Acetic acid	2.94±0.09^{a}	5.03±0.06^{b}	9.25±0.03^{c}	13.35±0.09^{d}	13.15±0.06^{d}
	Propionic acid	1.41±0.19^{b}	0.75±0.7^{a}	1.97±0.10^{c}	2.42±0.11^{d}	1.63±0.10^{b}
	Isobutyric acid	ND^{a}	0.67±0.12^{a}	ND^{a}	ND^{a}	0.88±0.09^{a}
	Butyric acid	14.40±0.05^{d}	14.30±0.02^{d}	11.44±0.05^{c}	7.90±0.07^{b}	3.96±0.05^{a}
RS type II	Acetic acid	3.00±0.10^{a}	7.46±0.9^{b}		12.23±0.10^{c}	6.90±0.11^{b}
	Propionic acid	1.32±0.09^{a}	4.70±0.05^{bc}	4.03±0.08^{bc}	6.21±0.09^{d}	3.53±0.06^{b}
	Isobutyric acid	ND^{a}	ND^{a}	ND^{a}	0.91±0.16^{b}	2.50±0.08^{c}
	Butyric acid	11.19±0.04^{d}	4.94±0.09^{c}	1.26±0.08^{ab}	1.73±0.08^{b}	0.91±0.09^{a}
Gallic acid	Acetic acid	3.09±0.16^{a}	9.15±0.11^{b}	16.65±0.12^{c}	17.14±0.04^{d}	19.85±0.12^{e}
	Propionic acid	ND^{a}	ND^{a}	ND^{a}	6.26±0.07^{b}	9.11±0.11^{c}
	Isobutyric acid	0.11±0.04^{a}	0.11±0.06^{a}	0.19±0.03^{a}	1.20±0.06^{b}	2.45±0.13^{c}
	Butyric acid	14.67±0.12^{a}	13.11±0.10^{a}	16.06±0.14^{b}	18.32±0.09^{c}	21.65±0.10^{d}
BS	Acetic acid	2.91±0.02^{a}	19.98±0.05^{b}	22.82±0.02^{bc}	23.62±0.08^{c}	26.35±0.06^{d}
	Propionic acid	20.81±0.04^{b}	17.57±0.02^{a}	18.45±0.07^{ab}	18.70±0.03^{ab}	18.90±0.07^{ab}
	Isobutyric acid	ND^{a}	0.01±0.06^{b}	3.20±0.09^{c}	3.84±0.06^{d}	4.48±0.04^{d}
	Butyric acid	3.71±0.09^{c}	2.49±0.10^{b}	0.78±0.06^{a}	0.83±0.09^{a}	0.41±0.11^{a}
S_{24}	Acetic acid	3.63±0.12^{a}	26.31±0.09^{b}	29.07±0.11^{c}	30.46±0.10^{d}	35.27±0.09^{e}
	Propionic acid	30.93±0.04^{c}	21.72±0.08^{a}	22.93±0.04^{ab}	23.44±0.05^{b}	23.83±0.04^{b}
	Isobutyric acid	ND^{a}	0.88±0.011^{b}	1.91±0.09^{c}	2.50±0.10^{cd}	2.00±0.10^{c}
	Butyric acid	4.42±0.08^{b}	ND^{a}	1.15±0.09^{a}	1.23±0.06^{a}	1.83±0.08^{a}
S_{24}+ G36	Acetic acid	3.18±0.03^{a}	23.52±0.03^{b}	23.86±0.06^{b}	26.58±0.04^{c}	29.90±0.06^{d}
	Propionic acid	29.20±0.07^{c}	17.74±0.03^{a}	18.42±0.07^{a}	21.97±0.09^{b}	20.65±0.06^{b}
	Isobutyric acid	ND^{a}	0.54±0.11^{a}	1.62±0.09^{b}	2.06±0.14^{bc}	2.75±0.11^{c}
	Butyric acid	4.40±0.06^{d}	1.04±0.03^{b}	0.54±0.05^{b}	ND^{a}	2.70±0.03^{c}

RS II- Resistant starch type II; BS - Before soaking; S24 - 24 h soaking; S24+G36 - 24 h soaking + 36 h germination. Sample means not having the same letter in superscripts are significantly different ($P < 0.05$).

4.4.6 Identification of phenolic metabolites from fecal fermentation analyzed by Liquid Chromatography -Mass Spectrometry (LC-MS)

The gut metabolites from phenolic compounds were identified from the fecal fermentation products of raw, soaked and germinated rice samples are present in Tables 15, 16 and 17, respectively with the chromatograms shown in Figure 30. The fermented purple rice samples were selected for identification in order to focus on the possible gut metabolites that could be produced from these substrates and to detect if there could be any changes in the metabolites among them. The metabolites in these fermented samples were identified at an absorbance of 280 nm as most organic acids including phenolic compounds is usually shown at this wavelength (Zhang *et al.*, 2013). The results showed that the fermented samples contained different metabolites from amino acids and protein (L-proline, aminocaproic acid, L-phenylalanine, 2-(o- carboxybenzamido)glutaramic acid and (S)-(-)-2-hydroxyisocaproic acid), carbohydrate and glucose (2-methyl-3-hydroxyethylenepyran-4-one and (also known as maltol) and 2,3-butanediol glucoside), phenolic metabolites (pyrocatechol, 3-(4-hydroxyphenyl)propionic acid, salicylic acid and *trans*-cinnamic acid).

The metabolites profiling was carried out in the negative mode of the LC-MS as improved resolutions has been recorded for LC/MS done at negative mode when compared with the positive mode (Daud *et al.*, 2017). Also, the metabolites were identified according to the molecular ion peaks, m/z and the fragment ions (Banerjee and Mazumdar, 2012). A total 9 gut metabolites were detected in fermented raw, while the fermented soaked and germinated rice samples exhibited 10 metabolites. Some of the metabolites which were observed in the fermented raw and soaked rice samples were not found in the fermented germinated purple rice fermented germinated rice sample.

Of all the phenolic metabolites identified, 3-(4-hydroxyphenyl) propionic acid (also known as 4-hydroxyphenylpropionic acid) was the most abundant, followed by *trans*-cinnamic acid, salicylic acids and pyrocatechol, respectively. The gut bacterial production of these organic compounds from the non-digestible rice samples confirms the presence of polyphenolic compounds in the substrates. Zhu *et al.* (2018), in their study on the *in vitro* metabolism and prebiotics properties of anthocyanins from Thai black rice (*Oryza sativa* L.), revealed several cyanidin-3-O-glucoside (C3G) metabolites produced in the fermentation medium after *in vitro* colonic fermentation. These metabolites include phenylpropanoic acid, phenylalanine, phenyllactic acid and 4-hydroxyphenylpropionic acid which were produced by bifidobacteria and lactobacilli groups. They reported that after incubation, C3G resulted into a substantial increase in the phenyllactic acid, which was the most abundant metabolite obtained after 48 h. C3G, which is known as a major anthocyanin is linked by β-glucoside bonds with glucoside, gut bacteria have ability to secrete β-glucosidases in order to degrade anthocyanins, leading to more energy for the gut bacteria growth (Zhu *et al.*, 2018). In addition, the main skeleton of anthocyanidin, 2-phenylbenzopyrylium is made up to three benzene rings which can be further broken down into phenolic acids and SCFAs such as acetate, propionate and butyrate (Cheng *et al.*, 2016). These results agree with the findings from this study which earlier reported the presence of anthocyanin, source of the major pigment in the purple rice variety. Purple rice anthocyanins could be a remarkable prebiotic potential in promoting the gut microflora health. Phenyllactic acid has been recognized for its ability to inhibit the growth of fungi and other pathogenic Gram negative bacteria such as *Escherichia coli* and *Staphylococcus aureus* as confirmed by studies of Wang *et al.* (2010).

Table 15: Identification of phenolic metabolites from non-digestible fecal fermentation of raw purple rice analyzed by Liquid Chromatography - Mass Spectrometry (LC-MS)

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (DB)	Abundance ($x10^4$)
1	1.579	L-proline	115.0633	115.0632	$C_5 H_9 N O_2$	0.71	87.27	3.2
2	2.264	Aminocaproic acid	131.0946	131.0951	$C_6 H_{13} N O_2$	-3.02	86.89	0.6
3	2.606	L-phenylalanine	165.079	165.0794	$C_9 H_{11} N O_2$	-2.35	86.83	4.1
4	3.860	Pyrocatechol	110.0368	110.0371	$C_6 H_6 O_2$	-3.25	82.36	1.1
5	4.373	3-(4-hydroxyphenyl)propionic acid	166.176	166.1758	$C_9 H_{10} O_3$	-0.15	92.97	4.2
6	6.141	Salicylic acid	138.0317	138.0318	$C_7 H_6 O_3$	-0.5	84.29	1.5
7	10.076	(S)-(-)-2-hydroxyisocaproic acid	132.0786	132.0789	$C_6 H_{12} O_3$	-1.77	85.65	4.5
8	14.866	*Trans*-cinnamic acid	148.0524	148.0526	$C_9 H_8 O_2$	-1.25	85.82	2.5
9	30.035	2-Methyl-3-hydroxyethylenepyran-4-one	152.0473	152.0475	$C_8 H_8 O_3$	-2.15	87.26	2.6

Table 16 Identification of phenolic metabolites from non-digestible fecal fermentation of soaked purple rice analyzed by Liquid Chromatography - Mass Spectrometry (LC-MS)

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance ($x10^4$)
1	1.579	L-proline	115.0633	115.0635	$C_5 H_9 N O_2$	-1.07	86.77	2.6
2	2.263	Aminocaproic acid	131.0946	131.0949	$C_6 H_{13} N O_2$	-1.87	87.28	1.2
3	2.605	L-phenylalanine	165.079	165.0794	$C_9 H_{11} N O_2$	-3.88	85.58	4.1
4	3.403	2,3-Butanediol glucoside	252.1209	252.121	$C_{10} H_{20} O_7$	-0.4	99.93	1.0
5	3.860	Pyrocatechol	110.0371	110.0368	$C_6 H_6 O_2$	-3.25	80.22	1.1
6	4.373	3-(4-hydroxyphenyl)propionic acid	166.176	166.1758	$C_9 H_{10} O_3$	-0.15	92.67	4.2
7	6.198	Salicylic acid	138.0318	138.0317	$C_7 H_6 O_3$	-0.5	87.01	1.5
8	10.132	(S)-(-)-2-hydroxyisocaproic acid	132.0786	132.079	$C_6 H_{12} O_3$	-2.77	86.79	8.3
9	14.979	*Trans*-cinnamic acid	148.0524	148.0526	$C_9 H_8 O_2$	-1.18	99.08	2.5
10	30.148	2-Methyl-3-hydroxyethylenepyran-4-one	152.0473	152.0476	$C_8 H_8 O_3$	-1.5	99.51	2.5

Table 17: Identification of phenolic metabolites from non-digestible fecal fermentation of germinated purple rice analyzed by Liquid Chromatography - Mass Spectrometry (LCMS)

