BREWERS' SPENT GRAINS AS POTENTIAL NOVEL FUNCTIONAL FOOD INGREDIENTS FOR FOOD SECURITY

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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.

11 Jan 2021

Date

Tan Yong Xing

Supervisor Declaration Statement

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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Date	Professor Chen Wei Ning, William

Authorship Attribution Statement

This thesis contains material from four papers published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

Chapter 1 and chapter 2 contain sections from 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,* (Vol. 120, pp. 155-168) https://doi.org/10.1016/j.tifs.2020.06.013

The contributions of the co-authors are as follows:

- I and Mok, W.K. compiled data and co-wrote manuscript (co-first authors).
- Prof Chen, W. N. edited the manuscript drafts.

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- I carried out the experiments and wrote the manuscript. Mok, W. K. and Lee, J.J. L. commented on the manuscript
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AI	Artificial intelligence
AO	Acid orange
AOAC	Association Of Official Analytical Collaboration
ATP	Adenosine triphosphate
AXOS	Arabinoxylooligosaccharides
GBF	Germinated barley food
BMI	Body mass index
BSG	Brewers’ spent grains
CFU	Colonies forming units
CH_4	Methane
CO_2	Carbon dioxide
DHA	Docosahexanoic acid
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DPPH	1,1-dipheny-2-picryl-hydrazil
FAMEs	Fatty acid methyl esters
FAO	Food and Agriculture Organization
GAE	Gallic acids equivalent
GC-MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal
GRAS	Generally recognized as safe
HCl	Hydrochloric acid
HDL	High density lipoprotein

HPLC	High performance liquid chromatography
IDF	Insoluble dietary fibre
IFAD	International Fund for Agricultural Development
IOT	Internet of things
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoproteins
LED	Light-emitting diode
MK	Menaquinone
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NADH	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
NH_3	Ammonia
N_2O	Nitrous oxide
OPLS-DA	Orthogonal Partial Least Square-Discriminant Analysis
PAR	Photosynthetically Active Radiation
PBS	Phosphate-buffered saline
PC	Principal component
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PLS-DA	Partial Least Square - Discriminant Analysis
Q-TOF MS	Quadrupole time-of-flight mass spectrometry
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
SCFAs	Short-chain fatty acids
SDF	Soluble dietary fibre

SSF	Solid State Fermentation
TCA	Tricarboxylic acid
TMCS	Trimethylchlorosilane
TQMS	Triple Quadrupole Mass Spectrometry
UDP	Uridine diphosphate
UV	Ultraviolet
WFP	World Food Program

THESIS ABSTRACT

With a growing population and increasing amount of food losses generated, food security is becoming a challenging issue. Facing the impending threat of food security, technology innovations are required to enhance food security. Strategies to enhance food security can be categorized into three main areas, urban farming, processing technology and alternative food sources. This thesis aims to explore the area of food waste processing technology, particularly development of feasible methodologies to sustainably manage brewers' spent grains (BSG). BSG are residues generated as side streams from beer production. Currently, BSG are used as animal feed or disposed in landfills and these massive amount of BSG generated annually are causing significant environmental, economic, and climatic issues. These disposed BSG are still of high nutritive value, containing compounds such as dietary fibres, fatty acids, proteins, amino acids, polyphenols. Despite previous studies performed on the usage of BSG, there lies an issue of remaining residual waste materials after utilization. Hence, there is an urgent need to utilize BSG and sustainably manage these residues.

In this work, bacterial fermentation was employed to harvest the remaining nutrients in BSG and enhance its nutritional profile. BSG were fermented using a generally recognized as safe (GRAS) bacteria, *Bacillus subtilis* WX-17. The first work seeks to investigate the changes which occurred during fermentation using untargeted metabolomics and pathway analysis. Based on the findings, bacterial fermentation gave a 2-fold increase in the total amount of amino acid from 0.859 ± 0.05 to 1.894 ± 0.1 mg per g of BSG. Also, the total amount of unsaturated fatty acid increased by 1.7 times and the total antioxidant quantity remarkably increased by 5.8 times after fermentation. The increase in various compounds enhanced BSG nutritional profile.

In order to assess the potential of fermented BSG as functional food ingredients, an *in vitro* digestion-fermentation setup was employed. According to the findings, *in vitro* digestion of fermented BSG had higher amounts of various nutritional components. Vitamin K_2 MK7 was detected with concentration of 1.2 x 10^{-4} ± 5 x 10^{-6} mg/ mL. Probiotics, *Bacillus subtilis* WX-17 were observed to be available for absorption. Various short chain fatty acids namely acetic acid, propionic acid and butyric acid were produced at higher amounts for fermented BSG. The concentrations obtained were 124.11 ± 18.72 mM, 13.18 ± 1.38 mM and 46.25 ± 7.57 mM respectively. Lastly, differential genera such as *Bacteroides, Odoribacter* were detected and correlates to beneficial effects on the intestinal microbiota. This study evaluated the potential of fermented BSG to serve as novel functional food ingredients.

The last study explored the possibility of submerged stage fermentation using *Bacillus subtilis* WX-17 on BSG as the sole substrate to produce a potential novel nutritional beverage as its application. *Bacillus subtilis* WX-17 was still viable after a period of 6 weeks with a final cell count of 9.86 log CFU/ mL. The other nutritional property such as phenolic content, increased from 125.7 ± 0.74 µg/ mL to 446.74 ± 1.26 µg/ mL.

In conclusion, this thesis demonstrates sustainable, zero waste food processing methods to enrich and harvest the nutrients present in underutilized BSG through fermentation using *Bacillus subtilis* WX-17.

Chapter 1. Introduction

1.1: Background

According to FAO, IFAD and WFP, 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 important concern. 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 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 must 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 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.

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," 2020). 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 (zero waste food processing) as well as platform technology to develop alternative and unconventional food sources.

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 amount of 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 shows 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 food production, 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 and there is a need to manage these food wastes. To mitigate the environmental effects of food wastage, reduction of food wastage through technological means will be required as the most preferred action would be prevention and reduction of food wastage at source and subsequently least favourable action is disposal of food waste into landfills (Figure 1). If innovations can convert such food waste into food for consumers, they will provide another food source to enhance Singapore’s food security.

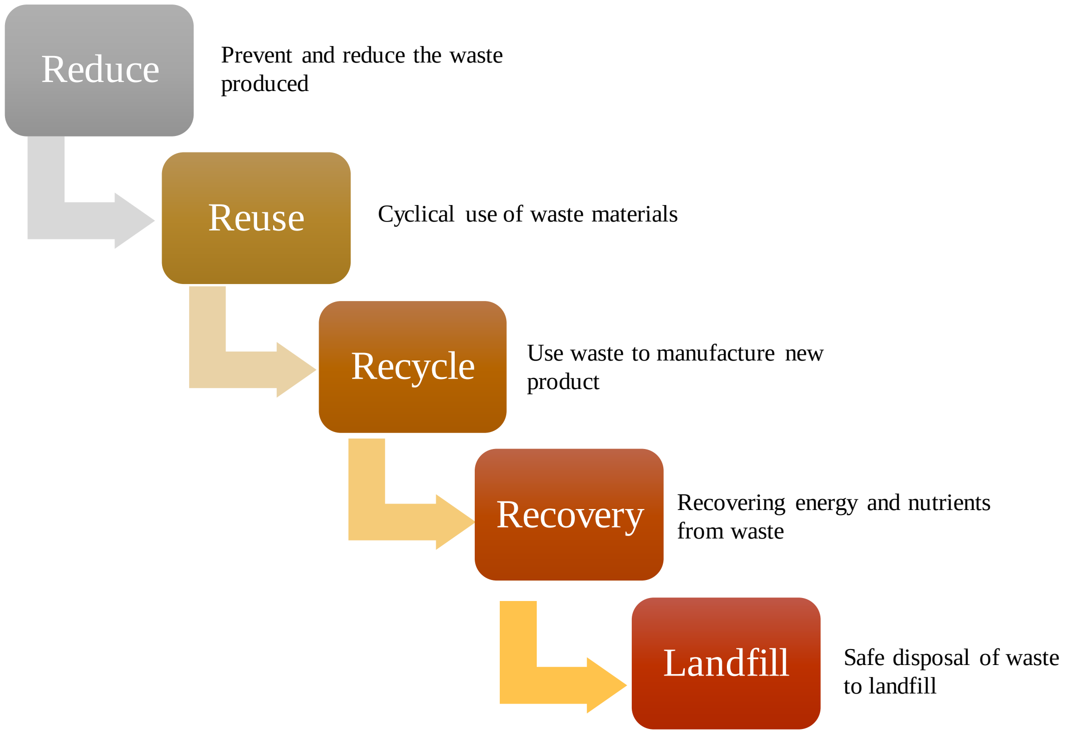


Figure 1: **Food waste hierarchy.** *Image content extracted from (Papargyropoulou, Lozano, K. Steinberger, Wright, & Ujang, 2014) with rights and permission obtained from Elsevier.*

Therefore, one of the approaches to achieve nutrient recovery, zero waste processing which is no remaining residual waste material, is through enhancement of the food wastes using fermentation. This approach provides sustainability, environmentally friendliness and reduced energy costs to manage the massive quantities of food waste generated. Fermentation is a metabolic process where microorganisms break down the elements in substrate enzymatically. The resulting metabolites after fermentation can serve as valuable compounds that provide higher value to the food waste.

1.2: Research gap and contribution

Providing food security to the population is becoming a challenge due to the huge amount of food losses generated and population rising continuously. Firstly, with higher amount of food losses, it will lead to wastage of resources such as fresh water, arable land, labour and capital. Secondly, food losses also generate more greenhouse gases and contribute to climate changes which can cause higher crop failure to happen due to higher frequency of natural disasters and in turn it will affect the global food production. Thirdly, disposed food wastes are materials that contain valuable compounds that can be further utilized. One of such food wastes is BSG, which contain amounts of beneficial components that are underutilized and being disposed. Lastly, the current approaches to utilize BSG have a common significant limitation of residues leftover, which would still require resources for disposal after the extraction of targeted compounds from BSG. The remaining residual can also cause detrimental effects on the environment and affect food security. There lies the existing gap to sustainably manage the food wastes to minimize the wastage of finite resources, having no residual waste material and converting it as a food source for the population. This study examines the utilization of BSG through fermentation processes so as to recover the remaining nutrients and providing a zero residual waste processing of BSG. Both solid state and submerged fermentation were examined to achieve the nutrient recovery outcome. An *in vitro* digestion-fermentation method was employed to evaluate the health effects of solid state fermented BSG as potential functional food ingredients. The enhanced BSG product and effects on human health were analyzed mainly through untargeted metabolomics, antioxidant assays and gut microbiota profiling.

1.3: Aims and thesis outline

Fermentation using GRAS bacteria can be an environmentally friendly and cost-effective methodology to ameliorate BSG by recovering the nutrients present and subsequently serve as functional food ingredients to achieve the objective of zero waste processing. Untargeted metabolomics was employed to allowed deep insights to the mechanism behind the fermentation of BSG and examined the range of compositional variations during the process. Identification of beneficial or detrimental compounds produced will be useful information for potential applications. In addition, the various metabolites will be mapped onto biochemical pathways will provide a more comprehensive view of the metabolic changes during fermentation process.

To investigate the health effects of fermented BSG after consumption, employing *in vitro* digestion-fermentation method will serve as an effective preliminary method. *In vitro* digestion-fermentation method mimics the complex gut environment and allows the study of microbiota responses to fermented BSG. The assessment was done in *in vitro* method instead *in vivo* method as *in vivo* studies are considered expensive, time-consuming and food materials have to be verified appropriately before the trials. *In vitro* evaluation of the food materials can serve as a cost-effective, simple, rapid and reliable preliminary assessment.

This thesis has been subdivided into 6 chapters. Chapter 1 is an introduction to the thesis which includes the background, aims and objectives of the research and an overview of the sections of the thesis is provided.

Chapter 2 presents the literature review of the topic, overview of the various studies to utilize food wastes, particularly BSG and evaluation of related biological processes. The review will also include the analysis methods such as metabolomics and *in vitro* method to evaluate foods.

Chapter 3 reports the study on solid state microbial fermentation using BSG. Untargeted metabolomics using gas chromatography-mass spectrometry (GC-MS), antioxidant assay, statistical analysis and pathway analysis were applied to analyze the changes during fermentation process and identify compounds that can be beneficial to human health.

Chapter 4 investigates the health effects of fermented BSG using an *in vitro* digestion-fermentation method. The analysis of the compounds present after *in vitro* digestion-fermentation were carried out through techniques such as high-performance liquid chromatography (HPLC), 16s rRNA sequencing to assess the health effects.

Chapter 5 demonstrates a potential nutritional beverage by employing submerged fermentation on BSG. The purpose of the work lies on the development of an alternate methodology and also a more scalable option to manage BSG. Viability of probiotics was investigated and quantified. Valuable compounds produced as a result of submerged microbial fermentation on the food waste were also identified and analyzed. Lastly, chapter 6 is a summary of key results of this study and potential future work is discussed.

