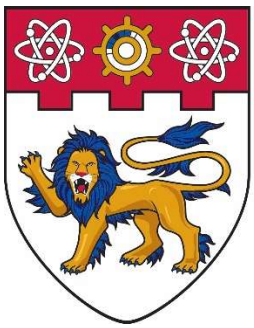

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

FERMENTATION OF SOYBEAN RESIDUE (OKARA): POTENTIAL IN FOOD APPLICATIONS

MOK WAI KIT
SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2020

FERMENTATION OF SOYBEAN RESIDUE (OKARA): POTENTIAL IN FOOD APPLICATIONS

MOK WAI KIT

School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

13/07/2020
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Mok Wai Kit

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 1 and 2 contain excerpts from the manuscript which was published as Mok, W.K., Tan, Y.X., Chen, W.N. (2020). Technology innovations for food security in Singapore: A case study of future food systems for an increasingly natural resource-scarce world Trends in Food Science & Technology, 102, 155-168. doi: https://doi.org/10.1016/j.tifs.2020.06.013

The contributions of the co-authors are as follows:

- I and Tan, Y. X. researched, compiled prior data and co-wrote the manuscript (co-first authors)
- Chen, W. N. conceived the framework and revised the manuscript

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The contributions of the co-authors are as follows:

- I designed and performed the experiments, analyzed the data as well as wrote the manuscript
- Tan, Y.X provided technical advice as well as assisted in data analysis
- Kim, J provided technical advice on experimental set-up and data analysis as well as revised the manuscript
- Lee, J revised the manuscript
- Chen, W.N conceived the motivation behind the project and revised the manuscript

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The contributions of the co-authors are as follows:

- I designed and performed the experiments, analyzed the data as well as wrote the manuscript
- Yong Xing Tan provided technical advice and assisted in data analysis
- Xiao Mei Lyu provided technical advice on experimental set-up and data analysis as well as revised the manuscript
- Wei Ning Chen conceived the motivation behind the project and revised the manuscript

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List of Abbreviations

ATP	Adenosine Triphosphate
A*STAR	Agency for Science, Technology and Research
***B. subtilis* WX-17**	*Bacillus subtilis* WX-17
BSG	Brewer's Spent Grain
BTI	Bioprocessing Technology Institute
COVID-19	Coronavirus Disease 2019
CRC	Colorectal Cancer Cells
CVD	Cardiovascular Diseases
DHA	Docosahexanoic Acid
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
DPPH	1-1,-diphenyl-2-picryl-hydrazil
E. coli	*Escherichia coli*
FAME	Fatty Acid Methyl Esters
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal Bovine Serum
FO	Fermented Okara Probiotic Beverage
FOS	Fructooligosaccarides
GABA	Gamma-Aminobutyric Acid
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography Mass Spectrometry
GCN2	General Control Non-Derepressible Protein 2

GOS	Galactooligosaccharides
GRAS	Generally Recognized As Safe
G6P	Glucose 6-Phosphate
HDL	High-Density Lipoprotein
HPLC	High Performance Liquid Chromatography
IDF	Insoluble Dietary Fibre
IFAD	International Fund for Agricultural Development
IOT	Internet of Things
IS	Internal Standard
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography Mass Spectrometry
LDL	Low-Density Lipoprotein
LED	Light-Emitting Diode
MH	Mueller-Hinton
MIT	Massachusetts Institute of Technology
MK-7	Menaquinone-7
MOX	Methoxamine Hydrochloride
MSTFA	N-methyl-N-(trimethylsilyl) Trifluoroacetamide
PAR	Photosynthetically Active Radiation
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate

PLS-DA	Partial Least Squares Discriminant Analysis
RAS	Recirculating Aquaculture System
RO	Unfermented Control
S. aureus	*Staphylococcus aureus*
SCFA	Short-Chain Fatty Acids
SDF	Soluble Dietary Fibre
SLF	Submerged Liquid Fermentation
SMART	Singapore-Massachusetts Institute of Technology (MIT) alliance for Research and Technology
SSF	Solid-State Fermentation
TCA	Tricarboxylic Acid
TMCS	Trimethylchlorosilane
tRNA	Transfer Ribonucleic Acid
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UN	United Nations
WFP	World Food Program

Thesis Summary

With global population projected to reach 9.20 billion in 2050, food security is becoming an increasingly important issue. However, rapid industrialization and urbanization taxes heavily on the finite natural resources that we have. As such, traditional methods of enhancing food security will no longer be feasible due to resource constraints. Therefore, technologically advanced techniques have to be employed to enhance food security and, in these aspects, Singapore, with its little land space and natural resources is the perfect testbed and case study for our increasingly resource-scarce world. Singapore's strategies for enhancing food security can be redefined to include 3 main areas: urban farming, processing technology and alternative food sources.

This thesis would focus on the area of food waste processing technology. It aims to develop a strategy to enhance the nutrient profile of soybean residue (okara), thereby utilizing it as a food ingredient to enhance food security. The methodologies developed should also not generate secondary waste since food wastage has been found to contribute significantly to climate change, which can in turn affect food security.

Solid-state fermentation, which is a low-cost microbial technique, was utilized to valorise okara using the bacterial strain *Bacillus subtilis* WX-17, which is a probiotic found in the human body. Results showed that after fermentation, valorisation was achieved. Total amino acids content increased from 3.04 ± 0.140 mg/g in unfermented okara to 5.41 ± 1.21 mg/g in fermented okara. Total fatty acids content increased from 153 ± 5.10 to 166 ± 2.41 mg/g okara after fermentation. 0.382 mg/100g of menaquinone-7 was also detected in fermented okara compared to almost nothing in unfermented okara. Total phenolic content and antioxidant content (DPPH) also increased by 4.09 and 6.40 times respectively. The findings of this work demonstrated the potential of using *Bacillus subtilis* WX-17 fermentation to enhance the nutritional profile of okara.

This could serve as a potential low-cost food ingredient, which can be incorporated into the human diet.

Following the successful valorisation of okara, an *in vitro* digestion and fermentation model was used to evaluate its potential effects in the human body. Bioaccessibility of nutrients, probiotic viability, cytotoxicity, total phenolic content, antioxidant activity as well as the microbial community of fermented and unfermented okara were evaluated. Fermented okara showed increased bioaccessibility of amino acids, fatty acids and menaquinone-7. *Bacillus subtilis* WX-17 also remained viable after *in vitro* digestion. Phenolic compounds and antioxidant activities were higher in fermented okara in both simulated small and large intestines. Production of SCFA was higher in the model using fermented okara. The gut microbiota was also found to be different between fermented and unfermented okara. These data suggest that the use of fermented okara as a potential functional, probiotic and prebiotic food ingredient is feasible. It would also confer more health benefits compared to unfermented okara.

After proving the feasibility of using fermented okara as a food ingredient, an alternative method of fermentation was explored to expand its application as well as enhance the scalability of the process. Here, submerged liquid fermentation of okara by *Bacillus subtilis* WX-17 was used to produce a novel functional probiotic beverage. Metabolomics analysis showed that amino acids as well as short chain fatty acids were significantly ($p<0.05$) upregulated. Total phenolic content and antioxidant content (in terms of DPPH radical scavenging activity) increased by 6.32 and 1.55 times respectively. After 6 weeks, probiotic viability remained unchanged when stored at 4°C and the cell count is above the minimum dosage to confer health benefits. Antimicrobial activity was also detected against gram-positive bacteria. The findings of this work

showed the potential of submerged liquid fermentation of *Bacillus subtilis* WX-17 using okara as sole substrate to produce a functional and low-cost probiotic beverage.

The results achieved within this thesis successfully demonstrated low-cost methodologies to utilize fermented okara as food ingredients. Large-scale adoption of these strategies can potentially enhance the food security of Singapore. At the same time, the strategies proposed also do not generate secondary waste.

1. Introduction

This introductory chapter presents an outline pertaining to the research, providing information on the background as well as the research problem. It will also highlight the aims of the study along with the significance and contribution. Section 1.1 introduces the background of the research topic, which would also include the current problems related to the research. Section 1.2 would outline the aims and objectives of the research while section 1.3 discusses the significance and contribution of the research towards the research topic. Section 1.4 presents the overview of this thesis and lastly, section 1.5 summarises the overall framework of this study.

"Chapter 1 and 2 contain excerpts from the manuscript published as: Mok, W.K., Tan, Y.X., Chen, W.N. (2020). Technology innovations for food security in Singapore: A case study of future food systems for an increasingly natural resource-scarce world. Trends in Food Science & Technology, 102, 155-168. doi: https://doi.org/10.1016/j.tifs.2020.06.013"

1.1. Research Background

According to FAO (Food and Agriculture Organization of the United Nations), IFAD (International Fund for Agricultural Development) and WFP (World Food Program), food security is defined as "a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (McGuire, 2015).

With the world's population projected to increase from the current 7.7 billion to 9.2 billion in 2050, food security is becoming an increasingly important global issue. Apart from the increase in population, changing consumer palate, climate change and

natural resource scarcity make meeting the increased demand for food even more challenging.

Food demand estimates across 10 global economic models were compared and it was found that food demand increases by 59 to 98% from 2005 to 2050 (Valin et al., 2014). This is a slightly higher figure from the most recent projection from FAO of 54% from 2005 to 2007. The authors also noted that the food demand for animal calories varies even more from 61% to 144% due to differences in income, price elasticities as well as demand system specifications. Although the projections of food demand by 2050 vary greatly across different studies, the fact that we are facing an imminent increase in food demand is undeniable.

China is the world's largest food producer that accounted for 29.1% of global rice production, 20% of maize production as well as 16.9% of wheat production in 2009. In the last 50 years, China was able to increase its crop yield per unit area through the use of planting technologies such as chemical fertilizers, pest and weed control, and irrigation (Fan et al., 2011). However, over the past 10 years, yields of rice and maize have been steadily declining due to factors such as poor soil quality, nutrient usage efficiency and water management (Dawe et al., 2000). Similarly, according to Takle et al. (2013), agriculture in USA is also facing constraints such as availability of arable land and freshwater. Another challenge faced in USA is in coping with climate change, which can directly affect crops and livestock productivity or indirectly affect income from agricultural production and food prices due to food availability. Fan et al. (2011) also noted that moving forward; it would be challenging to continue increasing crop yields through the methods mentioned previously due to decreasing arable land that can be attributed to rapid industrialization and urbanization. In this regard, a study conducted by Bren d'Amour et al. (2017) showed that urban expansion would result in a 1.8 to 2.4%

loss of global croplands. In addition, usage of chemical fertilizers has to be reduced as their overuse has led to environmental pollution, which can aggravate climate issues. More recently, the Coronavirus Disease 2019 (COVID-19) pandemic also highlighted an urgent need to enhance food security. The United Nations (UN) remarked that the pandemic would "unleash a food security crisis not seen since the Great Recession" (Tiensin, Kalibata, & Cole, 2020). Therefore, against the impending threat of food security, countries can no longer rely on traditional methods such as the increase of primary production using traditional farming techniques. Instead, more creative and technologically advanced methods must be adopted to maximise diminishing natural resources. Singapore is a good case study of a small city-state with limited natural resources that is striving to increase its own self-production of food using technology.

In Singapore, rapid economic development has seen its population increased by 87% from 3.047 million to 5.7 million in 2019 (Department of Statistic Singapore, 2019). This increase has been met by a rapid decline in the land allocated for agriculture. In 1965, Singapore was partially self-sufficient in food supply with farmlands occupying approximately 25% of land. However, by 2014, farmlands occupied less than 1% of the land in Singapore. Hence, Singapore is reliant on the 160 countries which it imports food from (Ludher, 2016).

According to a study titled "Environmental Impact of Key Food Items in Singapore" conducted by the Agency of Science Technology and Research (A*STAR) and Deloitte that was published in 2019, the total food consumption per capita in 2019 is approximately 365 kg compared to 363 kg in 2009. Although the overall increase was minimal, a breakdown of the food consumptions across different categories showed that consumption of vegetables, fruits, chicken, pork and eggs increased significantly while that of rice reduced drastically. This showed that the population is increasingly health

conscious and eating more healthily. Therefore, the food security strategies adopted should not only focus on quantity but the quality of the food as well. Apart from the changing food demand, an estimated 763,100 tonnes of food waste were generated within Singapore in 2018. To mitigate the environmental effects of food wastage, reduction of food wastage through technological means will be required. In addition, conversion of such food waste into food for consumers through the use of technology would provide another source of food to enhance Singapore's food security.

The key elements of Singapore's food security include availability of food from either domestic production or global market, accessibility of food by consumers, affordability, and safety as well as nutrition standards for consumers. According to the Global Food Security Index 2019, Singapore is ranked top based on the criteria of food affordability, availability, quality and safety. However, its rank would drop to 12 if climate change and natural resource risk were taken into consideration ("Global Food Security Index," 2019). This is due to the fact that Singapore imports over 90% of its food supply which leaves it vulnerable to trade and supply chain disruptions that can cause food prices to increase (Ludher, 2016). The current COVID-19 pandemic perfectly reflects Singapore's vulnerability in food security with supermarkets running short of essential items and general increase in food prices. Similarly, climate change may cause severe flooding and droughts in neighbouring countries such as Thailand and Indonesia, which can cause crop failure and in turn affect supply. According to the latest data in March 2020, Singapore imported S$2.093 billion and S$1.087 billion worth of food from Indonesia and Thailand respectively, which makes up almost 10% of its total food import when combined ("Singapore Imports of Food & Live Animals," 2020).

Therefore, technology innovations are key to enhance food security in Singapore. Such technologies may include vertical farming, aquaponics and internet-

driven agriculture, technology-driven food waste management (the focus of this thesis) as well as platform technology to develop alternative and unconventional food sources.

Taken together, Singapore's strategies for enhancing food security can be redefined to include 3 main areas: urban farming, processing technology and alternative food sources (Figure 1.1).

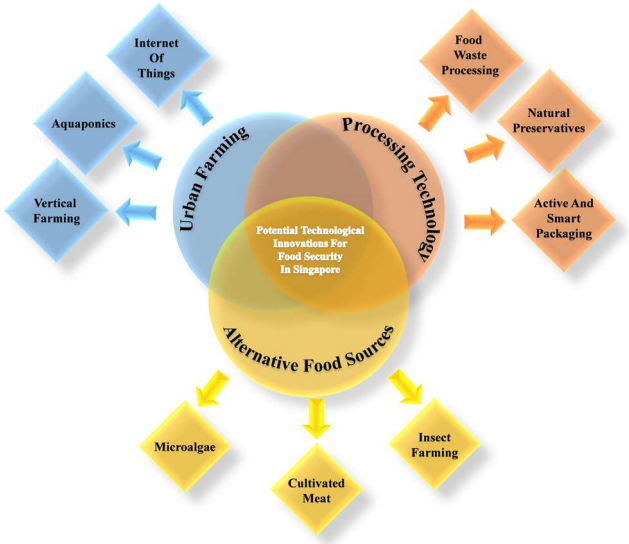


Figure 1.1. Overview of the main areas in enhancing food security in Singapore

Urban farming encompasses vertical farming, aquaponics and internet of things while processing technology would focus on food waste valorisation, natural preservatives and smart packaging. Lastly, alternative food sources would look into the areas of insect farming, microalgae and cultivated meat. Despite limited land available for agriculture, technology-driven farming practices should provide the nation with a buffer zone to tide over sudden disruption in food supply from other countries. Processing technology should lead to less food wastage and thus reduce its impact on climate change and secure food resources, while alternative food and nutrition sources can potentially reduce reliance on food import. Table 1.1 provides a snapshot of the technology innovations that Singapore has adopted for food security as well as challenges and future prospective.

Table 1.1. Summary of technology innovations and their impacts on food security in Singapore

Area of Innovation	Techniques	Materials	Challenges	Future Prospective
Urban farming	• Vertical farming	• Vegetables	• Energy consumption • High capital cost	• Higher yield per unit area
	• Aquaponics	• Vegetables and Fish	• Efficient fish waste solubilisation • Pest and disease control • pH stabilisation	• Sustainability and cost effective
	• IOT	• Nanosensors • Integrated control systems	• Durability of equipment • Energy consumption • Connectivity • Data Management	• Better monitoring of crop growth • More efficient usage of resources
Processing technology	• Food waste valorisation	• BSG • Okara	• Upscaling feasibility • Cost of production	• Reduction in food waste disposal
	• Biodegradable packaging	• Durian rinds	• Cellulose purity	• Reduction in plastic waste
	• Natural preservatives	• Flavonoid from yeast	• Upscaling feasibility	• Reduction in use of synthetic preservatives
	• Smart packaging with nanotechnology	• Chemical, gas and biosensors	• Performance of thin film electronics	• Increased food safety
Alternative food sources	• Insect farming	• Insects such as black soldier fly, crickets and mealworms	• Reliance on manual labour • Microbial degradation of insects	• Alternative protein source
	• Microalgae culture	• Microalgae	• Practical harvesting techniques	
	• Cultivated meat	• Stem cells	• Low-cost culture media	

One of the key prongs in enhancing Singapore's food security is processing technology and it encompasses food waste processing which will be the main focus of this thesis. Food waste processing involves the recovery of valuable components from food processing by-products and recycling them within the food chain. According to FAO, approximately 1.3 billion tonnes of food produced for human consumption goes to waste annually. This staggering figure amounts to more than one-third of the total food produced worldwide. These losses would lead to wastage of resources such as water, land space, labour and capital. Typically, food wastage and losses occur due to inefficiencies in harvesting techniques as well as inadequate storage and transportation facilities (FAO, 2019).

As previously indicated, in Singapore, roughly 763,100 tonnes of food waste were generated in 2018. Of these, only 17% was recycled while the rest was incinerated and disposed of in landfills (NEA, 2019). Disposal of such huge quantities of food waste would lead to undesirable effects on the environment since incineration of municipal solid waste which contains waste from biological origin emits CO_2 (Rabl, Spadaro, & Zoughaib, 2008). Therein lies the biggest problem that food waste contributes to: climate change. Venkat (2011) noted that avoidable food waste produces greenhouse emissions that are at least equivalent to 113 million metric tonnes of CO_2, which makes up 2% of the total greenhouse emissions in US alone. Similarly Hiç, Pradhan, Rybski, and Kropp (2016) reported that greenhouse gases due to food wastage had increased by 300% between 1965 to 2010. In the context of food security, climate changes can adversely affect global primary production due to higher frequency of natural disasters, which can lead to increased crop failure in turn affecting worldwide food supply. As such, there is a need to reduce the amount of food waste disposed through the use of technology. However, it is important to note that fibrous food wastes such as okara,

brewer's spent grain, bamboo shoots and vegetables still contain residue after reuse due to the presence of insoluble dietary fibre (cellulose and lignin) which would leave a carbon footprint when disposed. Moreover, it is important to note that according to the food waste hierarchy (Figure 1.2), disposal of food waste is the least desired mode of action. Therefore, zero-waste processing technologies have to be adopted in order to minimize the carbon footprint of food waste.

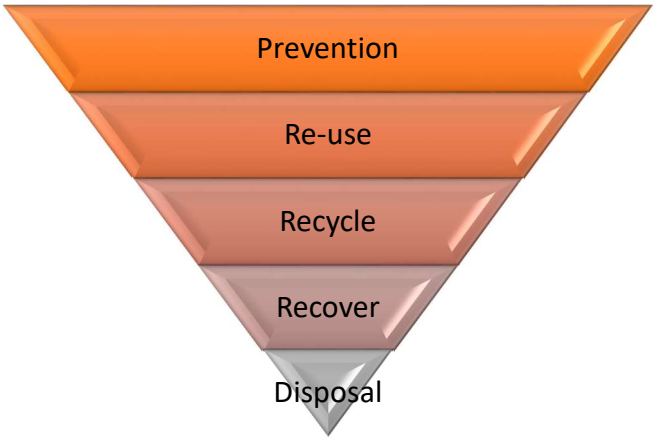


Figure 1.2. Food waste hierarchy

1.2. Aims and Objectives

The overarching aim of this study is to contribute towards food security through the area of processing technology that encompasses food waste valorisation by developing methodologies that enhance or utilize the nutritional value in okara without producing secondary waste after processing. This is important because disposal of large amount of okara in landfills can lead to climate issues due to the emission of greenhouse gases (methane, a gas that is 25 times more potent than CO_2 in terms of global warming potential) which can have devastating impacts on the planet. Okara was chosen as the substrate of interest in this thesis as it is produced in large quantities in Singapore (approximately 10,000 tonnes annually). Current methods of utilizing okara focus on producing high value compounds such as iturin A (Mizumoto, Hirai, & Shoda, 2006).

However, due to the presence of insoluble dietary fibre (IDF) in okara, most of these novel techniques still produced secondary waste (Figure 1.3).

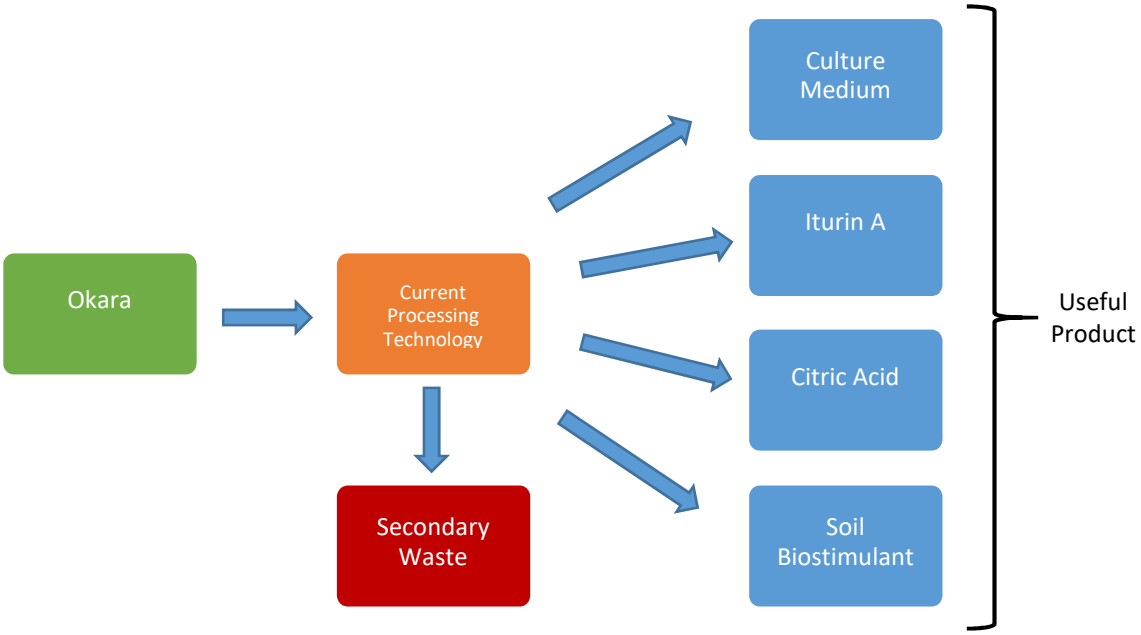


Figure 1.3. Current processing technology for okara

Our strategy is to explore the feasibility of using fermented okara as a food ingredient by evaluating its nutritional quality as well as its potential health benefits. The study would begin with a more traditional solid-state fermentation (SSF) approach to examine how this technology can enhance the nutritional value of okara using a food grade bacterium. Next, the effects of this fermented okara on the human body in terms of the bioaccessibility of nutrients and cytotoxicity would be investigated through an *in vitro* digestion and fermentation study. The effects on the gut microbiota will also be evaluated.

Since it is well known that SSF poses numerous problems in terms of scaling up (which would be elaborated on in chapter 2), the final part of this study would examine an alternative method of fermenting okara using submerged liquid fermentation (SLF) to create a nutritious, functional probiotic beverage. The motivation behind this

study is to provide an alternate method of utilizing okara as a food ingredient in a way that is more scalable for industrial applications.

In addition, this thesis would also explore on the use of a metabolomic approach to detect and evaluate changes in the metabolome of various biological processes. By nature, biological processes are complex and difficult to fathom. Therefore, a deep understanding of these processes would allow for optimization of specific metabolites to enhance the potential usage. Many of the previous studies in this field of utilizing okara focused on the changes in specific compounds of interest and very rarely on a global untargeted scale to investigate the changes in a broader range of metabolites. However, it is important to study the changes in the metabolic profile on a global untargeted level to provide a more holistic view of the changes occurring in biological processes. Metabolomics is a powerful tool that can identify a large cluster of compounds individually. As such, employing the use of metabolomics in the series of studies highlighted earlier would allow us to attain a deeper understanding of the different biological processes by studying the mechanism through pathway analyses as well as the value-added metabolites produced during the processes.

1.3. Significance and Contribution

Food waste disposal is a serious problem that can cause irreversible damage to our planet, which in turn can directly affect global food security due to more frequent occurrence of natural disasters. Intuitively, food waste can also lead to wastage in natural resources, which are already heavily taxed by increasing urbanization. Hall, Guo, Dore, and Chow (2009) noted that municipal solid food waste accounted for approximately 30% of the total wasted food energy in the US alone. The authors also remarked that a quarter of the total freshwater used in agriculture is accounted for by

wasted food. On top of that, food waste accounts for over 300 million barrels of oil per year, which is approximately 4% of the total oil consumption in the US in 2003.

Therefore, the successful findings of this thesis could potentially increase global okara usage leading to a reduction in food wastage. At the same time, the value-added okara would provide more nutritional values to consumers, which would have positive benefits on their health.

1.4. Thesis Outline

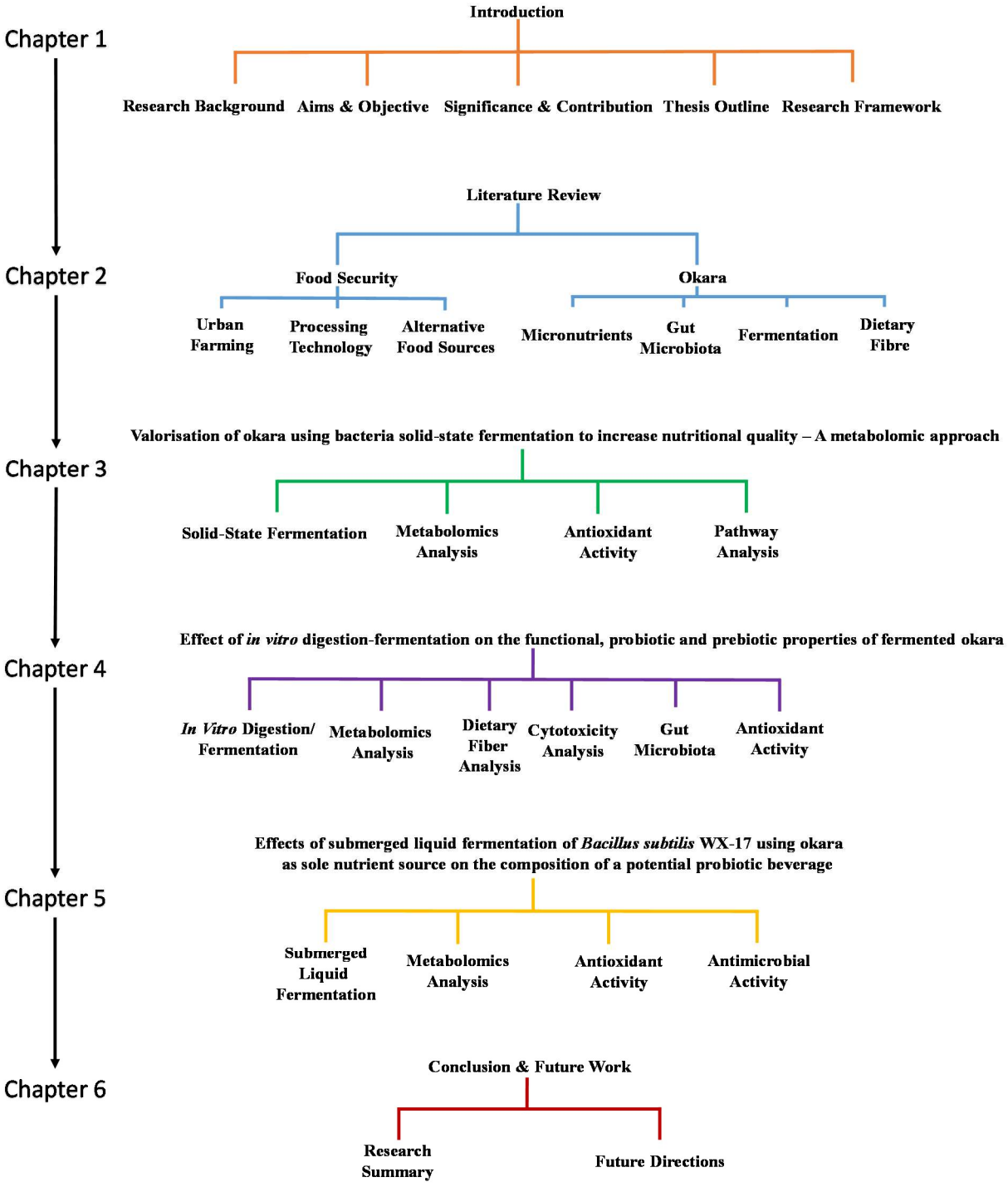
This section would provide a brief outline of this thesis.

- Chapter 1 provides an introduction to the thesis highlighting the research background as well as the research problem. This is followed by the aims and objectives, as well as the significance and contribution of the research. Lastly, the framework of this research would be presented.
- Chapter 2 presents a literature review on important topics related to this research. It would start off by highlighting some of the technological innovations towards enhancing food security, which Singapore has employed that can be used as a case study for other countries around the world. After which, basic information related to important factors in this research such as micronutrients, gut microbiota, dietary fibres and fermentation techniques would be presented.
- Chapter 3 investigates the changes in the nutritional value of okara after fermentation by *Bacillus subtilis* WX-17 (*B. subtilis* WX-17) through a metabolomic approach. A combination of global untargeted and targeted metabolomics approach using gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) were employed to detect these changes. Pathway

analysis was also performed to understand the flow of the metabolic flux throughout the fermentation process.

- Chapter 4 examines the effects of okara fermented by *B. subtilis* WX-17 on the human body through an *in vitro* digestion and fermentation model. The study will consist of a combination of untargeted and targeted metabolomic approach using GC-MS and high-performance liquid chromatography (HPLC) to detect changes in the bioaccessibility of metabolites. In addition, the viability of *B. subtilis* WX-17 after digestion would be evaluated. Changes in the dietary fibre profiles in fermented okara as well as the bioaccessibility of phenolic compounds would also be assessed. Toxicity study would be carried out to understand the cytotoxicity of the food ingredient. Lastly, the gut microbiota would be investigated to understand the prebiotic potential of fermented okara.
- Chapter 5 explores the feasibility of fermenting okara using *B. subtilis* WX-17 through SLF to create a probiotic beverage, which would be more scalable, compared to SSF. The study would look at the metabolic profile through an untargeted approach using GC-MS. On top of that, the viability of *B. subtilis* WX-17 after prolonged storage would be evaluated. Amount of total phenolic compounds present would also be studied. Lastly, antimicrobial activity of the beverage would be evaluated which would be important in determining the potential usage of artificial preservatives in the beverage.
- Chapter 6 would summarize the findings of this research as a whole. Future works required for the current researches involving okara would also be discussed.