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance ($x10^4$)
1	1.579	L-proline	115.0633	115.0632	$C_5 H_9 N O_2$	0.71	89.55	3.2
2	2.263	Aminocaproic acid	131.0946	131.0951	$C_6 H_{13} N O_2$	-3.02	87.48	0.6
3	2.605	L-phenylalanine	165.079	165.0791	$C_9 H_{11} N O_2$	-0.99	99.57	4.1
4	3.404	2,3-Butanediol glucoside	252.1209	252.1213	$C_{10} H_{20} O_7$	-1.55	98.72	1.1
5	3.860	Pyrocatechol	110.0371	110.0368	$C_6 H_6 O_2$	-3.25	80.22	1.1
6	4.373	3-(4-hydroxyphenyl)propionic acid	166.176	166.1758	$C_9 H_{10} O_3$	-0.14	95.96	4.2
7	6.198	Salicylic acid	138.0318	138.0317	$C_7 H_6 O_3$	-0.5	87.01	1.5
8	10.076	(S)-(-)-2-hydroxyisocaproic acid	132.0786	132.0789	$C_6 H_{12} O_3$	-1.77	87.21	4.5
9	26.955	2-(o-Carboxybenzamido)glutaramic acid	294.0852	294.0863	$C_{13} H_{14} N_2 O_6$	-3.89	92.93	3.0
10	30.091	2-Methyl-3-hydroxyethylenepyran-4-one	152.0473	152.0477	$C_8 H_8 O_3$	-2.15	86.26	2.5

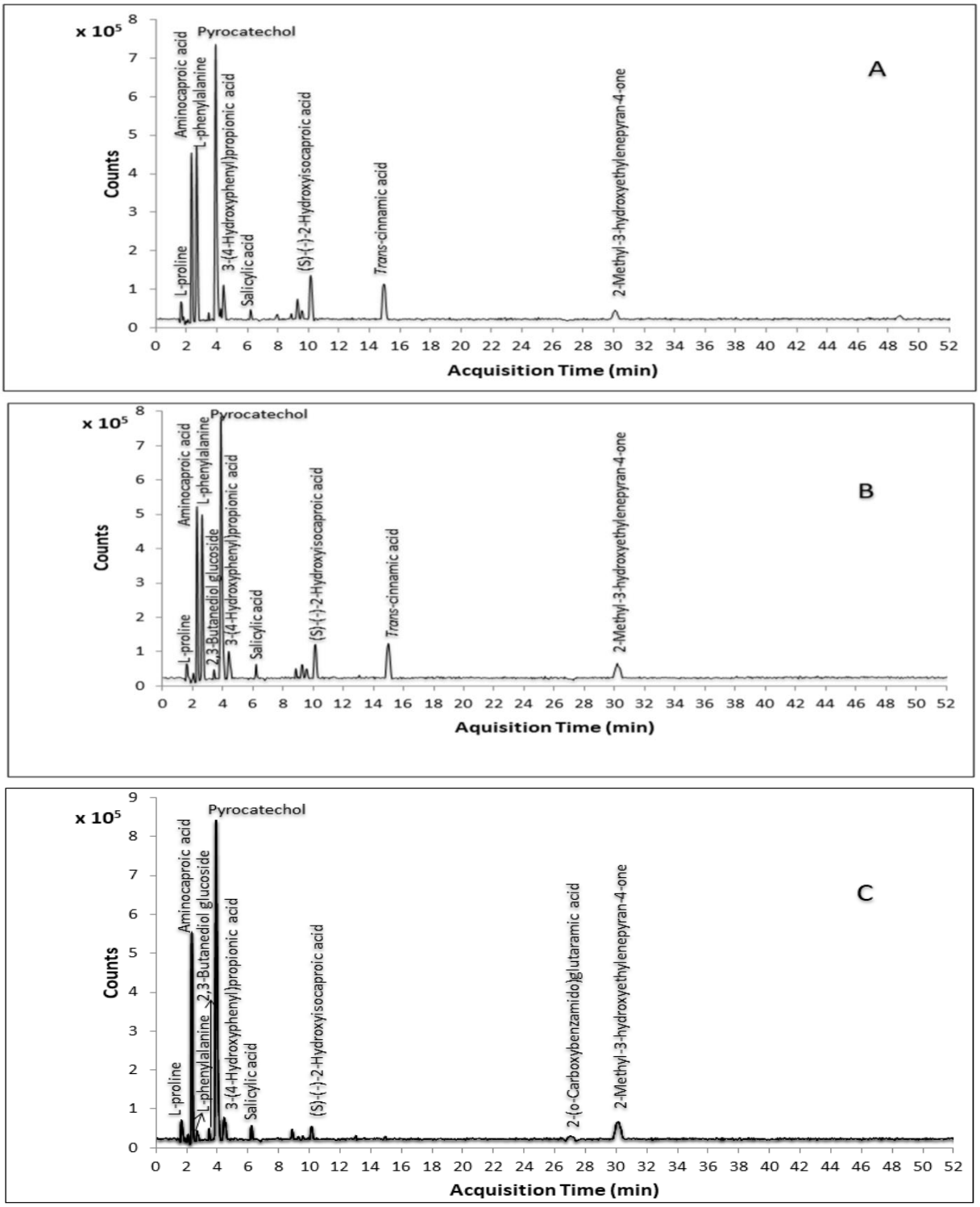


Figure 30: Chromatograms of gut metabolites identified from fermented non-digestible raw, soaked and germinated purple rice by LC-MS. A - Before soaking; B - 24 h soaking; C - 24 h soaking + 36 h germination.

Figure 31, 32 and 33 show the proposed biotransformation of 3-(4-hydroxyphenyl) propionic acid, L-phenylalanine, *trans*-cinnamic acid, salicylic acid and pyrocatechol, respectively by gut bacteria. From this study, the presence of phenyllactic acid (Figure 31) has been observed to be related to the existence of L-phenylalanine which was also detected in our study and several derivatives of phenylpropionic acid could be related to the L-phenylalanine metabolic pathway (Sánchez-Patán *et al.*, 2012). As shown in Figure 31, C3G degradation involves several reactions including hydrolysis, dihydroxylation and methylation. In the first step, C3G loses glucoside due to the presence of β-glucosidase produced by *Bifidobacterium* and *Lactobacillus* and subsequently degraded into cyanidin (Keppler and Humpf, 2005). Furthermore, the heterocycle fission of cyanidin was then executed by *Bifidobacterium* and *Lactobacillus* resulting into the production of chalcone pseudobase (Chen *et al.*, 2017). Lastly, the cyaniding and chalcone pseudobases were as well broken down into phenolic acids (including phenylpropionic acid, 3-(4-hydroxyphenyl) propionic acid, phenyllactic acid, etc.) via oxidation, dihydroxylation, α-oxidation, β-oxidation, rearrangement reactions and also, methylation. The phenolic acids produced lead to immediate decrease in the pH of the media (Kim and White, 2009). According to Zhu *et al.* (2018), dehydroxylation of 3-(3,4-dihydroxyphenyl)-propionic acid would result into the synthesis of 3-(4-hydroxyphenyl) propionic acid. Sánchez-Patán *et al.* (2012), in their study on *in vitro* fermentation of red wine, observed changes in microbial populations with some phenolic metabolites also identified including 3-(4-hydroxyphenyl) propionic acid, phenylpropionic acid and pyrocatechol, among other metabolites. They also agreed that 3-(4-hydroxyphenyl) propionic acid must have resulted from the dihydroxylation of C3G. Hydroxyphenyl propionic acid and other derivatives of phenylpropionic acids degraded from actions of colonic bacteria could pass through the liver and absorbed in the kidney where they

are excreted in the urine afterwards. Other studies involved in the production of 3-(4-hydroxyphenyl) propionic acid reported that it was produced by *Bacteroides* spp., *Eubacterium hallii,* and *Clostridium barlettii* (Rowland *et al.,* 2018). Antimicrobial, anti-inflammatory activities and the ability of 3-(4-hydroxyphenyl) propionic acid to reduce lipid cell formation has been evaluated (Zhang *et al.,* 2018). The bifidobacteria and lactobacilli groups have been reported to produce appreciable amounts of 3-(4-hydroxyphenyl) propionic acid (Beloborodova *et al.,* 2012). 3-(4-hydroxyphenyl) propionic acid have also been studied for their ability to reduce ROS (reactive oxygen species) and could function as a natural antioxidant (Beloborodov *et al.,* 2009).

Trans-cinnamic acids (*t*CA) is known to exhibit a wide range of biological activities such as antioxidant, anti-inflammatory and anti-cancer activities (Foti *et al.,* 2004). As described by (De Barros *et al.,* 2008), it is a main component of Brazilian green propolis and it exhibits an excellent gastric protective ability and anticancer properties. Additionally, *t*CA exhibits antiproliferative properties against melanoma cells (De Oliveira Niero and Machado-Santelli, 2013) and lung cancer carcinoma cells (Tsai *et al.,* 2013). Though *t*CA can be isolated from plants, its can as well be an endogenous compound in human body, mainly in the colon. It has been confirmed that *t*CA is one of the dietary phenolic metabolites found in the intestines (Jenner *et al.,* 2005). Waldecker *et al.* (2008) revealed that butyrate together with other SCFAs and derivatives of *t*CA were produced during the degradation of dietary polyphenolic constituents of fruits and vegetables by the gut microflora and inhibited the activity of global histone deacetylase (HDAC) in the nuclear extracts from HT-29 colon carcinoma cells in humans. *Trans*-cinnamic acid, a gut microbial metabolite produced by the deamination of phenylalanine (produced from non-digestible starch of the samples) as shown in Figure 32, is known to serve as a major intermediate compound in the synthesis of other

antibiotic or anti-fungal compounds such as 5-dihydroxy-4-isopropyl-stilbene, enterocin and soraphen (Kong, 2015). Colonic bacteria including *Bacteroides, Enterococcus, Bifidobacterium, and Lactobacillus* that produce different origins of phenylalanine ammonia-lyase (PAL) help to breakdown the toxic phenylalanine in the colon to non-toxic byproduct, *t*CA (Vamanu *et al.*, 2018). Watson *et al.* (2018), reported that clinical trials revealed that cinnamic acids were present in urine and plasma after dietary phenolic supplementation and consumption of black tea was known to promote the excretion of hippuric acid in human urine. Study of *in vitro* human gut microbiome response to bioactive extracts from edible wild mushrooms also recorded the production of cinnamic acid, following *in vitro* simulation in three-stage culture system (Vamanu *et al.*, 2018).

Salicylic acid (SA), also known as 2-hydroxybenzoic acid has been studied as one of phenolic compounds synthesized in plants. Beyond its functions in plants, SA and its derivative (aspirin) are known as important pharmacological compounds in humans in the treatment of acne, warts as well as aspirin used as pain reliever, prevention of inflammation and reducing the prevalence of heart attack, stroke and cancers (Cuzick *et al.*, 2014). Possible pathway of SA synthesis (Figure 32) suggests that some colonic bacteria that produce several origins of PAL convert phenylalanine to *t*CA, while *t*CA is converted to SA through the intermediate, *ortho*-coumaric acid (Dempsey and Klessig, 2017). Study on the *in vitro* colonic fermentation of grape extracts also identified different phenolic metabolites during the fecal fermentation such as SA, vanillic acid, 3-O-methylgallic acid and 4-hydroxybenzoic acid (Gil-Sánchez *et al.*, 2017). Salicylic acid could function directly as an anti-inflammatory and topical antibacterial agent due to its capacity to aid exfoliation (Dempsey and Klessig, 2017).

Pyrocatechol (also known as catechol) was also detected as a microbial metabolite produced by *Bifidobacterium, Atopobium, Ruminococcus, Roseburia, Eubacterium,* and *Faecalibacterium prausnitzii*with several of its derivatives recommended for therapeutic applications (Yam *et al.*, 2009). Several studies including Roowi *et al.* (2009) on colonic fermentation have reported the appearance of pyrogallol and pyrocatechol after colonic degradation of green tea flavan-3-ols. They concluded that these compounds were produced after the incubation of the epigallocatechin gallate constituent which resulted into the appearance of pyrogallol together with its dihydroxylation product, pyrocatechol. Epigallocatechin gallate (EGCG) is known as the ester of epigallocatechin and gallic acid and it is regarded as a type of catechin (Hanrahan *et al.*, 2011). This could be confirmed by the polyphenolic profiling of this rice sample in the previous study. From the results, catechin was found to be the most abundant with the methanolic rice extracts (1.38 – 3.12 mg g^{-1}). This is similar to the works of Huang and Ng (2012), who studied the polyphenolic profiles of ten Taiwan rice varieties extracted with 80% aqueous methanol and observed that catechin presented highest concentrations especially with the bran, seeds and husk or the red rice. In the same vein, Rao *et al.* (2018) that catechin exhibited highest concentrations among other polyphenols in most of the Australian non-pigmented, red and purple rice extracts studied.