Chapter 2. Literature Review

2.1: Technology innovations for food security in Singapore

Food security is becoming an increasingly important global issue and facing impending threat of food security, the world can no longer rely on traditional methods to meet its needs. Instead, more creative and technologically advanced methods must be adopted to maximise diminishing natural resources. Singapore’s strategies for enhancing food security can be redefined to 3 main areas: urban farming, processing technology and alternative food sources. Firstly, the technology innovations in urban farming encompasses vertical farming, aquaponics and internet of things while processing technology would focus on food waste valorization, 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 provides a snapshot of the technology innovations that Singapore has adopted for food security as well as challenges and future prospective. This would serve as a case study for the increasingly natural resource-scarce world that we are living in.

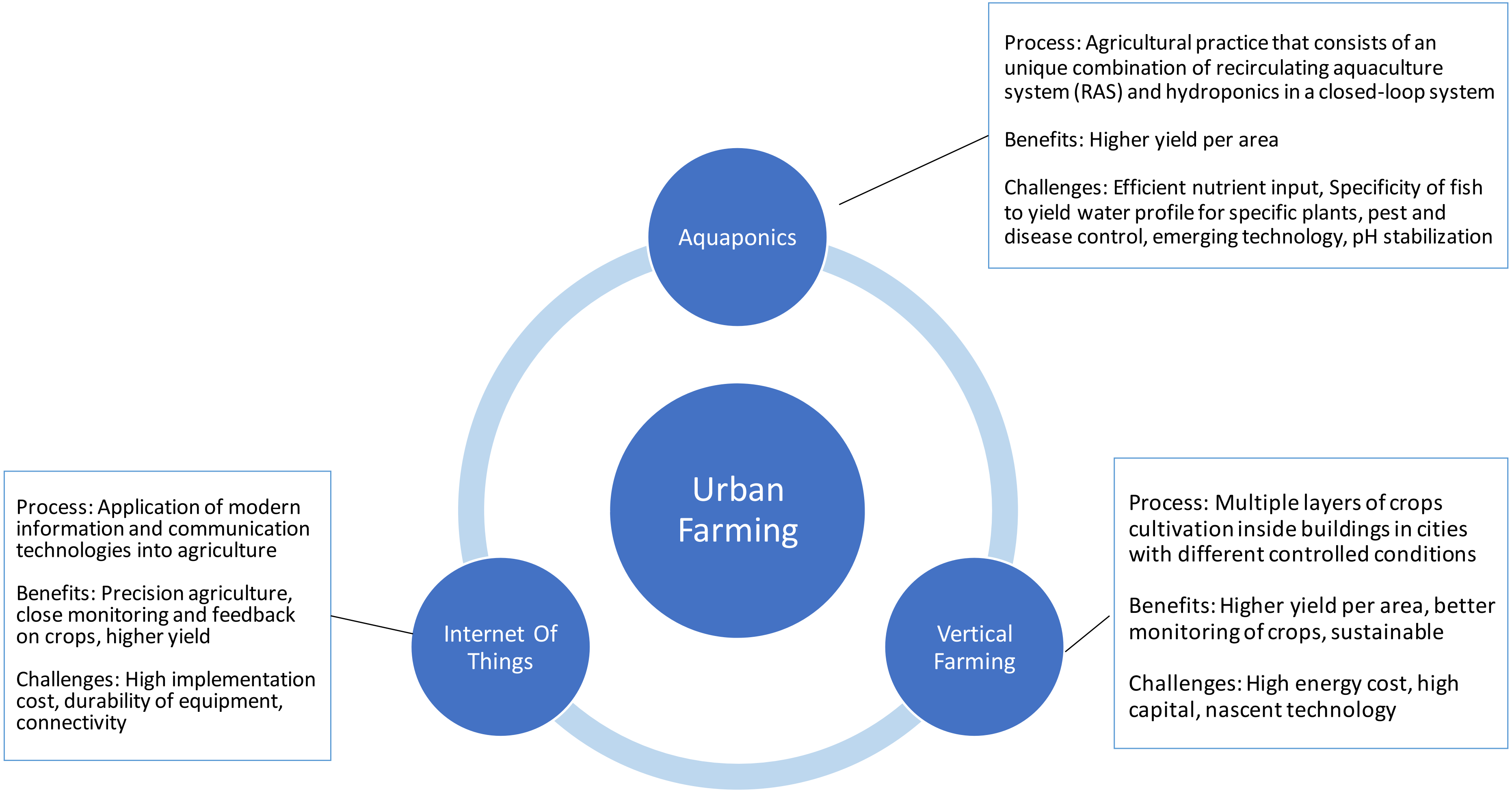
Table 1: Summary of technology innovations and their impacts on food security in Singapore

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 Higher yield per unit area
	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 valorization	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	

2.1.1 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 has already been adopted by the nation while others such as aquaponics and AI assisted smart agriculture are in their infancy (Figure 2).



Process: Agricultural practice that consists of an unique combination of recirculating aquaculture system (RAS) and hydroponics in a closed-loop system

Benefits: Higher yield per area

Challenges: Efficient nutrient input, Specificity of fish to yield water profile for specific plants, pest and disease control, emerging technology, pH stabilization

Process: Application of modern information and communication technologies into agriculture

Benefits: Precision agriculture, close monitoring and feedback on crops, higher yield

Challenges: High implementation cost, durability of equipment, connectivity

Process: Multiple layers of crops cultivation inside buildings in cities with different controlled conditions

Benefits: Higher yield per area, better monitoring of crops, sustainable

Challenges: High energy cost, high capital, nascent technology

Figure 2: Summary of urban farming.

2.1.1.1 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 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. Firstly, 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, light-emitting diode (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 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 the 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.

2.1.1.2 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 sulfur (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.

2.1.1.3 Internet of things (IOT) 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 (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).

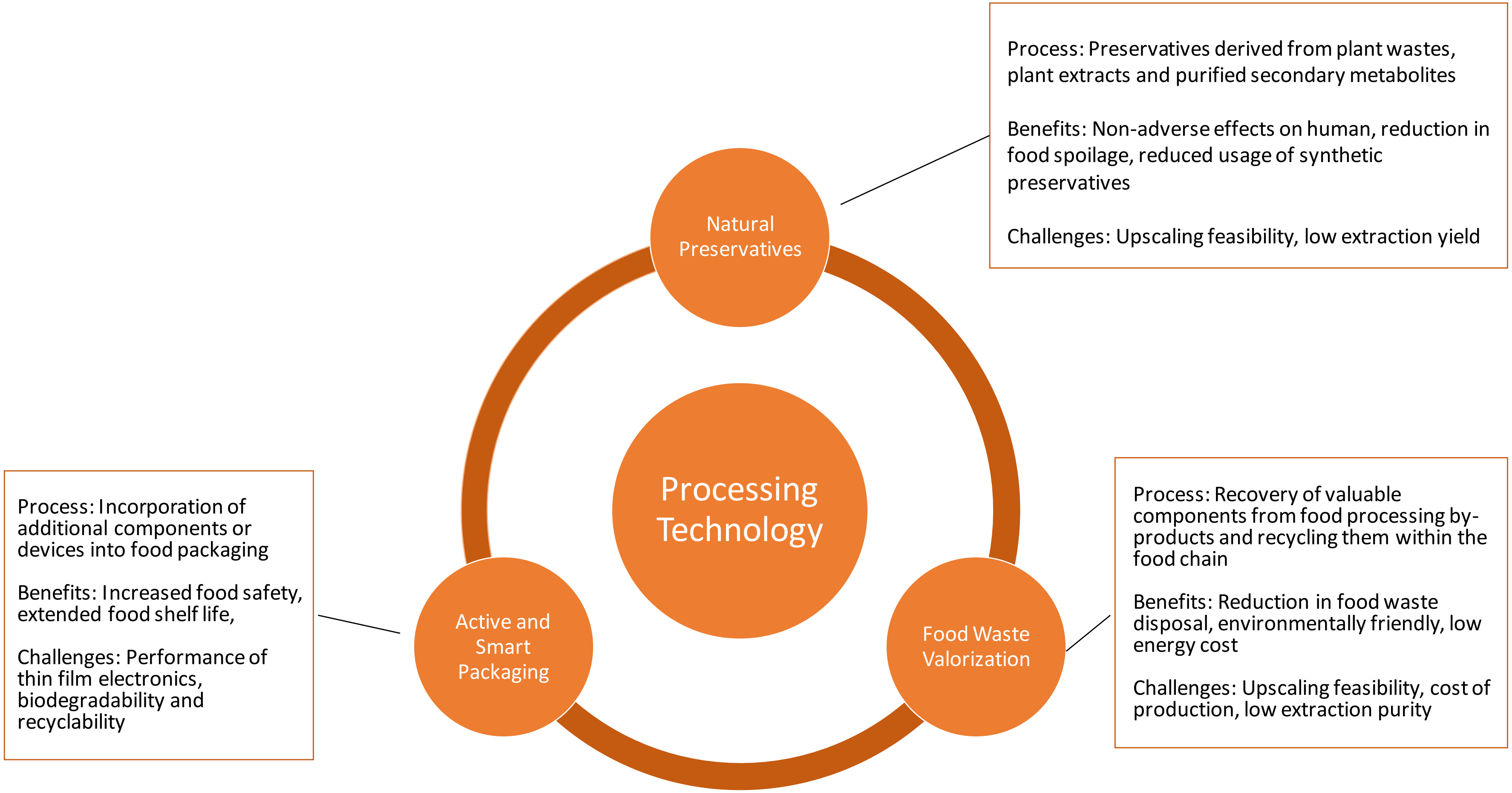
With its "30 by 30" goal in sight, Singapore has started to incorporate IOT into its urban farming scene. For example, researchers from the Singapore-Massachusetts Institute of Technology (MIT) alliance for Research and Technology (SMART) have found a method of monitoring the growth of plant at a molecular 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 expertises 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, 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.

2.1.2 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, this review would focus on the aforementioned areas. Figure 3 provides a graphical summary of the processes, challenges and benefits of each area.



Process: Preservatives derived from plant wastes, plant extracts and purified secondary metabolites

Benefits: Non-adverse effects on human, reduction in food spoilage, reduced usage of synthetic preservatives

Challenges: Upscaling feasibility, low extraction yield

Process: Incorporation of additional components or devices into food packaging

Benefits: Increased food safety, extended food shelf life,

Challenges: Performance of thin film electronics, biodegradability and recyclability

Process: Recovery of valuable components from food processing by-products and recycling them within the food chain

Benefits: Reduction in food waste disposal, environmentally friendly, low energy cost

Challenges: Upscaling feasibility, cost of production, low extraction purity

Figure 3: Summary of processing technology.

2.1.2.1 Food waste valorization

Food waste processing involves the recovery of valuable components from food processing by-products and recycling them within the food chain. As mentioned, more than one-third of the total food production goes to waste. These food 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).

In Singapore, roughly 763,100 tonnes of food waste were generated in 2018. Of these, only 17% were recycled while the rest were 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 using of technology. The reduction of food waste disposed can be achieved through the use of technology, however it is important to note that fibrous food wastes such as okara, BSG, bamboo shoots and vegetables still contains residue after reuse due to the presence of insoluble dietary fibre such as cellulose and lignin which would leave a carbon footprint when disposed. Therefore, zero-waste food processing technology has to be adopted in order to minimize the carbon footprint of food wastes. The following section provides examples about the processing technologies that can be applied to food waste that are developed in Singapore such as the extraction of nutrients, production of a probiotic beverage, fabricating of biodegradable food packaging using durian rinds and natural preservatives.

The most cost effective method to valorize 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 globally. For example, Vong and Liu (2019) used a combination of different biocatalyst in the fermentation of okara to create a novel probiotic beverage. Firstly, carbohydrase was added to convert the insoluble fibres into soluble fibres. The okara hydrolysate was then fermented with *Lactobacillus paracasei* and *Lindnera saturnus* to increase its free amino acids, isoflavone aglycones and fruity esters content. The probiotics were also able to remain viable when stored at 5°C for 6 weeks. In another work, 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-wastes (Khare et al., 1995). In another application, okara was fermented with *Bacillus 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, Hirai, & Shoda, 2006). In a study conducted by (Orts et al., 2019), okara was submitted to a combination of enzymatic hydrolysis treatment followed by fermented 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.

2.1.2.2 Biodegradable packaging

Although valorization 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, and 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).

2.1.2.3 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).

In summary, the various types of food waste generated in different processes had been explored widely to be reused in nutrient recovery through several methods. Food waste has also been used to create biodegradable food packaging which can reduce global plastic waste. In addition, development of natural preservatives would potentially help to extend shelf life of food and contribute to improving food security.

2.1.2.4 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.