1.5. Research Framework and Overall Workflow



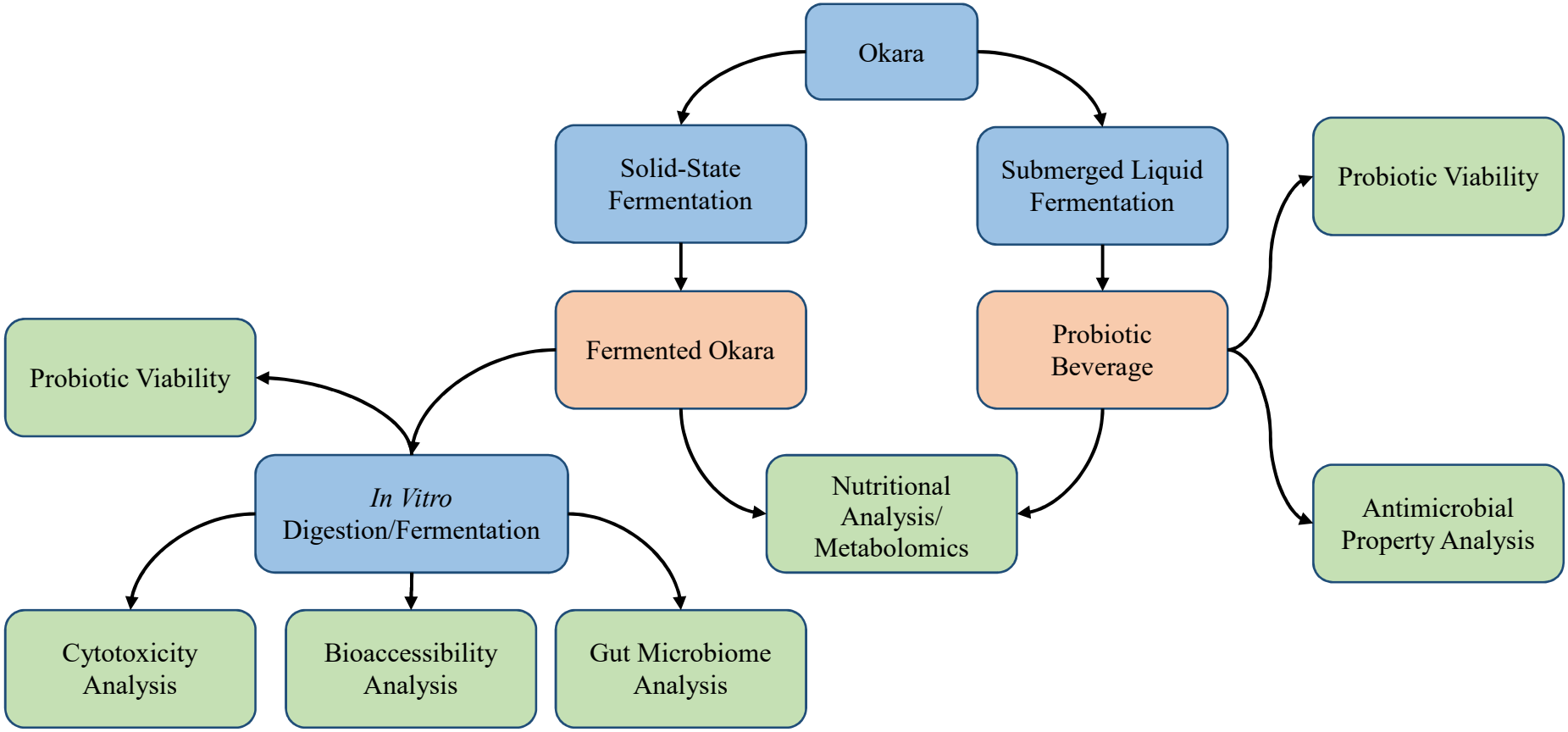


Figure 1.4. Overall workflow of this thesis to establish a methodology of reusing okara to enhance food security by 2050 in a way that does not produce secondary waste

2. Literature Review

Part I – Overview of the Areas in Enhancing Food Security in Singapore

Part I of this chapter would look at some of the key technologies that Singapore has adopted across all 3 areas of its food security strategies, namely, urban farming, processing technology and alternative food sources.

2.1. Urban Farming

Light, temperature, plant nutrition, air relative humidity and composition are important physiological and environmental factors that dictate plant quality and productivity. Over the past 50 years, urban farming had undergone significant evolution from simple covers, to greenhouses, and finally to sophisticated, environmentally controlled plant factories (Ting, Lin, & Davidson, 2016).

In March 2019, the Singapore government announced the "30 by 30" strategy which aims to increase its food production from 10% to 30% by 2030 (Paul Teng & Montesclaros, 2019). To meet this target, Singapore would have to adopt new technologies to maximise crop yields from the limited land spaces. Some of these innovations such as vertical farming are already adopted by the nation while others such as aquaponics and AI assisted smart agriculture are in their infancy (Figure 2.1).

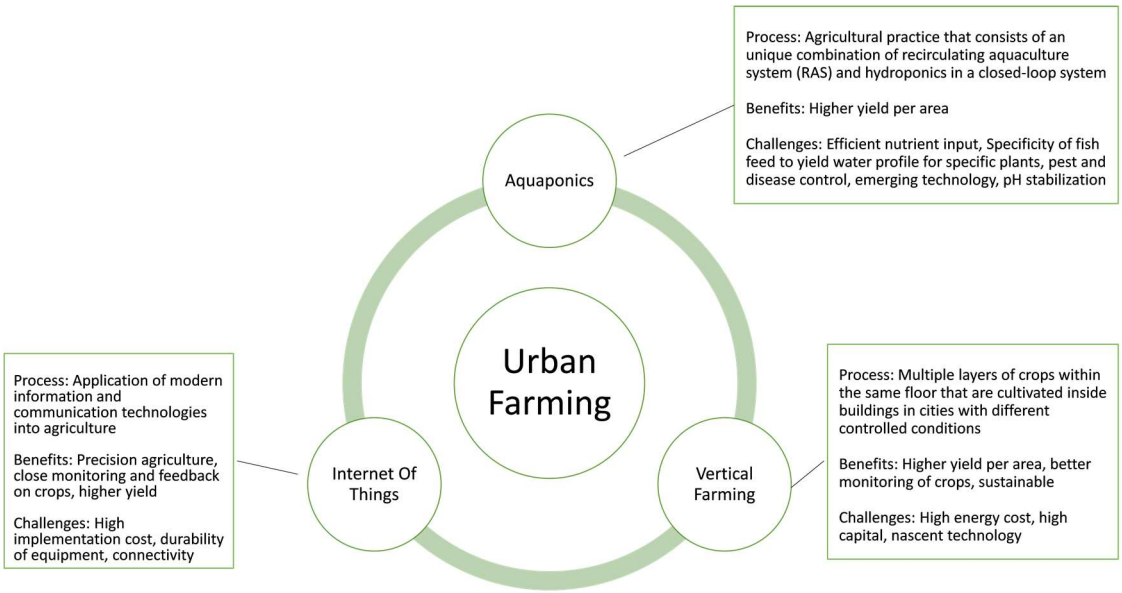


Figure 2.1. Overview of the process, benefits and challenges of different technologies in urban farming utilized in Singapore

2.1.1. Vertical Farming

Vertical farming refers to the cultivation of vegetables, fruits and grains in vertically stacked layers inside of a building in cities and urban areas in which the conditions of different floors are controlled to grow different types of crops (Al-Chalabi, 2015). Due to its limited land space, vertical farming is especially relevant to the primary production in Singapore. The adoption of this technology is gaining traction as the number of indoor vertical farms has increased from 6 in 2016 to 26 in 2018 (Lou, 2018).

Typically, vertical farms employ a combination of recycled water, air-temperature and humidity control, solar panel lighting or controlled 24-hour light-emitting diode (LED) lighting to minimize seasonality and reduce cost of production. In certain cases, plants are grown under soilless conditions with nutrients fed through a solution that flow past the plant roots (Benke & Tomkins, 2017). In Singapore, different companies employed slightly different techniques in the execution of vertical farming although the general concepts are the same. For instance, Sky Green, Singapore's first commercial vertical farm utilizes the award winning "A-Go-Gro" technology for its

vertical farms. Customizable modular towers are used to house the vegetables which are in turn planted on rotating racks powered by recycled water-pulley system that deploy rainwater collected from its overhead reservoirs. The rotating system helped to ensure equal distribution of sunlight, air flow and irrigation (Al-Kodmany, 2018). In another example, Sustenir Agriculture also uses a modular tower design with LED lightings. Nutrients are tube-fed to the vegetables while CO_2 is provided from the air-conditioning ducts with temperatures being controlled to be between 14 °C to 22 °C (Khew, 2016).

Although still a nascent technology, there are numerous benefits and opportunities to vertical farming that could significantly change the agricultural landscape. Due to its vertical nature, productivity per unit area of cultivated land is enhanced. It was reported that lettuce production was 13.8 times higher when grown using vertical farming compared to traditional farming (Touliatos, Dodd, & McAinsh, 2016). Similarly, the Den Bosch verti-farm was reported to be able to achieve 3 times more crop yields compared to traditional farming methods (Besthorn, 2013). On top of that, vertical farming could also produce multiple types of crops simultaneously on different levels while in traditional farming, only 1 crop can be produced at a time.

Another advantage of vertical farming is its resistance to seasonal climate changes and natural disasters. This is because in vertical farming, not only are the crops grown indoors where they are shielded from the environment as well as hazardous pests, the ideal conditions required for optimum growth such as heating, lighting, moisture content, humidity and nutrients can be controlled and customized for different crops (as per the methodologies adopted by Sky Green and Sustenir Agriculture). This would allow for multiple harvest in a year compared to traditional farming where there is typically only 1 harvest a year (Germer et al., 2011).

Another area that vertical farming can benefit the environment is the reduction in usage of fossil fuels. Traditional farming consumes huge amount of fossil fuels during transportation and storage. For instance, Besthorn (2013) stated that in America, 20% of fossil fuels are consumed for farming activities. It is well known that combustion of fossil fuels contributes greatly to global warming. It was reported that in 2015, 45% of CO_2 emissions came from coal burning, 35% from oil burning, and 20% originated from natural gas burning (Al-Ghussain, 2019). Since the target consumers of crops produced by vertical farming are living near the farms, there would be less requirement for long haul transportation, which would cut down fuel consumption. Transportation of crops also brings along other potential problems such as spoilage and infestation which can affect the environment due to methane emission (Williams & Wikström, 2011).

Although there are many benefits to vertical farming compared to traditional farming, there are also challenges that need to be overcome for it to be fully embraced. One of it is the energy consumption, which is closely related to carbon footprint. Since vertical farming in buildings has less access to natural light on top of the fact that there exist a light intensity gradient from the top of the building to the bottom (Touliatos et al., 2016), artificial lighting would need to be supplemented which translates into higher capital and energy cost. Al-Chalabi (2015) reported that currently, vertically grown crops have a higher energy consumption compared to conventionally grown ones. A simulation performed by Banerjee and Adenaeuer (2014) postulated that vertically produced vegetables would likely require 14 GWh of power per hectare of land per year, while according to Himanshu, Kumar, A, and K (2012), traditional farming only requires 1.75 GWh of power per hectare of land per year. Similarly, Kalantari, Mohd tahir, Akbari Joni, and Fatemi (2017) mentioned that if the whole agricultural industry in the US adopts a vertical approach, the energy required would be 8 times that of all

the energy produced by all the power plants annually. Proper energy usage and planning would be needed for vertical farming to be fully feasible. For example, LED is the preferred choice for vertical farming due to lower energy consumption, better reliability and brightness as well as its suitability for greenhouse agriculture (Kozai, 2016). LED lights can also be switched on and off intermittently as required for the plants based on the relationship between Photosynthetically Active Radiation (PAR) and biomass which correlates the conversion of absorbed light energy into biomass for crops (Leblon, Guerif, & Baret, 1991). Al-Chalabi (2015) also hypothesized that if the energy required for vertical farms is from renewable sources such as solar energy, the carbon footprint generated could be comparable to conventional farming methods. Furthermore, the rotating vertical rack concept pioneered by Sky Green can help to ensure even distribution of sunlight/LED light for the vegetables. Another area that requires much attention is in the implementation of automation. This could potentially lead to a decrease in contamination due to less handling from workers. It can also reduce cost of production, as less workers are required to manage the farm. Automation requires different domains of information technologies such as perception (sensing and data acquisition), reasoning and learning (mathematical and statistical methodologies), communication (delivery platforms such as wireless and local area network), task planning and execution (involving control logic, robotics and flexible automation workcells), and systems integration (providing computation resources and capabilities of system informatics, modelling and analysis). Successful implementation of automation would require more research into the different domains and how they can be integrated to achieve system optimization. It is also important to understand the appropriate levels of machine intelligence required (Ting et al., 2016). In addition, consumer acceptance of vertically produced vegetables should also be evaluated. A

study conducted by Jürkenbeck, Heumann, and Spiller (2019) reported that there were 2 factors, namely sustainability and naturalness of the produce, affecting consumer acceptability. Most of the people surveyed were not aware of what vertical farming is. Despite the lack of knowledge, many of the participants rated vertical farming systems as sustainable. Participants also weakly agreed vertical agriculture is not too artificial, which is a critical factor in their tendency to purchase.

Overall, vertical farming holds great potential in terms of meeting the food demand of our rising population, although there are still teething issues due to its technical infancy. In terms of sustainability, the vertical farming model is able to achieve enhanced ease of maintenance, improved ergonomics, automation and space efficiency. However, there are also issues that can impact sustainability such as high capital costs requirement and profitability (mainly due to high energy requirements). The economic factors provide a significant barrier to a wider adoption of vertical farming and its sustainability. Further research and innovations would be required for vertical farming to be more widely accepted and practiced.

2.1.2. Aquaponics

Aquaponics is an agricultural method that leverages the symbiotic relationship between fish and plants in a unique combination of recirculating aquaculture system (RAS) and hydroponics in a closed-loop system (Goddek et al., 2015). In conventional hydroponics, required macro and micronutrients are supplied to the plants in a nutrient solution under soil-less conditions (Treftz, 2016). However, in an aquaponics system, fish sludge that is rich in nutrients is used for plant growth. The basic idea of aquaponics is to provide fish with feed of the right composition, ammonia from fish urine and gill excretion are then converted into nitrates via nitrification by nitroso-bacteria (convert

ammonia into nitrites) and nitro-bacteria (convert nitrites to nitrates). Nitrate rich water is then channelled to the hydroponic beds where the plants would essentially act as water reprocessing units by removing nitrates from the water for growth. The "depleted" water is transferred back into the aquaculture where the cycle repeats. Hence, in aquaponics, water is recirculated around the system in a close loop (Graber & Junge, 2009).

Aquaponics presents advantages such as reduced land usage due to potential for vertical implementation, less weeds growth, less ongoing maintenance, less usage of water due to circular nature and moveable infrastructure. From an economic standpoint, it has the potential to generate more profits from two components for the producers: fish and vegetables. Also, the fish and crops produced are appealing to the consumers' demand for safe food produced in an environmentally responsible way (Blidariu & Grozea, 2011).

According to Junge, König, Villarroel, Komives, and Jijakli (2017), aquaponics only started garnering widespread attention in 2010 and can be termed an "emerging technology", while Kotzen, Emerenciano, Moheimani, and Burnell (2019) considered it to be at the mid-stage of development. As such, worldwide adoption of aquaponics are modest at best (McHunu, Lagerwall, & Senzanje, 2019). In recent years, several companies in Singapore have started to adopt aquaponics technology. For example, according to its website, Metro Farm has successfully commercialised a full-scale aquaponics farm spanning 7000 ft^2 at Kranji as well as a 3000 ft^2 aquaponics prototype system at Punggol. In another example, Orchidville has implemented a 600 m^2 aquaponics farm at Sungei Tengah that can rear 8000 rosa and romaine lettuce heads as well as 8000 fish at any one time, the fresh produce and fish are subsequently served at a restaurant beside the farm (Boh, 2017). There are also 6 agrotechnology parks in Singapore spanning 1465 hectares that houses modern farms that utilize advanced

technologies for intensive farming practices. The country has further announced a new 18 hectares Agri-Food Innovation Park at Sungei Kadut that will consolidate the high-tech farms in Singapore (Ai-Lien, 2019; SFA, 2019). Co (2019) also reported that aquaponics farms were installed on the rooftop of both Fairmont Singapore and Swissotel The Stamford. The latter is said to be able to produce up to 1,200 kg of vegetables such as water spinach, different types of lettuces, numerous different mints and 350 kg of tilapias monthly for the hotel's kitchens, which is approximately 30% and 10% of the hotel's daily requirement for vegetables and fish respectively. That being said, the owner of the farm also remarked that aquaponics is difficult to sustain due to several factors such as temperature control, lack of sunlight, excessive wind and moisture of air. This is could possibly account for the relatively slow implementation of aquaponics around the world as although aquaponics is acknowledged as one of the 10 technologies that could change our lives by the European Union Parliament, there are still many challenges that need to be overcome for it to contribute significantly to food security (Junge et al., 2017).

The main challenge for commercial aquaponics is to overcome its multi-disciplinarity, since it requires expertise from environmental, civil, mechanical engineering as well as knowledge in biochemistry, biotechnology, aquatic biology, process control, economics, finance and marketing. Some of the main technical challenges are highlighted below.

Firstly, for aquaponics to be a sustainable system for food production, nutrients input have to be used efficiently with minimal discard to achieve a zero discharge recirculating system (Boxman, Nystrom, Ergas, Main, & Trotz, 2018). Insoluble materials such as fish excreta represent inefficiency in the current aquaponics system. As such, more research would be required on fish waste solubilisation, which is rich in

ammonia that is critical to the aquaponics system. Vermicomposting could be a solution in mineralizing organic materials (fish excreta) thereby achieving the objective of converting all fish feeds into plant biomass (Torri & Puelles, 2010). The composition of fish feed also plays an important role in the efficacy of aquaponics since it would affect the nutritional profile of the water (Martins, Eding, & Verreth, 2011). It has been reported that aquaponics systems relying solely on fish feed to supply nutrients have low levels of phosphorous, iron, potassium, manganese and sulphur (Roosta & Hamidpour, 2011). A study conducted by Nozzi, Graber, Schmautz, Mathis, and Junge (2018) utilized 3 identical aquaponics set-up with different supplementation schemes. In general, it was found that different plants exhibited high yields under different schemes. For example, lettuce grew best when weekly supplementation of iron, potassium and phosphorus was provided, while mushroom herbs grew well without any nutrient supplementation. The goal in aquaponics is to find the perfect feed composition for specific types of fish that would yield a water profile that is as close as possible to the hydroculture requirements of specific plants. This is because, if the water lacks certain nutrients, inorganic minerals would need to be added into the system, which would translate into additional cost and affect its sustainability. Therein also lies the challenge of finding the perfect fish-plant couple where the nutrient profile provided by the fish excreta and the nutrients required by the plants overlaps significantly.

Pest and disease control is another challenging aspect of aquaponics that requires attention. By default, aquaponics systems contain more microflora compared to hydroponics due to the breeding of fish as well as the nitrifying autotrophic bacteria in the biofiltration units. Pesticides used in conventional hydroponics cannot be used in aquaponics due to their toxicity to the fish and the nitrifying bacteria (Blidariu & Grozea, 2011). At the same time, due to the need to maintain the nitrifying biofilm, antibiotics

and fungicides cannot be used for fish pathogen control. Furthermore, usage of antibiotics for plant applications is not permitted. These constraints necessitate the use of innovative pest control methods such as the use of microorganisms with biological control properties or plant extracts with antimicrobial properties (Gurjar, Ali, Akhtar, & Singh, 2012). Furthermore, According to Yavuzcan Yildiz, Radosavljevic, Parisi, and Cvetkovikj (2019), one of the main concerns for food safety in aquaponics is the fear of pathogen transfer in sludge from fish to plants. However, based on previous studies, there are minimal risks present. Potential microbes in aquaponics system include bacteria, archaea, fungi, viruses and protists in different compositions. To prevent the proliferation of pathogens, disinfecting protocol such as treating water with ultraviolet light combined with ozone can be employed. There is also the potential risk of having diseased fish in the aquaponics system. To mitigate the food safety risks due to diseased fish in the system, biological control methods such as the use of filter-feeding, filtering organism, beneficial microorganisms as probiotics in fish feed or use of effective medicinal plants against pathogens can be employed.

Another important facet of aquaponics is in pH stabilization. One of the most commonly reared fish species in aquaponics is nile tilapia (Oreochromis). This species is chosen for its robustness that allows it to tolerate wide environmental conditions. However, it is important to note that nile tilapia is also a relatively low value fresh water fish which is produced cheaply through non-aquaponic culture. Nile tilapia has optimum growth performance at pH from 7.0 to 9.0 while the nitrifying bacteria have optimum pH ranging from 7.5 to 8.3. Hydroponics plants perform optimally at pH 5.8 to 6.2 (Yep & Zheng, 2019). Such discrepancies in optimum pH mean that some organism's growth would have to be compromised in favour of others depending on which is more critical. In general, most reviewers recommended a more neutral pH from 6.8 to 7.0 in favour of

the nitrification process. pH of the aquaponics system tends to decrease overtime due to the acidity producing nitrification process which supersedes the increase in pH during root uptake of nitrates. The most commonly used method to maintain pH is the addition of carbonate and hydroxide to the system (Rakocy, 2012). Alternatively, some new technologies can be introduced into the field of aquaponics such as the introduction of the fluidized lime-bed reactor which involves the controlled addition of dissolved limestone into the acidic system to continuously raise its pH (Goddek et al., 2015).

Currently, aquaculture stands as the main method of fish farming. However, aquaponics has features and potential (such as its ability to go vertical) that are well suited for urban and land scarce area like Singapore as it allows for intensive production of fresh and high-quality plants and fish in small spaces such as rooftops. There are evidences of several local companies taking up the challenge of implementing more aquaponics farms around the country although as highlighted, there are still numerous issues and challenges which require further research before it can live up to its potential in alleviating the problems of food security.

2.1.3. Internet of Things Based Smart Agriculture

As the world becomes increasingly reliant on technology, internet of things (IOT) is a buzzword that is garnering more and more attention. It is estimated that IOT could potentially grow into a market worth 7.1 trillion by 2020 (Wortmann & Flüchter, 2015). The applications of IOT are broad and affect virtually all areas of life, for example the AI industry (development of intelligent product systems) and blockchain technology.

Agriculture is an industry that is beginning to adopt IOT technologies, which would enable farmers to enhance productivity and reduce wastage. Precision agriculture is one of the most promising concepts that has arisen in recent years and is expected to

enhance food security in a sustainable way (N. Zhang, Wang, & Wang, 2002). The main aim of precision engineering is to improve and optimize agricultural processes to maximise production. It requires fast, reliable and distributed measurements to give farmers holistic and detailed overview of the situation across the cultivation area as well as coordination of different automated hardware to optimize the use of energy, water and pest control measures for optimum plant growth (Tzounis, Katsoulas, Bartzanas, & Kittas, 2017).

Recently, wireless sensing technology is being used in agriculture to monitor environmental parameters such as temperature, humidity and illumination to provide optimal crop growth conditions (Srbinovska, Gavrovski, Dimcev, Krkoleva, & Borozan, 2015). For example, an IOT enabled garden system was developed whereby a controller is connected to light, temperature and soil moisture sensors together with an integrated Wi-Fi module. The system would be able to tell farmers what kind of vegetables grow best on the soil and send messages to the farmers' smart phones when in need of water and light. It also has voice-recognition capabilities as well as the ability to access specific information and make logical deductions (Ray, 2017).

With its "30 by 30" goal in sight, Singapore has started to incorporate IOT into its urban farming scene. For example, researchers from the Singapore-Massachusetts Institute of Technology (MIT) alliance for Research and Technology (SMART) have found a method of monitoring the growth of plant at a molecular level by injecting nanoparticles into the plant. These nanosensors would be able to detect minor changes in the plant ranging from temperature to growth impact by soil acidity to pest infestations and diseases. With this technology, urban farmers in Singapore would be able to detect diseases and pests before they are visible. Moreover with such real-time data available, farmers would be able to better monitor the growth of crops in terms of

what is working and what is not (Teh, 2019). CrowdFarmX is a local company that is the world's first cooperative farming platform on blockchain. It aims to connect farmers to the global market as well as provide them with the technological expertise to increase their productivity. These expertises include physical shared services hubs that provide IOT monitoring systems and data analysis on climates and soil condition. Farmers are also connected to agronomists and technologists through the platform to help them develop advanced farming protocols and automate their farming practices (Shiao, 2019).

Adoption of IOT in agriculture comes with its own set of challenges. Firstly, the sensors used at the cultivation sites have to be robust enough to endure harsh environmental conditions such as solar radiation, extreme temperatures (high temperature in Singapore), rain and humidity, winds as well as vibrations. Not only should they be durable enough to function for a prolonged period of time, they should be able to function well under those conditions as well. Power consumption can be an issue since these IOT equipment requires power sources, which can increase the production cost of the vegetables. Therefore, appropriate programming tools and low-power capabilities are required to reduce the overall production cost. Lastly, the large number of connected sensors and devices can produce a huge amount of data which can easily overwhelm small scale server infrastructure (Atzori, Iera, & Morabito, 2010).

These new technologies can be adopted into urban agriculture such as vertical farming and aquaponics (smart urban agriculture) which could potentially increase crop yield and reduce cost of production such as energy and water usage that can help Singapore inch closer to its "30 by 30" goals as well as minimize environmental impacts.

2.2. Processing Technology

Processing technology encompasses food processing, food waste processing as well as food packaging technologies. The technologies employed across multiple facets of the processes within the food industry seek to provide abundant, safe and nutritious food for the world. Food processing involves the deliberate altering of food before it becomes available for consumption. Additionally, food processing improves nutritional profile, extends shelf life, and enhances sensory characteristics and safety of food. Many food processing techniques such as pasteurizing, pickling, canning, salting, extrusion and milling are well known while new methods like high-pressure processing, pulses electric field, cool plasma and UV irradiation are getting increasing attention. However, in recent years, technology innovations in Singapore are more focused on the areas of food waste processing and packaging technologies. Therefore, the following sections would focus on the aforementioned areas.

2.2.1. Brewer's Spent Grain

One of the side stream products from food processing is known at brewer's spent grain (BSG). According to Mussatto (2014), in the beer manufacturing industry, large quantities of food by-products are generated of which 85% consist of BSG. The other by-products are mainly spent hobs and surplus yeast. The annual global production of BSG is estimated to be 38.6 million tonnes. On a dry basis, BSG is made up of fibres, which consist of cellulose, arabinoxylan, lignin, and protein. Currently, the bulk of BSG generated is managed by its usage as animal feed mainly for cattle as well as other alternative uses such as fuel source in energy combustion and mushroom cultivations (Mussatto, Dragone, & Roberto, 2006) while the rest is disposed of in landfills, which

as mentioned is an unsustainable option that can severely impact global food supply due to climate change (Buffington, 2014).

One of the challenges to valorise BSG lies in its husky physical property combined with high amount of cellulose and hemicellulose, which can bind onto proteins and other nutrients thereby making extraction difficult. Physical, biological, chemical pre-treatment or a combined treatment method can be employed to better harness the nutrients in BSG. Physical pre-treatment or sometimes a combination of physical and thermal pre-treatment are mainly used to reduce the size and deform the crystalline cellulose structure of BSG through extrusion, milling, grinding, microwave radiation and ultrasound (Buffington, 2014; Lynch, Steffen, & Arendt, 2016). The size reduction would increase the surface area of BSG, which would better allow enzyme or acid entry into the lignocelluloses. Chemical methods include steam explosion, ammonia fibre explosion, sulfur dioxide explosion as well as the addition of lime and acid (Ivanova et al., 2017). However, the main disadvantage of chemical methods is the formation of toxic compounds. Biological treatment involves the use of commercial enzymes or microorganisms. One advantage of enzymatic treatment is that its use does not generate toxic compounds (Sindhu, Binod, & Pandey, 2016). In a study by Niemi, Martins, Buchert, and Faulds (2013), it was reported that pre-treating milled BSG with a carbohydrase mix from *Humicola insolens* considerably improve the subsequent protein solubilisation in the residual biomass. However, it should be noted that in general, the main disadvantage of using commercial enzymes in pre-treatment is its high cost especially in large scale processing.

A lower cost option to utilize BSG through biological means is the use of fermentation using microorganisms. Employing the right strains of microorganisms that produce enzymes such as cellulases, proteases and lipases would achieve similar effects

to commercial enzymes at a fraction of the cost. In Singapore, various research institutions and local companies have been exploring the valorisation of BSG across various applications. For instance, Cooray, Lee, and Chen (2017) reported that fermentation of BSG by *Rhizopus oligosporus* was able to enhance its nutritional content which can be extracted into a liquid phase to produce a novel culture media for *Rhodosporidium toruloides* and *Saccharomyces cerevisiae.* The media derived from BSG was found to be competitive to commercial media in terms of supporting yeast growth. In another example, BSG was used as feed for microalgae culture as an alternate source of protein. Microalgae-based proteins have lower land requirement compared to other sources of proteins. For example, microalgae requires less than 2.5 m^2 per kg of protein compared to 47 – 64 m^2 per kg for pork, 42 – 52 m^2 for chicken and 144 – 258 m^2 for beef (Caporgno & Mathys, 2018). UglyGood, a local company is also exploring the use of BSG to produce bio-based cleaning products such as floor cleaners and multi-purpose solutions (Chiang, 2019).

On the whole, biological treatment methods are more environmentally friendly compared to chemical methods as they do not generate toxic compounds and also produce fewer inhibitors as a result of milder processing conditions on top of its lower energy requirement compared to physical methods.

2.2.2. Biodegradable Food Packaging

Although valorisation of food waste can be a good way to extract valuable compounds, the residues left behind still create carbon footprints. One strategy to mitigate this is the development of biodegradable food packaging through the extraction of compostable, biodegradable polymers such as fibres, starch, cellulose and lignin from plant-based food waste (Zhao, Lyu, Lee, Cui, & Chen, 2019). Not only would this

minimize food waste disposal, it would also alleviate the global problem of plastic waste disposal, which are getting widespread attention.