In vitro colonic fermentation of commercial apple varieties by Koutsos *et al.* (2017) revealed that 3-(4-hydroxyphenyl) propionic acid and pyrocatechol metabolites were produced in the Pink Lady apple variety. They also argued that pyrocatechol which has been identified as a native compound and microbial metabolite may be produced by the dihydroxylation of gallic acid. Figure 33 shows the proposed colonic synthesis of pyrocatechol. According to the literature, the colonic biotransformation of pyrocatechol synthesis was proposed. The synthesis of pyrocatechol reveals further degradation of gallic

acid which was generated from the hydrolysis of EGCG in the first step of the colonic degradation (Barroso *et al.*, 2014). The results on the gut metabolites are similar to the work of Beloborodova *et al.* (2012) who reported that phenyllactic and p-hydroxyphenyllactic acid were produced in higher quantities.

Maltol is known as a natural compound utilized in beverage, food, beverage etc., due to its flavor and antioxidant capacity (Johnson *et al.*, 2004). This compound exhibits antineoplastic properties which is attributed to the development of reactive oxygen species (ROS) (Hironishi *et al.*, 1996) as well as its ability to coordinate towards metal ions. For this reason, some ligands containing maltol have been produced and utilized as a novel potential metal-based antitumor drugs and oxovanadim (IV) complexes of ligands originated from maltol were developed recently as insulin-enhancing agents (Barve *et al.*, 2009). Various bacteria found in the gut have been identified as key producers of 2,3-butanediol glucoside such as *Klebsiella pneumoniae, K. oxytoca, Serratia marcescens, Enterobacter aerogenes,* and *Paenibacillus polymyxa* (Jung *et al.*, 2012). Finding on 2,3-butanediol glucoside indicates its sedative ability on the central nervous system which is similar to alcoholic beverages and this microbial metabolite can be a special biomarker for abuse of alcohol. Some colonic bacteria such as *Clostridium, Peptostreptococcus anaerobius* are known to be producers of protein and amino acids related colonic metabolites (Vernocchi *et al.*, 2016). Seventeen metabolites were found in the urine samples and nine of them were at increased concentration. These include syringic acid, pyrogallol, *trans*-cinnamic acid and vanillic acid (Jacobs *et al.*, 2012).

In general, several high molecular weight dietary polyphenols have ability to strongly react with protein, thereby forming complexes which influences their degradation and biotransformation in the intestines and may escape degradation in the small intestine until

they reach the colon (Sun *et al.*, 2017). They argued that polyphenolic compounds that are strongly bounded to protein may contain a pool of polyphenols with fates and efficacy very diverse from those that are not bounded to protein during digestion in the intestines. For example, epigallocatechin gallate (EGCG), among all the tannins studied in the polyphenols in green tea was observed to form very strong and stable complexes with bovine serum albumin (BSA) which could not be released by action of HCl, low pH or high temperature but may be degraded and transformed by bacteria in the colon. Thus, it is apparent that the gut microbiota and its corresponding inter-individual differences in makeup, perform the major responsibility in the biotransformation and bioavailability of polyphenols and corresponding metabolites (Cuervo *et al.*, 2015). In addition, some non-extractable polyphenols with higher molecular weights are usually not absorbed in the stomach and small intestine but go on to the colon nearly unaltered with the dietary fiber and some other food components that are non-digestible. Thus, these polyphenols perform a two-way relationship with the gut microbiota; they modulate the gut bacterial compositions and in turn, the gut bacteria also help to degrade these polyphenols. This interaction results into the biotransformation and release of polyphenolic metabolites with low molecular weights which are readily absorbed and proffer significant biological activities (Williamson and Clifford, 2010).

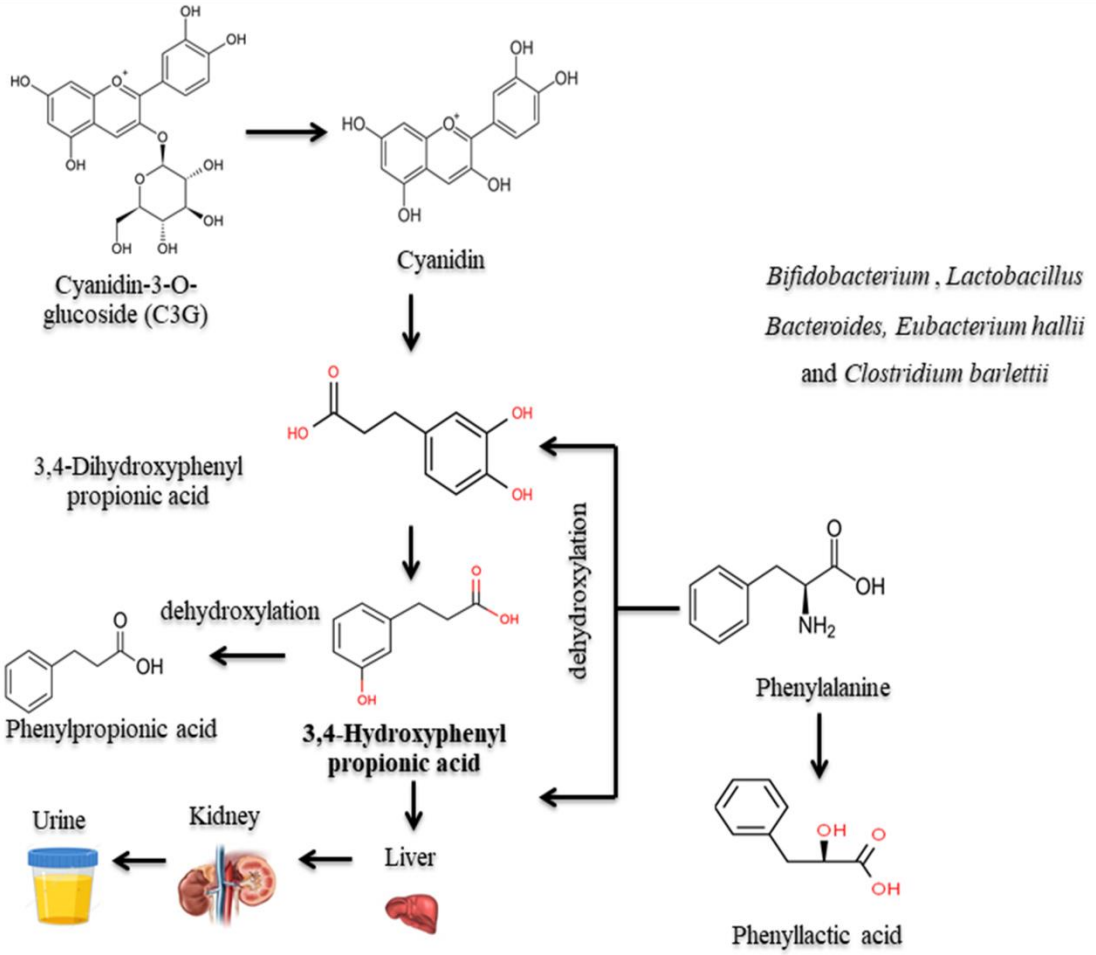


Figure 31: Proposed biotransformation of 3,4-hydroxyphenyl propionic acid from polyphenolic compound by gut bacteria.

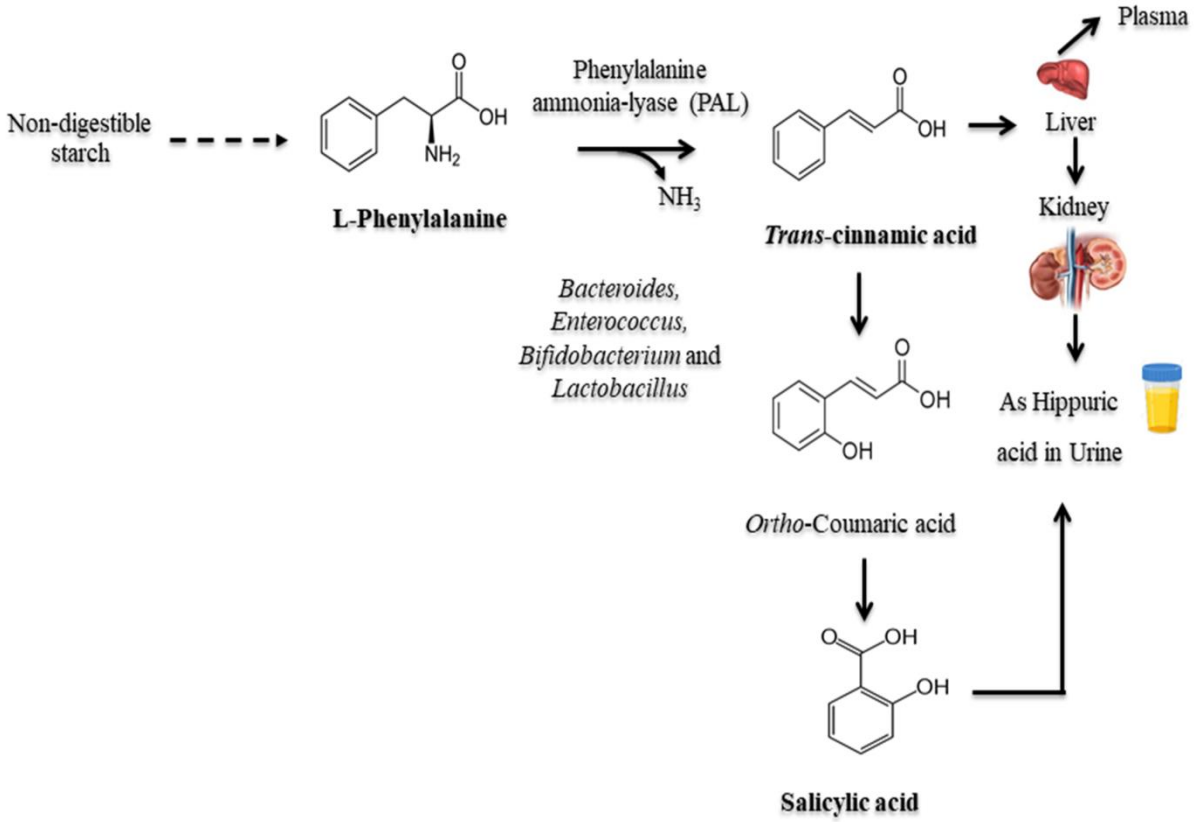


Figure 32: Proposed biotransformation of L-phenylalanine, *trans*-cinnamic and salicylic acid from non-digestible starch of samples by gut bacteria.