2.1.2.5 Challenges in processing technology

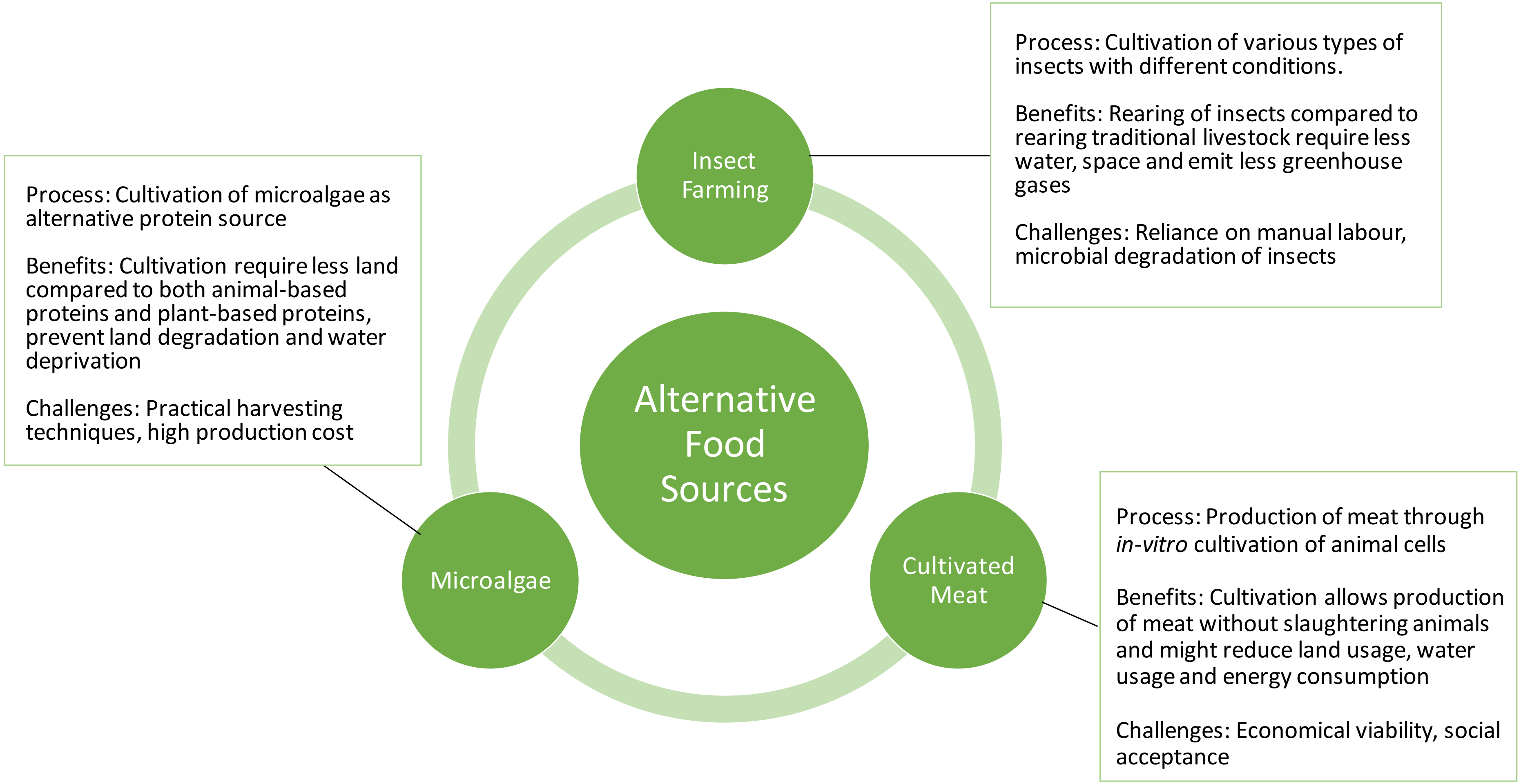
Most of the technologies highlighted are relatively new and generally performed at lab scale. Therefore, one of the main challenges is to scale up these processes. For example, food waste valorization is usually fermented under solid-state condition. At industrial scale, this would result in non-uniform fermentation due to the temperature gradient effect within the solid substrate. One way of mitigating this is the use of tray bioreactors where substrates are laid out onto each tray thinly which would minimize the effects of temperature gradient (Durand, 2003). However, such setups are space consuming which is unsuitable for countries like Singapore where spaces are at a premium.

Another important challenge 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 substrates. 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 is 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.

2.1.3 Alternative food sources

As mentioned, 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, moving forward, 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 to add on to existing food supply (Figure 4). 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 adversely affect the environment in the long run.



Process: Cultivation of various types of insects with different conditions.

Benefits: Rearing of insects compared to rearing traditional livestock require less water, space and emit less greenhouse gases

Challenges: Reliance on manual labour, microbial degradation of insects

Process: Cultivation of microalgae as alternative protein source

Benefits: Cultivation require less land compared to both animal-based proteins and plant-based proteins, prevent land degradation and water deprivation

Challenges: Practical harvesting techniques, high production cost

Process: Production of meat through *in-vitro* cultivation of animal cells

Benefits: Cultivation allows production of meat without slaughtering animals and might reduce land usage, water usage and energy consumption

Challenges: Economical viability, social acceptance

Figure 4: Summary of alternative food sources.

2.1.3.1 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, mineral, 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).

2.1.3.2 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 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). 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.

2.1.3.3 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 serum (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.

2.2: Utilization of brewers’ spent grains review

Noting that the massive production of food waste streams threatens the food security, there is a need to develop effective food processing technology innovations to manage such food wastes. One of the huge quantities of food wastes generated is the

BSG, which represent 85% of food wastes in the brewery industry (S. I. Mussatto, 2014). The annual global production of BSG is estimated to be 30 million tons (Combest & Warren, 2019). The massive amount of BSG generated would require sustainable methods to manage these wastes. The other by-products of beer manufacturing are mainly spent hobs and surplus yeast. BSG are generated from the first few steps, which includes malting, milling, in the brewing process in beer manufacturing (Figure 5).

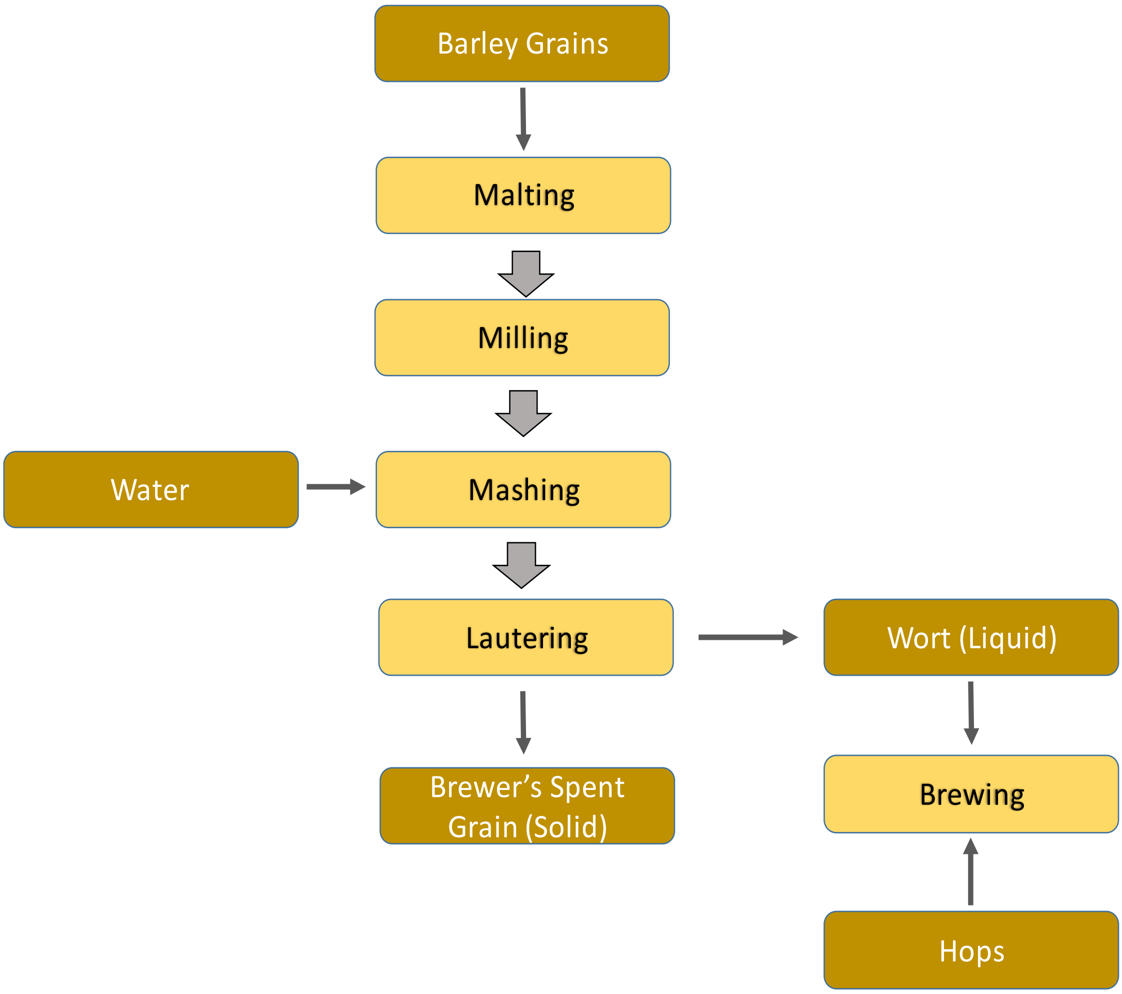


Figure 5: Production of BSG from beer brewing process.

BSG composed of barley malt grain husks, pericarp and seed coat layers of the grains (Lynch, Steffen, & Arendt, 2016; S. I. Mussatto, Dragone, & Roberto, 2006).

BSG are lignocellulosic materials containing about 17% cellulose, 28% non-cellulosic polysaccharides and 28% lignin (S. I. Mussatto et al., 2006) (Table 2). BSG are regarded as abundant in lignin, cellulose and non-cellulosic polysaccharides. Also, on a dry weight basis, 70% of BSG is recorded as fibre, which consists of cellulose, arabinoxylan and ligin, and 20% of BSG is protein (S. I. Mussatto et al., 2006).

Table 2: Composition of untreated BSG from (Solange I. Mussatto, Rocha, & Roberto, 2008)

Components	Composition (% dry weight)
Cellulose	16.8 ± 0.8
Hemicellulose	28.4 ± 2.0
Lignin	27.8 ± 0.3
Ashes	4.6 ± 0.2
Others	22.4 ± 1.2

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 (S. I. Mussatto et al., 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, 2014a). BSG fall under the category of organic crop residue such as fruits and vegetables. This category holds the highest potential for valorization among the other kind of waste (Figure 6) (Teuber & Jensen, 2016). One of the challenges to valorizing BSG is its husky physical property combined with high amount of cellulose and hemicellulose which can bind onto proteins and other nutrient 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, 2014a; Lynch et al., 2016). The size reduction would increase the surface area of BSG which would better allow enzyme or acid entry into the lignocelluloses.

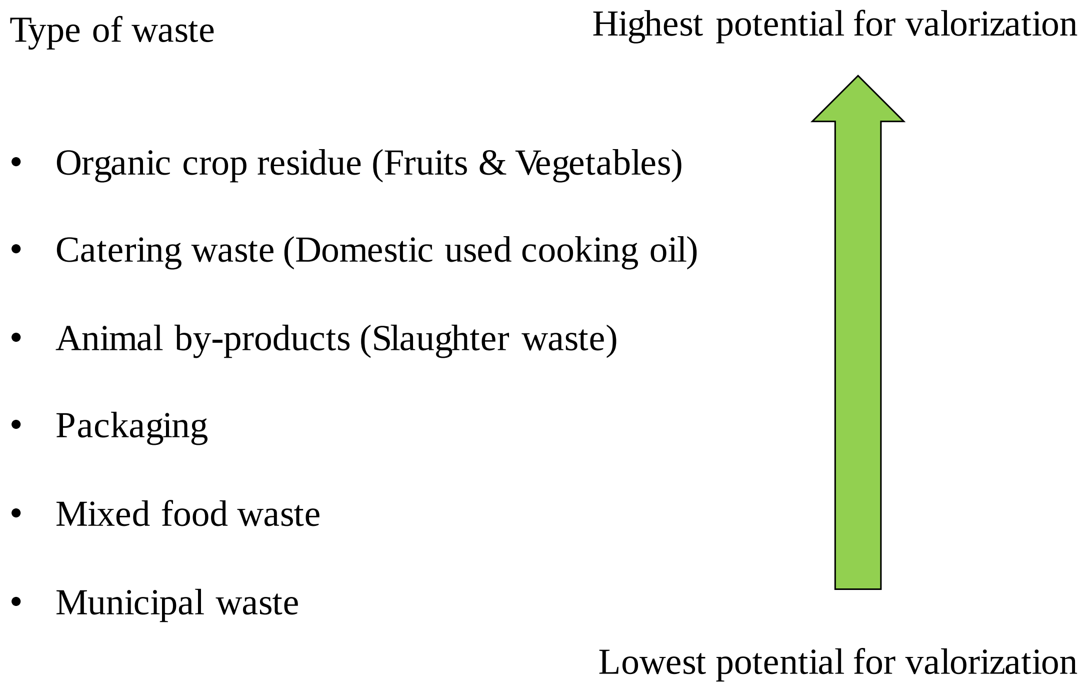


Figure 6: Classification of wastes.