Durian is a common fruit consumed in Southeast Asia countries and there is a huge amount of durian rinds disposed annually with up to 6 million of them consumed in Singapore alone annually (Khoe, 2018). Durian rinds, which are generally disposed, are rich in components such as hemicellulose, cellulose, lignin and phenolic compounds, which can serve as low-cost resources that can be used to produce biodegradable food packaging. On a dry basis, durian rind was reported to contain 31-36% cellulose, 10-11% lignin and 15-19% hemicellulose. A study in Singapore successfully extracted cellulose of high purity from durian rinds and utilized the cellulose to produce food packaging films (Zhao et al., 2019). Durian rind cellulose film was reported to have high tensile strength, high rigidity, smooth surface, excellent transparency and is also 100% biodegradable. Despite the advantages of converting durian rinds into films, a more thorough evaluation would be required to determine if it would actually prevent the deterioration of food quality. Furthermore, food migration tests would have to be conducted to ensure that no chemicals are migrated to the food. Similarly, a technology firm in Singapore recently developed fully biodegradable drinking straws from the bacterial fermentation of plant-based oils and sugars. Apart from biodegradable straws, the biopolymers can also be used to fabricate cutlery, cup lids as well as food packaging (V. Liu, 2019).

However, although these biodegradable packaging can potentially address the problems of secondary waste after processing, some of these techniques require costly and potentially harmful chemicals which might affect its suitability for mass production and widespread usage. For example, lithium chloride and dimethylacetamide are commonly used to increase the solubility of cellulose during biodegradable film

regeneration. However, although lithium toxicity on humans is considered low, it can still pose considerable amount of health risk for humans at certain concentration. Aral and Vecchio-Sadus (2008) reported that serum concentration of 20 mg/ L of lithium could potentially cause death. In a separate study, Baum and Suruda (1997) reported 2 cases of toxic hepatitis from dimethylacetamide among 25 employees on a production plant in the U.S. Hence, there still exist needs to develop a solution that utilizes the nutrients in food waste without producing secondary waste using methodologies that do not involve the use of potentially toxic chemicals.

2.2.3. Natural Preservatives

Apart from ensuring the abundance of food, food security also entails the provision of safe food for the population. The use of preservatives is one of the most common methods to prevent spoilage of food. Currently, most of the preservatives used in the food industry are synthetic such as benzoates, sorbates and nitrates. However, synthetic food preservatives were reported to have adverse effects on human health such as allergy reactions, headaches and even cancer (Bondi, Laukov, de Niederhausern, Messi, & Papadopoulou, 2017; Ng, Lyu, Mark, & Chen, 2019). On the other hand, natural preservatives, which can be derived from plant extracts, food waste, purified secondary metabolites, are perceived as better and safer compared to synthetic food preservatives (Erginkaya & Konuray, 2017; Ng et al., 2019). In a study conducted using a genetically engineered strain *Saccharomyces cerevisiae* Y26 that produces naringenin, Ng et al. (2019) was able to obtain antimicrobial phenolic metabolites that exhibited strong antimicrobial properties which can be used as natural food preservatives. Cherries and blackcurrants were also found to be able to produce natural preservatives. Nowak, Czyzowska, Efenberger, and Krala (2016) reported that 2 distinct groups of polyphenols present in blackcurrants and cherries were identified as phenolic acids and flavonoids

that include epigallocatechin and glycosides of quercetin as well as kaempferol. Other sources of natural preservatives from plant extracts include blueberry, garlic and mustard (Erginkaya & Konuray, 2017).

2.2.4. Active and Smart Packaging

As the population becomes increasingly affluent and well informed, there is a growing concern for better food safety, which drives the need for innovations in food packaging. With new technologies in food packaging, not only would there be safer food, there would also be a reduction in food spoilage thereby improving food security. Active packaging incorporates additional components into the packaging to provide safer food by maintaining or extending the food quality and shelf life (Biji, Ravishankar, Mohan, & Srinivasa Gopal, 2015). The techniques employed in active packaging include control of moisture, oxidation, microbial growth, ethylene removal and odour absorption (Ghoshal, 2018). For example, A*STAR in Singapore created a polymeric packaging material based on nanotechnology. By introducing silicate from natural sources into the gaps between the polymers, the oxygen barrier of food packaging can be enhanced which would prevent premature food spoilage. Oxygen-scavenging nanofillers can also be added into the packaging to remove remnant oxygen inside the packaging which can enhance shelf life (Neo, 2019).

Smart packaging is another technology in food packaging that has a different working principle compared to active packaging. According to Ghoshal (2018), smart packaging can be classified into simple smart packaging, interactive or responsive smart packaging. These packaging have devices such as sensors and indicators to judge the internal and external environment of package, identify the changes on food condition, as well as inform these changes to consumers. In addition to the common components,

interactive smart packaging also contains response mechanisms that can neutralize hazardous changes occurring in the food.

2.2.5. Challenges in Processing Technology

One of the main challenges concerning biodegradable food packaging is to obtain cellulose of high purity from the substrates. It is difficult to attain cellulose of high purity due to its conjunction with lignin and hemicellulose in the substrate. Zhao et al. (2019) suggested a 2-step purification process using sodium chlorite and hydrogen peroxide to remove lignin and hemicellulose. Using this method, cellulose of purity up to 90.4% was obtained. However, since the main application of cellulose extracted from food waste are for food packaging, the effects of chemicals added during the extraction process on the human body have to be investigated.

Active and smart packaging comes with its own set of challenges as well. According to Schaefer and Cheung (2018), smart packaging requires further development in terms of improving the performance of thin film electronics as well as its integration into food packaging. Most of the development on biosensors are limited to preliminary proof of concepts studies and require further works for practical implementation. More work would be required on the biodegradability and recyclability of the sensors and communication functionalities as the implementation of smart packaging will still generate waste (Schaefer & Cheung, 2018). For active packaging, more research work would be required in the development of active compounds to be incorporated into packaging.

2.3. Alternative food sources

With the rising population becoming increasingly affluent and educated, there is a need to produce not only more food, but also food of healthier origin. Therefore, it is important to cater to the changing dietary preferences of the population by using ingredients that are more natural. It is also important to develop alternative food and nutrition sources such as insect proteins, microalgae and cultivated meat (Figure 2.2) to add on to existing food supply. Insect farming may also be used to provide supplementary feed to livestock, which can indirectly impact food security. According to Hartmann and Siegrist (2017), animal protein production requires high amount of agricultural land, water and energy which would only increase as the global population and demand for food increase. Currently, meat production is estimated to be approximately 200 million tonnes which is slated to potentially increase to 470 million tonnes by 2050 (Liguori et al., 2015). The increase in animal protein production to meet the increasing demand would deplete resources rapidly and also adversely affect the environment in the long run.

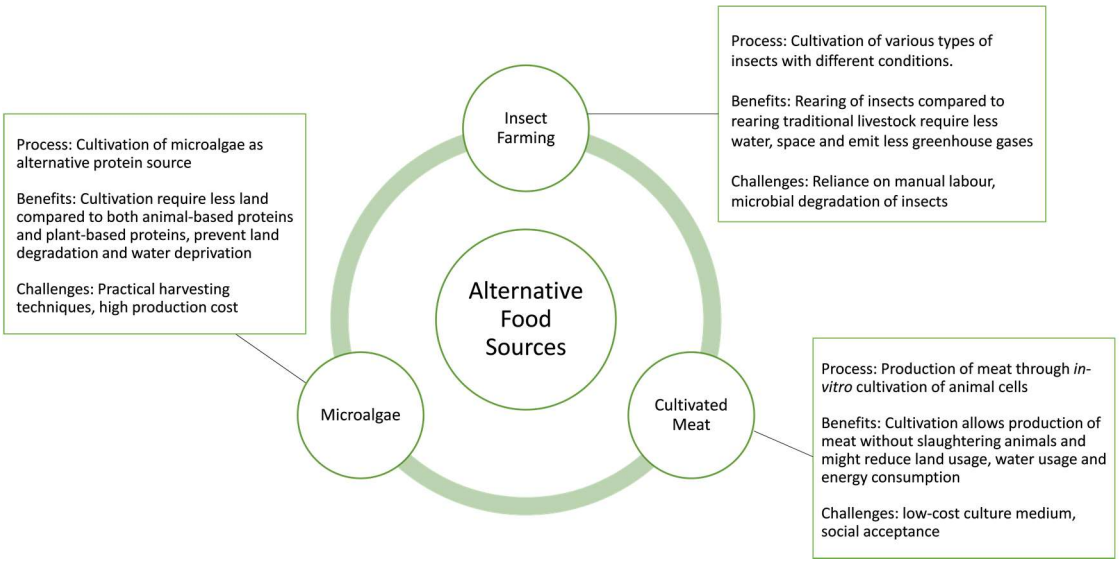


Figure 2.2. Overview of the process, benefits and challenges of different alternative food sources explored in Singapore

2.3.1. Insect Farming

Due to the huge demand in natural resources (land and water) required to grow livestock for protein, interest in insects as an alternative protein source has been increasing. Insects were found to be highly nutritious in terms of essential amino acids, vitamins, minerals, fats and have been consumed by humans since ancient times (Hartmann & Siegrist, 2017; B. A. Rumpold & Schluter, 2013). A study reported that the quality of insect as a protein source was comparable to soy protein (Vangsoe, Thogersen, Bertram, Heckmann, & Hansen, 2018). This will allow insects to be a potential solution to the issue of providing sufficient food for the populations.

There are a total of approximately 2000 edible insect species reported, which were consumed in eggs, larvae, pupae, nymphs or some in adult forms (Anankware, Fening, Osekre, & Obeng-Ofori, 2015; Dobermann, Swift, & Field, 2017). Insects can be obtained by harvesting from the nature or from insect farming (Dobermann et al., 2017). The more commonly consumed insects in the world are beetles, caterpillars, bees, wasps, ants, grasshoppers, locust, crickets, cicadas, leafhoppers, plant hoppers, scale insects, true bugs, termites and dragonflies (Van Huis et al., 2013). 31% of the total insect consumption globally was reported to be the consumption of beetles (Van Huis et al., 2013).

According to Anankware et al. (2015), apart from direct consumption of edible insects in the wild, insect farming can potentially serve as an alternative source of protein to traditional livestock. The cultivation of insects as protein source would have several advantages over traditional livestock. For instance, less CO_2, CH_4, N_2O, and NH_3 emissions were found from rearing insects compared to conventional livestock due to the insects' respiration, metabolism and their faeces (Van Huis & Oonincx, 2017). In terms of land usage, the production of mealworms only required about 10% of the

land used compared to that of beef (Oonincx & de Boer, 2012). Similarly, mealworm production requires approximately 5 times less water compared to beef production (Van Huis & Oonincx, 2017). In comparison to chicken production, mealworms require approximately 2 to 3 times less land and almost half the water footprint per gram of protein (Miglietta, De Leo, Ruberti, & Massari, 2015; Oonincx & de Boer, 2012).

Singapore has also taken its first step into the use of insects as alternative protein source. Insect farming in Singapore is still an emerging technology that requires further large-scale development. Asia Insect Farm Solutions is a local start-up that attempts to transform crickets into a nutritious flour-like product that can be used to replace conventional flour (Paulo & Ong, 2020). Crickets were chosen for several reasons. Firstly, they have lower carbon footprint compared to traditional livestock. They also require less water and land space compared to chicken. Crickets are also more efficient in converting feed into muscle mass due to them being poikilothermic, which means that they do not need to use energy from the feed to maintain their body temperature (Van Huis & Oonincx, 2017).

Apart from serving as alternate protein source, insects can also help to combat food wastage by converting them into other products. Insectta is a local black soldier fly farm established in 2018. Currently, approximately 500 kg of food waste from food suppliers, homes and food stalls are consumed and converted into plant fertilizers as well as fish and animal feed by 100 kg of black soldier fly larvae. St-Hilaire et al. (2007) reported that black soldier fly could replace up to 50% of fishmeal used to produce rainbow trout. Similarly, the fertilizers produced can be combined with a hydroponics system in a closed-loop to grow crops such as kale, lettuce and other vegetables (Boh, 2018). The conversion of food waste into animal feed and fertilizers could be a potentially effective method to reduce food wastage since the larvae are able to eat up

to 4 times their body weight. As this technique is relatively new in Singapore, the output is currently not at a significant scale. However, according to Surendra, Olivier, Tomberlin, Jha, and Khanal (2016), approximately 100,000 tonnes of food waste can be converted into 10,000 tonnes of animal feed based on a reported feed conversion ratio for black soldier fly larvae of approximately 10 to 15. As mentioned, reduction in food wastage can help to alleviate climate issues, which can affect primary production.

Although insect farming is a potentially viable choice to reduce food wastage, it has issues based on the optimization of farming techniques (Dobermann et al., 2017). The majority of insect farming is reliant on manual labour to feed, collect, clean and rehouse. The usage of manual labour instead of automation is costly and would lead to higher insect protein prices (Birgit A. Rumpold & Schlüter, 2013). Therefore, in order to reduce production costs, automation technologies have to be developed. Such technologies include monitoring devices, mechanical removal systems of dead or diseased insects, continuous rearing systems, harvesting devices, sanitation procedures for management of diseased and processing units for separation of proteins (Birgit A. Rumpold & Schlüter, 2013). Other means of cost reduction will include the development of cheap rearing substrates as well as innovations in production technologies incorporating cost-effective production systems. Another challenge in the execution of insect farming is the presence of potentially harmful ingredients or the microbial degradation of insects, which could present significant health risks for humans. Insects are vulnerable to microbiological hazards in the absence of proper heat treatment or storage facilities (Klunder, Wolkers-Rooijackers, Korpela, & Nout, 2012). To reduce the microbial contamination of insects, processes such as powdering of the insects, heating, drying, UV treating, acidifying, pasteurizing can be incorporated (Y. S. Wang & Shelomi, 2017).

2.3.2. Microalgae

Another interesting alternative protein source is microalgae. In fact, microalgae have been explored as food and proposed as possible alternative protein sources since the 1950s (Vigani et al., 2015). Microalgae are mainly autotrophic organisms found in marine and freshwater but some species have been found to be heterotrophic (Chacón-Lee & González-Mariño, 2010; Pleissner, Lam, Sun, & Lin, 2013). It is abundant in several nutrients such as essential amino acids, fatty acids, carotenoids, fibres, B vitamins, iron and calcium (Hayes et al., 2017; Vigani et al., 2015). It was also reported to possess antioxidant, antidiabetic, antiallergenic as well as anti-inflammatory properties (Hayes et al., 2017). The cultivation of microalgae-based proteins requires less land compared to both animal-based proteins and plant-based proteins (Caporgno & Mathys, 2018). It also contributes to the environment by preventing land degradation and water deprivation.

An interesting study by Pleissner et al. (2013) found that food waste hydrolysate can be used as culture medium in heterotrophic microalgae cultivation. A medium rich in nutrients through fungal hydrolysis of food waste was determined to be viable in the cultivation two heterotrophic microalgae species, *Schizochytrium mangrovei* and *Chlorella pyrenoidosa*. Kitchen wastewater was also reported to possibly serve as a nutrient source for cultivation of *Phaeodactylum strain E70* (X. Wang et al., 2020).

Microalgae products in the market come in form of dried algae, which are sold directly and used as sources of proteins and carbohydrates (Ruiz et al., 2016). Other high value compounds such as antioxidants, proteins, fatty acids and docosahexanoic acid (DHA) can also be extracted from microalgae (Borowitzka, 2013). The more commonly consumed microalgae species are the *Chlorella*, *Spirulina, Dunaliella, Haematococcus,*

and *Schizochytrium*, which are certified as Generally Recognized as Safe (GRAS) (Hayes et al., 2017; Vigani et al., 2015).

There are also recent developments in the microalgae scene in Singapore. It was reported that researchers were able to utilize the nutrients in a culture medium derived from okara to grow microalgae that can produce up to 3 times the yield when compared to commercial medium at a tenth of the cost. Most interestingly, the microalgae were able to grow in the absence of sunlight which is ideal for urban cities like Singapore as it allows for indoor farming (Zhuo, 2019). Such microalgae species can be cultivated in a dark environment as they utilize the organic carbon, such as glucose, that are available in the culture medium in the absence of sunlight (Yen, Hu, Chen, & Chang, 2014). Moreover, these microalgae are able to produce proteins, vitamins and minerals which many photosynthetic strains and plants are unable to (Zhuo, 2019). In another development, local start-up, Sophie's Bionutrients won the annual Liveability Challenge in 2019 for its technology in producing food grade microalgae as alternate protein source. The company is now actively developing the technology for commercialization (V. Liu, 2019).

The main challenge in large scale culturing of microalgae is in finding a low-cost, high-efficiency harvesting technique. This is due to a myriad of reasons such as the size of microalgae cells, small density differential between cells and culture medium which makes separation difficult, low cell concentration, high ionic strength in salt and brackish water as well as the need to manage large volume of culture medium (Chacón-Lee & González-Mariño, 2010). There is currently no single harvesting method that is suitable for every scenario. As such, there is much work ahead in terms of innovating and optimizing the systems to achieve higher productivity and cost effectiveness when harvesting the microalgae. However, Caporgno and Mathys (2018) noted that from an

economic standpoint, the lack of optimization in microalgae based protein production compared to traditional protein sources hinders its ability to attract investors to fund further developments. Nevertheless, despite the challenges, microalgae hold much economic attraction as the products that can be extracted such as β-carotene, astaxanthin and phycocyanin can fetch hundreds to thousands of euro per kg depending on purity.

2.3.3. Cultivated Meat

Cultivated meat refers to the production of meat through *in-vitro* cultivation of animal cells, rather than slaughtering of animals. In general, a biopsy is first taken from a live animal. Stem cells are then obtained by cutting the muscles. These stem cells have the ability to not only proliferate, but can also transform themselves into other types of cells such as muscle and fat cells. The stem cells are grown in culture medium, typically containing fetal bovine sereum (FBS). As the cells proliferate, they would form myotubes which can then grow into muscle tissues (Chriki & Hocquette, 2020).

Although still a nascent technology, cultivated meat, if successfully implemented, could be a potential environmentally sustainable protein source to satisfy the growing global demand for meat products (Verbeke et al., 2015). Based on a study by Post (2012), cultivated meat production can potentially reduce land usage, water usage and energy consumption by 99%, 90% and 40% respectively. In this regard, Singapore has also explored the potential of cultivated meat. Shiok Meats, a start-up in Singapore, is Southeast Asia's first cultivated meat company that focuses on crustaceans. The company was able to produce minced meat of shrimp using its stem cells and turn them into shrimp dumplings (Lawton, 2020). A*STAR's Bioprocessing Technology Institute (BFI) has also began trials on culturing meat using existing technology in stem cells bioengineering and bioproduction (Begum, 2019).

Although cultivated meat holds much potential in enhancing food security, there is still a major roadblock that needs to be overcome for it to be economically viable. Current methods of culturing stem cells utilize commercial culture medium such as L-15 and M-199 with supplementation of FBS. These media are prohibitively expensive and would greatly impede commercialization of cultivated meat. Despite decades of research into finding a low-cost, well-defined growth medium for expansion of stems cells, none have been identified till date (Thorrez & Vandenburgh, 2019). Another challenge in cultivated meat is in the difficulty in producing real muscles, which comprise of organized fibres, blood vessels, nerves, connective tissues and fat cells. The production of a thick piece of meat would be difficult due to the need to perfuse oxygen inside the meat to mimic the diffusion of oxygen in real tissues (Chriki & Hocquette, 2020). Apart from the technological challenges, cultivated meat also has social acceptance challenges. Cultivated meat can have associations with cloning, transgenesis and other unknown risks (Bhat & Fayaz, 2011). Also, some common objections to cultivated meats include unnaturalness, safety, inferior taste and texture (Bryant & Barnett, 2018).

All things considered, cultivated meat presents a promising look into a potential future where animal proteins are replaced or supplemented by lab-grown alternatives. However, it is important to note that this technology is extremely recent and the main challenge of finding a low-cost but yet effective culture medium has to be solved to achieve commercial viability.

Part II – Valorisation of okara

Part II of this chapter would focus on the key components and nutrients present in okara or can be produced when using okara as substrate during fermentation as well as some of the important technologies and concepts relevant to the studies.

2.4. Okara

Okara is the pulp left behind from soybean after soymilk and soybean curd processing. Global production of okara is estimated to be around 14 million tonnes every year with 10,000 tonnes being produced in Singapore annually (B. Li, Qiao, & Lu, 2012). Dry okara contains about 50% fibre, 25% protein, 10% lipids as well as other soy components such as isoflavones, phytosterols, lignans, saponins, coumestans, and phytates (B. Li, Qiao, et al., 2012). Similar to BSG, numerous methods such as chemical or enzymatic treatment, microorganism fermentation, high pressure and micronization treatments had been employed to valorise okara (B. Li, Qiao, et al., 2012). Although it is still highly nutritious, there are currently no major applications for okara apart from animal feed. Most of it is disposed of in landfills, which as highlighted earlier, can exacerbate existing climate issues.

The most cost-effective method to valorise okara is the use of microbial fermentation. This technique is able to convert insoluble fibres into soluble fibres, which would aid in the extraction of nutrients. For instance, fermentation of okara using *Lactobacillus* was shown to increase the amount of soluble fibres by 15%. This fermentation process provided an acidic environment in which the glycosidic linkages of the polysaccharides were broken down and hence insoluble fibres are converted into soluble fibres. Other nutritional contents such as isoflavones, crude protein and water soluble substances were also enhanced (Tu et al., 2007).

In recent years, okara has been the subject of much interest in Singapore. For example, J. Kim (2019) developed a nutrient-rich culture media using okara as substrate for the growth of *Phaeodactylum tricornutum*, a microalgae strain. The author reported that the biomass obtained in the okara culture media is twice the amount obtained when using commercial culture media.

Other applications of okara include its use to produce valuable compounds such as citric acid, iturin A, antioxidants and biostimulants. One such study is to employ solid-state fermentation of okara with co-culturing by *Aspergillus niger* and *Aspergillus terreus* in the production of citric acid (Khare, Jha, & Gandhi, 1995). It was reported that using *Aspergillus terreus* as a pre-culture to break down cellulose before fermenting with *Aspergillus niger* would increase yield of citric acid by 4 times which is comparable to yield obtained from fermentation with other agro-waste (Khare et al., 1995). In another application, okara was fermented with *B. subtilis* RB14-CS under solid-state condition to produce iturin A, which is a lipopeptide antibiotic that functions as a suppressor of plant pathogens such as *Rhizoctonia solani* which causes damping-off of tomatoes (Mizumoto et al., 2006). In a study conducted by Orts et al. (2019), okara was submitted to a combination of enzymatic hydrolysis treatment followed by fermentation using *Bacillus licheniformis* to produce soil biostimulants which are fast-acting fertilisers that does not require time to breakdown in order for the nutrients to be released. However, it is important to note that most of these technologies to utilize okara still produce secondary waste. Therefore, new methodologies that do not produce secondary waste will have to be developed.

2.5. Dietary Fibre

There are numerous different definitions of dietary fibre based on different organizations and associations. However, in general, it can be defined as the edible parts of the plant that are resistant to digestion and absorption in the human small intestines with partial or complete fermentation in the large intestines. The most widely accepted classification of dietary fibre is based on their solubility and gut fermentability. Thus, dietary fibre is most appropriately classified into water soluble/well fermented fibres and water insoluble/less fermented fibres. Water soluble/well fermented fibres include

pectin, gums and mucilages while water insoluble/less fermented fibres include cellulose, hemicellulose and lignin (Dhingra, Michael, Rajput, & Patil, 2012).

By nature, plants contain a mixture of soluble dietary fibres (SDF) and IDF. It is in our interest to convert more IDF into SDF so as to increase the bioaccessibility of metabolites such as amino acids and fatty acids in the small intestines. There are several ways to achieve this conversion, namely, heat, chemical and natural methods. Heating in general (simple processes such as cooking), changes the ratio of SDF to IDF, however such treatment might affect the quality of nutrients. Chemical methods include the use of commercial enzymes such as cellulases, which can effectively convert IDF to SDF. However, these enzymes are typically expensive and not suitable at a large scale. Natural methods involve the use of microorganisms that produce cellulases extracellularly such as *Bacillus subtilis* to ferment the plant products thereby converting IDF to SDF. Fermentation is a low-cost method that not only converts the dietary fibres, but also increases the nutritional value through the actions of other extracellular enzymes such as amylase, protease and lipase.

Dietary fibres also contain numerous health benefits when included in diets. Consumption of food rich in dietary fibres have been shown to reduce incidence of several diseases due to its beneficial properties such as the addition of bulk to faeces which improves bowel functions, decreasing the time of intestinal transit which has been found to reduce occurrence of bowel cancer as well as controlling cholesterol and glycaemic levels (Graham, Dayal, Swanson, Mittelman, & Wilkinson, 1978). Dietary fibres are also known to trap mutagenic and carcinogenic agents on top of stimulating the proliferation of the gut microbiota (Beecher, 1999).

2.6. Amino Acids

Amino acids are defined as organic materials that contain both amino and acid groups. Apart from glycine, all amino acids have an asymmetrical carbon and also exhibit optical activity. Similarly, other than proline, all amino acids have a primary amino and a carboxyl group bounded to the α-carbon atom. Amongst the hundreds of natural amino acids, only 20 of them serve as building blocks of proteins. Of these, some are classified as essential amino acids while others are classified as non-essentials. Essential amino acids are defined as those where the carbon skeletons cannot be adequately synthesized de novo by the body and have to be supplemented from diets to meet optimal requirements (Table 2.1). Non-essential amino acids are those that can be synthesized de novo in sufficient quantity by the body to meet ideal requirements (Guoyao Wu, 2009).

Amino acids are very important nutrients for the maintenance of health. Apart from being just building blocks of proteins and polypeptides, there are increasing scientific evidences, which suggest that amino acids also play important roles in regulating metabolic pathways that are essential for maintenance, growth, reproduction and immunity. For instance, Kilberg, Pan, Chen, and Leung-Pineda (2005) and Palii et al. (2009) reported that deficiency in either essential or non-essential amino acids can result in an increase in availability of uncharged transfer ribonucleic acid (tRNA) which binds and activates the general control non-derepressible protein 2 (GCN2) kinase. Enhanced activation of GCN2 will increase phosphorylation of eukaryotic translation initiation factor, which would in turn lead to a decrease in global protein synthesis. Amino acids have also been linked to immunity by regulating the expression of T lymphocytes, B lymphocytes, macrophages as well as natural killer cells (P. Li, Yin, Li, Woo Kim, & Wu, 2007).

Table 2.1. List of essential and non-essential amino acids for mammals. Information adapted from Guoyao Wu (2009)

Amino Acids for Mammals	
Essential Amino Acids	Non-Essential Amino Acids
Arginine[a]	Alanine
Histidine	Asparagine
Isoleucine	Aspartate
Leucine	Cysteine
Lysine	Glutamate
Methionine	Glutamine
Phenylalanine	Glycine
Threonine	Proline
Tryptophan	Serine
Valine	Taurine
	Tyrosine

[a] Arginine is an essential amino acids for young mammals. It may not be required to be supplemented through diets in adults

2.7. Fatty Acids

Fatty acids are carboxylic acids with a long aliphatic chain. They are typically segregated into saturated and unsaturated fatty acids. Typically, unsaturated fatty acids are known as the "good" fats while saturated fatty acids are known as the "bad" fats. This is due to the numerous purported health benefits of unsaturated fatty acids while saturated fatty acids are known to be detrimental to health. Studies have found that increased intake of unsaturated fatty acids is linked to a reduced risk of cardiovascular diseases (CVD). Elevated levels of plasma low-density lipoproteins (LDL) cholesterol are linked to increased risk of CVD. Replacing saturated fatty acids with mono- or polyunsaturated fatty acids reduces LDL cholesterol, which would reduce risk of CVD. At the same time, unsaturated fatty acids also increase high-density lipoprotein (HDL)

cholesterol, which assists in the removal of triacylglycerol from the bloodstream. High levels of triacylglycerol in the blood are also known to increase risk of CVD (Lunn & Theobald, 2006).

Another class of fatty acids that are beneficial to health are the short-chain fatty acids (SCFA). SCFA are fatty acids that contain lesser than 6 carbon atoms. They are the primary products of fermentation of dietary fibres by the gut microbiota (Morrison & Preston, 2016). The discovery of receptors in many cells and tissues of which SCFA are natural ligands have given rise to interest in SCFA as signalling ligands between the gut microbiota and the host. SCFA have been linked to regulatory roles in the maintenance of gut integrity, glucose homeostasis, lipid metabolism, appetite regulation as well as immune functions (Morrison & Preston, 2016).

2.8. Phenolic Compounds

Phenolic compounds are found throughout the plant kingdom with up to 8000 different phenolic structures. Their structure contain at least one aromatic ring with one or more hydroxyl group attached (Carocho & C.F.R. Ferreira, 2013). Typical synthesis of phenolic compounds involve the shikimate pathways, phenilpropanoid pathways and flavonoid pathways (Crozier, Clifford, & Ashihara, 2006). There are various groups within the phenolic compound family (Figure 2.3) with flavonoids constituting the largest group. Variations in the substitution patterns to the aromatic ring results in different flavonoid classes such as flavonols, flavones, isoflavones, flavanones, flavanols and anthocyanidins. Phenolic acids are another important class of phenolic compounds. They can be separated into 2 classes, hydroxybenzoic acids and hydroxycinnamic acids. The most commonly found hydroxybenzoic acids are gallic, p-hydroxybenzoic, syringic, vanillic and protocatechuic acids while the most common

hydroxycinnamic acids are ferulic, sinapic, p-coumaric and caffeic acids (S. Martins et al., 2011).

In recent years, phenolic compounds have garnered great attention from the scientific community due to their numerous health benefits such as the reduction in risk of cancer, diabetes, CVD as well as their antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory and antimicrobial properties (S. Martins et al., 2011). Perhaps, the most well-known property of phenolic compounds is their ability to scavenge free radicals in the body so as to reduce cellular damage, which in turn can potentially reduce the risk of cancer (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007).