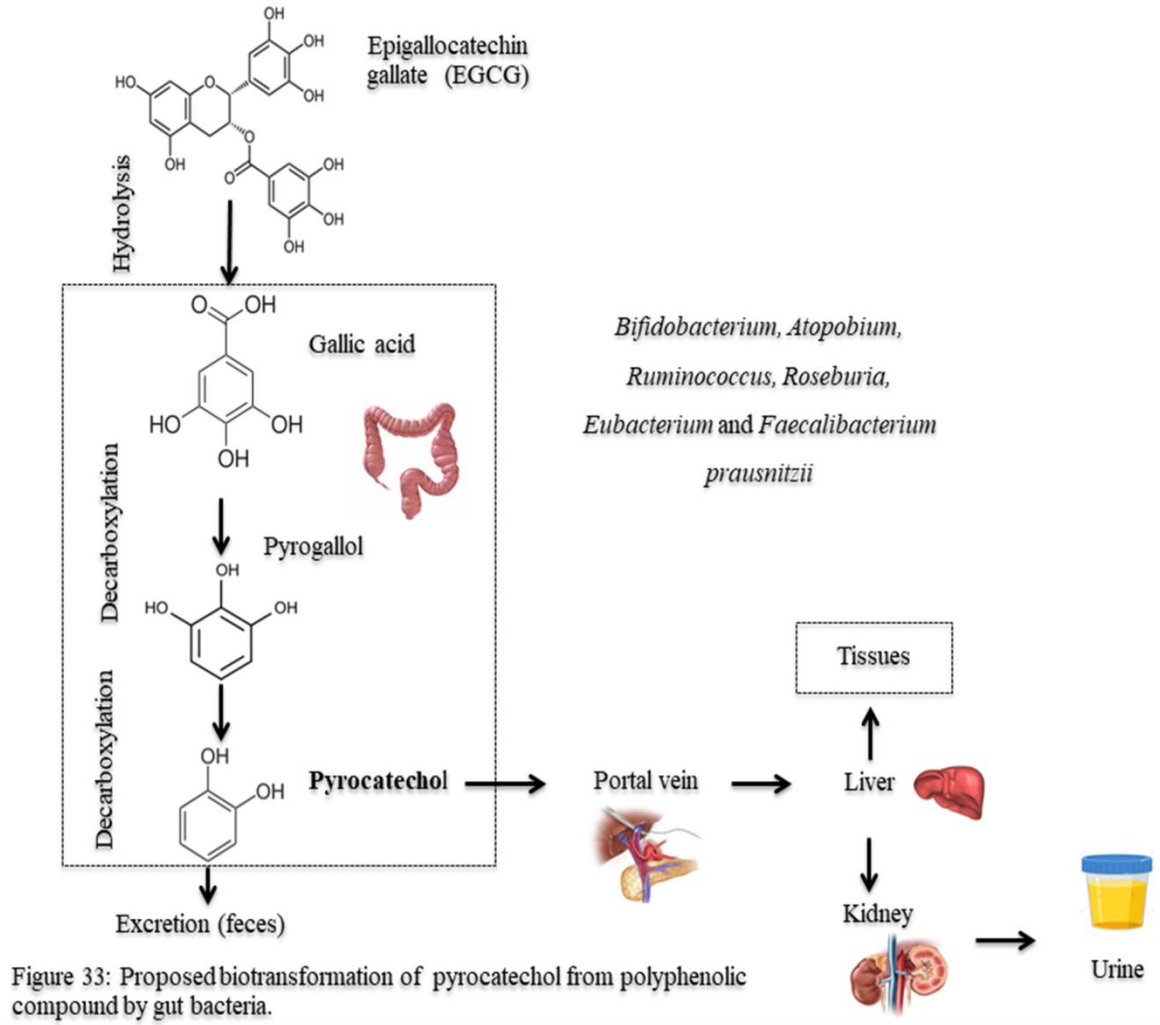


Figure 33: Proposed biotransformation of pyrocatechol from polyphenolic compound by gut bacteria.

4.5 Conclusion

Rice is a source of nutrition that could provide several health benefits to human. Pigmented rice varieties specifically are studied to contain phenolic compounds which may influence the starch digestibility, gut microbiota as well as the resulting phenolic metabolites, though the mechanism is not fully understood. The decrease in the starch digestibility and glycemic index of soaked purple rice from this study was found to be most substantial compared to the high glycemic index in the raw purple rice sample. Furthermore, soaking of pigmented rice and the possible application of products for diabetes could be further evaluated in human trials. All the purple rice substrates investigated for gut microbiota modulation exhibited positive PI scores with SCFAs produced, which suggest their significant potentials in improving the gut health. This study, to the best of our knowledge would be the first to report the effect of soaked/germinated purple rice on the gut microbiota modulation and their phenolic metabolites and discovers relationship between dietary polyphenolic compounds and their health benefits through the action of gut microbiota. It also reveals how an economic and easy innovative technique (soaking) of improving the rice quality could confer some health benefits.

In conclusion, this study has shown that the non-digestible soaked rice containing polyphenols and dietary fibers exhibited a notable ability of promoting the growth of the *Bifidobacterium* and *Lactobacillus* groups and remarkably inhibited the pathogenic bacteria (*Clostridia* and *Bacteroides*) with greater PI score. Therefore, supplementing diets with products from the soaked purple rice could be a promising intervention in modulating the gut microbiota and selectively promoting beneficial bacteria. This could be a novel approach in ameliorating intestinal disorders and interesting strategy in the prevention of diabetes, gut dysbiosis, obesity and related diseases.

4.6 References

Ashaolu, T. J., Saibandith, B., Yupanqui, C. T. and Wichienchot, S. (2019). Human colonic microbiota modulation and branched chain fatty acids production affected by soy protein hydrolysate. International Journal of Food Science and Technology. 54(1): 141-148.

Ayimbila, F. and Keawsompong, S. (2019). Effect of processing procedures on *in vitro* digestibility and colonic fermentation of riceberry rice. Journal of Microbiology, Biotechnology and Food Sciences. 8(3): 940-946.

Banerjee, S. and Mazumdar, S. (2012). Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. International Journal of Analytical Chemistry. 2012: 1-40.

Barroso, E., Van de Wiele, T., Jiménez-Girón, A., Muñoz-González, I., Martín-Alvarez, P., Moreno-Arribas, M., Bartolomé, B., Peláez, C., Martínez-Cuesta, M. and Requena, T. (2014). *Lactobacillus plantarum* IFPL935 impacts colonic metabolism in a simulator of the human gut microbiota during feeding with red wine polyphenols. Applied Microbiology and Biotechnology. 98(15): 6805-6815.

Barve, A., Kumbhar, A., Bhat, M., Joshi, B., Butcher, R., Sonawane, U. and Joshi, R. (2009). Mixed-ligand copper (II) maltolate complexes: synthesis, characterization, DNA binding and cleavage, and cytotoxicity. Inorganic Chemistry. 48(19): 9120-9132.

Begcy, K., Sandhu, J. and Walia, H. (2018). Transient heat stress during early seed development primes germination and seedling establishment in rice. Frontiers in Plant Science. 9: 1768-1781.

Beloborodov, N. V., Khodakova, A. S., Bairamov, I. T. and Olenin, A. Y. (2009). Microbial origin of phenylcarboxylic acids in the human body. Biochemistry (Moscow). 74(12): 1350-1355.

Beloborodova, N., Bairamov, I., Olenin, A., Shubina, V., Teplova, V. and Fedotcheva, N. (2012). Effect of phenolic acids of microbial origin on production of reactive oxygen species in mitochondria and neutrophils. Journal of Biomedical Science. 19(1): 89-89.

Bergillos-Meca, T., Costabile, A., Walton, G., Moreno-Montoro, M., Ruiz-Bravo, A. and Ruiz-López, M. D. (2015). *In vitro* evaluation of the fermentation properties and potential probiotic activity of *Lactobacillus plantarum* C4 in batch culture systems. LWT - Food Science and Technology. 60(1): 420-426.

Canfora, E. E., Jocken, J. W. and Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. Nature Reviews Endocrinology. 11(10): 577.

Chen, Z., Wang, P., Weng, Y., Ma, Y., Gu, Z. and Yang, R. (2017). Comparison of phenolic profiles, antioxidant capacity and relevant enzyme activity of different Chinese wheat varieties during germination. Food Bioscience. 20: 159-167.

Cheng, J. R., Liu, X. M., Chen, Z. Y., Zhang, Y. S. and Zhang, Y. H. (2016). Mulberry anthocyanin biotransformation by intestinal probiotics. Food Chemistry. 213: 721-727.

Chow, J., Lee, S. M., Shen, Y., Khosravi, A. and Mazmanian, S. K. (2010). Chapter 8 - Host–Bacterial Symbiosis in Health and Disease. In S. Fagarasan and A. Cerutti (Eds.), *Advances in Immunology* (Vol. 107, pp. 243-274). California, USA: Academic Press.

Chung, H.-J., Cho, D. W., Park, J. D., Kweon, D. K. and Lim, S. T. (2012). *In vitro* starch digestibility and pasting properties of germinated brown rice after hydrothermal treatments. Journal of Cereal Science. 56(2): 451-456.

Cornejo, F., Caceres, P. J., Martínez-Villaluenga, C., Rosell, C. M. and Frias, J. (2015). Effects of germination on the nutritive value and bioactive compounds of brown rice breads. Food Chemistry. 173: 298-304.

Cuervo, A., Reyes-Gavilán, C. G. D. L., Ruas-Madiedo, P., Lopez, P., Suarez, A., Gueimonde, M. and González, S. (2015). Red wine consumption is associated with fecal microbiota and malondialdehyde in a human population. Food and Function. 34(2): 135-141.

Cuzick, J., Thorat, M. A., Bosetti, C., Brown, P. H., Burn, J., Cook, N. R., Ford, L. G., Jacobs, E. J., Jankowski, J. A., La Vecchia, C., Law, M., Meyskens, F., Rothwell, P. M., Senn, H. J. and Umar, A. (2014). Estimates of benefits and harms of prophylactic use of aspirin in the general population. Annals of Oncology. 26(1): 47-57.

Daud, M. N. H., Fatanah, D. N., Abdullah, N. and Ahmad, R. (2017). Evaluation of antioxidant potential of *Artocarpus heterophyllus* L. J33 variety fruit waste from different extraction methods and identification of phenolic constituents by LCMS. Food Chemistry. 232: 621-632.

De Barros, M. P., Lemos, M., Maistro, E. L., Leite, M. F., Sousa, J. P. B., Bastos, J. K. and de Andrade, S. F. (2008). Evaluation of antiulcer activity of the main phenolic acids found in Brazilian green propolis. Journal of Ethnopharmacology. 120(3): 372-377.

De Guzman, M. K., Parween, S., Butardo, V. M., Alhambra, C. M., Anacleto, R., Seiler, C., Bird, A. R., Chow, C. P. and Sreenivasulu, N. (2017). Investigating glycemic potential of rice by unraveling compositional variations in mature grain and starch mobilization patterns during seed germination. Scientific Reports. 7(1): 5854-5868.

De La Hera, E., Rosell, C. M. and Gomez, M. (2014). Effect of water content and flour particle size on gluten-free bread quality and digestibility. Food Chemistry. 151: 526-531.

De Oliveira Niero, E. L. and Machado-Santelli, G. M. (2013). Cinnamic acid induces apoptotic cell death and cytoskeleton disruption in human melanoma cells. Journal of Experimental and Clinical Cancer Research. 32(31): 1-14.

Dempsey, D. M. A. and Klessig, D. F. (2017). How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans? BMC Biology. 15(1): 23-23.

Den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J. and Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of lipid research. 54(9): 2325-2340.

Dona, A. C., Pages, G., Gilbert, R. G. and Kuchel, P. W. (2010). Digestion of starch: *In vivo* and *in vitro* kinetic models used to characterise oligosaccharide or glucose release. Carbohydrate Polymers. 80(3): 599-617.

Dueñas, M., Muñoz-González, I., Cueva, C., Jiménez-Girón, A., Sánchez-Patán, F., Santos-Buelga, C., Moreno-Arribas, M. and Bartolomé, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. BioMed Research International. 2015: 1-15.

Dura, A., Błaszczak, W. and Rosell, C. M. (2014). Functionality of porous starch obtained by amylase or amyloglucosidase treatments. Carbohydrate Polymers. 101: 837-845.