Many studies have been carried out to explore a wide range of potential applications to utilize BSG. An aspect of utilizing BSG will be using it as substrates for bio surfactant production. Bio surfactants are viable alternatives to chemical surfactants in applications due to their high biodegradability. However, the disadvantage of using bio surfactants will be the high cost of large scale production. From (Moshtagh, Hawboldt, & Zhang, 2018), with the use of BSG as the carbon source for producing bio surfactant using *Bacillus subtilis* N3-1P strain, it can reduce the production cost. In addition, the set up would reduce the cost of disposing the BSG. Another potential application of utilizing BSG is incorporating BSG in the making of bricks. It is an area that was relatively unexplored. From (Russ, Mörtel, & Meyer-Pittroff, 2005), BSG can enhance the properties of the bricks by increasing thermal insulation ability. The fired finish bricks incorporated with BSG have higher strength, higher porosity and lower density. The characteristics of brick with a lighter weight and better thermal insulation ability will contribute into the future trend of moving into green technology of green building (Ferraz et al., 2013; Russ et al., 2005).

BSG being lignocellulosic materials, it can serve as low cost and viable adsorbents for removing dyes. In particular from (Kezerle, Velić, Hasenay, & Kovačević, 2018), BSG have been evaluated as an adsorbent for removing synthetic dyes and the removal percentages range from 70% to over 90%. The results showed that BSG can be potentially used as an adsorbent for coloured wastewaters. Further evaluation will be needed for the adsorption capacity. According to (Pedro Silva et al., 2004), AO7 dye which is used in the paper and textile industries was found to be removable by BSG . The presence of such dye in the liquid wastes cause environmental issues. The study demonstrated high levels of colour removal reached more than 90% with only less than 1 hour of contact.

Based on (Fontana, Peterson, & Cechinel, 2018), BSG have been evaluated to serve as biosorbents for removal of metallic ions such as, iron and manganese in groundwater and surface waters. As the concentrations of metallic ions in the groundwater are limited to a certain amount, necessary removal treatments are needed. BSG provide a low cost alternative to the removal treatments. The results showed that the removal percentages were around 87% and 71% for iron and manganese respectively. The maximum adsorption capacity for iron was 4 ± 1 mg/g and 0.96 ± 0.06 mg/g for manganese with about 5 hours of contact.

BSG can be incorporated into our daily food through blending it with flour (Öztürk, Özboy, Cavidoğlu, & Köksel, 2012; Özvural Emin, Vural, Gökbulut, & Özboy-Özbaş, 2009). Previously, high protein flour with BSG as one of the ingredients was successfully produced and made into foods such as breads, cookies, waffles, mixed grain cereals and other bakery products (Huige, 2006). Some benefits of using BSG flour in foods include reduction in calorie content compared to most cereal flours, higher fibre content in bread and higher protein content. With the addition of 10% BSG, it can increase the protein content of bread by 50% (Huige, 2006). However, there are also limitations of using flour with BSG which include its brownish colour and flavour. BSG would produce undesirable flavours caused by high heat of 100°C and 150°C. Such limitations resulted in only an addition of certain amount about 5-10% of heat treated (45°C) BSG in flour can be accepted by consumers (N. Prentice 1977).

BSG can also be made as a potential protein-rich fibrous food product, which is also known as germinated barley food (GBF) (O. Kanauchi & Agata, 1997). GBF can serve as a new potential prebiotic treatment in patients with ulcerative colitis. Patients with mild to moderate active ulcerative colitis who consumed GBF had notable clinical and endoscopic improvement (Bamba, Kanauchi, Andoh, & Fujiyama, 2002). It was observed that patients also had an increase in short chain fatty acids (SCFAs), butyrate, in their stool along with the improvement (Bamba et al., 2002; O. Kanauchi et al., 1999). The increased bacterial production of butyrate was most likely due to the increase in the amount of *Eubacterium* and *Bifidobacterium*, which was an observation in subjects' faecal samples after the consumption of GBF (O. Kanauchi et al., 1999). The increase in stool butyrate content was correlated to the improvement of ulcerative colitis as butyrate production from GBF can improve intestinal barrier function by enhancing the growth of colonic epithelial cells (O. Kanauchi et al., 1999; Osamu Kanauchi, Mitsuyama, & Araki, 2001).

Previous studies have reported that BSG can possibly serve as substrates for enzymes production. BSG was evaluated and gave positive results for the production of an enzyme, laccase using fungi known as *Trametes versicolor* (Marina, Anita, Ana, Mario, & Mirela, 2018). BSG mainly played the role as the nitrogen and carbon source for such enzymatic production process. Also, xylanase activity was observed when using BSG as the substrates and *Streptomyce* sp. AMT-3 strain (Nascimento et al., 2002). Production of microbial enzyme, cellulase by *Streptomyces malaysiensis* involving the use of BSG as one of the substrates was recorded (Nascimento, Junior, Pereira, Bon, & Coelho, 2009). BSG were investigated as viable substrates for α-amylase production using *Aspergillus oryzae* (A. Patel, Nampoothiri, Ramchandran, Szakacs, & Pandey, 2005).

The use of BSG as substrates extends to other kinds of production such as prebiotics, microorganisms. BSG are rich in arabinoxylan (Sajib et al., 2018) where BSG contain approximately arabinoxylan, which can be further hydrolysed to arabinoxylooligosaccharides (AXOS). AXOS are also known as functional food ingredients, prebiotics. One of the fermentation processes used probiotic strain, *Bifidobacterium adolescentis* (ATCC 15703) and Pentopan mono BG, a GH11 enyzme to produce AXOS (Sajib et al., 2018). Another fermentation of BSG to produce AXOS is through direct fermenting with *Bacillus subtilis* 3610 (Amorim et al., 2018).

Chemical methods have been explored to recover nutrients from BSG 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 where biological treatment involving the use of commercial enzymes or microorganisms does not generate toxic compounds (Sindhu, Binod, & Pandey, 2016). A study conducted by (Faulds et al., 2009) reported that the use of microbial proteases were able to release over 50% of protein from BSG. 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 produces enzymes such as cellulases, proteases and lipases would achieve similar effects to commercial enzymes at a fraction of the cost. In summary, biological treatment methods are more environmentally friendly compared to chemical methods as it does not generate toxic compounds and also produce fewer inhibitors as a results of milder processing conditions on top of its lower energy requirement compared to physical methods. BSG were utilized as basic substrates for fermentation of various microorganisms such as, *Pleurotus ostreatus* (oyster mushroom) (D. Wang, Sakoda, & Suzuki, 2001). Fermentation can provide additional value to the substrates. By mixing 10% of BSG with other materials such as 20% wheat bran, 68% beech sawdust and 2% CaCO3, the substrate mixture gave the fastest mycelium growth (Gregori, Svagelj, Pahor, Berovic, & Pohleven, 2008). The *Pleurotus ostreatus* grown using BSG showed higher protein content and higher biological efficiency (D. Wang et al., 2001). Liquid fermentation using *Aspergillus flavus* and *Aspergillus tamarii* on BSG was able to produce ascorbic acid, which is also known as vitamin C (Temitope Banjo, 2018). The highest ascorbic acid yields obtained were 6.25 g/L and 7.25 g/L by *Aspergillus flavus* and *Aspergillus tamarii* respectively (Temitope Banjo, 2018).

These studies have been investigating the different methods to utilize BSG and seek a viable large scale solution to manage the BSG waste issue. The objective of this study is to adopt a ‘zero waste’ approach through microbial fermentation to recover the nutrients in BSG, add value to the biomaterial and use the valorized product for food related application. To investigate the effect of the microbial fermentation process, a feasible method will be analyzing the compounds produced as a result of the fermentation. This can be carried out through untargeted metabolomics, which allows identification and quantification of the metabolites and able to account for the nutritional changes in BSG.

2.3: Metabolomics

Metabolomics is the study that identifies and quantifies all endogenous and exogenous low-molecular weight (<1 kDa) small molecules known as metabolites, which are the intermediates and end products of metabolism within biological systems (Dayalan, Xia, Spicer, Salek, & Roessner, 2019; Nalbantoglu, 2019). The measurements of the metabolites would provide insight about the metabolic changes in the biological systems and define varying phenotypes effectively (Roberts, Souza, Gerszten, & Clish, 2012). There are other types of omics including the genomics, transcriptomics, proteomics. Metabolomics stands closest to the phenotypes among other types of omics as it is the study of metabolome, which is the end product of the genome (Figure 7) (Nalbantoglu, 2019).

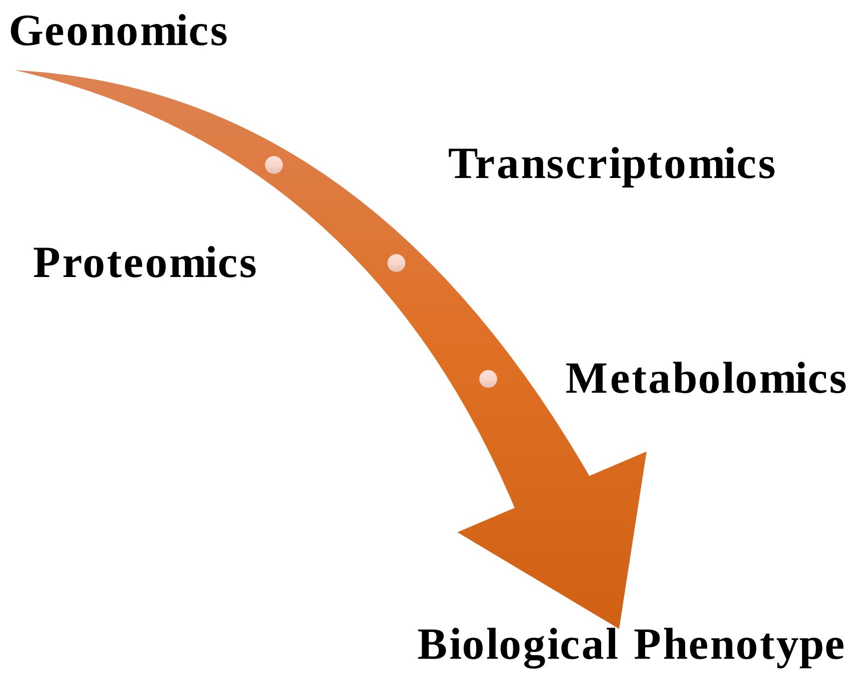


Figure 7: The different 'omics' in the 'omics' family. *Image content extracted from (Steuer, Brockbals, & Kraemer, 2019).*

According to (Roberts et al., 2012), there are two distinct groups of metabolomics, which are known as targeted or untargeted metabolomics. Targeted metabolomics has sets of defined metabolites to be quantified and analyzed while untargeted metabolomics is a comprehensive analysis and measurement of all metabolites in a sample. Metabolomics applied in fermentation of food is able to overcome the difficulty of characterization and comprehensive monitoring of the metabolic variations occurring (Adebo, Njobeh, Adebiyi, Gbashi, & Kayitesi, 2017). There are two types of metabolites can be categorized as primary and secondary metabolites. Primary metabolites are involved in common metabolism pathways for organism survival whereas secondary metabolites are organic compounds that are not associated with the metabolism pathways required for organism survival (Singh, Kumar, Mittal, & Mehta, 2017). Primary metabolites include carbohydrates, amino acids, fatty acids, enzymes and organic acids and secondary metabolites include vitamins, phenolics (Pott, Osorio, & Vallarino, 2019; Singh et al., 2017).

According to (Emwas, 2015), the common analytical methods to detect metabolites include nuclear magnetic resonance (NMR), GC-MS and liquid chromatography- mass spectrometry (LC-MS). Comparing the analytical methods, MS-based metabolomics approach has the advantage of providing sensitivity and selectivity over NMR. As for the MS-based methods, LC-MS is able to identify and measure a broader range of compounds whereas GC-MS is more commonly used to detect volatile compounds such as the primary metabolites and organic compounds (Perez et al., 2016). Also, LC-MS requires minimal sample preparation and it is a quicker method whereas GC-MS requires more extensive extraction procedures.

2.4: *In vitro* digestion-fermentation methodology

Digestion and absorption of nutrients are complex processes that are necessary for health where GI tract breaks down the ingested food into nutrients that can be used by the human body to function and survive. GI tract consists of the stomach, small intestine, large intestine, and organs such as gallbladder, liver and pancreas. The breakdown of food is done through both mechanically reducing the sizes of the food components and enzymatically breaking down the large molecules into smaller molecules that can be absorbed and transported to the liver (Carlson, 2019).

According to F. Kong and Singh (2008), food disintegration begins in the mouth by chewing, which is a rapid process and mouth secretes saliva containing an enzyme, amylase that catalyse hydrolysis of starch. The food is then transported to stomach through the oesophagus. Then, stomach contraction and presence of gastric juice would aid grinding of the food. Gastric juice contains gastric acid, bile salts and digestive enzymes. After the digestion in the stomach, multiphase slurry is further enzymatically digested in the small intestine and most nutrients will be absorbed through the small intestine as well. The remaining unabsorbed nutrients will move to the large intestine. In the large intestine, several processes such as reabsorption of water and electrolytes, formation and elimination of faeces, fermentation of soluble-fibre polysaccharides and phytochemicals by gut microbiota will take place.