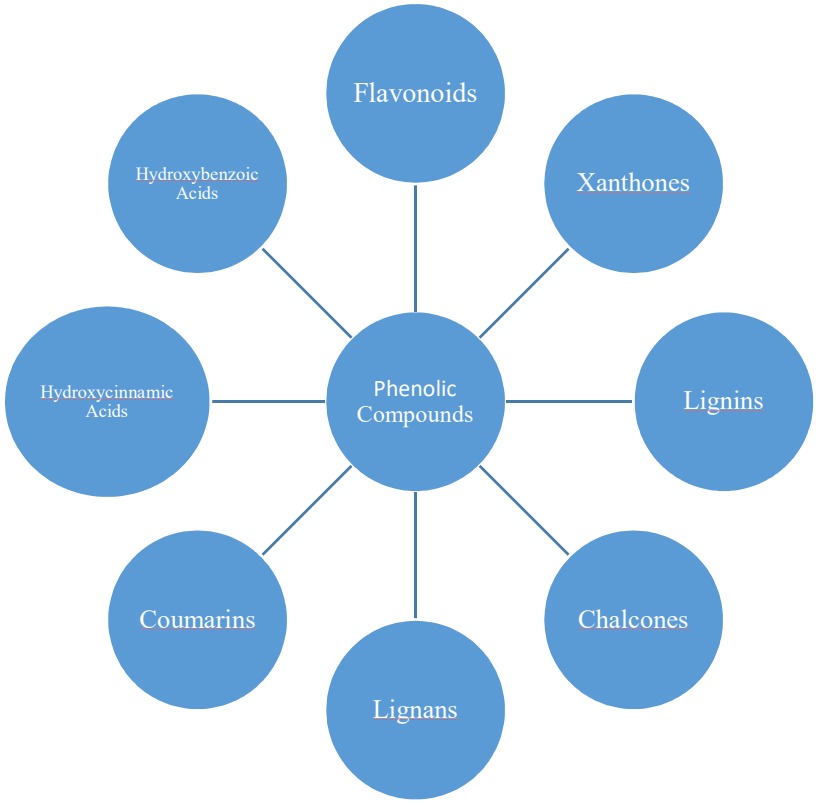


Figure 2.3. Shows the different groups within the phenolic compound family

2.9. Menaquinone-7 (MK-7)

There are 2 major forms of Vitamin K, namely, Vitamin K1 and K2. Vitamin K1 is also known as phylloquinone, phytomenadione or phytonadione. Vitamin K1 is naturally produced by plants and readily available in leafy green vegetables. On the other hand, MK-7 (a natural form of Vitamin K2) can only be produced by microorganisms and therefore is usually only available in fermented food. Hence, it is much easier to meet the dietary requirements of Vitamin K1 compared to Vitamin K2 (Mahdinia, Demirci, & Berenjian, 2016).

MK-7 is most well-known for its effects against osteoporosis and is therefore especially important for the elderly (Beulens et al., 2013). The most abundant and well-known source of MK-7 is *Natto*, a japanese fermented soybean dish with approximately 1103.4 µg/100 g. After which the next best source of MK-7 are the cheeses where the amount ranges from about 10 µg/100 g to 75 µg/100 g (Schurgers & Vermeer, 2000) . Although *Natto* provides abundant amount of MK-7, its unique taste, smell and slimy texture might prove to be unpalatable for many people.

MK-7 is typically produced through either SSF or SLF of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *lactococcus lactis* ssp. *cremoris* or *Lactococcus lactis*.

2.10. Fermentation

Fermentation is an age-old technique that has been used since ancient times in the production of food. Some examples include the fermentation of rice by *Aspergillus oryzae* during koji fermentation process, *penicillium roquefortii* for cheese production, beer brewing, vinegar and *Natto*. Typical microorganisms involved in food fermentation include lactic acid bacteria such as *Lactobacillus* sp., *Bacillus* sp., *Pseudomonas* sp.,

Streptococcus sp., *Saccharomyces cerevisiae, Rhizopus* sp, *Aspergillus* sp and *Mucor* sp. SSF and SLF are 2 of the most commonly used techniques in the fermentation of food.

Over the past decade, SSF had received increasing attention from the scientific community due to its ability to produce higher yield and better product characteristics compared to SLF (Couto & Sanromán, 2006). Castilho, Polato, Baruque, Sant'Anna, and Freire (2000) conducted a detailed economic analysis of lipase production by *Penicillium restrictum* in SSF and SLF and found that for a production scale of 100 m^3, total capital investment required for SLF is 78% higher than SSF. On top of that, unitary production cost of SSF is also 47% lower than the selling price. The solid substrate in SSF serves as both the support and source of nutrient, which would help to lower the cost of production since there would be no need for the addition of commercial medium.

However, although SSF certainly has many advantages in its usage during food production, one of its biggest drawbacks is the difficulty in scaling up due to the problems of temperature gradient. Since solid substrates are porous, there exist countless numbers of air pockets within the substrates, which would hinder heat transfer. This is because, the heat transfer coefficient of air is lower than liquid, and hence there are both axial and radial temperature gradients in SSF. In other words, temperature nearer to the core of the substrate would be higher compared to the temperature nearer to the reactor wall. Similarly, since most SSF reactors involve a static bed aerated by humidified air from the bottom, temperature at the top of the reactor would be much higher than the inlet air temperature. Such disparity in temperatures throughout the reactor can severely affect yield. Furthermore, since most microorganisms used in SSF grow optimally at temperatures between 30 °C to 37 °C, a temperature runaway could stop fermentation prematurely and affect yield (Ashley, Mitchell, & Howes, 1999).

2.11. Gut Microbiota

The large intestines in humans are home to hundreds of bacterial species that are collectively known as the gut microbiota. The dominant bacterial phyla in healthy adult humans are known to be Firmicutes, Bacteroidetes and Actinobacteria. Although the gut microbiota is generally considered to be stable, certain factors can effect a temporary change in the composition. Such factors include acquired species at birth, genetics, immunological factors, age, antibiotic usage and diet (Scott, Gratz, Sheridan, Flint, & Duncan, 2013).

Diet plays an important role in the composition of the gut microbiota and can greatly affect factors such as transit time and pH. For instance, Agus et al. (2016) reported that western diets, which are high in fat and sugar can aggravate the inflammatory process in Crohn's disease. Studies carried out on mice models showed that a high fat/high sugar diet created specific inflammatory environment in the gut that is correlated with intestinal mucosa dysbiosis, which is characterized by an increase in pro-inflammatory Proteobacteria, a decrease in protective bacteria as well as a decrease in SCFA concentrations.

On the other hand, consuming diets with higher amount of functional ingredients such as amino and fatty acids, probiotics and prebiotic properties can exert beneficial effects on the hosts. Lin et al. (2014) postulated that the benefits of probiotics lie in their capacity to produce vitamins, antioxidants and defensins against pathogens. Prebiotics restore the balance of the gut microbiota by promoting the growth of beneficial gut bacteria while stunting the growth of pathogenic bacteria.

2.12. Summary and Research Gap

Rapid urbanization and industrialization taxes on the world's natural resources, which can affect global food security. Singapore, with its lack of land space and natural resources, has adopted various strategies to enhance its food security and should be regarded as a good case study for the world in embracing the use of technologies to enhance self-production of food.

With that in mind, Singapore has adopted a 3-pronged approach towards enhancing its food security, namely, urban farming, processing technology and alternative food sources. Processing technology, which encompasses food waste valorisation is a viable method in waste management and can directly enhance food security through its reintroduction back into the food chain. It is gaining increasing attention from the scientific community and industrial players and can be employed to produce valuable materials without consuming the depleting natural resources. However, most current methods and applications of food waste valorisation still produce secondary waste, which are again disposed and contribute to the problems of climate change. Therefore, methods of utilizing food wastes without the production of secondary wastes have to be developed. One possible strategy is to valorise food waste and use them as food ingredients.

Okara is an underutilized biomaterial that is rich in dietary fibres and nutrients. It is generated as a food-processing residue in the soybean industry. It is also produced in large quantities especially in Asian countries but is not utilized in any significant applications apart from animal feed. The bulk of it is disposed of in incineration plants and landfills. As a result of the inherent richness in nutrients, okara is an ideal and appealing raw material to be used in waste valorisation to produce valuable compounds or be used as food ingredients. However, okara is a fibrous material with high amount

of IDF such as cellulose, hemicellulose and lignin. These fibres can impede the release of nutrient through physical obstructions or hydrogen bondings. Microbial fermentation using strains that produces extracellular enzymes such as cellulases, amylases, proteases and lipases is a cost-effective method of increasing the bioaccessibility of nutrients in okara.

SSF is generally cheaper and produces higher yield than SLF, however upscaling would be challenging due to temperature gradient issue. Therefore, SLF was also explored to provide a low-cost alternative method that is scalable. Most of the studies investigating the potential of fermented okara as a functional food or food ingredients involved the quantification of metabolites such as amino and fatty acids as well as other compounds like polyphenols after fermentation. However, their effects on health and bioaccessibility were not accessed. There are also no previous studies on the prebiotic effects of fermented okara on the gut microbiota.

Therefore, this thesis would focus on the use of processing technology on okara as a potential way to enhance food security while reducing food wastage. The proposed strategy is to employ SSF to valorise okara and evaluate its nutritional profile, potential health benefits and feasibility through an *in vitro* digestion and fermentation model. Subsequently, SLF of okara would also be explored to expand its application as well as to overcome the various problems currently present in SSF. These techniques would also not produce secondary waste, which would be beneficial to the climate.

3. A Metabolomic Approach to Understand the Solid-State Fermentation of Okara Using *Bacillus subtilis* WX-17 for Enhanced Nutritional Profile

Abstract

This chapter would look into the characterization of okara to understand its nutritional profile after fermentation. Okara is a major agro-waste produced from the soybean industry. To hydrolyse the okara and enable nutrient release, a strategy to valorise okara using solid-state fermentation with food grade *B. subtilis* WX-17 was carried out. The study showed that fermentation of okara with *B. subtilis* WX-17 improved its overall nutritional content. The total amino acids content increased from 3.04 ± 0.14 mg/g in unfermented okara to 5.41 ± 1.21 mg/g in okara fermented with *B. subtilis* WX-17. Total fatty acids content increased from 153.04 ± 5.10 mg/g okara to 166.78 ± 2.41 mg/g okara, after fermentation. Menaquinone-7 levels also increased from less than 0.0125 mg/100g to 0.382 mg/100g after fermentation. Total phenolic content and antioxidant content (DPPH) increased by 4.09 and 6.4 times respectively after fermentation. To gain an insight into the mechanism, gas chromatography mass spectrometry analysis was carried out. The decrease in carbohydrate metabolites showed that glycolysis was upregulated. This would have provided the energy and metabolic flux towards the amino acid and fatty acid pathways. This is in line with the increased amino acids and fatty acids production seen in okara fermented with *B. subtilis* WX-17. The findings of this work demonstrated the potential of using *B. subtilis* WX-17 fermentation, to enhance the nutritional profile of okara. This could serve as a potential low-cost food ingredient that can be incorporated into the human diet.

3.1. Introduction

Okara, also known as soy pulp is a major agro-waste produced from the soybean industry, which produces soymilk and bean curd. Approximately 1.1 kg of okara is produced from 1 kg of soybean (O'Toole, 1999). Okara is highly nutritious. It contains approximately 50% fiber, 25% protein, 10% lipid as well as a myriad of other high-value compounds such as isoflavones, coumestans, saponins, phytosterols, lignins and phytates. These compounds have been shown to exhibit numerous physiological and therapeutic functions such as the prevention of CVD in humans. However, a large amount of okara is being disposed of in landfills and incineration plants annually due to its unpalatable and insoluble nature (B. Li, Qiao, et al., 2012). It is estimated that around 14 million tonnes of okara are generated worldwide annually. The countries include Japan, Korea, China and Singapore, which contribute 800,000 tonnes, 310,000 tonnes, 2.8 million tonnes and 11,000 tonnes respectively (B. Li, Qiao, et al., 2012; Seong Choi, Gyu Kim, Kyun Jung, & Bae, 2015).

To utilize the highly nutritive compounds in okara, pre-treatment is required to release the nutrients from the insoluble okara. Previous studies have shown that enzymes secreted by microorganisms during fermentation can hydrolyse complex macromolecules such as fats, proteins and fibres into smaller and more soluble nutrients. It also reduces the amount of antinutritional factors present in okara including trypsin, phytic acid, lectin and tannin (Paredes-Lopez & Harry, 1989). Various microorganisms such as *Aspergillus sp., Aspergillus niger* and *Aspergillus ficuum* had been studied for their ability to produce phytases which inhibited the antinutritional factor phytate. This had been shown to reduce the bioavailability of calcium, zinc and iron (Pandey, Szakacs, Soccol, A. Rodriguez-Leon, & Thomaz-Soccol, 2001; Schlemmer, Frolich, Prieto, & Grases, 2009).

SSF is the culture of microorganism using solid substrate in the absence of liquid to produce desirable products. SSF has been shown to be effective in enhancing the nutritional content of a complex substrate. For example, a recent study showed that fruit and vegetable wastes that underwent SSF using *Aspergillus niger* and *Rhizopus oryzae* increased in succinic acid which is an important metabolite in the tricarboxylic acid (TCA) cycle and have numerous health benefits such as antioxidant properties and strengthening of the immune system (Dessie et al., 2018; Saif & Fumio Hashinaga, 2005). This is because microorganism secretes abundant amount of enzymes, which catabolize complex macromolecules into simpler forms, leading to increased amino acid and antioxidants. Bacteria, yeast and fungi are commonly used in SSF. *Rhizopus*, *Lactobacillus, Streptococcus, Aspergillus and Bacillus* are some of the most common microorganism used in SSF of food material (Hesseltine, 1987).

It is considerably cheaper and more environmentally friendly as compared to the more commonly used SLF. Some of the advantages of SSF over SLF include reduced probability of contamination due to lack of moisture and simple media composition since most nutrients are in the solid substrate. SSF also allows the use of simple reactor design due to the concentrated nature of solid substrates (Mienda & Idi, 2011).

In recent years, SSF has received increasing amount of attention from researchers and industrial players since several studies performed on colourings, flavourings, additives and other desirable products for the food industry had shown that SSF can achieve higher yield compared to SLF (Rodriguez-Couto & Sanromán, 2006).

B. subtilis is a microorganism of interest for fermentation of okara, due to its ability to secrete enzymes, which can break down the macromolecules in okara, as well as the ability to increase antioxidant activity. The objective of this study is to investigate

the effects of SSF on okara using a strain of food grade *B. subtilis* WX-17, which was isolated in this study, from *Natto*. Then, an untargeted metabolomic approach using GC-MS was carried out to analyse the value-added products produced in okara fermented with *B. subtilis* WX-17. Menaquinone-7 analysis and quantification were outsourced to Eurofins Scientific. The mapping of metabolites unto the metabolomic pathways would also provide an important insight into the mechanisms behind SSF of okara with *B. subtilis* WX-17. Till date, based on our knowledge, there exists a gap in the utilization of bacteria in valorising okara from a metabolomic perspective. The findings of this study could open up the possibility of using fermented okara as a low-cost food ingredient that can contribute towards alleviating the global food security issue.

3.2. Materials and Methods

3.2.1. Chemicals

Glycerol, nutrient broth, methanol, ribitol, methoxamine hydrochloride (MOX), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), sodium chloride, acetic acid, heptadecanoic acid, ethanol, chloroform, BF3-methanol, hexane, γ-aminobutyric acid, dimethylformamide (DMF), 1-1,-diphenyl-2-picryl-hydrazil (DPPH).

3.2.2. Microorganism

B. subtilis WX-17 was isolated from Marumiya Kyushu Ichiban *Natto*. This strain has been deposited in NCIMB with the accession number NCIMB 15204. The isolation was carried out according to C.-H. Liu, Chiu, Ho, and Wang (2009) with some modification. The protocol began by adding 20 mL of sterile water to 3 *Natto* beans in a falcon tube and vortexed for 5 mins to extract the microorganism. The cell suspension

was serial diluted and plated onto nutrient agar plates and incubated at 37˚C for 24 hours. A single colony was inoculated into 5 mL of nutrient broth, incubated at 37˚C for 24 hours, and subsequently stored in aliquots containing 50% glycerol at -80˚C.

3.2.3. Bacterium Identification

Bacterium identification was performed according to C.-H. Liu et al. (2009) with some modifications. A single colony was inoculated in 5 mL of nutrient broth and incubated overnight. The bacterial deoxyribonucleic acid (DNA) was then isolated using Bio Basic EZ-10 Spin Column Fungal Genomic DNA Mini-Prep Kit. Next, polymerase chain reaction (PCR) was carried out to amplify the 16S rDNA gene of the bacteria using the forward and reverse primer 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') respectively. PCR was performed with the following parameters: 35 cycles at 98°C 10 seconds for denaturation, 55°C 5 seconds for annealing, 72°C 2 mins for elongation and 68°C 10 mins for extension followed by cooling to 4°C. Gel electrophoresis was then carried out on the PCR sample to remove other contaminants and the DNA purified using QIAquick Gel Extraction Kit (250) before sequencing. 16s rRNA sequencing (Genbank accession number MK559744) was outsourced to Bio Basic Asia Pacific Pte Ltd using Sanger dideoxy sequencing technology. The obtained 16s rRNA sequence (GenBank accession number MK559744) was then compared with other 16s rRNA sequences using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.4. Source of Okara

Fresh okara samples were kindly provided by Vitasoy International Singapore Pte Ltd, Singapore. Okara was separated into aliquots, sealed in airtight polyethylene bags, and stored at -20˚C in the dark.

3.2.5. Fermentation

B. subtilis WX-17 was inoculated into 5 mL of nutrient broth and incubated at 37˚C for 24 hours, which served as the stock culture. Ten g of okara was inoculated with *B. subtilis* WX-17 at a concentration of 10^6 CFU/g of okara in a petri dish. The petri dish was then covered with 2 layers of cling film. The first layer was pressed onto the inoculated okara and the second layer was wrapped across the surface of the petri dish. Both layers were punctured with numerous holes using a sterile pin to maintain aeration and moisture content within the petri dish and subsequently fermentation was carried out at 37˚C for 72 hours. A beaker of water was placed in the incubator to maintain the moisture content. Fermentation was carried out in triplicates. The fermented okara was then freeze-dried and stored at -20˚C until further analysis.

3.2.6. Samples Preparation for Metabolomics, Fatty and Amino Acids Analyses

For the metabolomic analysis, 3 mL of methanol was added to 900 mg of fermented okara and fresh okara (control) respectively. The samples were then homogenized using Fastprep-24™ 5G Homogenizer. Homogenizing was carried out for 30 seconds at 5 mins interval for 5 times. The tubes were placed in a box of ice after each homogenizing cycle to cool the samples. Next, the samples were centrifuged at 9000 g for 10 mins at 4˚C. The supernatant was extracted and filtered through a 0.22 µm filter. Ten µL of ribitol (2 mg/mL) was added into 1.5 mL of filtered supernatant as the internal standard (IS). Samples were then vortexed for 30 seconds and allowed to dry in a heat block at 30˚C, overnight. Then, 100 µL of MOX (20 mg/mL pyridine) was added to the lyophilized samples for methoximation to protect the carbonyls and incubated at 37˚C for 60 mins. Next, silylation was carried out by adding 200 µL of MSTFA with 1% TMCS and subsequently incubated at 70˚C for 30 mins. The samples

were then centrifuged for 30 mins at 15330 g and 150 µL of supernatant was transferred to glass vials and sent for GC-MS analysis.

For fatty acids analysis, 10 mg of fermented okara and 10 mg of fresh okara (control) were weighed and placed into eppendorf tubes. One thousand µL of 0.9% NaCl solution and 200 µL of acetic acid were then added. Ten µL of 10 mg/ml heptadecanoic acid dissolved in ethanol was added to the extraction solvent to serve as IS. The solvents were then homogenized as described above. Then, 3 mL of a chloroform-methanol 2:1 mixture was added, and the samples were inverted several times, vortexed vigorously for 5 mins, and centrifuged at 10000 g for 10 min at 4°C. The chloroform layer (bottom, 1 mL) was collected and dried overnight at 30°C. The dried lipid residue was re-dissolved in 500 µL BF3-methanol 10% (FLUKA, 15716) and incubated in a sealed vial in a 95°C heater for 20 min. Fatty acid methyl esters (FAME) were extracted with 300µL n-hexane after the addition of 300 µL saturated NaCl in water. Samples were vortexed for 5 mins and centrifuged at 14800 rpm for 5 mins. Finally, 150 µL of sample (top layer) was transferred into glass vials for GC-MS analysis.

For amino acids analysis, 4 mg of fermented okara and 4 mg of fresh okara (control) were resuspended in 200µL of 6 M HCl. Twenty µL of γ – aminobutyric acid (10 mg/mL) was added as IS. The tubes were sealed and baked for 24 hours in an oven at 105°C. The cell hydrolysate was dried at 95°C in a heat block. After drying, 20 µL of DMF and 20 µL of MSTFA were added. The tubes were sealed and incubated at 85°C for 1 hour. Samples were then centrifuged at 14800 rpm for 5 mins and supernatant was transferred to glass vials. Forty µL of DMF was added into the glass vials and the vials were inverted a few times before sending for GC-MS analysis.

3.2.7. GC-MS Method for Metabolomics, Fatty and Amino Acids Analyses

Metabolomic analysis including carbohydrates and TCA cycle metabolites was carried out via GC-MS. The GC-MS system (Agilent Technologies 7890A-5975C) was equipped with a HP-5MS, 5% Phenyl-Methyl-Silox capillary column (30 m × 0.250 mm id.; 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA, USA). One μL of samples were injected into the system by the autosampler in splitless mode. The injector temperature and ion source temperature were set at 250 °C and 230 °C, respectively. The oven temperature was as follows: 75 °C for 4 min, ramped to 280 °C at the rate of 4 °C/min, and held at 280 °C for 2 mins. Data were acquired in full scan mode from 35 to 600 m/z with a 0.3 s of scan time. Metabolites were identified using the NIST08 mass spectral library based on mass spectral similarity. Samples were normalized with respect to the IS, ribitol, before comparison.

For fatty acid analysis, the injector temperature and ion source temperature were set at 250 °C and 230 °C, respectively. The oven temperature was as follows: 80 °C for 1 min, ramped to 250 °C at the rate of 7 °C/min, and held at 250 °C for 8 mins. Data were acquired in full scan mode from 50 to 600 m/z at 2.66 scans per second. Metabolites were identified using the NIST08 mass spectral library based on mass spectral similarity. Samples were normalized with respect to the IS, heptadecanoic acid before comparison.

For amino acid analysis, solvent delay was set at 2 mins 30 seconds. The injector temperature and ion source temperature were set at 250 °C and 230 °C, respectively. The oven temperature was as follows: 160 °C for 1 min, ramped to 290 °C at the rate of 20 °C/min, ramped again to 310 at 20°C/min and held at 310 °C for 1 min. Data were acquired in full scan mode from 180 to 550 m/z at 3.85 scans per second. Metabolites were identified using the NIST08 mass spectral library based on mass

spectral similarity. Samples were normalized with respect to the IS, γ – aminobutyric acid before comparison.

3.2.8. Menaquinone-7

MK-7 analysis and quantifications were outsourced to Eurofins Scientific.

3.2.9. Total Phenolic Content Analysis

Analysis of total phenolic content was carried out according to the protocol described by Kamtekar, Keer, and Patil (2014). Briefly, 1 g of sample was added with 5 mL of deionized water, 0.5 mL of Folin Ciocalteu's reagent and shaken vigorously. After 5 mins, 1.5 mL of 20% sodium carbonate was added and made up to 10 mL before incubation for 2 hours. Gallic acid with different concentrations was used as standards for quantification. The absorbance of the mixture was measured at 750 mm with deionized water as blank using Nanodrop 2000c Spectrophotometer.

3.2.10. Antioxidants Analysis

Three hundred µL of ethanol was added to 100 mg of sample and homogenized as described above. The samples were then centrifuged at 10,000 rpm for 5 mins. Next, 150 µL of the supernatant was transferred to new tubes and added with 100 µL of DPPH solution and 250 µL of ethanol. The samples were then incubated in a dark place for 30 mins at room temperature. The absorbance of the mixture was measured at 515 nm with ethanol as blank using Nanodrop 2000c Spectrophotometer. The activities of the samples were evaluated with respect to trolox equivalent-% signal inhibition calibration curve whereby % signal inhibition is defined as: $\% \, Signal \, Inhibition = (1 - \frac{A_S}{A_o}) \text{ X } 100$. A_s is defined as the absorbance of the samples and A_o is defined as the absorbance of pure DPPH.

3.2.11. Statistical Analysis

All experiments were conducted in triplicates. Statistical analysis was carried out using MetaboAnalyst 4.0 (Xia, Mandal, Sinelnikov, Broadhurst, & S Wishart, 2012; Xia & S Wishart, 2011; Xia & S. Wishart, 2016). Data scaling was carried out using mean-centering and divided by the standard deviation of each variable prior to partial least squares discriminant analysis (PLS-DA) and heat map analysis. The heat map was also constructed using Euclidean distance measurement and ward clustering algorithm.

3.2.12. Nucleotide Sequence Accession Number

The 16s rRNA sequence of WX-17 was deposited in the GenBank database with the accession number MK559744.

3.2.13. Metabolic Pathway Analysis

The pathway analyses were performed with reference to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Minoru Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017; M. Kanehisa & Goto, 2000; Minoru Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016; Ogata et al., 1999).

3.3. Results

3.3.1. Metabolic Profiles of Fermented and Unfermented Okara

A metabolomics analysis using GC-MS provided an overview on the metabolic profiles between the fermented and unfermented okara. Statistical analysis (PLS-DA and heat map) was carried out to understand the changes between the samples. These changes observed in the level of each metabolite, helped to shed light on the effects of *B. subtilis* WX-17 fermentation on okara. In total, 49 metabolites were detected. Figure 3.1 showed the PLS-DA score plot of the fermented and unfermented

okara. The green and red highlights denoted the 95% confidence region. The first principal component accounted for 76.5% of the total variance while the second principal component accounted for 5.30% of the total variance, which combined to explain a total of 81.8% of the variance. This showed that the first component largely explained most of the variance between the samples. From the PLS-DA score plot (R^2 = 99.8% and Q^2 = 98.3%), clear and distinct separations between the unfermented okara and the fermented okara along the first principal component were observed. This showed that during fermentation, the metabolic profile of okara had changed significantly.

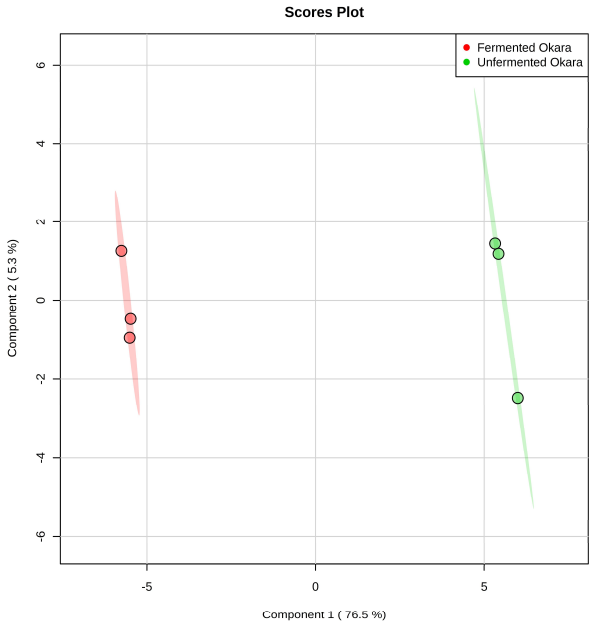


Figure 3.1. PLS-DA score plot of all metabolites found for fermented and unfermented okara. The green and red highlights denoted the 95% confidence region. Explained variance are shown in brackets

Although the PLS-DA score plot provided a visual representation of the difference in the metabolic profile between fermented and unfermented okara, it did not provide details about the specific metabolites. Hence, a clustering heat map was constructed to provide a visual breakdown of the metabolites that changed after

fermentation (Figure 3.2). From the heat map, unfermented okara had high amount of carbohydrates such as fructose, ribose, glucose, galactose, mannose and maltose. In comparison, the levels of carbohydrates were lower in fermented okara. In addition, fermented okara had higher amounts of amino and fatty acids as compared to unfermented okara. This suggested that *B. subtilis* WX-17 consumed the carbohydrates in okara for its growth and might have produced proteases and lipases to break down proteins and lipids in okara, into simpler amino acids and fatty acids (Lesuisse, Schanck, & Colson, 1993; Yang, Shih, Tzeng, & Wang, 2000a).

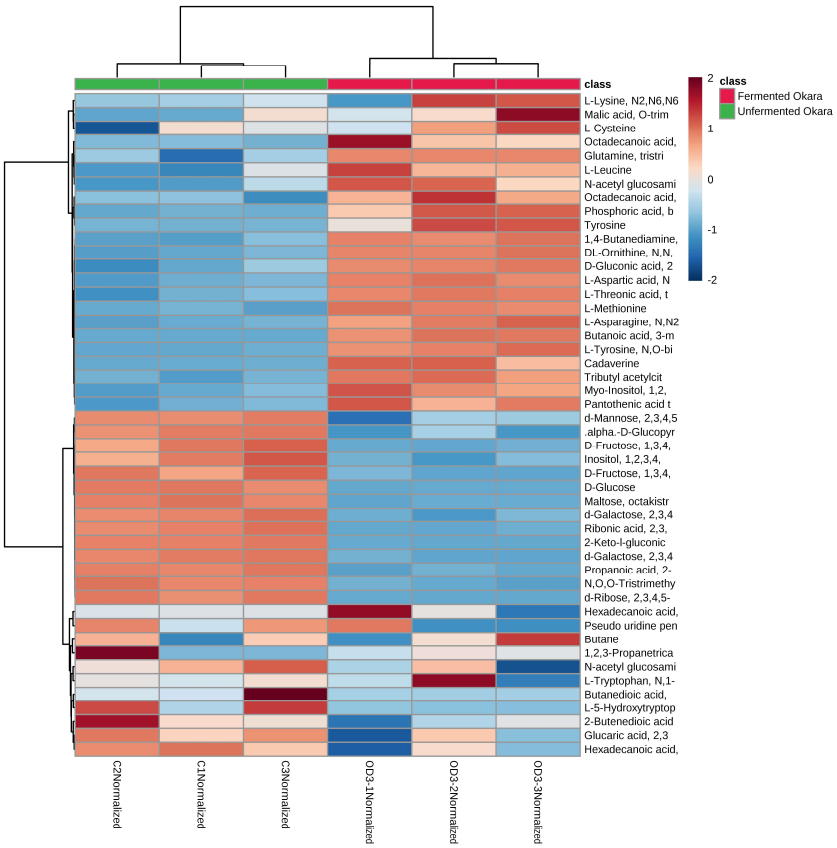


Figure 3.2. Heat map analysis correlating the metabolites of fermented and unfermented okara. Metabolites in brown are up regulated while those in blue are down regulated

3.3.2. Carbohydrates and TCA Cycle Metabolites

To better illustrate the effects of fermentation on fermented and unfermented okara, the results for carbohydrate metabolites and metabolites involved in the TCA cycle were presented in Figure 3.3 and 3.4 respectively. In total, 7 types of carbohydrates were detected. They were ribose, fructose, mannose, galactose, glucose, sucrose, and maltose. This result agreed with previous findings by B. Li, Lu, Nan, and Liu (2012) and Hou, Chen, Shi, Zhang, and Wang (2009) which showed that the polysaccharides in soybeans and okara were ribose, galactose, glucose, fructose, sucrose, mannose and other minor components such as verbascose, pinitol and myo-inositol. The abundance of all the different types of carbohydrates was reduced after fermentation (Figure 3.3).