Fernández, J., Redondo-Blanco, S., Gutierrez-del-Rio, I., Miguélez, E. M., Villar, C. J. and Lombo, F. J. J. O. F. F. (2016). Colon microbiota fermentation of dietary prebiotics towards short-chain fatty acids and their roles as anti-inflammatory and antitumour agents: A review. Journal of Functional Foods. 25: 511-522.

Fernando, W. M. A. D. B., Flint, S., Zou, M., Brennan, C. S., Ranaweera, K. K. D. S. and Bamunuarachchi, A. (2011). The effect of rice fibre fractions on the growth of co-cultures of probiotics. Journal of Food Science and Technology. 48(1): 14-25.

Foti, M. C., Daquino, C. and Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH• radical in alcoholic solutions. The Journal of Organic Chemistry. 69(7): 2309-2314.

Friedman, M. (2013). Rice brans, rice bran oils, and rice hulls: Composition, food and industrial uses, and bioactivities in humans, animals, and cells. Journal of Agricultural and Food Chemistry. 61(45): 10626-10641.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N. N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J. M., Topping, D. L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K. and Ohno, H. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 504: 446-450.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A. and Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews. 17(2): 259-275.

Gil-Sánchez, I., Ayuda-Durán, B., González-Manzano, S., Santos-Buelga, C., Cueva, C., Martín-Cabrejas, M. A., Sanz-Buenhombre, M., Guadarrama, A., Moreno-Arribas, M. V. and Bartolomé, B. (2017). Chemical characterization and *in vitro* colonic fermentation of grape pomace extracts. Journal of the Science of Food and Agriculture. 97(10): 3433-3444.

Goñi, I., Garcia-Alonso, A. and Saura-Calixto, F. (1997). A starch hydrolysis procedure to estimate glycemic index. Nutrition Research. 17(3): 427-437.

Gullón, B., Gullón, P., Tavaria, F., Pintado, M., Gomes, A. M., Alonso, J. L. and Parajó, J. C. (2014). Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. Journal of Functional Foods. 6: 438-449.

Hanrahan, J. R., Chebib, M. and Johnston, G. A. R. (2011). Flavonoid modulation of GABA(A) receptors. British Journal of Pharmacology. 163(2): 234-245.

Hara, A. M. and Shanahan, F. (2006). The gut flora as a forgotten organ. EMBO Reports. 7(7): 688-693.

Hidalgo, M., Oruna-Concha, M. J., Kolida, S., Walton, G. E., Kallithraka, S., Spencer, J. P. and de Pascual-Teresa, S. (2012). Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. Journal of Agricultural and Food Chemistry. 60(15): 3882-3890.

Hironishi, M., Kordek, R., Yanagihara, R. and Garruto, R. M. (1996). Maltol (3-hydroxy-2-methyl-4-pyrone) toxicity in neuroblastoma cell lines and primary murine fetal hippocampal neuronal cultures. Neurodegeneration. 5(4): 325-329.

Hooda, S. and Jood, S. (2003). Physicochemical, rheological, and organoleptic characteristics of wheat-fenugreek supplemented blends. Food/Nahrung. 47(4): 265-268.

Huang, S.-H. and Ng, L. T. (2012). Quantification of polyphenolic content and bioactive constituents of some commercial rice varieties in Taiwan. Journal of Food Composition and Analysis. 26(1): 122-127.

Inpun, R. (2014). *Studies of Glycemic index of rice and flour rice of indigenous Southern Thai Rice: Sangyod Phatthalung.* Prince of Songkla University,

Jacobs, D. M., Fuhrmann, J. C., van Dorsten, F. A., Rein, D., Peters, S., van Velzen, E. J., Hollebrands, B., Draijer, R., van Duynhoven, J., Garczarek, U. J. J. o. a. and chemistry, f. (2012). Impact of short-term intake of red wine and grape polyphenol extract on the human metabolome. Journal of Agricultural and Food Chemistry. 60(12): 3078-3085.

Jenner, A. M., Rafter, J. and Halliwell, B. (2005). Human fecal water content of phenolics: the extent of colonic exposure to aromatic compounds. Free Radical Biology and Medicine. 38(6): 763-772.

Jensen, I.-J., Abrahamsen, H., Maehre, H. K. and Elvevoll, E. O. (2009). Changes in antioxidative capacity of saithe (*Pollachius virens*) and shrimp (*Pandalus borealis*) during *in vitro* digestion. Journal of Agricultural and Food Chemistry. 57(22): 10928-10932.

Johnson, V. J., Kim, S.-H. and Sharma, R. P. (2004). Aluminum-maltolate induces apoptosis and necrosis in neuro-2a cells: potential role for p53 signaling. Toxicological Sciences. 83(2): 329-339.

Jood, S., Khetarpaul, N. and Goyal, R. (2012). Effect of germination and probiotic fermentation on pH, titratable acidity, dietary fibre, β-glucan and vitamin content of sorghum based food mixtures. Journal of Nutrition and Food Sciences. 2(164): 1-4.

Jung, M.-Y., Ng, C. Y., Song, H., Lee, J. and Oh, M. K. (2012). Deletion of lactate dehydrogenase in Enterobacter aerogenes to enhance 2, 3-butanediol production. Applied Microbiology and Biotechnology. 95(2): 461-469.

Kandil, A., Sharief, A. and Sheteiwy, M. (2013). Seedling parameters of soybean cultivars as influenced with seed storage periods, conditions and materials. International Journal of Agriculture Sciences. 5(1): 330-338.

Kaur, B., Ranawana, V. and Henry, J. (2016). The glycemic index of rice and rice products: a review, and table of GI values. Critical Reviews in Food Science and Nutrition. 56(2): 215-236.

Kelly, T., Yang, W., Chen, C. S., Reynolds, K. and He, J. (2008). Global burden of obesity in 2005 and projections to 2030. International Journal of Obesity. 32(9): 1431-1437.

Keppler, K. and Humpf, H. U. (2005). Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. Bioorganic and Medicinal Chemistry. 13(17): 5195-5205.

Kim, H. J. and White, P. J. (2009). *In vitro* fermentation of oat flours from typical and high β-glucan oat lines. Journal of Agricultural and Food Chemistry. 57(16): 7529-7536.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P. and Bäckhed, F. (2016). From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. Cell. 165(6): 1332-1345.

Kong, J.-Q. (2015). Phenylalanine ammonia-lyase, a key component used for phenylpropanoids production by metabolic engineering. RSC Advances. 5(77): 62587-62603.

Koutsos, A., Lima, M., Conterno, L., Gasperotti, M., Bianchi, M., Fava, F., Vrhovsek, U., Lovegrove, J. and Tuohy, K. (2017). Effects of commercial apple varieties on human gut microbiota composition and metabolic output using *in vitro* colonic model. Nutrients. 9(6): 533-556.

Kushi, L. H., Doyle, C., McCullough, M., Rock, C. L., Demark-Wahnefried, W., Bandera, E. V., Gapstur, S., Patel, A. V., Andrews, K. and Gansler, T. (2012). American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: reducing

the risk of cancer with healthy food choices and physical activity. CA: a Cancer Journal for Clinicians. 62(1): 30-67.

Levy, M., Thaiss, C. A. and Elinav, E. (2016). Metabolites: messengers between the microbiota and the immune system. Genes Development. 30(14): 1589-1597.

Macfarlane, S. and Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. Proceedings of the Nutrition Society. 62(1): 67-72.

Matthan, N. R., Ausman, L. M., Meng, H., Tighiouart, H. and Lichtenstein, A. H. (2016). Estimating the reliability of glycemic index values and potential sources of methodological and biological variability. The American Journal of Clinical Nutrition. 104(4): 1004-1013.

Megat Rusydi, M., Noraliza, C., Azrina, A. and Zulkhairi, A. (2011). Nutritional changes in germinated legumes and rice varieties. International Food Research Journal. 18(2): 705-713.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31(3): 426-428.

Owolabi, I. O., Chakree, K. and Takahashi Yupanqui, C. (2019). Bioactive components, antioxidative and anti-inflammatory properties (on RAW 264.7 macrophage cells) of soaked and germinated purple rice extracts. International Journal of Food Science and Technology. 54: 2374–2386.

Owolabi, I. O., Saibandith, B., Wichienchot, S. and Yupanqui, C. T. (2018). Nutritional compositions, polyphenolic profiles and antioxidant properties of pigmented rice varieties and adlay seeds enhanced by soaking and germination conditions. Functional Foods in Health and Disease. 8(12): 561-578.

Pham, T., Teoh, Savary, J. B., Chen, M. H., McClung, A. and Lee, S. O. (2017). *In vitro* fermentation patterns of rice bran components by human gut microbiota. Nutrients. 9(11): 1237-1252.

Prosky, L., Asp, N.-G., Furda, I., DeVries, J. W., Schweizer, T. F. and Harland, B. F. (1985). Determination of total dietary fiber in foods and food products: collaborative study. Journal Association of Official Analytical Chemists. 68(4): 677-679.

Rao, S., Callcott, E. T., Santhakumar, A. B., Chinkwo, K. A., Vanniasinkam, T., Luo, J. and Blanchard, C. L. (2018). Profiling polyphenol composition and antioxidant activity in Australian-grown rice using UHPLC Online-ABTS system. Journal of Cereal Science. 80: 174-179.

Rivière, A., Selak, M., Lantin, D., Leroy, F. and De Vuyst, L. (2016). Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. Frontiers in Microbiology. 7: 979.

Roowi, S., Stalmach, A., Mullen, W., Lean, M. E., Edwards, C. A. and Crozier, A. (2009). Green tea flavan-3-ols: colonic degradation and urinary excretion of catabolites by humans. Journal of Agricultural and Food Chemistry. 58(2): 1296-1304.

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I. and Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. European Journal of Nutrition. 57(1): 1-24.

Rueangwatcharin, U. and Wichienchot, S. (2015). Digestibility and fermentation of tuna products added inulin by colonic microflora. International Food Research Journal. 22(5): 2068-2077.

Sacks, F. M., Carey, V. J., Anderson, C. A., Miller, E. R., Copeland, T., Charleston, J., Harshfield, B. J., Laranjo, N., McCarron, P. and Swain, J. (2014). Effects of high vs

low glycemic index of dietary carbohydrate on cardiovascular disease risk factors and insulin sensitivity: the OmniCarb randomized clinical trial. Jama. 312(23): 2531-2541.

Sánchez-Moya, T., López-Nicolás, R., Planes, D., González-Bermúdez, C. A., Ros-Berruezo, G. and Frontela-Saseta, C. (2017). *In vitro* modulation of gut microbiota by whey protein to preserve intestinal health. Food and Function. 8(9): 3053-3063.

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., Moreno-Arribas, M. V. and Bartolomé, B. (2012). Gut microbial catabolism of grape seed flavan-3-ols by human faecal microbiota: Targetted analysis of precursor compounds, intermediate metabolites and end-products. Food Chemistry. 131(1): 337-347.

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson M, G. R., Quintanilla-López, J. s. E., Lebrón-Aguilar, R., Martin-Alvarez, P., Moreno-Arribas, M. V. and Bartolomé, B. a. (2012). *In vitro* fermentation of a red wine extract by human gut microbiota: changes in microbial groups and formation of phenolic metabolites. Journal of Agricultural and Food Chemistry. 60(9): 2136-2147.

Shaik, S. S., Carciofi, M., Martens, H. J., Hebelstrup, K. H. and Blennow, A. (2014). Starch bioengineering affects cereal grain germination and seedling establishment. Journal of Experimental Botany. 65(9): 2257-2270.

Sharif, M. K., Butt, M. S., Anjum, F. M. and Khan, S. H. (2014). Rice Bran: A Novel Functional Ingredient. Critical Reviews in Food Science and Nutrition. 54(6): 807-816.