Gut microbiota refers to the full complement of microorganisms colonising the human gastrointestinal (GI) tract gut (Thursby & Juge, 2017). Gut microbiota provides vital functions to the host such as adjustment of individual immune system, extraction of energy from food and maintenance of proper intestinal functions (Smirnov et al., 2016). It also plays a pivotal role in homeostasis, protection from diseases throughout the body (Whiteside, Razvi, Dave, Reid, & Burton, 2015). According to (Flint, Duncan, Scott, & Louis, 2014), the gut microbiota and its metabolites interact with the host and influence the individual health outcome. The gut microbiota and metabolic product such as the SCFAs, are affected by the intake of dietary components. In other words, diet has a considerable effect on the composition of individual gut microbiota. Studies have shown that composition and metabolic products of gut microbiota have effects on individual immune and inflammatory responses (Maslowski & Mackay, 2011).

The composition of gut microbiota is also extremely diverse, varying greatly between individuals and can fluctuate over time. Investigating the effects of diet on gut microbiota is vital to correlate with the effects of the diet on individual health. In order to simulate the human digestion, nutrient absorption and obtain gut microbiota profile without the actual use of human subjects, an *in vitro* gut digestion-fermentation method is feasible. According to (Payne, Zihler, Chassard, & Lacroix, 2012), it allows insights on both the presence of various gut microbial species and their related functionality. *In vitro* gut fermentation methods are each characterized by inoculation of faecal microbiota and operated under body temperature, respective pH and anaerobic conditions. The *in vitro* digestion-fermentation method consists of the digestion phase including the oral, gastic and small intestinal phase and fermentation phase representing large intestine phase (Figure 8). Thus far, *in vitro* digestion-fermentation evaluation studies have been more focused on targeted compounds of interest such as SCFAs and phenolic compounds. Investigation of other available nutrients will contribute to a more detailed assessment of functional food ingredients.

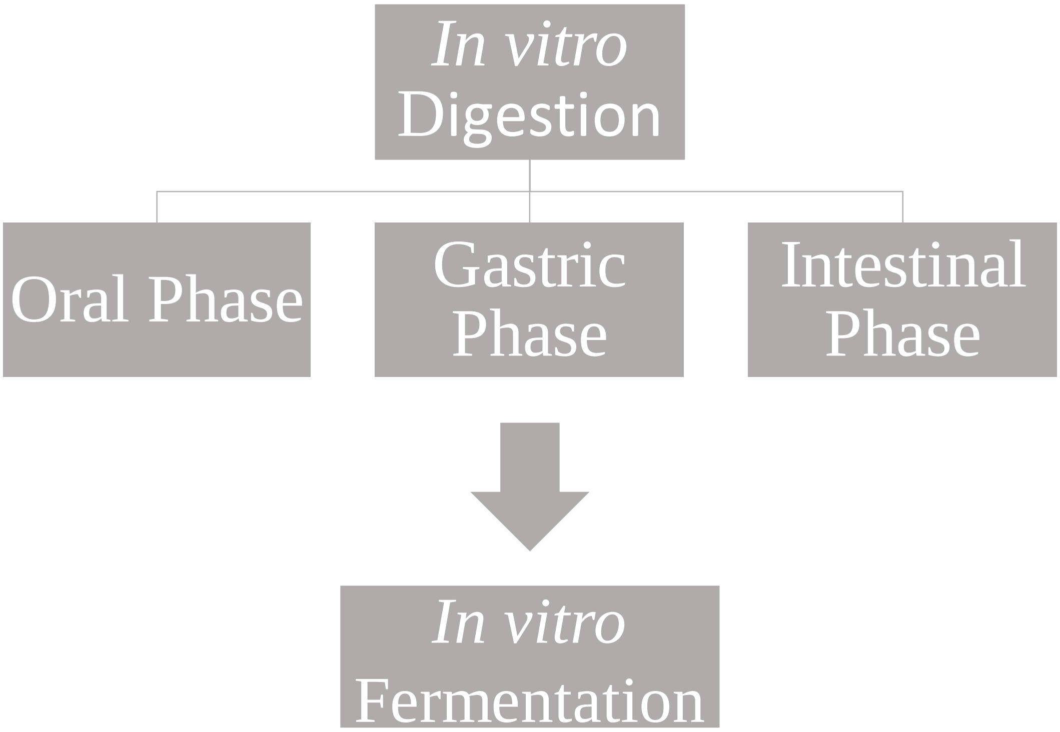


Figure 8: Overview of *in vitro* digestion-fermentation method.

In summary, remaining nutrients in BSG were underutilized and disposed. Disposal of food by product streams in massive amount contributes to climate change issues, which is detrimental to food security. To provide a sustainable solution to manage the wastes, a zero waste approach using microbial fermentation will be employed. Analysis of fermented BSG through metabolomics will be useful for choice of food related applications and evaluation of fermented BSG through an *in vitro* digestion-fermentation model would provide results indicating the bioavailability of the nutrients and potential health effects of fermented BSG.

Chapter 3. Solid state fermentation of brewers' spent grains for improved nutritional profile using *Bacillus subtilis* WX-17

Abstract

BSG are underutilized food waste materials produced in large quantities from the brewing industry. In this study, solid state fermentation of BSG using *Bacillus subtilis* WX-17 was carried out to improve the nutritional value of BSG. Fermenting BSG with the strain WX-17, isolated from commercial natto, significantly enhanced the nutritional content in BSG compared to unfermented BSG, as determined by the marked difference in the level of metabolites. In total, 35 metabolites showed significant difference, which could be categorized into amino acids, fatty acids, carbohydrates and tricarboxylic acid cycle intermediates. Pathway analysis revealed that glycolysis was upregulated, as indicated by the drop in the level of carbohydrate compounds. This shifted the metabolic flux particularly towards the amino acid pathway, leading to a 2-fold increase in the total amount of amino acid from 0.859 ± 0.05 to 1.894 ± 0.1 mg per g of BSG after fermentation. Also, the total amount of unsaturated fatty acid increased by 1.7 times and the total antioxidant quantity remarkably increased by 5.8 times after fermentation. This study demonstrates that novel fermentation processes can value-add food by-products, valorized food waste could potentially be used for food related applications. In addition, the study revealed the metabolic changes and mechanisms behind the microbial solid state fermentation of BSG.

3.1 Introduction

An estimated one third of the food produced globally is lost during processing or wasted. Global food production is expected to rise due to expanding population in the world. This would lead to huge amounts of food wastages annually which cause significant environmental, economic and climate issues. The nutritional contents remaining in these food processing residues can be harnessed to potentially become materials for other processes. Utilizing these residues would be a viable and cost-effective solution to the issues caused by massive amounts of food wastes generated worldwide (Waqas, Rehan, Khan, & Nizami, 2019).

In the beer industry, large quantities of by-products are generated. In particular, BSG represent 85% of the total by-products (Solange I. Mussatto, 2009). The remaining by-products includes spent hops and surplus yeast. The annual global production of BSG is estimated to be 38.6 x 10^6 tonnes (S. I. Mussatto, 2014). It was reported that approximately 14-20 kg of BSG could be generated from 100 litres of beer (Mathias, Fernandes de Aguiar, Batista de Almeida E Silva, Moretzsohn de Mello, & Sérvulo, 2017). Currently, most of the BSG are used as animal feed, which contribute to methane gas production, while the minority are disposed in landfills (Kerby & Vriesekoop, 2017). Hence, other more economical and environmental friendly uses for BSG are needed.

The components in BSG consist of the barley malt grain husks, pericarp and seed coat layers of the grains (Lynch et al., 2016; S. I. Mussatto et al., 2006). BSG are lignocellulosic materials that contain cellulose, non-cellulosic polysaccharides and lignin (S. I. Mussatto et al., 2006). Also, BSG were found to be abundant in proteins, essential amino acids, fibres and phenolic compounds (Ikram, Huang, Zhang, Wang, & Yin, 2017; S. I. Mussatto, 2014). Nutrients such as lipids, fatty acids and polyphenols were also found to be present in BSG (Fărcaş et al., 2015; Salihu & Bala, 2011). The predominant lipids identified were triglyceride and the fatty acids were linoleic, palmitic, oleic, α-linoleic and stearic acids. Other fatty acids including myristic and vaccenic acids were present in lower amounts (Fărcaş et al., 2015). One of the main challenges in the utilization and extraction of useful components in BSG is that the proteins and nutrients are bound to the cellulose and hemicellulose. Hence, in order to extract these compounds, physical, biological, chemical pre-treatments, or a combined pre-treatment, are required (Behera, Arora, Nandhagopal, & Kumar, 2014). Biological pre-treatment would be a more environmentally friendly option as it does not require chemicals or solvents, and has added advantage of not generating toxic compounds (Sindhu et al., 2016). For agricultural by-products, solid state fermentation (SSF) commonly serves as a biological pre-treatment method, which is also convenient and economical. This is because SSF requires less energy, produces less wastewater, and hence is overall more environmentally friendly (Pandey, 2003). The amount of polysaccharide, along with the other nutritional contents in BSG also makes it a suitable substrate for SSF (Salihu & Bala, 2011). It has been successfully carried out with fungi and some bacteria on food such as soybean waste, rice, cassava, corn corbs, bagasse (Couto & Sanromán, 2006; Madamwar, Patel, & Parikh, 1989). Some of the targeted products generated by SSF studies were antioxidants, protein content and lipids (Lizardi-Jimenez & Hernandez-Martinez, 2017; Queiroz Santos et al., 2018). Up till now, some microorganisms, such as *Trametes versicolor* and *Streptomyces* sp. strain AMT-3, have also proved to be able to grow using BSG as the carbon and nitrogen sources for production of xylanase, laccases, and polyphenols under SSF conditions(Nascimento et al., 2002; Tišma, Jurić, Bucić-Kojić, Panjičko, & Planinić, 2018). These studies showed the potential of using microorganisms to valorize BSG and hence generating products that can be used in food related processes.

Bacillus are GRAS species, which are well known to secrete abundant amount of extracellular enzymes (Schallmey, Singh, & Ward, 2004). In this study, we used *Bacillus subtilis (B. subtilis)* WX-17, which was previously isolated from natto (traditional food in Japan where cooked soybeans were fermented using *B. subtilis* giving beneficial effects in health) (Nishinari, Fang, Nagano, Guo, & Wang, 2018), as the host for SSF to enrich nutritional content of BSG. GC-MS was applied for metabolomics analysis to provide important insights into the mechanism behind BSG fermentation with *B. subtilis* WX-17. Till date, few studies have been carried out on metabolic profiling of fermented BSG. This study would help to shed light on the metabolic changes and mechanisms behind the microbial SSF of BSG. With enrichment of nutritional content in BSG, fermented BSG could possibly be used in food related applications such as culture medium, functional food ingredients in human diet and value-added animal feed.

3.2 Materials and Methods

3.2.1 Microorganism for fermentation

B. subtilis WX-17 previously isolated from natto (accession number NCIMB 15204) was maintained on nutrient agar plates at 37 °C. The bacterial strain, *B. subtilis* WX-17, was extracted by adding 25 mL of sterile water to 5 natto beans from Marumiya Kyushu Ichiban in a falcon tube and shaking vigorously for 2 minutes. Serial dilution was carried out on the liquid suspension and plated on nutrient agar. The agar plates were incubated at 37 °C for 24 hours. A single colony was obtained from a serial diluted plate and inoculated in 5 mL of nutrient broth. The nutrient broth was incubated at 37 °C for 24 hours. The bacterial DNA were then processed using Bio Basic EZ-10 Spin Column Fungal Genomic DNA Mini-Prep Kit. Then, PCR was performed to amplify the 16S rDNA gene of the bacterial strain with forward and reverser primer 27F (5′AGA GTT TGA TCM TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) under the following parameters: 35 cycles at 98 °C 10 s for denaturation, 55 °C 5 s for annealing, 72 °C 2 min for elongation and 68 °C 10 min for extension followed by cooling to 4 °C. Gel electrophoresis was applied to purify the DNA and purified DNA was processed using QIAquick Gel Extraction Kit (250) for 16s rRNA gene sequencing. Sequencing was outsourced to Bio Basic Asia Pacific Pte Ltd. The obtained 16s rRNA gene sequencing result was then uploaded on the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison.

3.2.2 Brewer's spent grains and fermentation conditions

BSG were taken from Asia Pacific Breweries (Singapore) Pte, Ltd. and stored in sealed polyethylene bags at −80 °C until used. *B. subtilis* WX-17 was cultured in sterile falcon tube with 5 mL of nutrient broth for 24 hrs at 37°C.

Ten g of BSG was placed in boiling water for 20 minutes and cooled. The BSG were then inoculated with *B. subtilis* WX-17 (10^6 CFU/g) and 5 mL of sterile water. The plates of BSG were incubated at 37 °C for two days. The inoculated samples were wrapped with two layers of cling wrap. Both cling wrap layers were punctured with tiny holes using a sterile needle. Samples were collected at 0 hours and 48 hours to represent unfermented and fermented BSG, respectively. The collected BSG was then lyophilized and stored at –20 °C until further analysis.