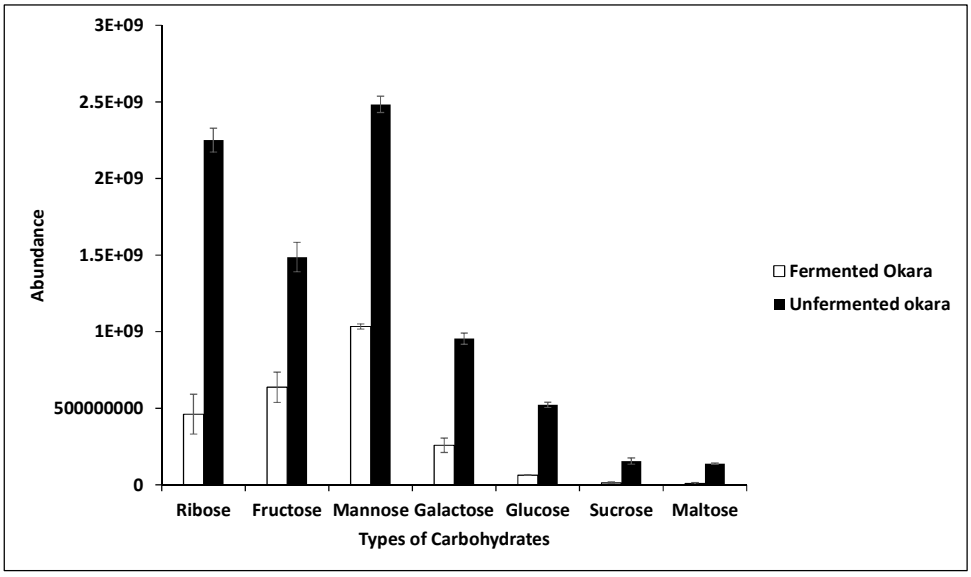


Figure 3.3. Abundance of all the carbohydrates detected during analysis

From the metabolomics analysis, it was shown that isocitric acid and malic acid increased while succinic acid and fumaric acid decreased (Figure 3.4). Most notably, isocitric acid increased by approximately 3 times. A study conducted by Markuszewski, Otsuka, Terabe, Matsuda, and Nishioka (2003) showed that isocitric acid levels in medium containing *B. subtilis* was higher than medium without the microorganism.

Recent studies have postulated on the potential benefits of isocitric acid. For example, M.E. Abdel-Salam et al. (2014) suggested that isocitric acid contains antioxidant properties that can help to combat oxidative stress of the brain and liver in mice by decreasing the brain lipid peroxidation and inflammation, liver damage as well as DNA fragmentation. Another study suggested that isocitric acid can help in combating hypoxia or hypoxic conditions such as fatigue, dizziness to more serious conditions such as hypercapnia and organ failure as it is the only metabolite in the TCA cycle that can unblock succinate dehydrogenate which would promote cell respiration even under stressful environment (Kamzolova et al., 2018).

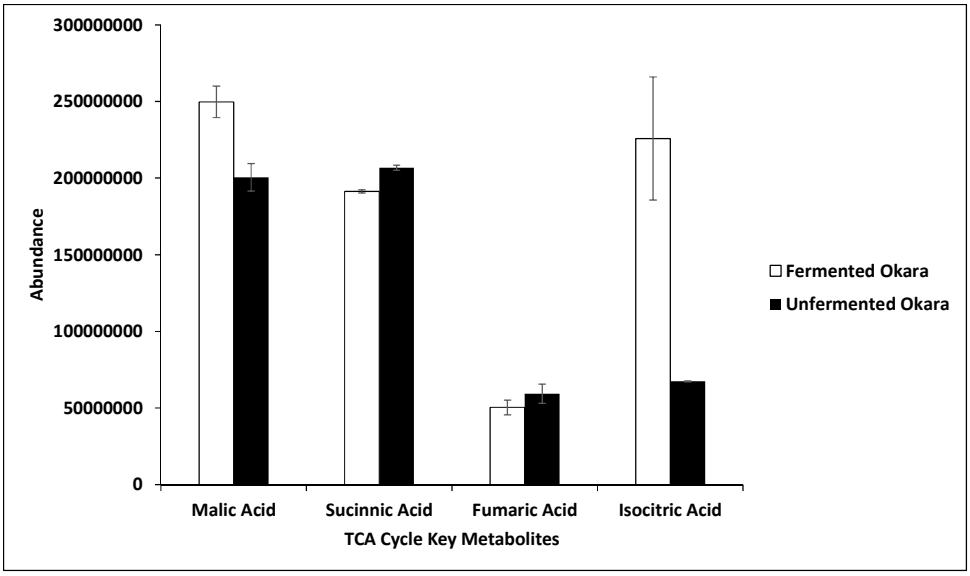


Figure 3.4. Abundance of all the TCA cycle key metabolites detected

3.3.3. Amino Acids and Fatty Acids Metabolites

From the heat map, it was shown that amino acids and fatty acids levels increased after fermentation. To reaffirm these findings, the amount of amino acids and fatty acids before and after fermentation was determined using GC-MS protocols, which are specific for amino and fatty acids. These specific protocols are more sensitive towards their respective target metabolites. Table 3.1 showed the absolute value of amino acids (mg/g okara) in fermented and unfermented okara. The results in Table 3.1

showed that all amino acids increased after fermentation with the total amount increasing by almost two-fold after fermentation. Notably, the essential amino acids leucine, phenylalanine and glutamic acid increased the most at 2.26, 2.42 and 2.12 times respectively.

Table 3.1. Changes in amino acids in absolute value (mg/g dried okara) for fermented and unfermented okara

mg/g okara	**Control**	**Fermented okara**
Glycine	0.183 ± 0.0441	0.329 ± 0.104
Valine	0.0228 ± 0.00291	0.0458 ± 0.00457
Proline	1.28 ± 0.442	2.15 ± 0.591
Leucine	0.303 ± 0.0684	0.685 ± 0.175
Serine	0.130 ± 0.0309	0.141 ± 0.0188
Threonine	0.138 ± 0.0391	0.151 ± 0.00157
Phenylalanine	0.0799 ± 0.0306	0.194 ± 0.0179
Aspartic Acid	0.200 ± 0.0703	0.292 ± 0.0278
Glutamic Acid	0.611 ± 0.0211	1.30 ± 0.182
Lysine	0.0694 ± 0.00989	0.0856 ± 0.0156
Tyrosine	0.0235 ± 0.00215	0.0439 ± 0.00352
Total Amino acids	3.04 ± 0.136	5.41 ± 1.21

Results are as *mean ± standard deviation (3 replicates)*

Fatty acids specific analysis suggested that there was negligible change in stearic and palmitic acids levels. However, linoleic and oleic acids levels were shown to increase by 2.93 and 2.37 times, respectively (Table 3.2).

Table 3.2. Changes in fatty acids in absolute value (mg/g dried okara) for fermented and unfermented okara

mg/g okara	Control	Fermented okara
Stearic Acid	60.3 ± 2.00	55.0 ± 5.24
Oleic Acid	3.39 ± 1.02	8.04 ± 2.87
Linoleic Acid	9.61 ± 3.31	28.2 ± 9.55
Palmitic Acid	79.8 ± 1.93	75.6 ± 5.37
Total Fatty acids	153 ± 5.09	166 ± 2.41

Results are as *mean ± standard deviation (3 replicates)*

3.3.4. Menaquinone-7

MK-7 was found to have increased significantly after fermentation by *B. subtilis* WX-17. Fermented okara contained 0.382 mg/100g of MK-7 while it is virtually undetected in unfermented okara with less than 0.0125 mg/100g (Figure 3.5).

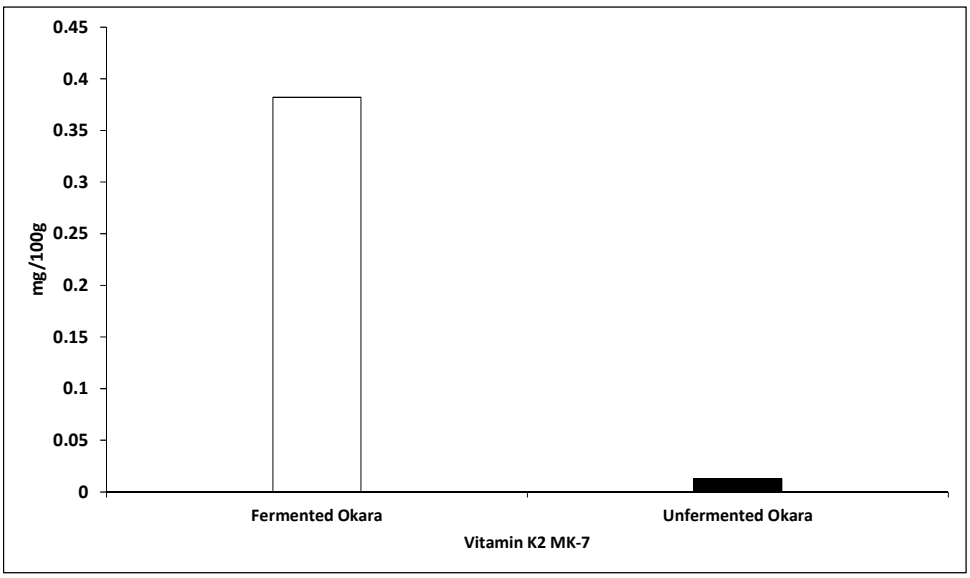


Figure 3.5. Amount of MK-7 detected in both fermented and unfermented okara (mg/100g)

3.3.5. Total Phenolic Content

Total phenolic content of fermented okara was found to have increased significantly compared to unfermented okara (Figure 3.6). Fermented okara contained 9.56 mg/g okara gallic acid equivalent (GAE) while unfermented okara contained 2.34/g okara GAE. This represented an increase in total phenolic content by 4.09 times.

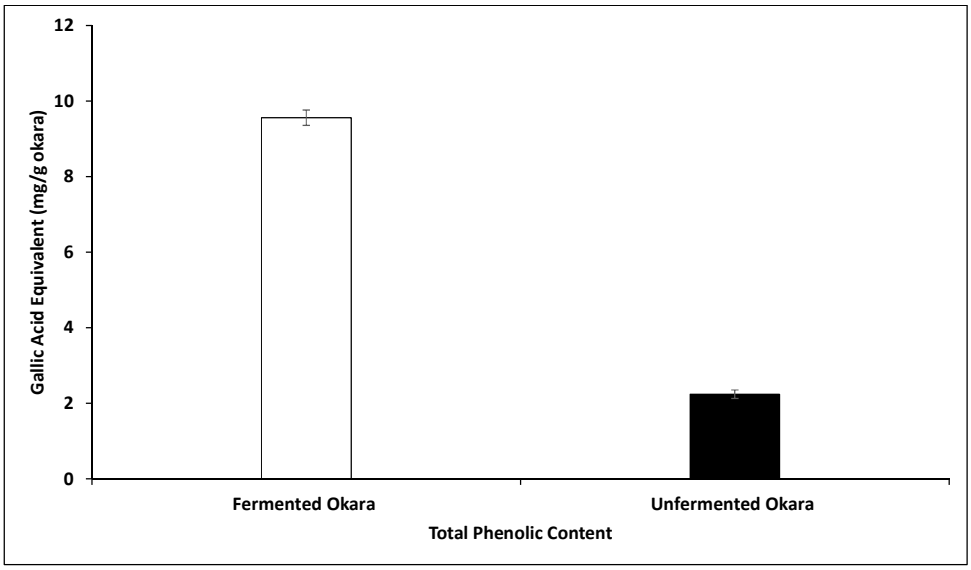


Figure 3.6. Total phenolic content of fermented and unfermented okara expressed in terms of gallic acid equivalent (mg/mL). Each data point represents the triplicate mean. Error bars represent standard deviation

3.3.6. Antioxidant Activity

The DPPH radical scavenging activity of fermented okara was shown to have increased during fermentation (Figure 3.7). Fermented okara showed the highest DPPH radical scavenging activity at 43.7 µg Trolox equivalent/g dried okara after 72 hours of fermentation. As compared to the DPPH radical scavenging activity of unfermented okara at 6.79 µg Trolox equivalent/g dried okara, fermented okara displayed an increased in DPPH radical scavenging activity by approximately 6.40 times.

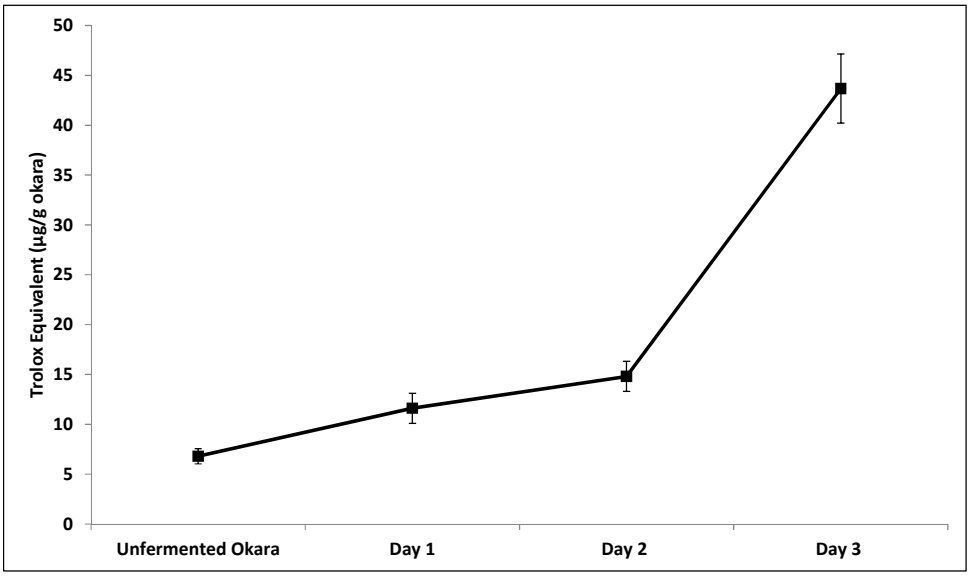


Figure 3.7. DPPH radical scavenging activity of fermented and unfermented okara across 72 hours expressed in terms of Trolox equivalent (µg/g okara)

3.4. Discussion

Metabolomics analysis showed that the overall metabolite profile of okara changed after *B. subtilis* WX-17 fermentation (Figure 3.1 and 3.2). Most of the carbohydrate metabolites decreased after fermentation. This indicated that they were being consumed by *B. subtilis* WX-17 which could utilize the carbohydrates through glycolysis, to produce increased amounts of the energy molecule acetyl-CoA. This precursor could then enter the amino acids and fatty acid pathways. This suggested that the microorganism was able to utilise the carbon source present within okara and use it for metabolism to produce other components. It had been suggested that glucose is the preferred carbon source for *B. subtilis* (Singh, Schmalisch, Stülke, & Görke, 2008; Tian, Fan, Liu, Xiangying, & Chen, 2015). In this study, analysis of the various sugar pathways suggested that most other forms of carbohydrate were converted into glucose before being used for other processes.

From the results, isocitric acid levels increased significantly after fermentation. This increase can be attributed to the actions of aconitase that catalyses the stereo-specific isomerization of citrate to isocitrate via cis-aconitate.

The increase in amino acids was confirmed as shown in Table 3.1. This could be due to *B. subtilis* producing extracellular proteases, which would breakdown the proteins in okara into amino acids thereby contributing to their increase after fermentation. The increase in amino acids is important as they can provide a rich source of nitrogen that is essential to living organisms. For example, yeasts such as *Rhodosporidium toruloides* and *Saccharomyces cerevisiae* require nitrogen for the synthesis of amino acids, proteins, DNA and ribonucleic acid (RNA) (Da Cruz, Cilli, & Ernandes, 2002; T. Evans & Ratledge, 1984).

Fatty acid levels also increased after okara fermentation (Table 3.2). Particularly, the increase in both linoleic acid and oleic acid levels, were desirable, as studies have reported various health benefits when these fatty acids were consumed. Linoleic acid is a polyunsaturated fatty acid that contains numerous purported health benefits such as anti-obesity, anti-carcinogenesis, anti-atherosclerosis, anti-diabetic, osteosynthetic and immunomodulation effects (Benjamin & Spener, 2009; Nagao & Yanagita, 2005). Likewise, oleic acid is a monounsaturated fatty acid that has been linked to a reduction in coronary heart disease due to its ability to reduce LDL-cholesterol, thrombogenicity, LDL-oxidative susceptibility as well as insulin sensitivity factors (Lopez-Huertas, 2010). The increase in linoleic acid and oleic acid was expected as *B. subtilis* is known to produce lipases that catalyse the hydrolysis of fatty acids (Ma et al., 2006; Sánchez, Prim, Rández–Gil, Pastor, & Diaz, 2002).

B. subtilis is well known to produce MK-7, which is an important nutrient with respect to bone health. An *in vitro* study conducted by Yamaguchi, Sugimoto, and

Hachiya (2001) concluded that MK-7 has an anabolic effect on bone tissue and osteoblastic cells which can stimulate osteoblastic bone growth. Okara that is fermented by *B. subtilis* WX-17 contained more than 30.6 times more MK-7 compared to unfermented okara (Figure 3.5). MK-7 can be converted from glucose through the glycolysis pathway, which generates the intermediate PEP. PEP then enters the shikimate pathway, which produces isochorismate through the addition of succinic semialdehyde-TPP anion derived from 2-ketoglutarate resulting in the formation of 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid (SEPHCHC). In the subsequent reaction, the pyruvoyl group is eliminated resulting in the prearomatic compound 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC). This is then aromatized to a benzenoid aromatic acid and used as the framework for the construction of the rest of the molecule.

Endogenous metabolic processes or exogenous chemicals in food systems may generate free radicals, which can cause oxidative damages by oxidizing biomolecules resulting in tissue damage or even cell death. Numerous traditional fermented food such as miso, tempeh, sufu and douche have free radical scavenging ability (Zhu, Fan, Cheng, & Li, 2008) due to presence of large amount of phenolic compounds. In the same vein, soybean fermented with *B. subtilis* B2 have shown increased DPPH radical-scavenging activity (Juan & Chou, 2010). Therefore, it is of interest to analyse if *B. subtilis* WX-17 fermented okara displayed the same ability. In this regard, analysis showed that total phenolic content increased by 4.29 times (Figure 3.6) and antioxidant activity (Figure 3.7) increased by 6.40 times which strengthened the case of fermented okara as a potential functional food.

To better understand the metabolic flux during fermentation, a metabolic pathway analysis was performed that would allow a hypothetical insight into the various

metabolic pathways which were up regulated or down regulated during fermentation (Figure 3.8).

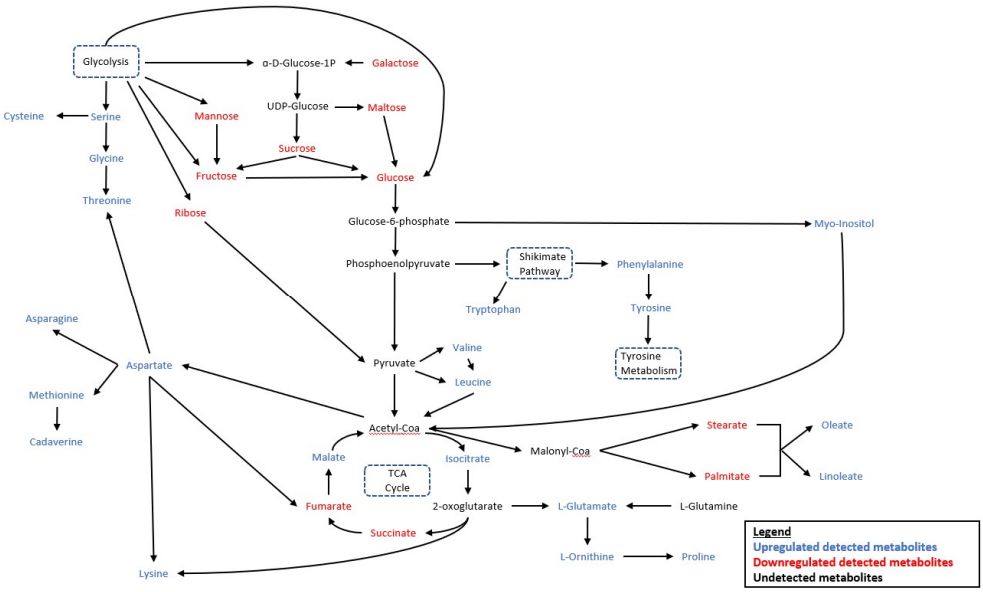


Figure 3.8. Metabolic pathway analysis of all detected carbohydrates, amino acids and fatty acids after fermentation of okara

The precursor for glycolysis is glucose. It is converted into pyruvate through the intermediate glucose 6-phosphate (G6P) and phosphoenolpyruvate (PEP). The analysis showed that apart from ribose, all other forms of carbohydrates could be converted into glucose. Mannose can be converted into fructose through the enzyme mannose isomerase in a reversible reaction. Fructose is in turn converted into glucose via xylose isomerase. Both mannose and fructose are also produced through glycolysis through the intermediates mannan and D-fructose-2-phosphate respectively. The overall decrease in both mannose and fructose suggested that the rate of consumption is greater than the rate of production.

Glycolysis also produces sucrose through the intermediate UDP-glucose and sucrose synthase. Sucrose can then be converted into fructose and glucose through sucrose alpha-glucosidase. UDP-glucose can also be converted to maltose via

maltodextrin, which is then converted to glucose through maltose phosphorylase. Galactose can be converted into UDP-glucose via UDP glucose pyrophosphorylase.

The KEGG database suggested no direct pathway for conversion of ribose to glucose. This suggested that the reduction in ribose after fermentation was due to direct consumption of ribose by *B. subtilis* WX-17. This was supported by studies which showed that *B. subtilis* exhibited increased sporulation when provided with a mixture of glucose and ribose as carbon source compared to a medium with glucose as the sole carbon source (Warriner & Waites, 1999).

One of the main intermediates of glycolysis, G6P can be converted into myo-inositol, a carboxylic sugar through myo-inositol 1-phosphatase (inositol phosphate metabolism pathway). During fermentation, *B. subtilis* WX-17 releases phytase that hydrolyses phytic acid, an antinutrient present in okara to produce myo-inositol, which might explain the increased amount of myo-inositol, detected after fermentation (Chen, Ye, Guo, Lv, & Yu, 2015; Kerovuo, Lauraeus, Nurminen, Kalkkinen, & Apajalahti, 1998). Myo-inositol can then be converted into acetyl-coA through malonate-semialdehyde dehydrogenase, which can then enter the TCA cycle (inositol phosphate metabolism pathway).

Another intermediate in glycolysis, PEP was involved in the shikimate pathway that produces tryptophan through a reversible reaction with (3-indole)-glycerolphosphate. The increased level of tryptophan detected suggested that the forward reaction (3-indole-glycerolphosphate to tryptophan) occurred at a higher rate than the backward reaction. Both phenylalanine and tyrosine are involved in the shikimate pathway as well and can be interconverted through prephenate, which is an intermediate in the shikimate pathway. Their increased levels after fermentation

suggested that reactions along the pathway are skewed towards phenylalanine and tyrosine metabolism rather than quinate.

Both valine and leucine, which are essential amino acids, are produced from the glycolysis intermediate, pyruvate (valine, leucine, isoleucine biosynthesis pathway). Valine is produced from the intermediate, 2-oxoisovalerate that can also be irreversibly converted into leucine. In addition, pyruvate can also be converted into leucine through pyruvate metabolism. Increased levels of valine and leucine suggested that their rate of synthesis was greater than their rate of degradation as leucine can be broken down into acetyl-coA. This implied that the bulk of the acetyl-coA was converted from pyruvate rather than leucine.

TCA cycle is a chain of reactions that are used by aerobic organisms to release stored energy in acetyl-coA through oxidation into adenosine triphosphate (ATP) and carbon dioxide. 4 of the key components in the TCA cycle were detected, of these both isocitrate and malate increased while fumarate and succinate decreased. Results also showed that isocitrate increased the most (3 times) after fermentation. This is in line with studies that had shown that *B. subtilis* produced the enzyme aconitate hydratase, which catalysed the stereo-specific isomerisation of citrate to isocitrate (Cox & Hanson, 1968; Dingman, Rosenkrantz, & Sonenshein, 1987). The large amount of isocitrate produced likely drove reactions forward to produce succinate and fumarate, which were consumed to produce malate.

The intermediates of the TCA cycle are involved in numerous reactions that produced important compounds. Isocitrate are broken down by the action of isocitrate dehydrogenase into 2-oxoglutarate which can then be converted into the non-essential amino acid, glutamate. Glutamate is in turn converted into ornithine via acetylornithine deacetylase, which is part of the urea cycle. Critically, ornithine cyclodeaminase

catalyses the conversion of ornithine to proline which is an essential amino acid (arginine and proline metabolism pathway). Glutamate, ornithine and proline were all upregulated after fermentation, which strengthened the hypothesis that isocitrate, was produced in excess. 2-oxoglutarate can also be converted into lysine through the lysine biosynthesis pathway.

From the pathway analysis, aspartate played a vital role in the synthesis of numerous important compounds. Firstly, in the lysine biosynthesis pathway, aspartate is converted into lysine through catalysis by diaminopimelate decarboxylase. Aspartate can also be directly converted into the TCA cycle intermediate, fumarate by aspartase (alanine, aspartate and glutamate metabolism pathway). Secondly, aspartate is also directly converted into asparagine, a non-essential amino acid by asparagine synthetase (alanine, aspartate and glutamate metabolism pathway). Thirdly, aspartate is also involved in the synthesis of the essential amino acid, methionine which had been linked with optimising the immune function of the human intestines (cysteine and methionine metabolism pathway)(Ruth & Field, 2013). Lastly, aspartate can be converted to threonine through threonine synthase (glycine, threonine and serine metabolism pathway). Threonine is also produced from the reversible reaction of glycine through catalysis by threonine aldolase (glycine, serine, threonine metabolism pathway). Glycine, another essential amino acid is in turn produced from the reversible reaction of serine through catalysis by glycine hydromethytransferase (glycine, serine, threonine metabolism pathway). In addition, serine can also be converted to cysteine through cysteine synthase (cysteine and methionine metabolism pathway).

It was also observed that all the amino acids detected were glucogenic amino acids, which mean that they can be converted into glucose through gluconeogenesis. This could explain why although all the amino acids were up regulated after

fermentation, the overall amount detected after fermentation were much lesser compared to fatty acids.

The metabolic pathways for the biosynthesis of fatty acids involved the reaction between acetyl-coA and malonyl-coA, which produces the saturated fatty acids, palmitate and stearate through the fatty acid biosynthesis pathway. Palmitate and stearate then underwent elongation and unsaturation process to yield oleate and linoleate (biosynthesis of unsaturated fatty acids pathway). Both palmitate and stearate were down regulated while oleate and linoleate were up regulated. This suggested that a larger proportion of the saturated fatty acids were converted into unsaturated fatty acids. The reduction in saturated fatty acids were desirable as it is well known that consumption of high amount of saturated fatty acids are associated with increased risk of coronary heart diseases (Zong et al., 2016).

Overall, from the metabolic pathway analysis (Fig. 3.8), it can be summarized that the amount of various carbohydrates in okara decreased. This suggested that carbohydrates were consumed and utilised by *B. subtilis* WX-17 through glycolysis, to produce energy for its metabolism and cellular processes. Subsequently, this led to the increased phenolic content, antioxidant amount, MK-7, amino acids and fatty acids in fermented okara.

In conclusion, with the world's population predicted to reach 9.2 billion by 2050, food security is becoming a rising global issue (Godfray et al., 2010). One strategy to combat this issue is the biovalorisation of food waste. With the growing popularity of soy-based products, the amount of okara produced is increasing rapidly (approximately 14 million tonnes globally every year). This work showed the possibility of reintroducing okara, a food waste back into the food chain through biovalorisation by fermentation. As the microorganism used is food grade, this study also presented the

potential application of fermented okara into human diets in various forms. The study revealed that fermentation of okara using food grade *B. subtilis* WX-17 enhanced its nutrient profile. GC-MS and metabolic pathway analysis showed that both amino acids and fatty acids production increased after fermentation due to the release of hydrolases by *B. subtilis* WX-17 to break down complex macromolecules into simpler molecules, which are easier to digest. Amount of MK-7 detected was also enhanced after fermentation. Furthermore, antioxidant analysis also showed that post fermentation, fermented okara displayed higher antioxidant activities as well as phenolic content. Future work will include characterising the enzymes produced by *B. subtilis* WX-17, such as nattokinase, as well as evaluating the bioaccessibility of nutrients, cytotoxicity and probiotic/prebiotic effects of fermented okara in the human body through *in vitro* models. In addition, it would be interesting to explore alternate methods of fermenting okara to broaden its applications as well as to enhance upscaling potential.

4. Evaluating the potential of *Bacillus subtilis* fermented okara as a functional food ingredient through in vitro digestion and fermentation

Abstract

While the previous chapter focused on the valorisation of okara, this chapter would look into its potential application as a food ingredient. In this study, *in vitro* digestion and fermentation was employed to simulate the consumption of fermented okara and hence, evaluate its potential as a functional food ingredient. This is so as to develop a method of utilizing okara without producing secondary waste. Fermentation increased amount of soluble dietary fibre by 187%. Bioaccessibility of amino acids, fatty acids and vitamin K2 MK-7 is higher in the digestion supernatant of fermented okara. *Bacillus subtilis* also remained viable after digestion. Erythrocyte haemolysis assay also showed that fermented okara is non-toxic to the human body. Supernatants of fermented okara exhibited higher bioaccessibility of total phenolic content and higher DPPH radical scavenging activity in the small and large intestines. Similarly, the concentrations of acetic acid, propionic acid and butyric acid were 44.4%, 46.9% and 51.9% higher respectively. The gut microbiota was also found to be different in the fermentation supernatants between fermented and unfermented okara. Results demonstrated the potential of fermented okara as a functional food ingredient.

4.1. Introduction

Okara is the pulp left behind during the food processing of soybeans when the water-soluble fractions are filtered to produce soymilk or bean curds. According to Khare et al. (1995), due to the absorption of moisture by the soybeans during processing, 1 kg of soybeans will yield approximately 1.1 – 1.2 kg of okara. As such, large amount of okara is produced worldwide annually, especially in Asian countries. For instance, the amount of okara generated by the soybean-curd industry is approximately 2,800,000 tonnes in China, 800,000 tonnes in Japan, 310,000 tonnes in Korea and 10,000 tonnes in Singapore (Vong & Liu, 2016).

The large volume of okara produced would not be a problem if it were utilized in meaningful ways. However, okara is generally disposed as waste due to its high moisture content, which causes it to go rancid rapidly. Therein lies the main issue since the disposal of okara in landfills can cause environmental problems such as pollution and climate change (Venkat, 2011). Although it is regarded as waste, okara is in fact still highly nutritious. Vong and Liu (2016) commented in their review that on a dry basis, okara contains 15.2 – 33.4% of proteins, 8.30 – 10.9% of lipids as well as a respectable amount of isoflavones. These isoflavones include glucosides (28.9%), aglycones (15.4%) and acetyl genistin (0.89%). Therefore, there is a real possibility of using these nutrients in okara for various purposes rather than disposing it.