Singh, R. K., Chang, H. W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T. and Liao, W. (2017). Influence

of diet on the gut microbiome and implications for human health. Journal of Translational Medicine. 15(1): 73-90.

Sirisena, S., Ajlouni, S. and Ng, K. (2018). Simulated gastrointestinal digestion and *in vitro* colonic fermentation of date (*Phoenix dactylifera* L.) seed polyphenols. International Journal of Food Science andTechnology. 53(2): 412-422.

Sucheta, S., Sakshi, G. and Satish, K. (2007). Changes in lipid and carbohydrate composition of germinating soyabean seeds under different storage conditions. Asian Journal of Plant Sciences. 6(3): 502-507.

Sun, X., Engstrom, M. T., Salminen, J. P. and Hagerman, A. E. (2017). Polyphenol antioxidants bind irreversibly to protein under oxidative conditions. The FASEB Journal. 646.649-646.649.

Teixeira, C., Nyman, M., Andersson, R. and Alminger, M. (2017). Application of a dynamic gastrointestinal *in vitro* model combined with a rat model to predict the digestive fate of barley dietary fibre and evaluate potential impact on hindgut fermentation. Bioactive Carbohydrates and Dietary Fibre. 9: 7-13.

Tsai, C. M., Sun, F. M., Chen, Y. L., Hsu, C. L., Yen, G. C. and Weng, C. J. (2013). Molecular mechanism depressing PMA-induced invasive behaviors in human lung adenocarcinoma cells by cis-and trans-cinnamic acid. European Journal of Pharmaceutical Sciences. 48(3): 494-501.

Tuohy, K., Rouzaud, G., Bruck, W. and Gibson, G. (2005). Modulation of the human gut microflora towards improved health using prebiotics-assessment of efficacy. Current Pharmaceutical Design. 11(1): 75-90.

Vamanu, E., Gatea, F. and Sârbu, I. (2018). *In vitro* ecological response of the human gut microbiome to bioactive extracts from edible wild mushrooms. Molecules. 23(9): 2128-2140.

Vardakou, M., Palop, C. N., Christakopoulos, P., Faulds, C. B., Gasson, M. A. and Narbad, A. (2008). Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. International Journal of Food Microbiology. 123(1): 166-170.

Venter, V. (2007). Prebiotics: an update. Journal of Consumer Sciences. 35(1): 0378-5254.

Vernocchi, P., Del Chierico, F. and Putignani, L. (2016). Gut microbiota profiling: metabolomics based approach to unravel compounds affecting human health. Frontiers in Microbiology. 7: 1144-1165.

Voreades, N., Kozil, A. and Weir, T. L. (2014). Diet and the development of the human intestinal microbiome. Frontiers in microbiology. 5: 494.

Voreades, N., Kozil, A. and Weir, T. L. (2014). Diet and the development of the human intestinal microbiome. Frontiers in Microbiology. 5: 494-503.

Waldecker, M., Kautenburger, T., Daumann, H., Busch, C. and Schrenk, D. (2008). Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. The Journal of Nutritional Biochemistry. 19(9): 587-593.

Wang, J., Lee, J., Yoo, J., Cho, J., Kim, H. and Kim, I. (2010). Effects of phenyllactic acid on growth performance, intestinal microbiota, relative organ weight, blood characteristics, and meat quality of broiler chicks. Poultry Science. 89(7): 1549-1555.

Watson, R. R., Preedy, V. R. and Zibadi, S. (2018). *Polyphenols: Prevention and Treatment of Human Disease*. London: Academic press.

WHO. (2016). *Global health estimates 2015: deaths by cause, age, sex, by country and by region, 2000–2015*: World Health Organization, 2016.

WHO. (2016). *Global report on diabetes* (Vol. 20-31): World Health Organization, 2016.

WHO. (2016). *Obesity and overweight. fact sheet* World Health Organization (WHO), Media Centre, 2016.

Wichienchot, S., Hemmaratchirakul, J., Jaturapiree, P. and Pruksasri, S. (2016). Evaluating prebiotic property of galactooligosaccharide produced by *Lactobacillus pentosus* var. *plantarum* BFP32 in fecal batch culture. International Food Research Journal. 23(5): 2241-2248.

Wichienchot, S., Jatupornpipat, M. and Rastall, R. (2010). Oligosaccharides of pitaya (dragon fruit) flesh and their prebiotic properties. Food Chemistry. 120(3): 850-857.

Wichienchot, S., Prakobpran, P., Ngampanya, B. and Jaturapiree, P. (2017). Production, purification and fecal fermentation of fructooligosaccharide by FTase from Jerusalem artichoke. International Food Research Journal. 24(1): 134-141.

Wichienchot, S., Prasertsan, P., Hongpattarakere, T., Gibson, G. and Rastall, R. (2006). *In vitro* fermentation of mixed linkage gluco-oligosaccharides produced by *Gluconobacter oxydans* NCIMB 4943 by the human colonic microflora. Current Issues in Intestinal Microbiology. 7(1): 7-12.

Williamson, G. and Clifford, M. N. (2010). Colonic metabolites of berry polyphenols: the missing link to biological activity? The British Journal of Nutrition. 104(S3): S48-S66.

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A. and Jenkins, D. J. A. (2006). Colonic health: fermentation and short chain fatty acids. Journal of Clinical Gastroenterology. 40(3): 235-243.

Woolnough, J. W., Monro, J. A., Brennan, C. S. and Bird, A. R. (2008). Simulating human carbohydrate digestion *in vitro*: a review of methods and the need for standardisation. International Journal of Food Science and Technology. 43(12): 2245-2256.

Xu, J., Zhang, H., Guo, X. and Qian, H. (2012). The impact of germination on the characteristics of brown rice flour and starch. Journal of the Science of Food and Agriculture. 92(2): 380-387.

Yam, K. C., D'Angelo, I., Kalscheuer, R., Zhu, H., Wang, J. X., Snieckus, V., Ly, L. H., Converse, P. J., Jacobs, W. R., Jr., Strynadka, N. and Eltis, L. D. (2009). Studies of a ring-cleaving dioxygenase illuminate the role of cholesterol metabolism in the pathogenesis of *Mycobacterium tuberculosis*. PLoS Pathogens. 5(3): e1000344-e1000344.

Zhang, A., Wan, L., Wu, C., Fang, Y., Han, G., Li, H., Zhang, Z. and Wang, H. (2013). Simultaneous determination of 14 phenolic compounds in grape canes by HPLC-DAD-UV using wavelength switching detection. Molecules. 18(11): 14241-14257.

Zhang, Y. Y., Li, X. L., Li, T. Y., Li, M. Y., Huang, R. M., Li, W. and Yang, R. L. (2018). 3-(4-Hydroxyphenyl)propionic acid, a major microbial metabolite of procyanidin A2, shows similar suppression of macrophage foam cell formation as its parent molecule. RSC Advances. 8(12): 6242-6250.

Zhao, K., Xue, P. j. and Gu, G. y. (2008). Study on determination of reducing sugar content using 3, 5-dinitrosalicylic acid method. Food Science. 29(8): 534-536.

Zhu, Y., Sun, H., He, S., Lou, Q., Yu, M., Tang, M. and Tu, L. (2018). Metabolism and prebiotics activity of anthocyanins from black rice *(Oryza sativa* L.) *in vitro*. PLOS ONE. 13(4): e0195754-e0195769.

Zuwariah, I., Fadilah, N., Hadijah, H. and Rodhiah, R. (2018). Comparison of amino acid and chemical composition of jackfruit seed flour treatment. Food Research. 2(6): 539-545.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

Prevention of occurrences of some prevalent diseases is more effective than curing. Therefore, consumption of functional foods high in polyphenols and antioxidants could be helpful in human health. From this study, soaking especially and some germination conditions led to an increase in the nutritional values, polyphenolic compounds and antioxidant properties in the pigmented rice varieties and adlay. Adlay though contains polyphenolic contents at lower concentrations compared with the pigmented rice varieties, the higher protein and fat contents it contains could be exceptional functional foods when combined with the rice varieties. Interestingly, the purple rice is an excellent source of polyphenols and flavonoids improved by soaking and germination conditions. Various food products derived from these cereals may not only help to prevent some various diseases such as gut related diseases and diabetes in humans, but also improve the market for these indigenous pigmented rice varieties and adlay. Development of bioactive compounds from them could as well be an exquisite source of functional food products and nutraceuticals.

Soaking could be considered as an economic, less-time consuming and easy technique for improving polyphenolic compounds, other bioactive components and anti-oxidative properties in Thai rice varieties. The extracts of khao niaw dam peuak dam can also be classified safe as it caused no toxicity on the macrophage cells. In addition, 24 h soaking condition led to the cell proliferation and greatly inhibited NO production compared to other samples. This study would however be the first to observe the outstanding cell proliferation and NO inhibition activities of khao niaw dam peuak dam extracts especially at 24 h soaking condition. In conclusion, soaked and germinated khao niaw dam peuak dam may be produced as functional foods and supplements such as functional drinks, confectionaries and as deserts.

They could also be added to functional food products for lower risks of inflammatory diseases and oxidative stress occurrences.

Rice is a source of nutrition that could provide several health benefits to human. Pigmented rice varieties specifically are studied to contain phenolic compounds which may influence the starch digestibility, gut microbiota as well as the resulting metabolites, though the mechanism is not fully understood. The decrease in the starch digestibility and glycemic index of soaked purple rice samples from this study was found to be most substantial compared to the high glycemic index in the raw sample. Furthermore, soaking of pigmented rice and the possible application of products in the diets to diabetic mechanism could be further evaluated in human studies. All the purple rice substrates investigated for gut microbiota modulation exhibited positive prebiotic index (PI) scores with SCFAs produced, which suggest their significant potentials in improving the colon health.

This study has shown that the fermented soaked rice substrate exhibited a notable ability of promoting the growth of the bifidobacteria and lactobacilli groups and remarkably inhibited the pathogenic bacteria (clostridia and bacteroides) with greater PI score. Therefore, supplementing diets with products from the soaked purple rice could be a promising intervention in modulating the gut microbiota and selectively promoting beneficial bacteria. This could be a novel approach in ameliorating intestinal disorders and interesting strategy in the prevention of diabetes, gut disorders, obesity and related diseases. In conclusion, the best of our research knowledge obtained from this study would be about the process of ‘soaking’ which is an easy, innovative and economic technique of increasing bioactive components of rice with improved anti-inflammatory properties, lower glycemic index and gut microbiota modulation potential that could confer more health benefits to humans.

5.2 Suggestions

It is therefore recommended that soaked khao niaw dam peuak dam sample should be further evaluated in *in vivo* studies for assessing the anti-inflammatory properties and its ability to lower the blood glycemic index in humans. Food products and additives produced from soaked purple rice should also be further investigated in clinical trials in order to establish its prebiotic property and gut microbiota modulation potential. This will not only confirm the appropriate effective dosage which can confer health benefits but also will guarantee the production of a standard functional food product that is capable of lowering the risk of diabetes, obesity and cancer incidences.