3.2.3 GC-MS conditions

Amino acids, fatty acids, carbohydrates and TCA cycle intermediates were detected via GC-MS. The tests were done by the GC-MS system (Agilent Technologies 7890A-5975C Inert MSD) equipped with a HP-5MS capillary column (30 m × 0.250 mm i.d, 0.25 µm film thickness). Samples of 1µL were injected to the system by the auto-sampler in splitless mode. Carrier gas (helium) was set to flow at 1.1 mL/min. The samples were processed accordingly as described in Chapter 3, Section 3.2.5.

3.2.4 Analysis of amino acids using GC-MS

Based on (Zamboni, Fendt, Rühl, & Sauer, 2009), four mg of samples were re-suspended in 200µL of 6 M HCl. 20 µL of γ – aminobutyric acid (10 mg/ mL) were added as the internal standard. The tubes were sealed and incubated for 12 – 24 hours in an oven at 105°C. The cell hydrosylate were then dried in a heat block set at 95°C. After drying, 20 µL of DMF and 20µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane purchased from Sigma-Aldrich were added. The tubes were then sealed and incubated at 85°C for 1 hour. Samples were then centrifuged at 14800 rpm for 5 minutes and supernatant were transferred to glass vials. 40 µL of DMF were added into the glass vials and the solution is well mixed before sending for GC-MS analysis. Each sample was measured in triplicates.

For amino acids detection, the solvent delay was set to 2.5 minutes. The ion source temperature and injector temperature were set at 230 °C and 280 °C respectively. The oven temperature set to hold at 160 °C for 1 minute then ramped to 310 °C at the rate of 20 °C/min, and finally held at 310 °C for 0.5minutes. Data were obtained in full scan mode from 180 to 550 m/z with a 2-4 scan per sec. The identification of amino acids was carried out accordingly by using NIST08 mass spectral library. Samples were normalized using γ – aminobutyric acid before comparison.

3.2.5 Derivatization for metabolomics study

Three g of fermented BSG were weighed and 10 mL of methanol was added. The samples were homogenized 6 times, for 30 secs each run, using FastPrep-24 MP homogenizer. In between the homogenizing, the samples were placed in ice bath to cool the sample. The samples were then centrifuged at 9000g for 10 minutes at 4 °C. The supernatant containing the metabolites, both carbohydrates and TCA cycle intermediates, were pressed through a syringe attached with a 0.22 µm filter. 1.5 mL of filtered supernatant was added with 10 µL of 2mg/ mL ribitol dissolved in ultrapure water as the internal standard. Samples were dried in a heat block at 30 ºC overnight. According to the method in (Chen & Chen, 2014), the dried samples were then derivatized with 50 µL of methoxamine hydrochloride in 20 mg/ mL pyridine and incubated at 37 °C for 1 hour. Then, silylation was carried out by adding 100 µL of MSTFA with 1% TMCS to the precipitate and incubated at 70 °C for 30 minutes. Each sample was measure in triplicate.

The ion source temperature and injector temperature were set at 230 °C and 250 °C, respectively. The oven temperature was as follows: 75 °C for 4 minutes, ramped to 280 °C at the rate of 4 °C/min, and held at 280 °C for 2 minutes. Data were acquired in full scan mode from 35 to 600 m/z with a 0.3 s of scan time. The identification of carbohydrates was carried out accordingly by using NIST08 mass spectral library. Normalization was done using ribitol, before comparison.

3.2.6 Analysis of fatty acids using GC-MS

Ten mg of fermented BSG and 10mg of fresh BSG (control) were weighed and placed into Eppendorf tubes following a method by (Chen & Chen, 2014). Then, 1 mL of 0.9% NaCl solution and 200 µL of acetic acid were added to each sample. 10 µL of 10 mg/ mL heptadecanoic acid dissolved in ethanol was then added as internal standard. The samples were sonicated for 30 seconds each. Then, 3 mL of a chloroform-methanol 2:1 solution was added and the samples were inverted several times, vortexed vigorously, and centrifuged at 10,000 g for 10 minutes at 4°C. 1 mL of the chloroform layer, which is the bottom layer, 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 heat block set at 95°C for 20 minutes. FAMEs were extracted with the addition of 300 µL saturated NaCl in ultrapure water then an addition of 300µL n-hexane. Samples were vortexed for 5 minutes and centrifuged at 14800 rpm for 5 minutes. 200 µL of samples (top layer) were transferred into glass vials for GC-MS analysis. Each sample was measured in triplicate.

The injector temperature was set at 250°C and MS source temperature was set at 230°C. The oven was held at a temperature of 80°C for 1 minute, then elevated to 250°C at a rate of 7°C/min, and finally maintained at 250°C for 10 minutes. Data were acquired in full scan from 35–600 m/z. The identification of fatty acids was carried out by using NIST08 mass spectral library. Normalization was done by using heptadecanoic acid, before comparison.

3.2.7 Vitamin K_2 MK-7 Quantification

Analysis of the composition of BSG before and after fermentation was outsourced to Eurofins Food Integrity & Innovation, Singapore. Vitamin K_2 MK-7 content was determined by LC-MS.

3.2.8 Antioxidant Assay

Free radical-scavenging activity of the samples was investigated by DPPH with some modifications (Wan et al., 2011). 0.6 mM of DPPH solution was prepared in ethanol. Samples for the test were prepared by adding 100mg of fermented BSG after 2 days to 300 µL of ethanol. 150 ul of each sample was drawn and added to a solution of 250 µL of ethanol and 100 µL of DPPH. The tubes were then incubated in a dark place for 30 minutes at room temperature. The absorbance of each sample was measured at 515 nm against ethanol as the blank using Thermo Fisher NanoDrop 2000c spectrophotometer. The antioxidant activity was quantified in signal inhibition percentage. A standard curve was generated by Trolox standards to correlate the weight of Trolox to the signal inhibition (%). The signal inhibition % obtained from each sample was then converted into weight of Trolox.

3.2.9 Statistical Analysis

MetaboAnalyst 4.0 was utilized to construct clustering heatmap and partial least square - discriminant analysis (PLS-DA) for statistical analysis (Chong et al., 2018). Heatmap was clustered with calculated Euclidean distance and ward clustering algorithm. All experiments were conducted in triplicates and standard deviation was calculated.

3.3. Results

3.3.1 Analysis on untargeted extracellular metabolic profiling

An untargeted metabolomics study was carried out using GC-MS. This was to provide insights into the changes which occurred during fermentation. A total of 35 differential metabolites were identified, which could be categorized into carbohydrates, TCA cycle intermediates, fatty acids and amino acids. To make sense of the metabolomics changes, the metabolites were mapped on biochemical pathways. Also, to gain an overall view on the changes in metabolites abundance, analysis was carried out using heatmap and partial least square - discriminant analysis (PLS-DA) (Gromski et al., 2015). Based on the heatmap (Figure 9), the carbohydrate levels were shown to have decreased and the amino acid, fatty acid and TCA cycle intermediate levels were found to increase after fermentation for 48 hrs.

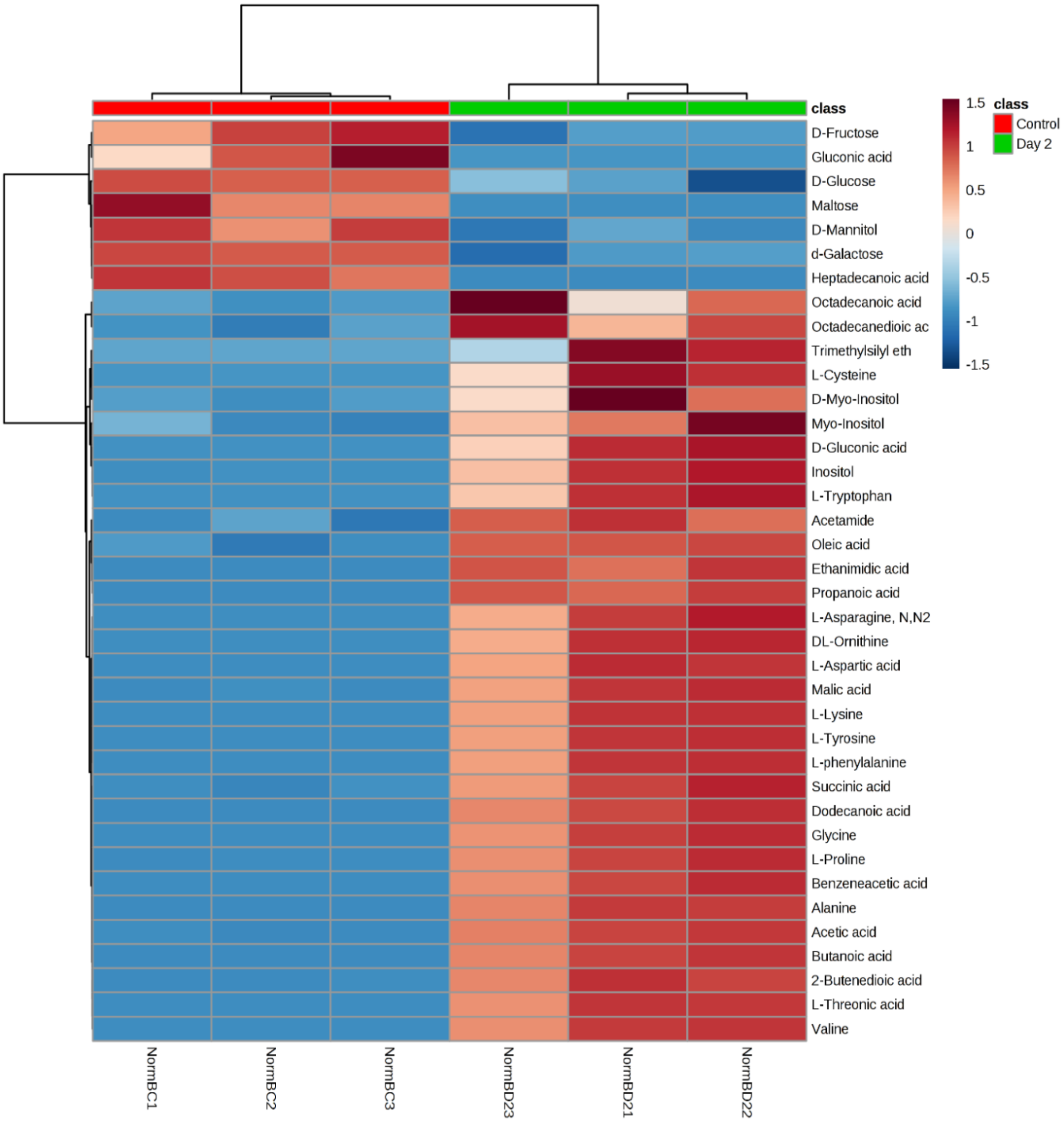


Figure 9: A heatmap analysis of metabolites variations. *Fermentation was carried out for 2 days and metabolites were analyzed by GC-MS. The unfermented BSG samples, in triplicates, are displayed from the left, followed by the fermented BSG samples, in triplicate. Red boxes indicate metabolites upregulated whereas blue boxes indicate metabolites downregulated. Norm BC1, Norm BC2, Norm BC3 stand for the unfermented samples. NormBD21, NormBD22, NormBD23 stand for samples after 2 days of fermentation.*

In addition, the respective changes in the metabolites across 3 days of fermentation were shown on the heatmap (Figure 10). In general, after 2 days of fermentation, amino and fatty acids were upregulated while carbohydrates were downregulated. However, after 3 days of fermentation, fatty acids were downregulated dramatically which might be due to the lack of carbon source for *B. subtilis* WX-17 leading to the beta-oxidation of fatty acids to generate additional carbon source. Hence optimum valorization was achieved after 2 days of fermentation and the analysis of results were based on 2 days accordingly.