In recent years, there have been increasing interests in the reusing of okara. Most of these studies involve the production of bioactive compounds using okara as substrate. For instance, Japakaset, Chakamas, and Vichien (2009) fermented okara with *Monascus purpureus* IFRPD 4046 for 7 days to produce monacolin K which is a cholesterol-lowering agent that has been approved for sale in the US and Europe. In another study, okara was fermented by *Bacillus subtilis* NB22 under solid-state

condition to produce iturin A which is a lipopeptide antifungal antibiotics that had proven to be effective in the biological control of plant diseases (Mizumoto et al., 2006). In the same vein, X. Liu et al. (2018) developed a novel two-stage solid-state fermentation using okara as substrate and buckwheat husk as inert support fermented by *Mucor flavus* (pre-fermentation) and *Yarrowia lipolytica* to produce erythritol. Although these techniques to produce valuable compounds are undoubtedly novel and achieved the aim of reusing okara, wastes are still produced at the end of the processes, which would again lead to problems related to waste disposal.

Hence, there is a need to explore other means of using okara without the production of resultant wastes. One strategy is to utilize okara as a food ingredient. This strategy is feasible as okara is already in use in some Asian countries as a food ingredient. For example, unohana is a traditional side dish in Japan where okara is stir-fried in soy sauce. In South Korea, kongbiji jjigae is a traditional porridge made using okara. Other uses of okara include its inclusion in flour during the baking of bread (Wickramarathna & Arampath, 2003) as well as in beef patties to increase its nutritional values (Turhan, Temiz, & Sagir, 2007). However, although nutritious, okara contains antinutrients such as saponins, phytates as well as trypsin inhibitors. On top of that, okara also contains oligosaccharides that can cause flatulence. However, studies have shown that microbial fermentation reduces the amount of antinutrients as well as flatulence causing oligosaccharides such as raffinose and stachyose (Egounlety & Aworh, 2003; Reddy & Pierson, 1994). As such, the use of fermented okara as a food ingredient could be an ideal method to harness the nutrients within without the added downsides compared to its unfermented counterpart. Previous studies had also proposed the use of fermented okara as substrate for probiotic beverages or as animal feed (Gupta, Lee, & Chen, 2018; Vong & Liu, 2019).

Most of the studies investigating the potential of fermented okara as a functional food involve the quantification of metabolites such as amino and fatty acids as well as other compounds like polyphenols after fermentation. Previous study had shown that okara fermented by *Bacillus subtilis* WX-17 (*B. subtilis* WX-17) exhibited increased levels of amino and fatty acids as well as 1-1,-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activity (Mok, Tan, Lee, Kim, & Chen, 2019). Vong and Liu (2019) also reported that okara pre-treated with carbohydrase before being fermented by *Lactobacillus paracasei* showed increased levels of amino acids, isoflavone aglycones and fruity esters.

Although these studies showed that nutritional values of okara are enhanced after fermentation, the effects and bioaccessibility of these nutrients in the human body are not evaluated. Typical studies on okara digestion through an *in vitro* model focus on digestibility in the sense of its fermentability by the gut microbiome to produce short-chain fatty acids (SCFA), phenolic compounds as well as its prebiotic effects (Espinosa & Rupérez, 2009; Pérez-López, Cela, Costabile, Mateos-Aparicio, & Rupérez, 2016). Prebiotics are officially defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Davani-Davari et al., 2019). Okara is well known to exert prebiotic effects on the gut microbiota with numerous studies reporting on it (M.-J. Villanueva-Suárez, Pérez-Cózar, Mateos-Aparicio, & Redondo-Cuenca, 2016; M. J. Villanueva-Suárez, Pérez-Cózar, & Redondo-Cuenca, 2013). For instance, Pérez-López et al. (2016) reported that okara that is treated with high hydrostatic pressure together with food-grade enzymes exhibited potential prebiotic effects by increasing the growth of *lactobacilli* and *bifidobacteria* as well as inhibiting *clostridia*. In another study, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* was shown to

be able to ferment okara *in vitro* and proliferate (Espinosa & Rupérez, 2009). Hence, it is important to determine if fermented okara will exhibit similar or better prebiotic effects compared to unfermented okara. However, such studies on fermented okara are currently lacking. Moreover, the bioaccessibility of nutrients such as amino acids, fatty acids, phenolic compounds and vitamins in the small intestines have not been evaluated.

Therefore, the purpose of this work is to study the functional, probiotic as well as prebiotic properties of fermented okara as a food ingredient through an *in vitro* digestion and fermentation model to better understand its characteristics in the human body.

4.2. Materials and Methods

4.2.1. Okara

Fresh okara samples were kindly provided by Vitasoy International Singapore Pte Ltd, Singapore. Okara was separated into aliquots, sealed in airtight polyethylene bags, and stored at -20˚C in the dark.

4.2.2. Fermentation

SSF of okara with *B. subtilis* WX-17 was carried out according to the method described previously by Mok et al. (2019). Ten g of okara was inoculated with *B. subtilis* at a concentration of 10^6 CFU/g of okara and incubated at 37°C for 72 hours. Isolation and identification of *B. subtilis* WX-17 were also detailed within the work. Briefly, *B. subtilis* WX-17 was isolated from Marumiya Kyushu Ichiban Natto. A single colony was isolated and PCR was carried out to amplify the 16S rDNA gene. Sequencing was performed via Sanger dideoxy sequencing technology.

4.2.3. Dietary Fibre Analysis

Soluble and insoluble dietary fibres were tested using defatted samples (solid samples before *in vitro* digestion and fermentation) according to the AOAC 991.43 enzymatic-gravimetric method (Vong, Hua, & Liu, 2018). The analysis was done using the Total Dietary Fibre Assay Kit from Megazyme (Product code: K-TDFR-200A).

4.2.4. *In Vitro* Digestion

Both fermented okara and unfermented okara (control) were subjected to a two-stage *in vitro* digestion and fermentation process following the protocol described by Pérez-Burillo, Rufián-Henares, and Pastoriza (2018) to mimic the physiological processes in the human gut. The *in vitro* digestion portion consisted of 3 phases. Oral phase (5g sample, alpha-amylase 75 U/ mL, pH 7.0 at 37 °C for 2 mins), gastric phase (pepsin 2000 U/ mL, pH 3.0 at 37 °C for 2 hours) and the intestinal phase (pancreatin 13.37 mg/ mL, bile 20 mmol/L, pH 7.0 at 37 °C for 2 hours).

4.2.5. *In Vitro* Fermentation

In vitro fermentation was carried out using faecal samples from 3 healthy donors (non-smoker, healthy BMI within 18 to 25, absence of drug and supplement intake, no antibiotic usage in the past 1 month prior to donation). One g solid sample from the *in vitro* digestion step was mixed with 1 mL of the digestion supernatant and fermented by the gut microbiome in the faecal samples anaerobically. Three fractions were obtained after both *in vitro* digestion and fermentation processes. They were digestion supernatant (fraction available for absorption in the small intestines), fermentation supernatant (fraction available for absorption in the large intestines) and solid residue (simulated faeces).

4.2.6. Metabolites and Amino Acids Analysis

Analysis of the metabolites including amino acids in the samples were carried out according to the method as described previously by Mok et al. (2019) with some modifications. Briefly, 10 µL of ribitol (2 mg/mL) were added to 1.5 mL of digestion supernatant and freeze-dried. Samples were then derivatised with 100 µL of MOX and silylation was carried out using 200 µL of MSTFA with 1% TMCS. Samples were then centrifuged and 120 µL were transferred to glass vials for GC-MS analysis. The column and method used were as per described in the preceding work.

4.2.7. Fatty Acids Analysis

Lipids were extracted from the samples using the chloroform-methanol 2:1 protocol detailed previously (Mok et al., 2019) with some modifications. Digestion supernatant of 1.5 mL was added with a chloroform-methanol mixture (2:1 v/v) and the samples were inverted several times, vortexed vigorously for 10 mins, and centrifuged at 10000 g for 10 min at 4°C. The chloroform layer (bottom, 1 mL) was collected and dried overnight at 30°C. The dried lipid residue was re-dissolved in 500 µL BF3-methanol 10% (FLUKA, 15716) and incubated in a sealed vial in a 95°C heater for 20 min. FAME was extracted with 300µL n-hexane after the addition of 300 µL saturated NaCl in water. Samples were vortexed for 5 mins and centrifuged at 14800 rpm for 5 mins. One hundred and fifty µL of sample (top layer) was transferred into glass vials for GC-MS analysis. Column and GC-MS method used were as described.

4.2.8. Enumeration of *Bacillus subtilis* WX-17

Hundred µL of sample were added to 900 µL of sterile water and serial diluted 10 times. Hundred µL from each dilution were plated onto nutrient agar plates and

incubated at 37 °C for 24 hours. After which the cell counts were recorded. This experiment was performed at inoculation, after fermentation and after *in vitro* digestion.

4.2.9. Total Phenolic Content Analysis

Total phenolic content analysis was carried out according to the protocol described by Kamtekar et al. (2014). One mL of sample (both digestion supernatant and fermentation supernatant) was added with 5 mL of deionized water, 0.5 mL of Folin Ciocalteu's reagent and shaken vigorously. After 5 mins, 1.5 mL of 20% sodium carbonate was added and made up to 10 mL before incubation for 2 hours. Gallic acid with differing concentrations (10, 20, 40, 60, 80, 100 µg/mL) was used as standards for quantification. The absorbance of the mixture was measured at 750 nm with deionized water as blank using Nanodrop 2000c Spectrophotometer.

4.2.10. DPPH Scavenging Activity Analysis

The DPPH radical scavenging activity of the samples were evaluated using the method described by Gjorgievski, Tomovska, Dimitrovska, Makarijoski, and Shariati (2014) with minor modification. Six hundred µL of sample (both digestion supernatant and fermentation supernatant) was added with equal volume of DPPH solution and vortexed before incubation in the dark for 30 mins. Absorbance was measured at 515nm using ethanol as blank and the activities of the samples were evaluated with respect to trolox equivalent-% signal inhibition calibration curve whereby % signal inhibition is defined as: $\% \, Signal \, Inhibition = (1 - \frac{A_S}{A_O})$ X 100. A_s is defined as the absorbance of the samples and A_o is defined as the absorbance of pure DPPH.

4.2.11. Menaquinone-7 Analysis

Analysis of MK-7 was carried out in accordance to the protocol described by Berenjian et al. (2014) with minor modifications. One mL of digestion supernatant was added with 5 mL of 2-propanol and hexane mixture (1:2 v/v) and was mixed vigorously for 10 mins. The mixture was then centrifuged for 10 mins at 3000 rpm. The organic layer was collected and dried before adding 1 mL of 2-propanol and hexane mixture (1:2 v/v) and vortex for 20 seconds (concentration process). The samples were then filtered through a 0.45 μm nylon membrane filter into a glass vial for HPLC analysis. HPLC (Agilent 1260 Infinity) with variable wavelength UV-vis detector was used for quantification of MK-7. The HPLC column (RESTEK, C18, 4.6 mm × 150 mm, 5μm) was maintained at 40 °C. Methanol was used as the mobile phase at a flow rate of 1 mL/min for 40 mins. The absorbance of MK-7 was detected at 220 nm.

4.2.12. Erythrocyte Haemolysis Assay

Toxicity of the samples were conducted with reference to the protocol described by S. Liu and Huang (2015) with some modifications. One mL of each samples (digestion supernatant) was freeze-dried, weighed (take note of concentration) and re-suspended in 1 mL of phosphate buffer saline (PBS). Human blood was washed with sterile PBS (pH 7.4, 10mM) 3 times. Centrifugation was done at 1500 g for 5 mins for the first 2 times and 1500 g for 10 mins for the 3rd time. Excess PBS was drained off, 100 μL of red blood cells were added to 200 μL of each sample (concentration of 20, 10, 5, 2.5, 1.25 mg/mL), and the total volume is made up to 500 μL with PBS. Two hundred μL of de-ionized water and PBS were used as positive and negative control respectively. The samples were then incubated at 37 °C for 3 hours (200 rpm). After

incubation, the samples were diluted with 8 mL of PBS and centrifuged for 10 mins at 1041 g. Absorbance readings were then taken at 550 nm using PBS as blank.

4.2.13. Short-Chain Fatty Acids Analysis

Analysis of SCFA in the fermentation supernatant was conducted based on the protocol described by Delgado-Andrade et al. (2017) with some modifications. The samples (fermentation supernatant) were centrifuged at 13,000 g and 10 mins before being filtered through 0.22 µm nylon filter into glass vials for HPLC analysis. The system used for detection is the Agilent 1100 HPLC with a variable wavelength UV-vis detector and a C18 column (RESTEK, 4.6 mm × 150 mm, 5µm). The mobile phase consists of a mixture of phosphate buffer at concentration of 50 mmol/L and pH at 2.8 as well as acetonitrile at a ratio of 99:1 (v/v). Flow rate was set at 1.25 mL/min running in isocratic conditions for 30 mins. Absorbance of SCFA was detected at 210 nm. Quantifications were performed using acetic, propionic and butyric acid standards at concentration ranging from 100 mmol/L to 1 mmol/L.

4.2.14. Gut Microbiome Analysis

16S amplicon sequencing was outsourced to NovogeneAIT, Singapore. DNA samples were extracted from the solid residue using the QIAamp Fast DNA Stool kit. The concentration of the DNA samples was measured using Nanodrop 2000c Spectrophotometer. Purity of DNA samples were monitored on 1% agarose gels. DNA was diluted to 1 ng/µL. For each sample, 16S rRNA genes of the V3 – V4 region were amplified. The primer set that corresponds to the primer 341F and 806R with the barcode was used for amplification. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Loading buffer containing SYBR green was mixed with PCR products. Gel electrophoresis was ran on 2% agarose gel for

detection. Samples with bright main strip between 400 bp to 450 bp were chosen for further experiments. PCR products were mixed at equal density ratios and purified with Qiagen Gel Extraction Kit.

The sequencing libraries were generated using NEBNext® UltraTM DNA Library Prep Kit and quantified via Qubit 2.0 and Q-PCR. Amplicon was sequenced on Illumina paired-end platform to generate 250 bp paired-end reads.

4.2.15. Statistical Analysis

All experiments were conducted in triplicates. Statistical analysis was performed using Metaboanalyst 4.0 (Xia & S. Wishart, 2016). Data scaling was done using Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). Heat map was constructed using Euclidean distance measurement and ward clustering algorithm.

4.3. Results and Discussion

4.3.1. Soluble and Insoluble Dietary Fibre

Before undergoing *in vitro* digestion and fermentation, okara samples were tested for the amount of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). It was found that after fermentation, the amount of SDF in fermented okara increased by 187% compared to unfermented okara from 3.99 to 7.47 g/100g dry weight okara. On the other hand, IDF in fermented okara decreased by 130% compared to unfermented okara from 50.4 to 38.8 g/100g dry weight okara (Table 1). Amount of IDF and SDF as well as conversion percentages are similar to that as reported by Vong et al. (2018). The bioconversion of IDF to SDF is due to the production of cellulases by *B. subtilis* WX-17 (Vong et al., 2018). Y. K. Kim, Lee, Cho, Oh, and Ko (2012); F.-C. Wu, Chang, and Shih (2013) reported that *Bacillus subtilis* could produce multiple cellulases such as

endoglucanase, avicelase, β-glucosidase and xylanase, which act in synergy for the hydrolysis of cellulose. The authors also noted that endoglucanase, avicelase and xylanase are extracellular while β-glucosidase is cell membrane bound. The production of enzymes that are extracellular are important as they allow *B. subtilis* WX-17 to act on the IDF in okara, which consist of mainly cellulose, hemicellulose and pectin and convert them into soluble oligosaccharides, disaccharides (sucrose and maltose) and other monosaccharides such as arabinose, galactose and glucose.

The conversion of IDF to SDF is also critical to the enhanced functional properties of fermented okara compared to unfermented okara. This is because; decrease in IDF increases the bioaccessibility of metabolites such as amino acids and fatty acids due to less entrapment effects. Parada and Aguilera (2007) commented that fermentation could increase the bioaccessibility of nutrients due to the disruption of cell wall structure and dissociation of nutrient-matrix complex. Proteins, amino acids as well as lipids can also form hydrogen bonds with cellulose, which can affect its dissolution into the small intestinal fluid (Arola & Linder, 2016; Kostritskii, Tolmachev, Lukasheva, & Gurtovenko, 2017).

Table 4.1. Changes in menaquinone-7, amino acids and fatty acids in absolute value (mg/mL digestion supernatant) for fermented and unfermented okara. Changes in IDF and SDF were also shown in absolute value (mg/100 g dry weight okara)

Compounds	mg/mL digestion supernatant		
	Fermented okara	Unfermented okara	P values
Vitamin			
Menaquinone-7	0.000110 ± 0.0000100	Undetected	NA
Amino acids			
Alanine[a]	1.13 ± 0.00800	0.564 ± 0.0430	5.12E-05
Glycine[a]	0.0450 ± 0.00500	0.0170 ± 0.00100	0.00100
Leucine	0.0120 ± 0.00100	0.00900 ± 0.00200	> 0.05
Valine[a]	0.0420 ± 0.00100	0.0220 ± 0.00200	0.000200
Isoleucine[a]	0.0300 ± 0.00400	0.0210 ± 0.00300	0.0370

Serine[a]	0.0450 ± 0.00500	0.0190 ± 0.00500	0.00500
Threonine[a]	0.0330 ± 0.00100	0.0160 ± 0.00100	1.29E-05
Aspartic Acid[a]	0.0390 ± 0.00300	0.0160 ± 0.00200	0.000900
Methionine[a]	0.0270 ± 0.00500	0.0150 ± 0.00100	0.0150
Proline[a]	0.510 ± 0.0820	0.287 ± 0.0590	0.0170
Lysine[a]	0.0600 ± 0.00700	0.0300 ± 0.00400	0.00700
Tyrosine[a]	0.0350 ± 0.00200	0.0170 ± 0.00100	0.000600
Fatty acids			
Palmitic acid[a]	4.52 ± 0.303	1.76 ± 0.106	0.0110
Linoleic acid[a]	6.90 ± 0.194	4.01 ± 0.150	0.00400
Oleic acid[a]	1.84 ± 0.136	0.828 ± 0.0370	0.00200
Stearic acid[a]	2.72 ± 0.234	1.06 ± 0.0630	0.0110
	g/100g dry weight okara		
Dietary fiber			
IDF[a]	38.8 ± 0.155	50.4 ± 2.36	0.00100
SDF[a]	7.47 ± 0.528	3.99 ± 0.0110	0.000300

[a] denote compound that are significantly different with $p < 0.05$. Values are mean ± standard deviation (n = 3). IDF = insoluble dietary fiber. SDF = soluble dietary fiber. NA = not applicable

4.3.2. Metabolic Profiling of Digestion Supernatant

A metabolic profiling was performed on the digestion supernatant between fermented and unfermented okara to gain a broad overview. PLS-DA was used to discern if the 2 sets of data were significantly different (Figure 4.1A). The first principal component accounted for the majority of the variances in the dataset (93.1%) while the second component accounted for 2.40%, which totals to 95.5% of the total variances. From the score plot (R^2 = 99.7% and Q^2 = 95.5%), there were clear and distinct separation between the 2 groups of data across the first principal component. The red and green highlights denote the 95% confidence internal (95% confident that all data points for each particular sample are within the highlighted region).

A clustering heat map (Figure 4.1B) was constructed to allow better visualization on the changes to the metabolic profile in the small intestines when digesting fermented and unfermented okara. A total of 32 metabolites were detected. Eleven amino acids and 4 fatty acids were detected and quantified (Table 4.1).

Of the amino acids detected across both sets of samples (digestion supernatant of both fermented and unfermented okara), 6 were essential amino acids (leucine, isoleucine, valine, methionine, lysine, threonine), which means that the human body is unable to produce them and they would need to be supplemented from food (Adibi & Gray, 1967). As mentioned previously, the increased amount of amino and fatty acids detected in the digestion supernatant is likely due to the conversion of IDF to SDF in fermented okara which reduced entrapment and hydrogen bonding. This is significant as these nutrients are essential to humans. For example, amino acids are known to play important roles in human health. Transformation of amino acids in the small intestines plays an important role in regulating endogenous synthesis of non-essential amino acids such as proline and alanine as well as modulating the availability of dietary amino acids to extra-intestinal tissues. Other well-known functions of amino acids include their role in regulating gene expression, cell signalling, antioxidative responses and immunity. Of the essential amino acids, leucine is known to activate rapamycin to stimulate protein synthesis and inhibit proteolysis (G. Wu, 2010). It has also been proposed as a potential pharmaconutrient in the prevention of type 2 diabetes and sarcopenia (Loon, 2012). Jordan, Brunner, Hunt, and Berry (1985) also reported that oral administration of leucine, isoleucine and valine could reduce the concentration of phenylalanine in the cerebrospinal fluid of humans thereby reducing the risk of phenylketonuria. Valine also plays a role in mental functioning by stimulating the central nervous system. Cusick, Koehler, Ferrier, and Haskell (1978) suggested that rats deficient in valine showed

neurological symptoms such as retracting head, aimless circling and staggering. Another essential amino acid, methionine had been linked to cellular functions such as methylation reactions, polyamine synthesis, folate coupling metabolism and redox maintenance (Sanderson, Gao, Dai, & Locasale, 2019). Lysine had also been found to be useful for treatment of stress and anxiety using animal models due to their modulatory effects on neurotransmitter systems such as gamma-aminobutyric acid (GABA) and serotonin (Camfield, 2017). Threonine is linked to the synthesis of mucin lining along the intestines which prevents the absorption of allergy-provoking proteins that are incompletely digested (Faure et al., 2005).

Fatty acids were detected in the digestion supernatant due to the presence of bile salts in the *in vitro* digestion model, which encapsulated the fatty acids thereby allowing them to stay soluble in the aqueous phase. More importantly, both linoleic acid and oleic acid, which are unsaturated fatty acids, were up regulated. Linoleic acid is known to exhibit health benefits ranging from anti-obesity, anti-carcinogenesis, anti-atherogenesis, anti-diabetic, osteosynthetic as well as immunomodulation effects (Benjamin & Spener, 2009). Recently, a study conducted by Zong et al. (2019) also reported that intake of linoleic acid would lower the risk of type-2 diabetes. Similarly, oleic acid is a monounsaturated fatty acid that reduces risk of CVD due to factors such as thrombogenesis, LDL susceptibility and sensitivity to insulin. Lopez-Huertas (2010) reported that reducing 5% of energy from saturated fatty acids with oleic acid could reduce the risk of CVD by 20 – 40%.

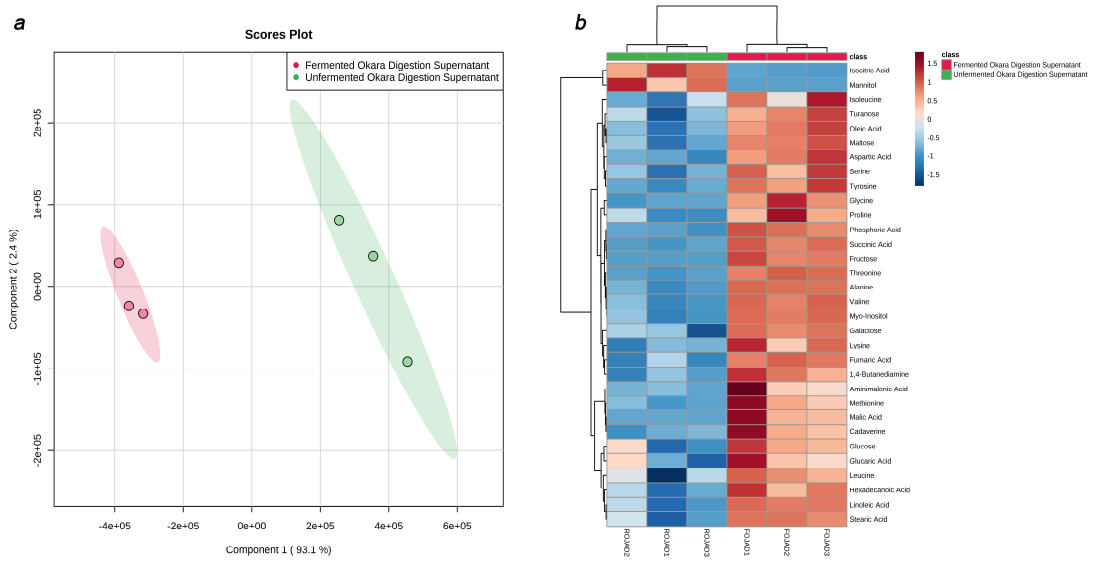


Figure 4.1. Panel A shows the PLS-DA score plot of all metabolites found for the digestion supernatant of fermented and unfermented okara. The green and red highlights denoted the 95% confidence region. Explained variance is shown in brackets. Panel B shows the heat map analysis correlating the metabolites in the digestion supernatant of fermented and unfermented okara. Metabolites in red are up-regulated while those in blue are down-regulated

4.3.3. Menaquinone-7

B. subtilis is well known to produce MK-7 which is a highly bioactive homologue of Vitamin K (Sato et al., 2001). Results showed that 0.0110 µg/mL of MK-7 was detected in the digestion supernatant of fermented okara (Table 4.1). MK-7 was not detected for unfermented okara. Typically, serum concentration of MK-7 is in the ng/mL range (Sato, Schurgers, & Uenishi, 2012). MK-7 functions as a cofactor for carboxylase which is a microsomal enzyme that facilitates the posttranslational modification of glutamic acid residues into γ-carboxyglutamyl residues (Sato et al., 2001). Proteins that contain γ-carboxyglutamyl residues are abundant in bone tissues and 80% of these residues in mature bones are osteocalcin. Numerous mechanistic data point suggests that osteocalcin plays an important role in bone mineral maturation. Bone formation was found to be enhanced in an experiment conducted on osteocalcin knock-

out mouse model. MK-7 is also reported to have a positive influence in calcium balance as well as working synergistically with vitamin D on bone metabolism (Weber, 2001). Beulens et al. (2013) also reported that carboxylation of matrix γ-carboxyglutamate protein by MK-7 may reduce coronary calcification which would help to reduce the risk of CVD. This observation was also reported by Knapen et al. (2015) who suggested that intake of MK-7 reduces arterial stiffness in postmenopausal women which reduces their risk of CVD.

4.3.4. *B. subtilis* WX-17 Viability

Viability of the probiotic strain *B. subtilis* WX-17 was tested after *in vitro* digestion to ensure its survival as it passes through the gastrointestinal tract. Results showed that cell count remained relatively unchanged after *in vitro* digestion with an average of 10.5 log CFU/mL of digestion supernatant (Figure 4.2). This figure is above the recommended amount of probiotic intake (9 log CFU) to confer health benefits (Hill et al., 2014). This survivability is due to the unique ability of *B. subtilis* WX-17 to form endospores under harsh growth conditions such as the stomach which has a pH of 3 (McKenney, Driks, & Eichenberger, 2012). Abel-Santos (2015) also noted that bacterial endospores such as those of *B. subtilis* are able to better survive the acidic conditions in the stomach compared to other non-endospore forming probiotic species. *B. subtilis* is a probiotic that is commonly found in soil, however there are evidences that showed *B. subtilis* can be found in the human gut and should be considered as gut commensal (H. A. Hong et al., 2009). *B. subtilis* is also safe for consumption as it is commonly used during the production of *Natto*. Clinical studies were also conducted to evaluate the *in vitro* and *in vivo* safety of *B. subtilis* as a food supplement and found it to be safe for oral consumption (Cuentas, Deaton, Khan, Davidson, & Ardita, 2017; H.A. Hong et al., 2008). It is important for *B. subtilis* WX-17 to remain viable as it passes through the

gastrointestinal model due to the health benefits that it confers. For instance, a study had shown that *B. subtilis* may improve gut health by strengthening intestinal barrier as well as limiting inflammatory responses (Rhayat et al., 2019). Another study conducted by Cuentas et al. (2017) found that *B. subtilis* DE111 when administered in capsule form orally, is effective in improving constipation or diarrhoea in healthy humans.

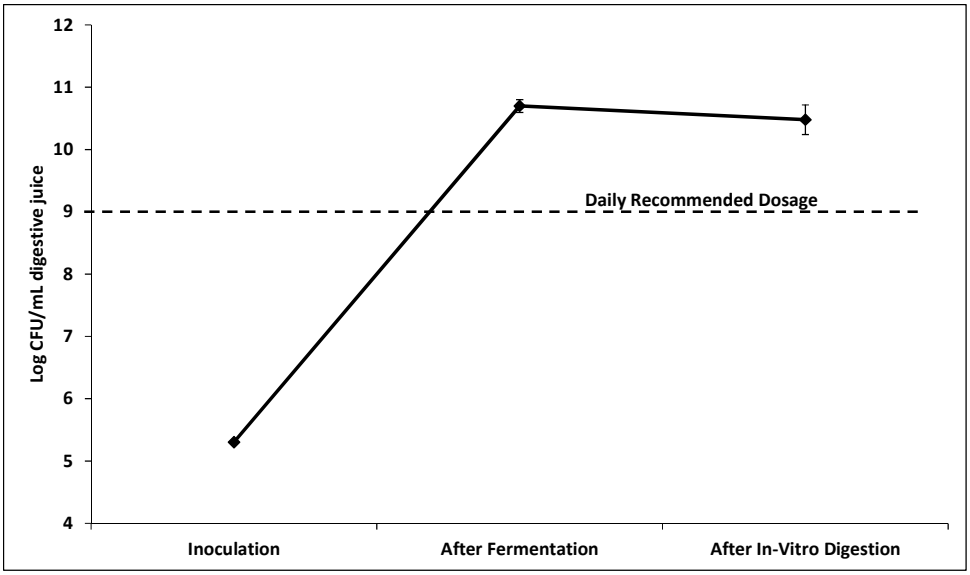


Figure 4.2. Cell count of *B. subtilis* WX-17 from inoculation to after *in vitro* digestion

4.3.5. Erythrocyte Haemolysis – Toxicity Testing

The cytotoxicity of fermented and unfermented okara on erythrocytes at different concentrations was assessed after *in vitro* digestion (Figure 4.3). Both digestion supernatants were found to be toxic at concentrations of 10 and 20 mg/mL, which caused 100% haemolysis of the erythrocytes. At 5 mg/mL, digestion supernatant of fermented okara exhibited percentage haemolysis of 5.29% while unfermented okara had a percentage haemolysis of 1.13%. Both digestion supernatants did not exhibit any cytotoxicity on erythrocytes at concentrations of 2.50 and 1.25 mg/mL respectively. Although both the digestion supernatants of fermented and unfermented okara showed cytotoxicity at concentrations above 5 mg/mL, classifying them as toxic to the human body would be highly inaccurate. This is because typical serum concentrations of

nutrients are at least 50 times lower than what was administered (typically in the nmol/mL range). For instance, amino acids and free fatty acids concentrations in the blood was reported to be approximately 0.0500 mg/mL (Bo et al., 2017; Campollo, 2018). These estimates concurred with studies by Duplessis, Cue, Santschi, Lefebvre, and Girard (2018) and Strømmen et al. (2017). Henceforth, it is reasonable to assume that okara fermented by *B. subtilis* WX-17 is safe for consumption.