APPENDIX 1

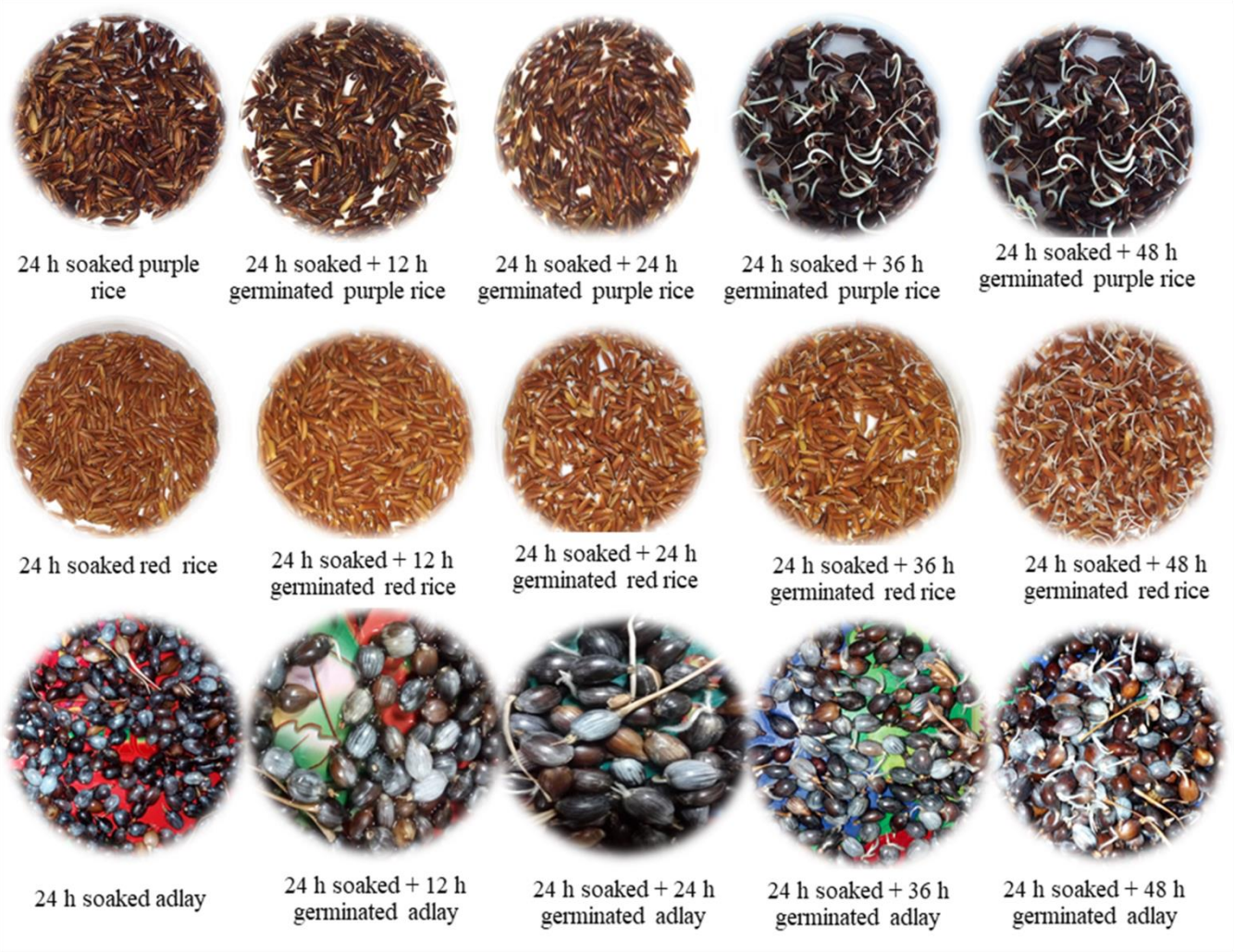


Figure A1: Pictures of soaked and germinated samples used in this study.

APPENDIX 2

Table A2: Glycemic Index calculation example of 24 h soaked (S_{24}) purple rice sample

	Glucose concentration (C) **(mg/mL)**				Glucose concentration (C) **(mg/mL)**		
		AUC linear	**AUC (SUM)**		White bread (WB)	AUC linear	**AUC (SUM)**
	Rep 1				Rep 1		
0	1.14	35.67		0	2.68	99.48	
30	1.23	40.35		30	3.96	117.69	
60	1.46	49.03		60	3.89	127.52	
90	1.81	58.21		90	4.61	143.77	
120	2.07	63.03		120	4.97	153.05	
150	2.13	69.18		150	5.23	155.11	
180	2.48		315.46	180	5.11		796.62
	Rep 2				Rep 2		
0	1.14	35.67		0	2.61	98.92	
30	1.23	40.35		30	3.99	117.92	
60	1.46	49.03		60	3.87	126.61	
90	1.81	58.66		90	4.57	142.59	
120	2.10	63.18		120	4.94	154.01	
150	2.11	68.88		150	5.33	156.44	
180	2.48		315.76	180	5.10		796.49
	Rep 3				WB3		
0	1.13	35.53		0	2.60	98.22	
30	1.24	42.02		30	3.95	116.04	
60	1.56	50.81		60	3.79	125.21	
90	1.83	57.98		90	4.56	143.01	
120	2.04	62.18		120	4.98	155.56	
150	2.11	68.52		150	5.39	159.76	
180	2.46		317.04	180	5.26		797.81
	Rep 4				WB4		
0	1.18	36.55		0	2.87	100.77	
30	1.26	43.25		30	3.85	115.58	
60	1.63	50.77		60	3.85	126.02	
90	1.76	57.13		90	4.55	142.32	
120	2.05	63.68		120	4.94	154.18	
150	2.20	66.96		150	5.34	157.49	
180	2.27		318.34	180	5.16		796.37
							796.83

$$\text{AUC linear} = \frac{C_1+C_2}{2} \text{ x } t_2 - t_1$$

AUC (Sum) = Sum (t_0+ t_{30}……..t_{180})

E.g.

$$\text{AUC linear} = \frac{1.14 + 1.23}{2} \text{ X } 30 - 0$$

= 35.67

AUC (Sum) = 35.67 + 40.35 + 49.03 + 58.21 + 63.03 + 69.18

= 315.46

AUC White Bread	AUC Sample 2
796.83	315.46
HI	39.59
eGI_G	61.44

AUC White Bread	AUC Sample 2
796.83	315.46
HI	39.59
eGI_G	61.44

AUC White Bread	AUC Sample 2
796.83	317.04
HI	39.79
eGI_G	61.55

AUC White Bread	AUC Sample 2
796.83	318.34
HI	39.95
eGI_G	61.64

$$\text{HI (\%)} = \frac{\text{AUC sample}}{\text{AUC white bread}} \text{ x } 100$$

E.g.

$$\text{HI (\%)} = \frac{315.46}{796.83} \text{ x } 100$$

HI (%) = 39.59

GI = (0.549 x HI) + 39.71

GI = 61.44

For total starch hydrolyzed;

$$\text{Total starch hydrolysis (\%)} = \frac{\text{Released glucose weight (\%) x 0.9}}{\text{Total starch weight (\%)}} \text{ x 100}$$

Total starch hydrolyzed $t_0 = 0$

Total starch hydrolyzed $t_{30} = \frac{6.15\% \text{ x } 0.9}{63.9\%}$ x 100 = 8.67%

Total starch hydrolyzed $t_{60} = \frac{7.30\%6 \text{ x } 0.9}{63.9\%}$ x 100 = 10.28%

Total starch hydrolyzed $t_{90} = \frac{9.15\% \text{ x } 0.9}{63.9\%}$ x 100 = 12.82%

Total starch hydrolyzed $t_{120} = \frac{10.35\% \text{ x } 0.9}{63.9\%}$ x 100 = 14.65%

Total starch hydrolyzed $t_{150} = \frac{10.65\% \text{ x } 0.9}{63.9\%}$ x 100 = 15.00 %

Total starch hydrolyzed $t_{180} = \frac{12.40\% \text{ x } 0.9}{63.9}$ x 100 = 17.46%

Note: to get the glucose weight (%),

E.g. 1.23 mg/mL $= \frac{1.23 \text{ mg}}{0.02 \text{ g}}$ = 61.5 mg/g = 6.15 %

Weight of sample used = 50 mg

Total volume = 25mL

Therefore, weight of sample/mL = 2 mg or 0.02g

Therefore C_∞ (equilibrium percentage of starch hydrolyzed after 180 min) = 69.86%

For k value;