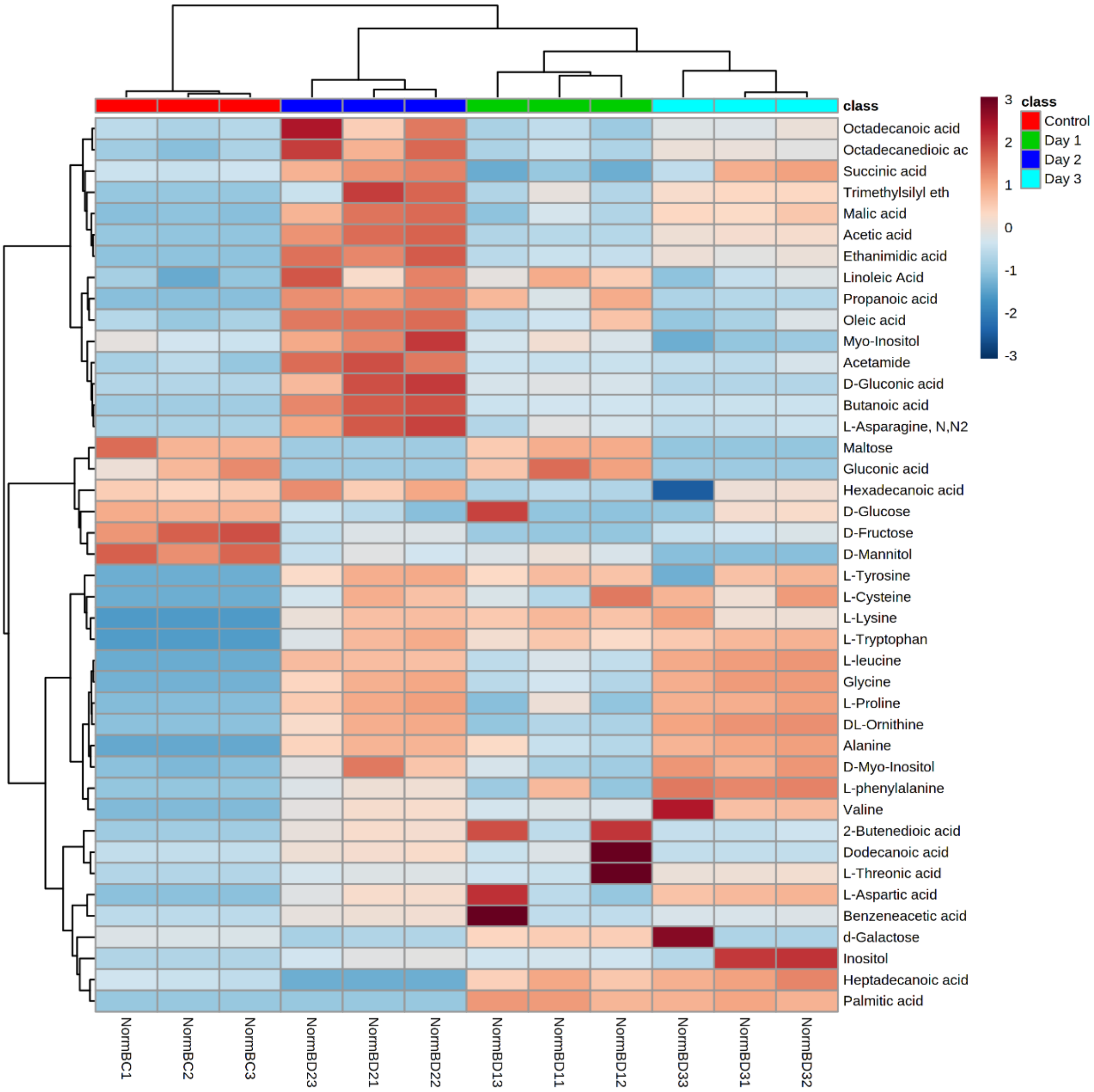


Figure 10: A heatmap analysis of metabolites variations for day 1, day 2 and day 3. *Fermentation was carried out for 3 days and metabolites were analyzed by GC-MS. The unfermented BSG samples, in triplicates, are displayed from the left, followed by the fermented. Norm BC1, Norm BC2, Norm BC3 stand for the unfermented samples. NormBD11, NormBD12, NormBD13 stand for samples after 1 day of fermentation. NormBD21, NormBD22, NormBD23 stand for samples after 2 days of fermentation. NormBD31, NormBD32, NormBD33 stand*

for samples after 3 days of fermentation. Red boxes indicate metabolites upregulated whereas blue boxes indicate metabolites downregulated.

Similarly, clear difference in the metabolites between fermented and unfermented BSG was observed using PLS-DA, as shown in figure 11. PC1 and PC2, displayed a 95.3% and 3.3% of variance, respectively. The PLS-DA plot had a variance R^2 value of 0.99, which is considered to be extremely substantial (Henseler, Ringle, & Sinkovics, 2009). The observed trend of having overall significant changes in the metabolites based on PLS-DA analysis, was congruent and correlated with the trend observed from the clustering heatmap. The metabolic profiles were further investigated by the use of respective component tests and biochemical pathways triggered during the fermentation of BSG with *B. subtilis* WX-17.

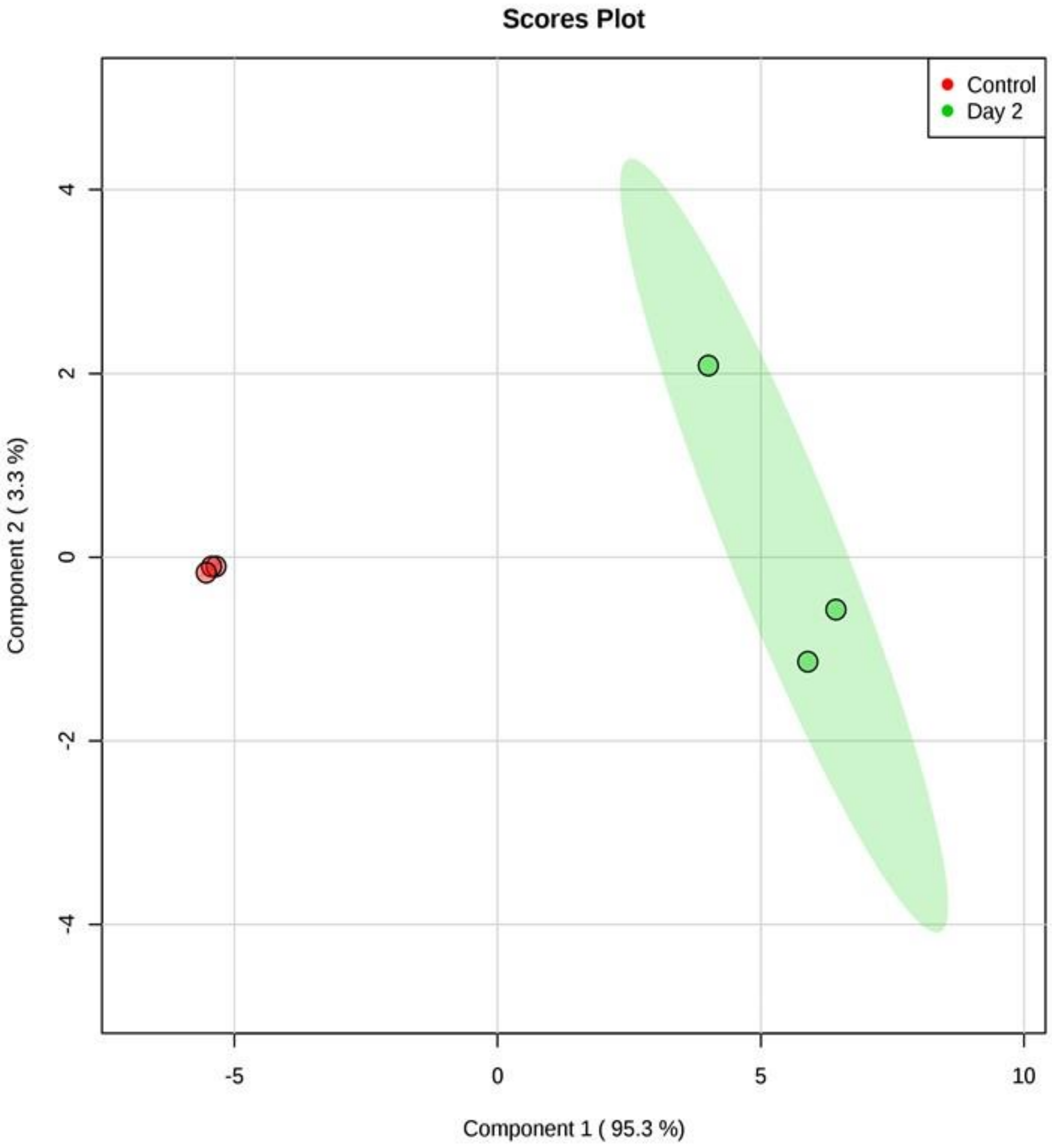


Figure 11: A PLS-DA score plot for metabolites analyzed by GC-MS for unfermented BSG (red) and fermented BSG samples (green), with a 95% confidence interval. *Each dot represented all the metabolites detected in each replicate.*

3.3.2 Amino acids analysis

The amino acid metabolites were found to increase in fermented BSG sample as compared to unfermented BSG (Table 3). Several amino acids metabolites were detected including leucine, phenylalanine, lysine, threonine, serine, proline, glutamic acid, aspartic acid and tyrosine. The increase in the respective amino acids were calculated and expressed as fold change (Table 3). Among the amino acids, proline was found to have the highest increase, at 3.5 times. In terms of total amino acids, the amount increased from 0.859 mg/ g in unfermented BSG to 1.894 mg/ g in fermented BSG after 2 days of fermentation.

Table 3: Amino acids results (mg/ g) before and after fermentation

	Unfermented BSG	**Fermented BSG(Day 2)**	**Fold Change**
Leucine	0.113 ± 0.031	0.134 ± 0.098	1.185
Serine	0.015 ± 0.005	0.017 ± 0.009	1.812
Aspartic Acid	0.024 ± 0.003	0.034 ± 0.007	1.542
Threonine	0.015 ± 0.005	0.026 ± 0.016	2.092
Phenylalanine	0.021 ± 0.003	0.027 ± 0.015	2.133
Proline	0.349 ± 0.182	1.230 ± 0.568	3.527
Glutamic Acid	0.304 ± 0.20	0.407 ± 0.376	1.625
Lysine	0.012 ± 0.006	0.011 ± 0.010	1.200
Tyrosine	0.006 ± 0.001	0.009 ± 0.001	1.560
Total amino acids	0.859 ± 0.049	1.894 ± 0.125	2.204

3.3.3 Fatty acids analysis

It was shown that the total fatty acid content of BSG increased in the fermented BSG sample as compared to unfermented BSG sample (Table 4). A total of four fatty acids were detected by GC-MS analysis. The fatty acids included hexadecenoic acid (palmitic acid), 9,12-octadecanoic acid (linoleic acid), 9-Octadecanoic acid (oleic acid) and octadecanoic acid (stearic acid). These could be categorized into saturated or unsaturated fatty acids. The essential unsaturated fatty acids were linoleic acid and oleic acid, whereas palmitic acid and stearic acid were the saturated fatty acids. Oleic acid was found to have the highest increase with a fold change of 2.366, whereas palmitic acid was found to decrease with by 0.844 fold, in the fermented BSG as compared to unfermented BSG (Table 4). Overall, increasing levels of essential unsaturated fatty acids, and decreasing or unchanged levels of saturated fatty acids after 2 days of fermentation were observed. Unsaturated fatty acids, such as linoleic acid or oleic acid would slightly increase the level HDL cholesterol also known as good cholesterol in human. HDL is reported to aid in the removal of triacylglycerols from the bloodstream. Hence, the increase in unsaturated fatty acids could be interpreted as a higher nutritional value in fermented BSG (Lunn & Theobald, 2006).

Table 4: Fatty acids results (mg/ g) in BSG before and after fermentation

	Unfermented BSG	**Fermented BSG (Day 2)**	**Fold Change**
Palmitic Acid	1.805 ± 0.003	1.523 ± 0.205	0.844
Linoleic Acid	0.445 ± 0.100	0.731 ± 0.220	1.643
Oleic Acid	0.041 ± 0.006	0.097 ± 0.053	2.366
Stearic Acid	4.596 ± 0.091	4.734 ± 0.131	1.03
Total Fatty Acids	6.89 ± 0.055	7.085 ± 0.152	1.028

3.3.4 Carbohydrates analysis

In total, 5 carbohydrates were detected. The carbohydrates detected from GC-MS were D-fructose, D-mannitol, D-glucose, D-galactose and maltose. After 2 days of fermentation, it was observed that D-fructose, D-mannitol, D-glucose, D-galactose and maltose decreased by 64.9%, 68.9%, 62.2%, 66.5% and 86.2% respectively (Figure 12).

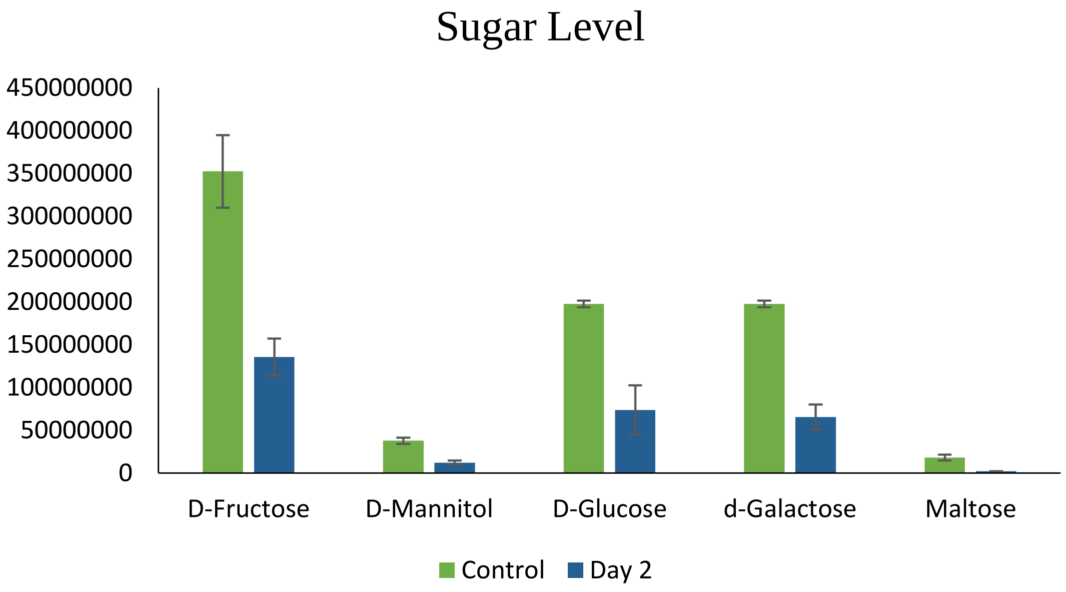


Figure 12: Metabolites belonging to the class of carbohydrate as shown in abundance analyzed by GC-MS. *Green bars represented the levels in the unfermented BSG samples whereas the blue bars represented the levels in the fermented BSG samples.*

There were 2 intermediate metabolites, malic acid and succinic acid detected and both metabolites increased significantly by 11.6 times and 1.35 times respectively after 2 days of fermentation (Figure 13).