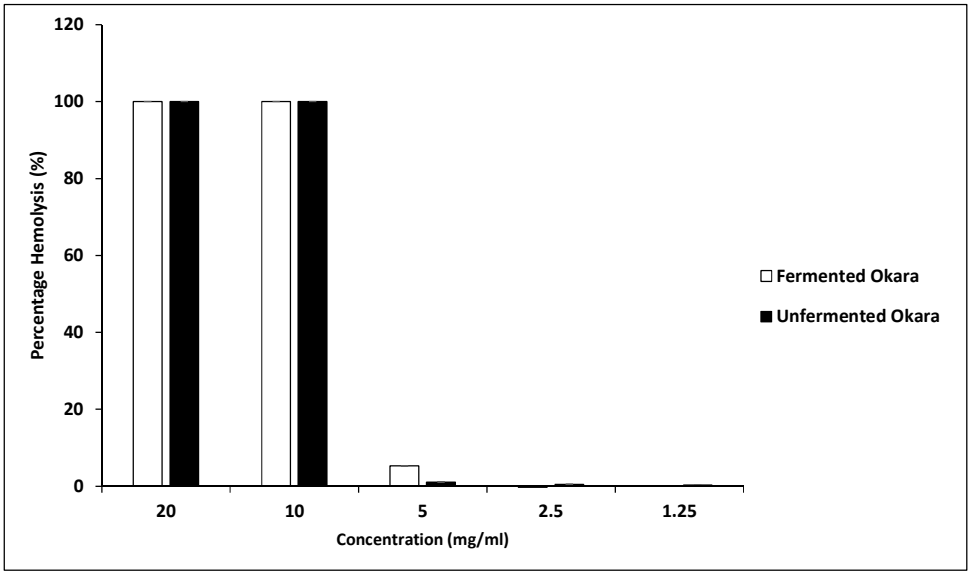


Figure 4.3. Percentage erythrocytes haemolysis caused by nutrients in the digestion supernatant of fermented and unfermented okara

4.3.6. Total Phenolic Content and Antioxidant Activity

As it had been reported by studies from Quiros-Sauceda et al. (2014) that phenolic compounds can be detected in both the small and large intestines, both digestion and fermentation supernatants were analysed for their total phenolic content as well as their DPPH radical scavenging activity (Table 4.2). In the digestion supernatant of fermented okara, 0.819 mg/mL gallic acid equivalent (GAE) of phenolic content was detected while the digestion supernatant of unfermented okara only contained 0.370 mg/mL GAE. Similarly, digestion supernatant of fermented okara displayed 3 times the amount of DPPH radical scavenging activity compared to the

digestion supernatant of unfermented okara. Rodriguez-Roque, Rojas-Grau, Elez-Martinez, and Martin-Belloso (2013) and Quintana et al. (2019) reported similar amount of total phenolic content in soymilk and okara respectively. Total phenolic content in the fermentation supernatant of fermented okara was 4.48 mg/mL GAE while the fermentation supernatant of unfermented okara contained 2.15 mg/mL GAE. In terms of DPPH radical scavenging activity, fermentation supernatant of fermented okara contained 0.0750 mg/mL trolox equivalent while the fermentation supernatant of unfermented okara contained 0.0360 mg/mL trolox equivalent. Phenolic compounds are most well known for their antioxidant properties as well as free radical scavenging activities that protects biomolecules against oxidative damages (Quiros-Sauceda et al., 2014). Hence, the trends for DPPH radical scavenging activity mirrored that of the total phenolic content.

Table 4.2. Changes in total phenolic content and DPPH radical scavenging activity in both digestion and fermentation supernatant for fermented and unfermented okara (mg/mL)

Compounds	mg/mL		
	Fermented okara	Unfermented okara	P values
Digestion supernatant			
Total phenolic content[a,b]	0.819 ± 0.0790	0.379 ± 0.0470	0.00300
DPPH radical scavenging[a,c]	0.0120 ± 0.00100	0.00400 ± 0.000200	0.000300
Fermentation supernatant			
Total phenolic content[a,b]	4.48 ± 0.342	2.15 ± 0.0640	0.000700
DPPH radical scavenging[a,c]	0.0750 ± 0.00400	0.0360 ± 0.00600	0.000400

[a] denote compound that are significantly different with $p < 0.05$. [b] denote compound expressed in gallic acid equivalent (GAE). [c] denote compound expressed in trolox equivalent. Values are mean ± standard deviation (n = 3). DPPH = 1-1,-diphenyl-2-picryl-hydrazil

In both the modelled small and large intestines, it can be seen that fermented okara provides more bioaccessible phenolic compounds compared to unfermented okara. This is reasonable and expected as mentioned above; *B. subtilis* secretes a myriad of extracellular cellulases that breaks down and convert IDF to SDF. This would increase the bioaccessibility of phenolic compounds as IDF may entrap them within the fibre matrix. Hence, a pre-fermentation step of okara before consumption can be a cost-effective method to increase the bioaccessibility of phenolic compounds (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011).

By comparing the total phenolic content in the digestion and fermentation supernatant, it is clear that the fermentation supernatant contains much more bioaccessible phenolic content compared to the digestion supernatant. This is again reasonable since it is well accepted that bioaccessibility of phenolic compounds are lower in the small intestines compared to the large intestines. The reason for this phenomenon is due to a number of factors. Firstly, due to the presence of IDF even after pre-fermentation (prior to consumption), the fibre matrix can cause physical entrapment of phenolic compounds. Secondly, the gastric fluids are higher in viscosity, which can affect the peristaltic mixing process that in turn hinders the movement of soluble phenolic compounds to the small intestinal walls. Lastly, phenolic compounds can interact with IDF in the form of hydrogen and convalent bondings, which would again affect bioaccessibility. That being said, phenolic compounds can be released from the food matrix in the small intestines through direct solubilisation in the intestinal fluids and/or the actions of digestive enzymes since it was reported that the hydrolysis of proteins, lipids and carbohydrates releases phenolic compounds (Pérez-Jiménez et al., 2009). However, due to the lack of cellulases in the human gut, these amounts are known to be small. These small amounts of accessible phenolic compounds are then partially

absorbed through the mucosa of the small intestines while the rest plus inaccessible phenolic compounds would travel to the large intestines where the fibres are more comprehensively fermented by the gut microbiome to produce large amount of phenolic compounds as well as SCFA. These phenolic compounds in the large intestines are released gradually in the intestinal lumen and absorbed by the gut epithelial cells. Thereafter, the non-absorbable or unreleased phenolic compounds would stay in the colonic tissues to scavenge free radicals and counteract the effects of dietary fibres pro-oxidants (Saura-Calixto, 2011).

4.3.7. Short-Chain Fatty Acids

Short-chain fatty acids are produced primarily when SDF are fermented by the gut microbiota. The fermentation supernatants were tested for the presence of SCFA and it was found that the fermentation supernatant of fermented okara produced more SCFA compared to unfermented okara (Table 4.3). Acetic acid production increased from 128 mmol/L to 231 mmol/L. Propionic acids increased by 46.9% from 10.3 mmol/L to 19.5 mmol/L. Butyric acid increased from 25.5 mmol/L to 52.9 mmol/L. The levels of SCFA detected were in line with those reported by Pérez-López et al. (2016).

Table 4.3. Changes in SCFA in absolute value (mM fermentation supernatant) for fermented and unfermented okara

Compounds	mM fermentation supernatant		
	Fermented okara	Unfermented okara	P values
SCFA			
Acetic acid[a]	231 ± 25.7	128 ± 43.3	0.0440
Propionic acid[a]	19.5 ± 3.29	10.3 ± 2.39	0.0340
Butyric acid[a]	52.9 ± 4.47	25.5 ± 7.35	0.0110

[a] denote compound that are significantly different with $p < 0.05$. Values are mean ± standard deviation (n = 3). SCFA = short-chain fatty acids

Unlike starch, non-starch polysaccharides cannot be digested by digestive enzymes in the human body. As such, they would pass through the digestive tract unaltered to the large intestines where they serve as energy source for the gut microbiota, which would in turn proliferate and generate healthy compounds such as SCFA. Examples of non-starch polysaccharides are inulin, fructooligosaccarides (FOS) and galactooligosaccharides (GOS), these are also known as SDF or prebiotic fibres (Fernández et al., 2016). Therefore, a higher amount of SDF should theoretically translate to a higher amount of SCFA.

As mentioned, *B. subtilis* WX-17 can convert IDF to SDF through its extracellular cellulases. This would provide additional energy source for the gut microbiota to generate more SCFA. Hence, more SCFA were detected in the fermentation supernatant of fermented okara.

Of the 3 most commonly produced SCFA (acetate, propionate and butyrate), butyrate is considered the most important since it is a major energy source of colonocytes and have numerous health benefits ranging from anti-inflammatory to anti-cancerous properties. For instance, normal colonocytes utilize butyrate as the main energy source. Butyrate also helps to regulate the proliferation rates of these colonocytes to ensure normal growth. At the same time, colorectal cancer cells (CRC) metabolize butyrate differently. In CRC, butyrate blocks growth, promotes differentiation and apoptosis (Fernández et al., 2016). Acetate is the primary SCFA in the large intestines and is readily transported to the liver. In the presence of acetyl-CoA synthetase in the adipose tissues and mammary glands, acetate can be used for lipogenesis. Furthermore, acetate can also be used by the gut microbiota to produce butyrate (Hijová & Chmelarova, 2007). Propionate has been reported to inhibit the synthesis of cholesterol in the liver tissues. Propionate is also known to enhance glycolysis by using hepatic

citrate. It can also affect hepatic glucose metabolism by lowering the plasma fatty acid concentration (J. M. W. Wong, de Souza, Kendall, Emam, & Jenkins, 2006). On the whole, SCFA also play an important role in modulating colonic and intracellular pH which can influence the composition of the gut microbiota, decrease bile solubility, increase mineral absorptions as well as reduce ammonia absorption (Hijová & Chmelarova, 2007).

4.3.8. Effects of Fermented Okara on the Gut Microbiota

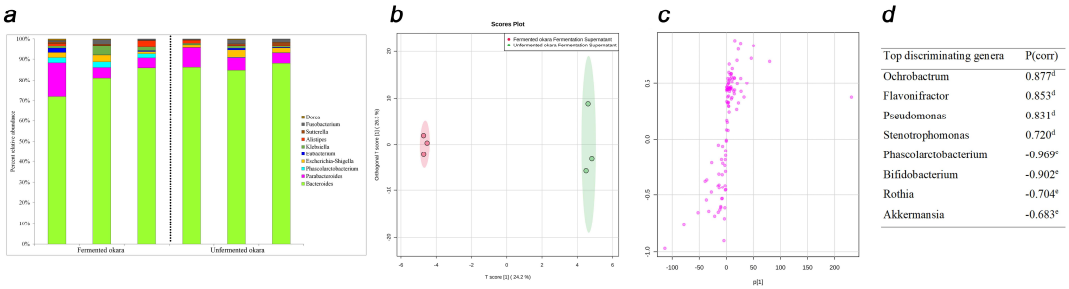
Prebiotics are officially defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Davani-Davari et al., 2019). Okara is well known to exert prebiotic effects on the gut microbiota with numerous studies reporting on it (M.-J. Villanueva-Suárez et al., 2016; M. J. Villanueva-Suárez et al., 2013). For instance, Pérez-López et al. (2016) reported that okara that is treated with high hydrostatic pressure together with food-grade enzymes exhibited potential prebiotic effects by increasing the growth of *lactobacilli* and *bifidobacteria* as well as inhibiting *clostridia*. In another study, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* was shown to be able to ferment okara *in vitro* and proliferate (Espinosa & Rupérez, 2009). Hence, it is important to determine if fermented okara, with its higher amount of SDF will exhibit similar or better prebiotic effects compared to unfermented okara.

The effect of fermented and unfermented okara on the microbiota community structure was determined after anaerobic *in vitro* fermentation for 24 hours. In general, the microbiota composition amongst all samples was comparable with *Bacteroides* and *Parabacteroides* being the most abundant genera (Figure 4.4A). Interestingly, the microbiota from fermented okara contained more *Phascolarctobacterium* which are

known to produce SCFA (F. Wu et al., 2017). The differences in genera were sufficient to separate the 2 sets of data in the OPLS discriminant analysis (Figure 4.4B). The separation was good with R^2Y value of 0.998 although the predictive ability of the model was modest with Q^2 of 0.441. The score plot (Figure 4.4C) shows the significance of each genera in separating the 2 sets of data. The y axis represents correlation which signifies the reliability of the effect while the x axis represents covariance which signifies contribution in terms of abundance (Wiklund et al., 2008). Base on the analysis, the top discriminating genera are *Ochrobactrum, Flavonifractor, Pseudomonas, Stenotrophomonas, Phascolarctobacterium, Bifidobacterium, Rothia* and *Akkermansia* (Figure 4.4D). Some of these discriminating genera that play important roles in human health were upregulated. For instance *Bifidobacterium* has numerous health benefits such as the reduction of plaque accumulation and gingival inflammation parameters (Kuru, Laleman, Yalnızoğlu, Kuru, & Teughels, 2017). Other benefits of *Bifidobacterium* include the alleviation of gastrointestinal disorder, modulating of the gut microbiota, modulating of immune functions and reduction in allergic rhinitis (C. B. Wong, Odamaki, & Xiao, 2019). *Rothia* is a probiotic that has been reported to be able to degrade gluten proteins, which would serve to protect genetically predisposed people from celiac disease (Zamakhchari et al., 2011). *Akkermansia* is another promising probiotic that participates in the immune regulation of the host, enhances the integrity of the intestinal epithelial cells as well as the thickness of the mucous layer hence promoting intestinal health (T. Zhang, Li, Cheng, Buch, & Zhang, 2019).

At the same time, certain discriminating genera, which are known to affect human health negatively, were downregulated. For example, *Ochrobactrum* is an unusual human pathogen that was reported to have caused septic shock in a healthy patient after administration of a venous infusion contaminated with said bacteria

(Kettaneh et al., 2003). In another report, *Ochrobactrum anthropi* was reported to have caused septic arthritis in a patient after infection during an accident (Battaglia, 2008). Similarly, a study by He, Shao, Li, Xie, and Wen (2016) found that *Flavonifractor* was among the genera significantly enriched in Chinese patients with systemic lupus erythematosus. *Pseudomonas* is also a well-known pathogen that can cause respiratory infections in patients with cystic fibrosis or chronic lung diseases (Debois, Degreef, Vandepitte, & Spaepen, 1975). Lastly, *Stenotrophomonas* is an emerging opportunistic pathogen that has a high mortality rate of 14% to 69% in patients with bacteremia. It is also found in the respiratory tracts of cystic fibrosis patients as a cocolonizer with *Pseudomonas anthropi* (Brooke, 2012).



Top discriminating genera	P(corr)
Ochrobactrum	0.877[d]
Flavonifractor	0.853[d]
Pseudomonas	0.831[d]
Stenotrophomonas	0.720[d]
Phascolarctobacterium	-0.969[e]
Bifidobacterium	-0.902[e]
Rothia	-0.704[e]
Akkermansia	-0.683[e]

Figure 4.4. Panel A shows the relative abundance of the top 10 most abundant microbial genera in the *in vitro* model of fermented and unfermented okara. Panel B shows the OPLS-DA score plot of all genera found in the *in vitro* model of fermented and unfermented okara. The green and red highlights denoted the 95% confidence region. Panel C shows the OPLS-DA loading plot for all genera in the *in vitro* model of fermented and unfermented okara. Y-axis represents the correlation while the X-axis represents covariance. Panel D shows the genera with the largest discriminating weight. [d] denote genera that are down-regulated after in-vitro fermentation of fermented okara. [e] denote genera that are up-regulated after in-vitro fermentation of fermented okara

4.4. Conclusion

This work reports the effect of *in vitro* digestion and fermentation of fermented okara and unfermented okara on the bioaccessibility of nutrients, probiotic viability, potential toxicity, total phenolic content, antioxidant activity, SCFA production as well as the microbial community. The increase in SDF after fermentation by *B. subtilis* WX-17 plays a big role in enhancing the nutritional value of fermented okara. Amino acids, fatty acids and MK-7 showed increased bioaccessibility in fermented okara although in the case of MK-7, none was detected in unfermented okara due to the absence of *B. subtilis* WX-17. *B. subtilis* WX-17 was also found to remain viable after *in vitro* digestion where it is acidic. Erythrocyte haemolysis analysis suggested that both fermented and unfermented okara are non-toxic when ingested. Total phenolic content and antioxidant activity were also higher in fermented okara compared to unfermented okara in both digestion and fermentation supernatant although fermentation supernatant contained higher amounts due to the more complete fermentation of the SDF by the gut microbiome. SCFA production was also higher in fermented okara due to the increased amount of SDF, which stimulated the metabolism of the gut microbiome in turn producing more SCFA. Finally, the gut microbiome community was also different between fermented and unfermented okara. These results suggest that the use of fermented okara as a potential functional, probiotic and prebiotic food ingredient is feasible and would confer more health benefits compared to unfermented okara. Future researches can focus on a larger scale *in vivo* study or animal study to gauge the effectiveness of fermented okara as a food ingredient.

5. Effects of Submerged Liquid Fermentation of *Bacillus subtilis* WX-17 Using Okara as Sole Nutrient Source on the Composition of a Potential Probiotic Beverage

Abstract

After understanding the effects of fermented okara in the human body through an *in vitro* digestion and fermentation model. This chapter would dive into an alternative method of fermentation to enhance scalability. This work aims to produce a functional probiotic beverage using okara as the sole nutrient source. Hence, okara was fermented with *Bacillus subtilis* WX-17 in submerged liquid fermentation and the supernatant was tested. Metabolomics analysis showed that the nutritional profile of the beverage was enhanced after fermentation. Essential amino acids as well as short chain fatty acids were significantly ($p<0.05$) upregulated. Total phenolic content and antioxidant content (in terms of DPPH radical scavenging activity) increased by 6.32 and 1.55 times respectively. After 6 weeks, probiotic viability remained unchanged when stored at 4°C and the cell count was above the minimum dosage to confer health benefits. Antimicrobial activity was also detected against gram-positive bacteria. The findings of this work showed the potential of submerged liquid fermentation of *Bacillus subtilis* WX-17 using okara as sole substrate to produce a functional and low-cost probiotic beverage.

5.1. Introduction

Okara is the by-product left behind after the processing of soybean typically in the soymilk and bean curd industry. Okara is generally disposed due to its unpalatable and fibrous nature. It is also prone to spoilage due to its high moisture content. However, it is still highly nutritious (okara contains approximately, 50% fibre, 25% of proteins, 10% of lipids as well as a plethora of other useful compounds such as isoflavones, phytates, lignins, saponins, coumestans as well as phytosterols) and studies have found that it is a source of antioxidants and can be potentially used as weight-loss dietary supplements. Every year, large amount of okara is disposed worldwide in incineration plants and landfills with more than 2.8 million tonnes produced in China's tofu industry alone (G. Li, Guo, Gao, Wang, & Sun, 2020). It is estimated that almost 14 million tonnes of okara are produced around the world annually (Mok et al., 2019).

In human nutrition, probiotics are defined as live microorganisms in food ingredients that have specific health benefits when consumed in adequate amount (Afzaal et al., 2020). For probiotics to perform, they must remain viable and various researches have shown that okara can support the growth of these microorganisms in model media (Albuquerque, Bedani, Vieira, LeBlanc, & Saad, 2016; Espinosa & Rupérez, 2009; Tu et al., 2014; Vieira, Bedani, Albuquerque, Biscola, & Saad, 2017) as well as under *in vitro* conditions (Bedani, Rossi, & Isay Saad, 2013). The consumption of fermented soy-okara products has been credited with numerous health benefits such as the increase in probiotics in the gut microbiota (Cheng et al., 2005) as well as lowering the liver weight, plasma cholesterol levels and hepatic triglyceride content in rats (Kitawaki et al., 2009).

In recent years, researchers are increasingly looking into producing novel probiotic beverages that confer more health benefits to consumers. For instance, Lu, Tan,

Chen, and Liu (2018) explored the use of *Lactobacillus helveticus* L10*, Lactobacillus paracasei* L26 and *Lactobacillus rhamnosus* HN001 to ferment star fruit juice and found that cell count were around 10^8 CFU/mL. On top of that, ketones, alcohols and fatty acids were enhanced which can confer various health benefits. In another study, quinoa, which is traditionally used in salads and stews was processed and fermented separately with *Lactobacillus plantarum* Q823, *Lactobacillus casei* Q11 and *Lactococcus lactis* ARH74. The resultant probiotic beverage (Pasankalla quinoa) was found to contain higher protein content and lower saponin concentration (Ludena Urquizo et al., 2017). Mukisa, Byaruhanga, Muyanja, Langsrud, and Narvhus (2017) utilized single and mixed starter cultures of lactic acid bacteria (LAB) to produce Obushera, a fermented sorghum beverage. It was found that co-culturing of LAB with *Saccharomyces cerevisiae* produced a profile flavour compounds that are closed to spontaneous Obushera hence potentially allowing for a more controlled production of Obushera. In the same vein, Men et al. (2019) employed a combination of enzymatic treatment followed by fermentation of jujube juice with *Pediococcus pentasaceus* PC-5 and *Lactobacillus plantarum* M. It was reported that after fermentation, gamma-aminobutyric acid as well as branched-chain and free amino acid levels were enhanced. On top of that, native functional components were also maintained.

The probiotic microorganisms used in fermented beverage studies typically consist of bacteria such as *Bifidobacterium*, *Lactobacillus* and *Streptococcus;* the latter two are commonly used in yoghurt. However, these microorganisms typically require additional carbon source such as MRS, the addition of enzymes or other physical processes to break down the cellulose in the food matrix for them to proliferate (Espinosa & Rupérez, 2009; Vieira et al., 2017). This would greatly increase the cost of production of these products on an industrial scale as well as introduce chemicals into

the products. As such, there is a gap in terms of the production of a novel probiotic beverage using a low-cost methodology with minimal addition of chemicals.

B. subtilis is a GRAS probiotic found in the human gut. It is commonly used in the production of *Natto,* a traditional Japanese fermented food made with soybean whereas rarely explored for the usage as other food products. Previous work had shown that *B. subtilis* was able to utilize okara as the carbon source to grow through solid-state fermentation (Ohno, Ano, & Shoda, 1996), Furthermore, *B. subtilis* is known to produce antioxidants and numerous extracellular enzymes such as cellulases, proteases and lipases which would help to break down cellulose to increase the accessibility of nutrients as well as macromolecules (proteins and lipids) in okara into amino and fatty acids (Lesuisse et al., 1993; Mawadza, Hatti-Kaul, Zvauya, & Mattiasson, 2000; Yang, Shih, Tzeng, & Wang, 2000b; Zhu et al., 2008). However, to the best of our knowledge, submerged liquid fermentation of *B. subtilis* using okara as sole nutrient source as well as its development for producing a probiotic beverage, has not been evaluated. In this work, submerged liquid fermentation of *B. subtilis* WX-17 with okara as sole nutrient source was performed to develop a potential low-cost and functional probiotic beverage. The viability of the probiotic *B. subtilis* WX-17 as well as the nutritional analysis, total phenolic content, antioxidant and antimicrobial activity assays of fermented product were investigated herein to verify the practicality of this approach. Moreover, the metabolic mechanism of *B. subtilis* WX-17 for submerged liquid fermentation of okara was analysed.

5.2. Materials and Methods

5.2.1. Materials

All chemicals including nutrient broth, methanol, ribitol, MOX, MSTFA, TMCS, Folin Ciocalteu's reagent, 20% sodium carbonate, DPPH, ethanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and gallic acid were purchased from Sigma-Aldrich. Fresh okara samples were kindly provided by Vitasoy International Singapore Pte Ltd, Singapore. Okara was separated into aliquots, sealed in airtight polyethylene bags, and stored at -20˚C in the dark.

5.2.2. Fermentation

Ten g of heat-treated okara was placed in a flask and inoculated with *B. subtilis* WX-17 which was isolated and identified as described previously in Chapter 3 at a concentration of 10^6 CFU/g of okara. Fifty mL of sterile water was added to the flask and subsequently submerged liquid fermentation was carried out for 72 hours at 37 °C and 200 rpm. After fermentation, okara was removed through filtration and the supernatant was stored in 4 °C until further analysis. Okara with 50 mL of sterile water and no inoculation of *B. subtilis* WX-17 was used as control.

5.2.3. Enumeration of *B. subtilis* WX-17

Hundred µL of sample was added to 900 µL of sterile water and serial diluted 10 times. Hundred µL from each dilution were plated onto nutrient agar plates and incubated at 37 °C for 24 hours. After which the cell counts were recorded. This is repeated weekly for a duration of 6 weeks.

5.2.4. Metabolomics Analysis

Analysis of the metabolites present in the samples was performed based on the method described by Cooray et al. (2017) with minor modifications. Briefly, 10 µL of ribitol was added to 1.5 mL of sample and freeze-dried. Samples were derivatised using 100 µL of methoxamine hydrochloride and silylation was carried out using 200 µL of MSTFA with 1% TMCS. Samples were then centrifuged and 120 µL was transferred to glass vials for GC-MS analysis. The column and method used were as per described by Cooray et al. (2017).

5.2.5. Total Phenolic Content Analysis

Total phenolic content analysis was carried out with respect to the protocol described by Kamtekar et al. (2014). One mL of sample was added with 5 mL of deionized water, 0.5 mL of Folin Ciocalteu's reagent and shaken. After 5 mins, 1.5 mL of 20% sodium carbonate was added and made up to 10 mL before incubation for 2 hours. The absorbance of the mixture was measured at 750 nm with deionized water as blank using Nanodrop 2000c Spectrophotometer.

5.2.6. DPPH Scavenging Activity Analysis

The DPPH radical scavenging activity of the samples was evaluated using the method described by Gjorgievski et al. (2014) with minor modification. Six hundred µL of sample was added with equal volume of DPPH solution and vortexed before incubation in the dark for 30 mins. Absorbance was measured at 515nm using ethanol as blank and the activities of the samples were evaluated with respect to trolox equivalent-% signal inhibition calibration curve whereby % signal inhibition is defined

as: $\% \, Signal \, Inhibition = (1 - \frac{A_S}{A_O})$ X 100.A_s is defined as the absorbance of the samples and A_o is defined as the absorbance of pure DPPH.

5.2.7. Agar Disc Diffusion for Antimicrobial Assay

The antimicrobial activity of the fermented okara probiotic beverage (FO) was evaluated by employing the agar disc diffusion assay as documented by Ng et al. (2019) with minor modifications. Firstly, *Escherichia coli* (*E. coli)* ATCC 25922 and *Staphylococcus aureus* (*S. aureus)* ATCC 29213 were streaked onto fresh LB agar plates and incubated overnight at 37˚C. After incubation, individual colonies from each culture were re-suspended in 1 mL of Mueller-Hinton (MH) broth and the OD600 was adjusted to approximately 0.5 McFarland standard which corresponds to 1-3 X 10^8 CFU/mL. A sterile cotton swab was then used to streak-inoculate each strain on fresh MH agar plates. Sterile filter paper discs of 6mm were inoculated with 5 and 10 µL of FO as well as the unfermented control (RO) and subsequently placed onto the inoculated plates. Thereafter all inoculated plates were incubated at 37˚C for 18 hours. The antimicrobial activity was determined by measuring the inhibition zones in millimetres (mm) on the agar plates less the diameter of the filter paper (6 mm). All disc diffusion assays were carried out in triplicates. Ampicillin and sterile water were used as positive and negative controls respectively.

5.2.8. Statistical Analysis

Statistical analysis was performed using Metaboanalyst 4.0 as described previously (Mok et al., 2019). Data scaling was carried out using mean-centering and divided by the standard deviation of each variable prior to PLS-DA and heat map analysis. The heat map was constructed using Euclidean distance measurement and ward clustering algorithm. All experiments were carried out in triplicates.

5.3. Results and Discussion

5.3.1. Viability of *B. subtilis* WX-17

In a probiotic beverage, it is important that the microorganism remain viable after prolonged period of storage. The viability of the probiotic strain *B. subtilis* WX-17 was tested for a duration of 6 weeks at storage temperature of 4 °C (Figure 5.1). Results showed that after 6 weeks of storage, the cell count of viable *B. subtilis* WX-17 remained relatively unchanged at an average of 10.7 log CFU/mL which is above the daily probiotic recommended intake amount of 9 log CFU (Hill et al., 2014). These results are due to the fact that *B. subtilis* WX-17 would form endospores under unfavourable growth conditions (McKenney et al., 2012). This property would come in handy in terms of increasing the shelf life of the beverage without the addition of other ingredients, which shows the practicality of the methodology in the development of a low-cost functional *B. subtilis* probiotic beverage.

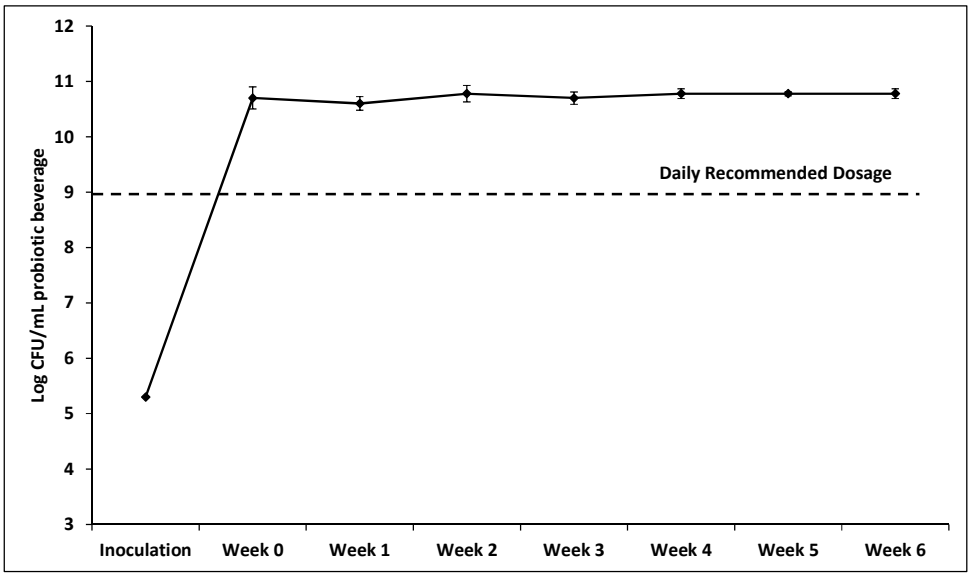


Figure 5.1. Changes in cell count of *B. subtilis* WX-17 using okara as sole nutrient source when stored at 4°C for 6 weeks

5.3.2. Metabolic Profiling of Fermented Okara Probiotic Beverage Control

A metabolomics analysis was carried out using the GC-MS between FO and RO to better understand the difference in the metabolic profiles during the fermentation process. Statistical analysis in the form of a PLS-DA and heat map was conducted to understand these changes better. A total of 31 metabolites were detected. PLS-DA analysis (Figure 5.2) was carried out to show the difference in the metabolic profiles. The green and red highlights represent the 95% confidence region. The first principal component accounted for 92.2% of the total variance while the second accounted for 2.20% for a total of 94.4%. This shows that the first principal component largely explained most of the variance within the dataset. The PLS-DA score plot (R^2 = 99.9% and Q^2 = 98.6%) showed a clear and distinct separation between the 2 sets of samples along the first principal component and not along the second principal component which is acceptable since most of the variance are accounted for by the first component.