Using = $kt = \ln \frac{C_\infty - C_t}{C_\infty}$

For 30 min, $-k\,(30) = \ln \frac{17.46 - 6.15}{17.46}$

$-k\,(30) = \ln 0.50 = -0.69$

For 60 min, $-k\,(60) = \ln \frac{17.46 - 10.28}{17.46}$

$-k\,(60) = \ln 0.41 = -0.89$

For 90 min, $-k\,(90) = \ln \frac{17.46 - 12.82}{17.46}$

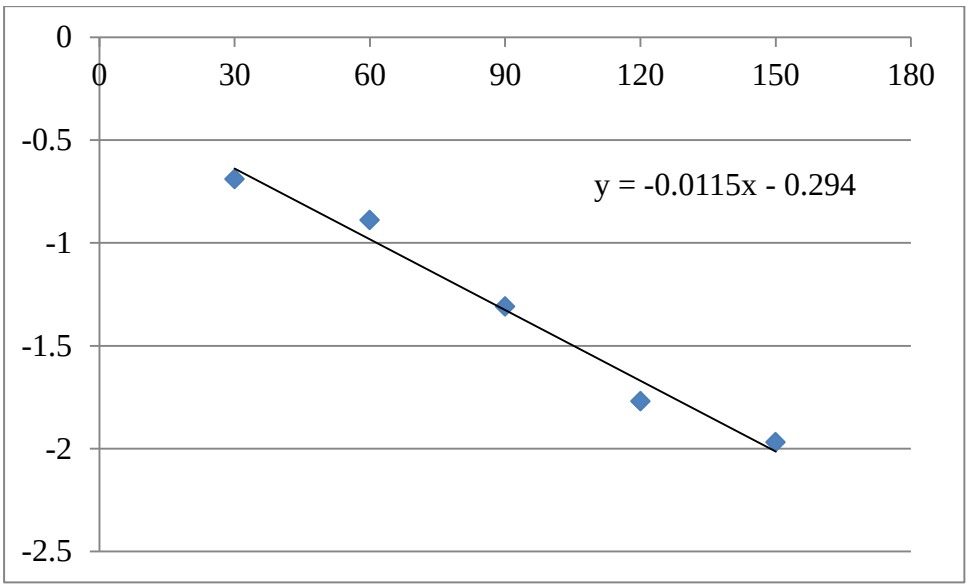
$-k\,(90) = \ln 0.27 = -1.31$

For 120 min, $-k\,(120) = \ln \frac{17.46 - 14.65}{17.46}$

$-k\,(120) = \ln 0.17 = -1.80$

For 150 min, $-k\,(150) = \ln \frac{17.46 - 15.00}{17.46}$

$-k\,(150) = \ln 0.14 = -1.96$



Therefore, k value (slope) = 0.01

APPENDIX 3

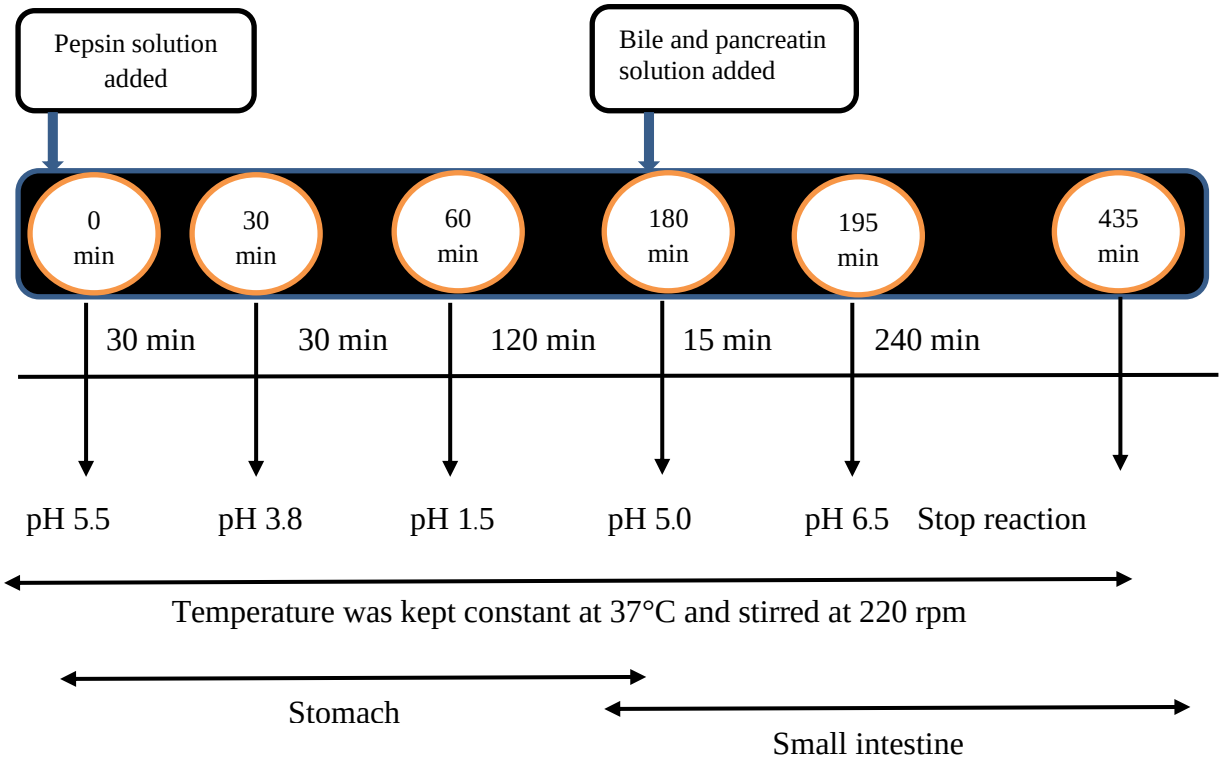


Figure A3: An illustration of a simulated *in vitro* upper-gut digestion protocol.

APPENDIX 4

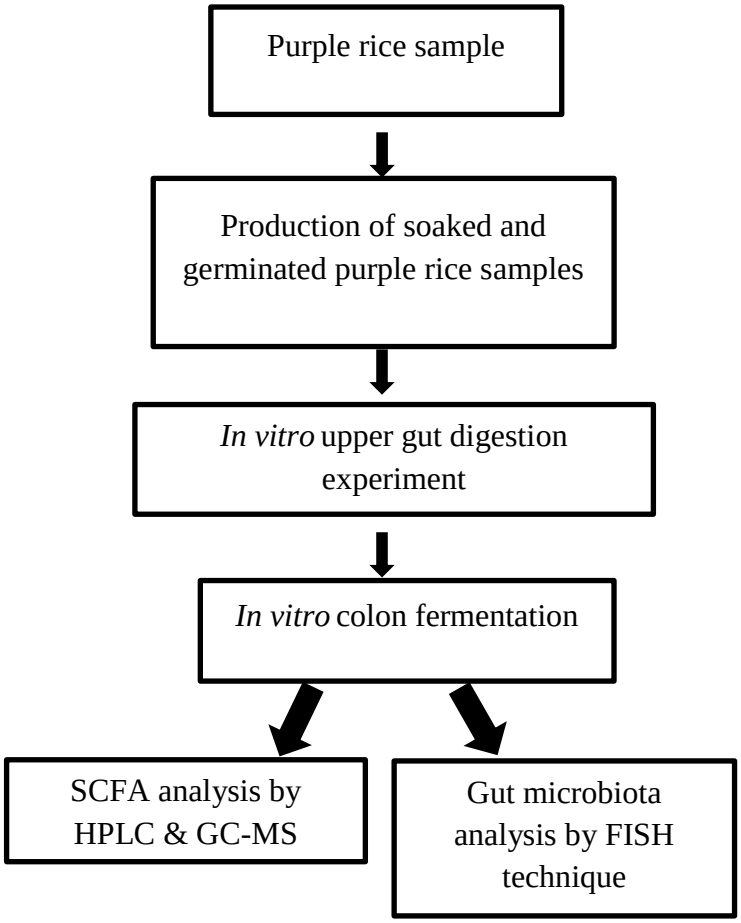


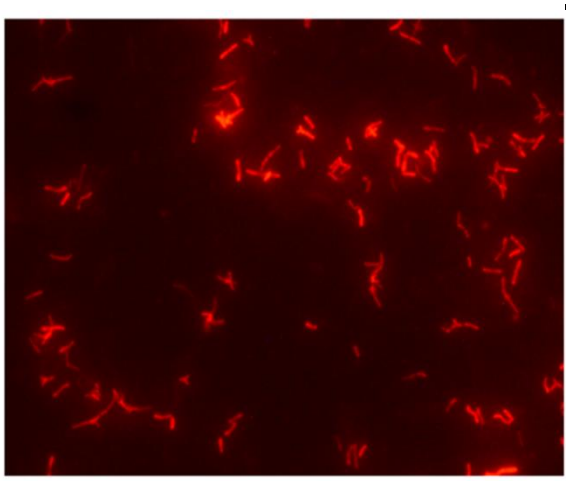
Figure A4: Summary of the *in vitro* colon fermentation experimental flow.

APPENDIX 5

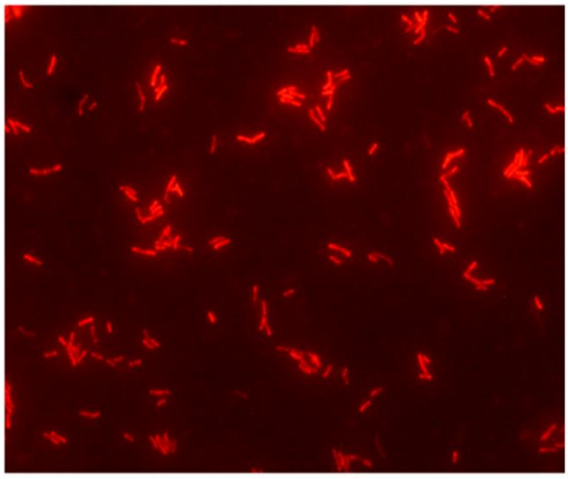
Table A5: 16S rRNA oligonucleotide probes and hybridization conditions used in FISH technique.

Probe name	Specificity	Sequence (5′ – 3′)	Temperature (°C)	
			Hybridization	**Washing**
Bac 303	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae* (*Bacteroide*s group)	CCAATGTGGGGGACCTT	46	46
Chis 150	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	TTATGCGGTATTAATCTYCCTTT	50	50
Lab 158	*Lactobacillus* spp. /*Enterococcus* spp.	GGTATTAGCAYCTGTTTCCA	50	50
Bif 164	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	50	50
Eub 338	Members of the domain Bacteria	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	46	46

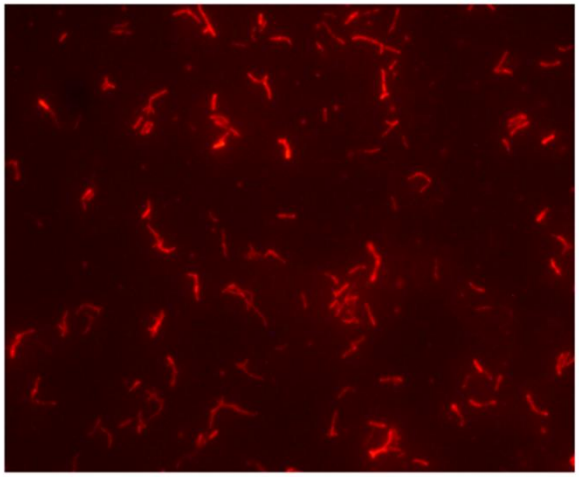
APPENDIX 6



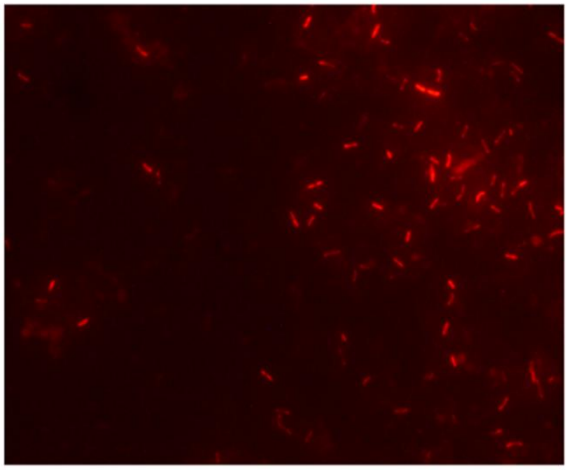
Eubacteria (100x dilution)



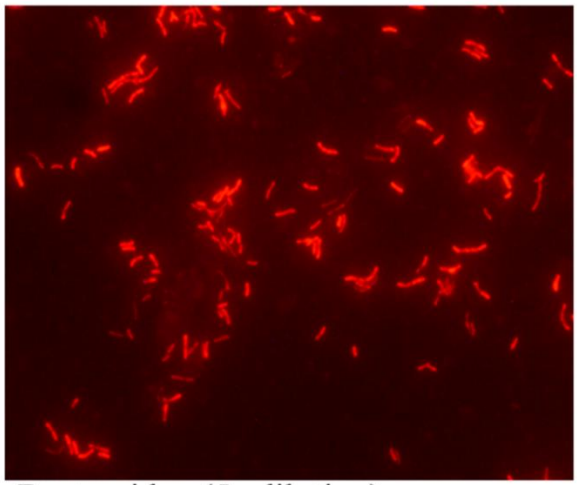
Bifidobacteria (20x dilution)



Lactobacilli (5x dilution)



Clostridia (5x dilution)



Bacteroides (5x dilution)

Figure A6: Selected fecal bacteria from batch culture fecal fermentation.

VITAE

Name Miss. Iyiola Oluwakemi Owolabi

Student ID 5911030003

Educational attainment

Degree	**Name of institution**	**Year of graduation**
Bachelor of Agriculture	University of Ilorin, Nigeria	2011
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Scholarships awards during enrolment

1. PSU Research Fund
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Work experience with position and address

2013 – 2016 Research assistant
Faculty of Agriculture, University of Ilorin, Nigeria

2013 -2014 Examiner
West African Examination Council

2011 - 2012 Agriculture officer
Ministry of Agriculture and Natural Resources, Kebbi, Nigeria

Publications

1. Owolabi, I.O., Saibandith, B., Wichienchot, S., Yupanqui, C.T. (2018). Nutritional compositions, polyphenolic profiles and antioxidant properties of pigmented rice varieties and adlay seeds enhanced by soaking and germination conditions. Functional Foods in Health and Disease. 8(12): 561-578.
2. Owolabi, I.O., Chakree, K., Yupanqui, C.T. (2019). Bioactive components, anti-oxidative and anti-inflammatory properties (on raw 264.7 macrophage cells) of

soaked and germinated Thai purple rice extracts. International Journal of Food Science and Technology. 54: 2374–2386.

3. Owolabi, I.O., Yupanqui, C.T., and Siripongvutikorn, S. (2018). Enhancing secondary metabolites (emphasis on phenolics and antioxidants) in plants through elicitation and metabolomics. Pakistan Journal of Nutrition. 17: 411-420.
4. Owolabi, I.O., Saibandith, B., Wichienchot, S., Yupanqui, C.T. (2018). Physicochemical, phenolic compounds and *in vitro* digestibility of Thai purple rice variety influenced by germination conditions. The 44th Congress on Science and Technology of Thailand (STT 44) conference proceeding. pp 747-754.
5. Owolabi, I.O., Dat-arun, P., Yupanqui, C.T., Wichienchot, S. *In vitro* starch digestibility, gut microbiota and metabolites modulation of soaked and germinated purple rice (Manuscript).