A clustering heat map (Figure 5.3) was constructed to provide a visual representation of the changes in each metabolite between FO and RO. From the heat map, it can be seen that the overall profile changed significantly after submerged liquid fermentation by *B. subtilis* WX-17 (Figure 5.2, 5.3 and Table 5.1). Firstly 11 amino acids were detected in the samples of which 7 were upregulated (tyrosine, tryptophan, lysine, methionine, proline, phenylalanine and valine) after fermentation while 4 were downregulated. Interestingly, the amino acids that were downregulated (alanine, aspartic acids, asparagine and ornithine) were non-essential amino acids (the amino acids naturally produced by the human body in adequate amount) (Eagle, 1959). Since these non-essential amino acids are glucogenic, it can be hypothesized that they were consumed by *B. subtilis* WX-17 for metabolism. This hypothesis is reinforced by the

observation that both glucose, maltose and fructose were upregulated after fermentation which suggested that the rate of consumption of carbohydrates by *B. subtilis* WX-17 were lower than the rate of production since the microorganism are well known to produce amylase. This hypothesis is supported in a study by Sheu, Konings, and Freese (1972) which suggested that the presence of SCFA would inhibit the uptake of carbohydrates. Therefore, it is possible that *B. subtilis* WX-17 was not able to fully utilize the carbohydrates available for metabolism and hence used the non-essential amino acids instead. Vong and Liu (2019) also related that essential amino acids increased when okara is fermented by *Lindnera saturnus* under submerged liquid condition.

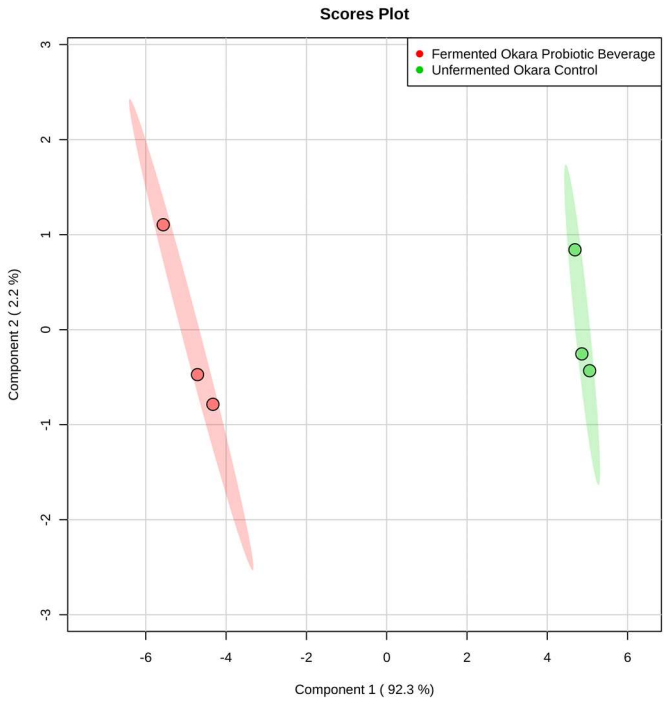


Figure 5.2. PLS-DA score plot of all metabolites found for fermented okara probiotic beverage and unfermented okara control. The green and red highlights denoted the 95% confidence region. Explained variance are shown in brackets

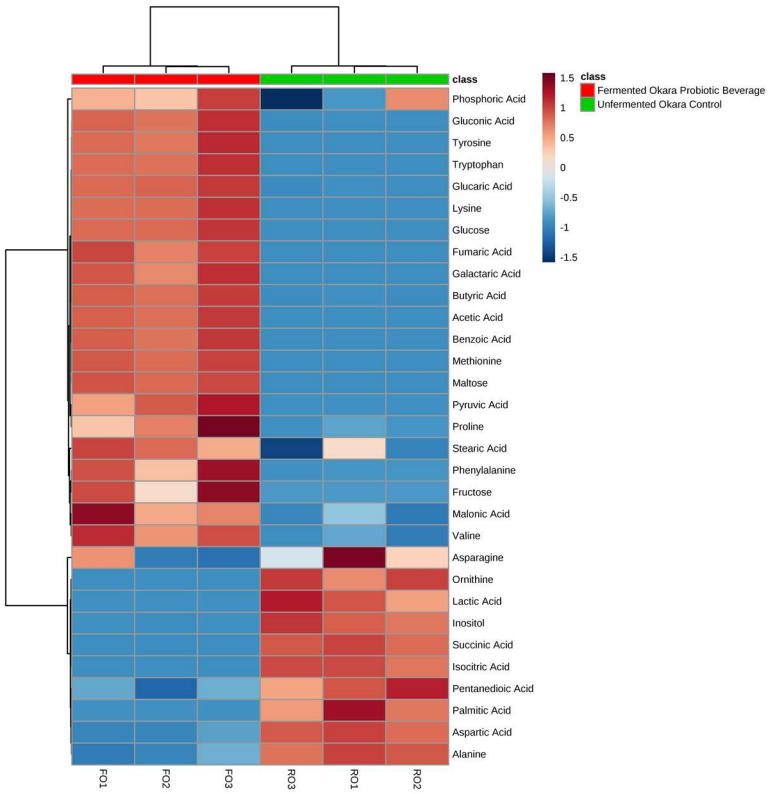


Figure 5.3. Heat map analysis correlating the metabolites of fermented okara probiotic beverage and unfermented okara control. Metabolites in brown are upregulated while those in blue are downregulated

Two important SCFA, acetic acid and butyric acid were also detected in FO. This is in agreement with studies conducted by Dirar, Harper, and Collins (1985) and Haq et al. (2018) which showed that *B. subtilis* can produce SCFA in the presence of dietary fibres. The upregulation of these SCFA is important, as they are known to confer various health benefits such as reducing the risk of colonic cancer, CVD as well as various gastrointestinal disorders (Hijová & Chmelarova, 2007; J. M. W. Wong et al., 2006).

On top of that, other important metabolites with numerous purported health benefits were also upregulated after fermentation. For example, both gluconic and glucaric acids are commonly found in *Kombucha* which have been linked with detoxification of toxins from the body (Martínez Leal, Valenzuela Suárez, Jayabalan, Huerta Oros, & Escalante-Aburto, 2018). Fumaric acid was found to be effective in the

treatment of psoriasis (Altmeyer et al., 1994). Studies have also shown that pyruvic acid exhibited angiogenic activity in both in vivo and in vitro models (Lee et al., 2001)

These results suggested that *B. subtilis* WX-17 were able to utilize okara as the sole nutrient source in submerged liquid fermentation and produce enzymes such as amylases, proteases and lipases to break down carbohydrates, proteins and lipids into their simpler form (Asgher, Asad, Rahman, & Legge, 2007; Lesuisse et al., 1993; Yang et al., 2000b).

The metabolites were also expressed in terms of relative percentage (Table 5.1) to gain a better context of the respective metabolic profiles. It was telling that many of the metabolites detected in FO were undetected in RO. The bulk of the metabolites in FO were made up of phenylalanine (17.5%), lysine (25.1%) and glucaric acid (15.1%) while RO consisted of mainly lactic acid (57.5%), isocitric acid (18.8%) and myo-inositol (15.6%).

Table 5.1. Changes in metabolites in terms of relative percentage for fermented okara probiotic beverage and unfermented okara control

Metabolites Relative %	Fermented Okara Probiotic Beverage	Unfermented Okara Control
Lactic Acid [a]	undetected	57.5±2.77
Acetic Acid [a]	2.47±0.0700	undetected
Pyruvic Acid [a]	0.160±0.0200	undetected
Butyric Acid [a]	2.78±0.0100	0.180±0.0200
Malonic Acid [a]	0.240±0.0200	0.0800±0.0100
Valine [a]	0.100±0.0100	0.0300±0.0100
Succinic Acid [a]	0.0900±0.0100	1.43±0.130
Fumaric Acid [a]	0.170±0.0100	undetected
Methionine [a]	0.500±0.0200	undetected
Proline [a]	0.640±0.0400	0.210±0.0200
Aspartic Acid [a]	0.120±0.0100	0.140±0.0100

Alanine [a]	0.310±0.0200	0.340±0.0400
Pentanedioic Acid [a]	1.72±0.0900	1.19±0.180
Benzoic Acid [a]	3.92±0.0900	undetected
Phenylalanine [a]	17.5±2.28	1.14±0.170
Lysine [a]	25.1±0.830	undetected
Phosphoric Acid	0.360±0.0100	0.150±0.0500
Ornithine [a]	undetected	1.20±0.190
Isocitric Acid [a]	0.380±0.0100	18.8±1.11
Fructose [a]	0.620±0.140	Undetected
Tyrosine [a]	6.40±0.120	Undetected
Galactaric Acid [a]	0.740±0.0200	Undetected
Gluconic Acid [a]	5.24±0.110	0.270±0.0300
Palmitic Acid [a]	undetected	0.390±0.0800
Tryptophan [a]	1.37±0.0400	undetected
Stearic Acid [a]	0.930±0.1000	0.350±0.0600
Asparagine	0.240±0.0500	0.150±0.0300
Myo-Inositol [a]	1.57±0.110	15.6±0.770
Glucaric Acid [a]	15.1±0.750	0.860±0.120
Glucose [a]	7.58±0.260	undetected
Maltose [a]	3.60±0.190	undetected

Results are as mean ± standard deviation (3 replicates)

[a] denotes metabolites that are significantly different ($p<0.05$)

5.3.3. Total Phenolic Content and Antioxidant Activity

Total phenolic content of FO (Figure 5.4) was found to have increased significantly compared to RO. The increase in total phenolic content after fermentation is likely due to the release of enzymes by *B. subtilis* WX-17 which hydrolysed the phenolic complexes that are combined or bound with sugars into soluble-free phenols (Queiroz Santos et al., 2018). FO was found to contain approximately 1.77 mg/mL GAE equivalent while RO contains only 0.280 mg/mL. This marked an increase in total phenolic content of 6.32 times. Similarly, Rashad, Mahmoud, Abdou, and Nooman

(2011) reported that okara fermented with various GRAS microorganism showed a significant increase in total phenolic content.

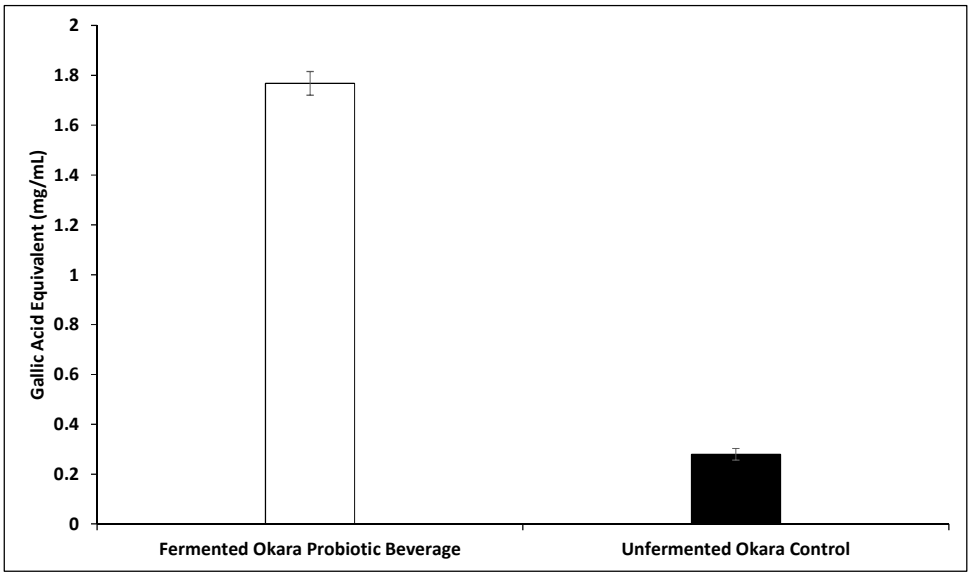


Figure 5.4. Total phenolic content of fermented okara probiotic beverage and unfermented okara control expressed in terms of GAE (mg/mL). Each data point represents the triplicate mean. Error bars represent standard deviation

Okara is well known to contain a large number of phenolic compounds. Studies had shown that phenolic molecules have numerous health benefits such as anti-oxidation, anti-aging, anti-inflammation, anti-carcinogenic and anti-atherosclerosis.

The antioxidant activity of FO (Figure 5.5) was also evaluated with regards to its DPPH radical scavenging activity. For FO, the DPPH radical scavenging activity was found to be 23.9 µg trolox equivalent/mL while RO contained only 15.5 µg trolox equivalent/mL which translates to an increase of 1.55 times.

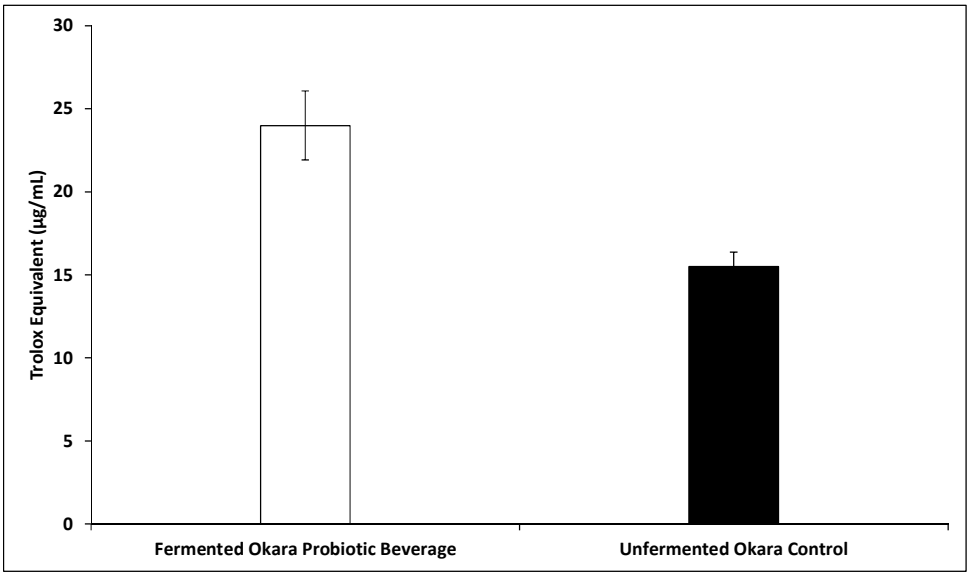


Figure 5.5. DPPH scavenging activity of fermented okara probiotic beverage and unfermented okara control expressed in terms of Trolox equivalent (µg/mL). Each data point represents the triplicate mean. Error bars represent standard deviation

Our body generates substantial amount of free radical through metabolic processes, which may result in oxidative damages to the tissues and cells in the human body. Therefore, the increase in DPPH radical scavenging activity (Figure 5.5) by 1.55 times after fermentation by *B. subtilis* WX-17 is ideal. This observation is in agreement with studies by Juan and Chou (2010) whom reported that soybean fermented with *B. subtilis* B2 exhibited an increase in DPPH radical scavenging activity.

5.3.4. Antimicrobial Activity

FO showed antimicrobial activity against *S. aureus* ATCC 29213, which is a gram-positive bacterium with mean inhibition zones of 5.3 mm and 6 mm for 5 µL and 10 µL respectively (Figure 5.6). No inhibition zones were detected for *E. coli* ATCC 25922 that indicated that there was no antimicrobial activity. RO did not exhibit any antimicrobial activity for both *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. These results were supported by Ghribi et al. (2012) and Yilmaz, Soran, and Beyatli (2006) which suggested that *B. subtilis* can exhibit antimicrobial properties due to the

production of broad-spectrum antibiotics or biosurfactants such as bacillomycins which are amphiphilic membrane-active biosurfactants with strong antimicrobial activities. However, Ghribi et al. (2012) also suggested that the type of substrate affects the production of biosurfactants, which could explain why FO only exhibited antimicrobial activity against gram-positive microorganisms. These findings suggest that less chemical preservatives might be required for the probiotic beverage from a commercialization point of view, which would reduce cost and public perceptions.

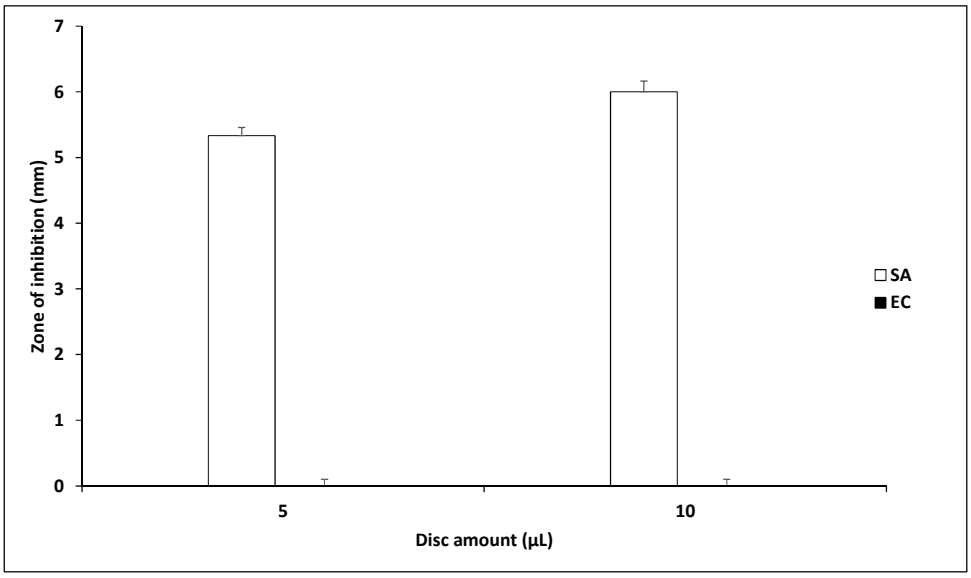


Figure 5.6. Agar disc diffusion results for *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. Each data point represents the triplicate mean. Error bars represent standard deviation. SA: *S. aureus* ATCC 29213. EC: *E. coli* ATCC 25922

5.4. Conclusion

This work showed that submerged liquid fermentation with *B. subtilis* WX-17 using okara, as the sole nutrient source is a viable approach. FO exhibited higher amount of amino acids, SCFA as well as other useful metabolites such as gluconic acid, glucaric acid and fumaric acids. It also provided an adequate amount of probiotic (more than 9 log CFU daily) on top of increased amount of phenolic content and antioxidant activity. Furthermore, FO displayed antimicrobial activity against gram-positive bacteria, which

could potentially reduce the need for chemical preservatives in the probiotic beverage. These results were achieved without the addition of other enzymes or chemicals which would greatly reduce the cost of production as well as minimize potential health concerns for our increasingly health-conscious population. It would be of significant interest to further investigate the health benefits, consumer acceptance of this novel probiotic beverage as well as modifications to the substrate such that the antimicrobial activity encompasses gram negative bacteria. Modification to the methodology or addition of natural emulsifiers can be explored to enhance solubility of fat-soluble nutrients such as MK-7 or fatty acids, which would further increase the nutritional value of the beverage.

6. Conclusion and Future Directions

6.1. Conclusion

This thesis set out to alleviate the rising global problems of food security by establishing methodologies of reusing okara to enhance said topic as well as to reduce its disposal, which can cause climate issues due to the release of greenhouse gases. With this in mind, processing technology (which is one of the 3 key prongs for enhancing food security in Singapore) was utilized to valorise okara in a manner that does not produce secondary waste (Figure 6.1).

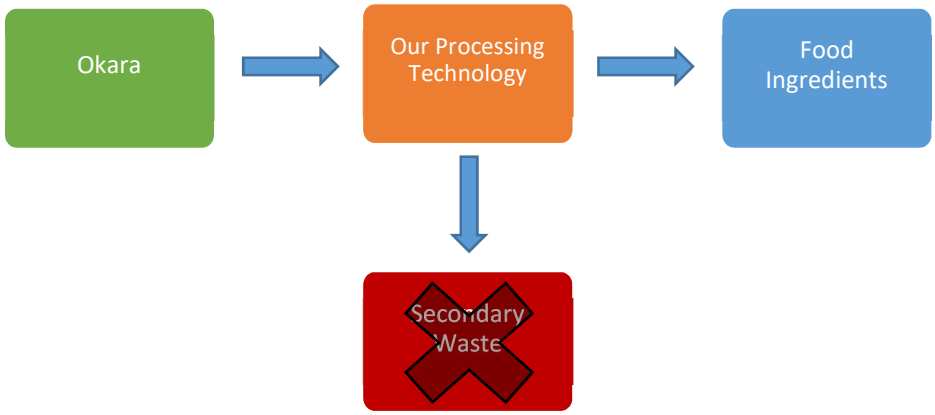


Figure 6.1. Our processing technology for okara

We proposed a solution where okara was fermented by *B. subtilis* WX-17 under solid-state conditions to increase its nutritional values and thereafter, be used as a food ingredient for consumption. Widespread adoption would not only enhance food security by reducing reliance on primary production, okara disposal would also be reduced since there is no secondary waste generated during the production cycle. This is unlike most of the current utilization techniques of okara, which produces secondary waste. In addition, SLF of okara was also explored to provide an alternative solution (probiotic beverage) that is more scalable compared to SSF based on current advancement in upscale technology.

In Chapter 3, okara was fermented by *B. subtilis* WX-17 under solid-state condition. Thereafter, its nutritional profile was evaluated using a combination of metabolomics and various assays. Through metabolomics, it was found that the different sugars in okara were consumed by *B. subtilis* WX-17 for metabolism, which would in turn produce extracellular proteases and lipases to break down proteins and fats into amino acids and fatty acids, which have numerous functional properties. As a result, all detected amino acids were upregulated. Interestingly, results showed that after fermentation, saturated fatty acids were down regulated while unsaturated fatty acids were upregulated. More importantly, fermented okara was found to contain high amount of MK-7, which have been reported by numerous other studies to be beneficial to bone health. This is significant because MK-7 is not easily attainable from other food apart from *Natto,* which is not widely consumed outside of Japan. Total phenolic content and consequently antioxidant activity of okara were also enhanced after fermentation. This study showed that a relatively simple and low-cost microbial fermentation of okara could dramatically increase its nutritional value, which would be more beneficial to health compared to unfermented okara.

In Chapter 4, the functional, probiotic and prebiotic properties of fermented okara in the human body were evaluated through an *in vitro* digestion/fermentation model to understand the bioaccessibility of nutrients in the small intestines as well as its probiotic and prebiotic effects in the large intestines. After *in vitro* digestion, the bioaccessibility of amino acids, fatty acids, MK-7 and total phenolic content in fermented okara were higher compared to unfermented okara. This is due to the conversion of IDF into SDF during the fermentation process. Fermented okara was also shown to have no cytotoxicity when consumed in reasonable amounts. *B. subtilis* WX-17 was also able to remain viable after passing through the acidic gastric conditions. In

the large intestines, presence of more SDF led to an increase in total phenolic content and SCFA, which have numerous health benefits. In addition, statistical analysis showed that some beneficial microorganisms in the gut were enhanced while non-beneficial ones were diminished. The findings of this work proved that fermented okara is potentially more beneficial to human health and is therefore an ideal way of utilizing okara to fulfil both objectives of enhancing food security while not contributing secondary waste through a low-cost methodology.

As mentioned, although SSF has numerous advantages over SLF in the food industry, its main problem is in upscaling due to heat transfer issues among other problems. Therefore, in Chapter 5, a low-cost alternative of utilizing okara using a scalable methodology was developed. Here, submerged liquid fermentation was employed using okara as sole nutrient source to develop a novel probiotic beverage. Results showed that *B. subtilis* WX-17 was able to remain viable in the probiotic beverage when stored for 6 weeks at 4°C. Metabolomics analysis also found that amino acids and SCFA were enhanced. Total phenolic content and antioxidant activity were also higher in the probiotic beverage. It was also found that the beverage contained antimicrobial properties against gram-positive bacterium. This result suggested that chemical preservatives usage could potentially be reduced for the probiotic beverage. The success of this study provided an alternative way of utilizing okara in a scalable method while at the same time meeting the overall aim of enhancing food security while not producing secondary waste.

Overall, the results obtained across all studies contained in this thesis had achieved the aims outlined. We were able to effectively enhance the nutritional profile of okara through low-cost fermentation techniques (both SSF and SLF) using *B. subtilis* WX-17 that was able to even produce significantly higher amount of MK-7 which are

difficult to obtain in diets. The bioaccessibility of these enhanced levels of nutrients, cytotoxicity and the effects of fermented okara on the gut microbiota were also evaluated and found to be generally beneficial to human health. Alternative method of valorising okara was also established. The findings of this thesis suggest that further crystallization of the techniques used and subsequent large-scale adoption of the methodologies detailed can go a long way towards enhancing global food security and at the same time reducing food wastage/disposal issues, which can help alleviate climate issues.

6.2. Future Directions

6.2.1. Fermentation Parameters

In order for the techniques detailed in this thesis to be more widely adopted and commercialized, it is important that various fermentation parameters be optimised both to maximise the nutrient content and also to reduce production time. Currently, in both SSF and SLF, okara was fermented by *B. subtilis* WX-17 for 3 days at 37°C. However, it is unclear if that is the optimum growth condition and duration. Further experiments should be carried out to determine if the duration of fermentation affects nutrient content, this could potentially shorten production cycles. Similarly, experiments should be carried out across various temperatures to determine if 37°C is the ideal temperature for *B. subtilis* WX-17 fermentation.

An optimisation study should be carried out as based on literature research, different studies conducted *B. subtilis* fermentation at different durations and temperatures. Therefore, it is difficult to determine the ideal culture condition since those studies are for different purposes. For example, Owens, Allagheny, Kipping, and

Ames (1997) conducted SSF using *B. subtilis* on soybeans at 35°C for 18 and 36 hours while Zhu et al. (2008) conducted their fermentation at 40°C for 48 hours.

6.2.2. Solid-State Fermentation

There are currently no commercial solid-state bioreactors. This is due to the heat transfer problems as mentioned earlier. Over the years, many studies had been conducted to identify the best configuration for solid-state bioreactor design. However due to its complexity, there is no consensus on the best configuration although the Zymotis packed bed design from ORSTOM is considered to be one of the most promising in recent years (Mitchell, Krieger, Stuart, & Pandey, 2000). The zymotis design consists of closely spaced internal heat transfer plates within a packed bed bioreactor that can effectively remove metabolic heat through radial conduction. However, current capacity of the zymotis bioreactor is at about 55 kg moist solid substrate which is not sufficient for large-scale commercial process (Desobgo Zangue, Mishra, Behera, & Panda, 2016). Therefore, much work is required to further develop the solid-state bioreactors such that it would become economically and technologically feasible at industrial scale.

6.2.3. Submerged Liquid Fermentation

SLF is a well-understood and highly developed technology currently. As such, the focus of improvement here is specific to the methodology described in this thesis. The current technique to produce the novel probiotic beverage uses okara as sole nutrient source and contains no other additives. This way, fat-soluble nutrients such as fatty acids and MK-7 were not detected in the beverage. However, based on the nutritional profile for SSF, it is reasonable to believe that both fatty acids and MK-7 can be produced using SLF and it would enhance the nutritional value of the probiotic

beverage if they were present. Therefore, the addition of natural emulsifiers such as beeswax (which is approved for use in food) can be considered to allow fatty acids and MK-7 to be soluble in the probiotic beverage, which would further increase its health benefits. It would also be interesting to evaluate the consumer acceptance of such the probiotic beverage as well as modification of its formulation to improve its taste profile. Lastly, further work is required for the inherent antimicrobial properties to encompass gram-negative bacteria.

6.2.4. Bioavailability and *In Vivo* Evaluation

The *in vitro* study carried out in this thesis provided a first indication about the benefits and safety of fermented okara. In order for fermented okara (by *B. subtilis* WX-17) to be widely accepted as a functional, probiotic and prebiotic food ingredient, further evaluations would have to be conducted. For instance, a bioavailability study can be conducted using a dynamic continuous-flow dialysis system to simulate the flow of nutrient through the small intestinal walls. This would allow us to better understand how much nutrients from fermented okara are actually available in the blood. Following which, an *in vivo* study can be carried out using animal models first to determine health benefits and potential side effects. After which, a large-scale dietary intervention study can be conducted where volunteers are provided with a fixed amount of fermented okara in their diet for a set duration. Samples (blood and stool) can be subsequently collected for analysis. Nutrient analysis (for example, amino acids, fatty acids and MK-7) can be conducted on the blood samples to determine if frequent consumption of fermented okara would increase serum concentration of nutrients. 16s rRNA analysis can be carried out on the stool samples to determine the effects of fermented okara on the gut microbiota.

Currently, *in vivo* studies on okara are sparse at best, with the limited studies focusing mainly on antioxidant properties. For example, J. Liu, Chang, and Wiesenborn (2005) evaluated the *in vivo* antioxidant properties of soybean isoflavone extract and found that diets high in isoflavone contents showed elevated enzymatic levels across multiple organs. Matsuo (1997) conducted a similar study on okara koji and found that rats that are fed with okara koji exhibited antioxidant abilities against lipid peroxides. Therefore, a carefully designed, comprehensive *in vivo* nutrient study can provide a more holistic view of the effects of fermented okara and potentially move one-step closer to widespread adoption in our daily diets.

6.2.5. Sensory evaluation

Fermented okara has a nutty and strong pungent flavour not dissimilar to natto. Therefore, extensive sensory evaluation would have to be performed to incorporate it into food products as a food ingredient. This is because, outside of Japan, natto is widely regarded to be an acquired taste. Even then, there is a sizable portion of Japanese that does not like the strong taste profile of natto. This is an area where food scientists and flavour specialists could potentially look into the flavour profile of fermented okara and thereafter, through the application of flavour chemistry, make it more palatable.

7. References

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