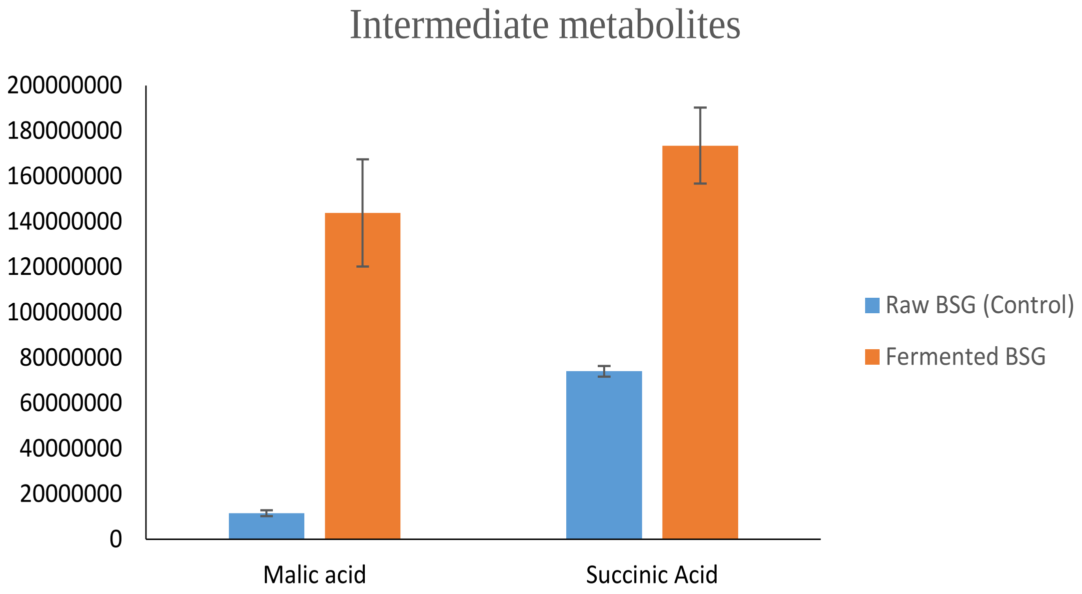


Figure 13: Intermediate metabolites in the TCA cycle detected in abundance by GC-MS for unfermented BSG represented by the blue bars and fermented BSG samples represented by the orange bars.

3.3.5 Antioxidant test

The antioxidant properties of the samples were evaluated through DPPH radical scavenging activity and quantified by Trolox equivalent (Table 5). The method used DPPH assay, which is a test of the amount of electron donation of antioxidants to neutralize the deep purple DPPH radical. The respective samples displayed a visual discoloration, which is reflected in the signal inhibition %. The conversion of signal inhibition % to weight of Trolox was done via calibration curve and it was observed that fermented BSG showed an increase in Trolox equivalent from 1.2 ± 0.03 µg/ g BSG to 6.94 ± 0.21 µg/ g BSG, an approximate 5.8 times increase in antioxidant activity.

Table 5: Trolox quantification for antioxidant activity before and after fermentation

	Signal Inhibition %	Weight of Trolox (μg/g BSG)
Unfermented BSG (Control)	4.72	1.2 ± 0.03
Fermented BSG (Day 2)	28.27	6.94 ± 0.21

3.3.6 Vitamin K_2 MK-7 quantification

A unique component produced as a result of fermentation by B. subtilis is vitamin K_2 MK-7. From the results, 0.227 mg/ 100g BSG of vitamin K_2 MK-7 was detected in the fermented BSG whereas unfermented BSG did not have any amount of vitamin K_2 MK-7 detected (Table 6).

Table 6: Vitamin K_2 MK-7 quantification before and after fermentation

	Weight of vitamin K_2 MK-7 (mg/ 100 g BSG)
Unfermented BSG (Control)	N.D.
Fermented BSG (Day 2)	0.227 ± 0.07

N.D. – Not detected (detection limit: 5 x 10^{-4} mg/ 100g BSG)

3.4. Discussion

The regulations in the metabolites after fermentation were hypothesized by analyzing them using various biochemical pathways. The respective biochemical pathways could have possibly triggered the changes in the metabolites during the fermentation.

3.4.1 Carbohydrate pathway analysis

In the carbohydrate analysis, it was observed that D-fructose, D-mannitol, D-glucose, D-galactose, maltose obviously decreased after fermentation (Figure 14). This result indicates that the various sugars were being used by *B. subtilis* WX-17 for growth. It is speculated that various microbial enzymes, such as invertase, amylase and pectinase, are first produced as bacterial fermentation progressed to hydrolyze long-chain polymeric sugars and starch molecules into simpler carbohydrates during fermentation by *B. subtilis* WX-17 (Ahlawat, Dhiman, Battan, Mandhan, & Sharma, 2009; Lincoln & More, 2017a; Raul, Biswas, Mukhopadhyay, Kumar Das, & Gupta, 2014; Sethi, Datta, Gupta, & Gupta, 2013).

For the inter-conversion among the carbohydrates, maltose can be catalysed by maltose-6'-phosphate glucosidase, to produce D-glucose (starch and sucrose metabolism pathway); D-glucose can be converted into alpha-D-glucose-1P and then converted into UDP-glucose with the aid of the enzyme, UDP glucose pyrophosphorylase (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2016); UDP-glucose would produce sucrose which in turn is converted into fructose through the enzyme, sucrose phosphorylase (starch and sucrose metabolism); fructose can be converted back into D-glucose or alpha-D-glucose, to produce galactose (galactose metabolism pathway). In the following step, glycolysis will convert glucose into pyruvate to supply ATP and NADH which trigger the generation of other primary or secondary metabolites that are important to cell growth. Besides, it has been reported that BSG contain phytic acid and fermentation reduces the level of phytic acid (Lynch et al., 2016). This would lead to an increase in the product, myo-inositol from phytic acid hydrolysis. Myo-inositol was also detected to have increased by the GC-MS analysis which reflected the reduction of the antinutrient, phytic acid.

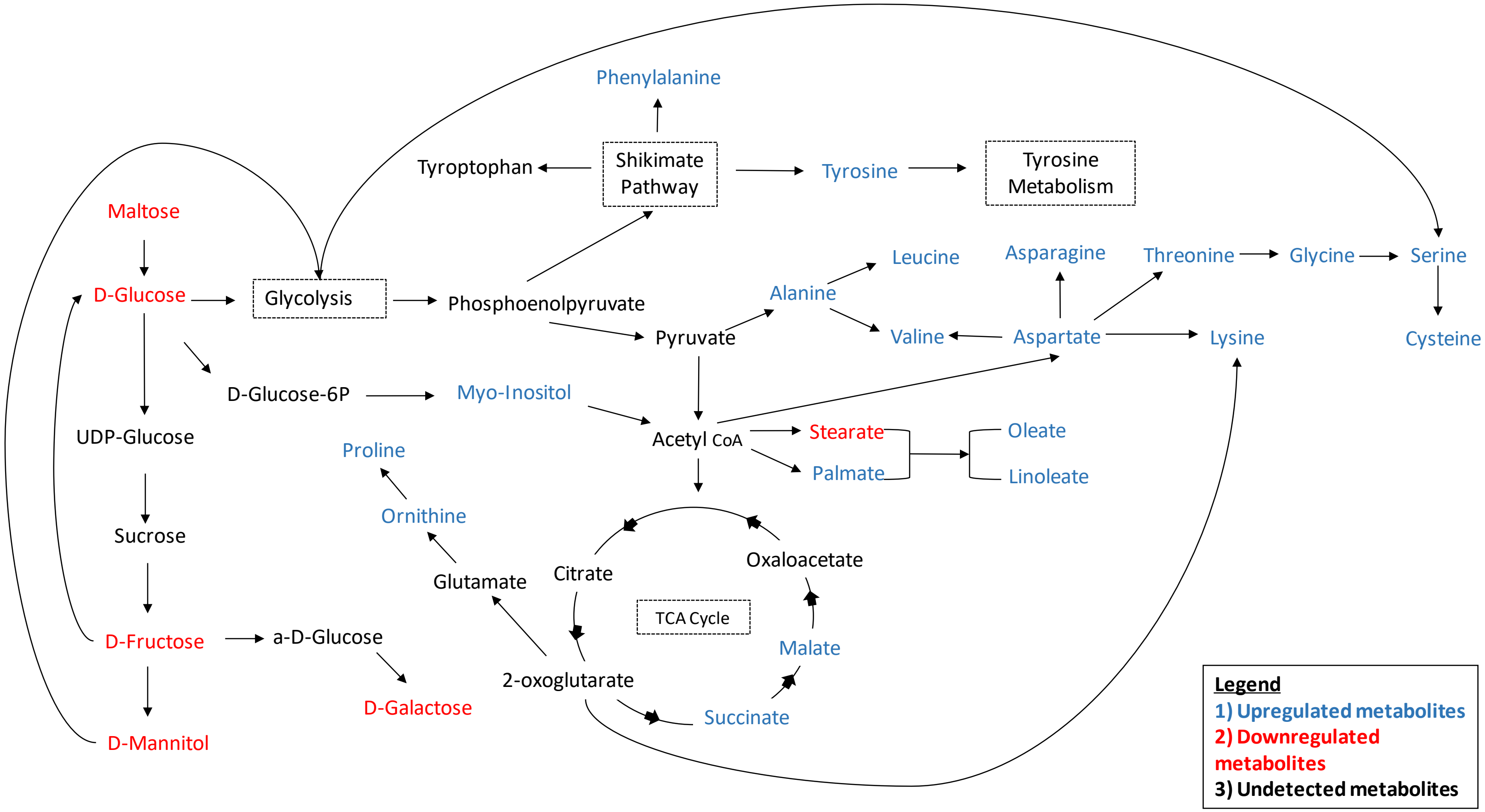


Figure 14: Metabolic changes mapped on metabolic pathways during fermentation of BSG by *B. Subtilis* WX-17. *Coloured labels indicate the respective increase, decrease or undetected levels of metabolites at each given time.*

3.4.2 Amino acids metabolism analysis

It was observed that the amount of amino acids increased in fermented BSG sample, as compared to unfermented BSG. This could be attributed to the enzymes, such as proteases which would be produced by *B. subtilis*. This would hydrolyze the complex proteins present in BSG, into simple amino acids (Oyeleke, Oyewole, & Egwim, 2012). The production of proteases from microorganisms for proteolysis during fermentation and releasing amino acids was also observed in another fermented food study (Lee et al., 2016). In addition, it is also possible that the amino acids were synthesized from or associated to the carbohydrate metabolic pathways.

For instance, serine could be synthesized through glycolysis. In turn this could aid the synthesis of glycine and threonine as reactions are reversible. The series of correlated serine, glycine and threonine pathways can be seen in figure 14. The production of serine would yield cysteine (cysteine and methionine metabolism pathway). The reversible reactions in alanine pathway would produce pyruvate, valine, leucine. Mainly, glycolysis and breakdown of amino acids contribute to the production of pyruvate. A decrease in the precursors for glycolysis such as D-fructose, D-mannitol, could possibly be due to the upregulation of glycolysis. The upregulation of glycolysis increased the production of amino acids (Figure 14). The product of glycolysis, pyruvate, is then converted into acetyl-CoA, which would enter the TCA cycle to produce energy, and also aspartic acid. Valine, alanine and asparagine would be produced by aspartic acid (alanine, aspartate and glutamate metabolism pathway). Also, lysine can be produced from aspartic acid (lysine biosynthesis pathway). Lysine would also be produced from 2-oxoglutarate, which is an intermediate of the TCA cycle. The increasing amount of intermediate after fermentation would drive the metabolic flux towards the production of lysine which explains its increase. Glutamate can also be produced when pyruvate enters the TCA cycle. Proline is produced from glutamate (alanine, aspartate and glutamate metabolism pathway). Proline could also be produced by arginine (arginine and proline metabolism pathway). Through the urea cycle, aspartic acid can be converted into arginine. The production of phenylalanine, tyrosine and tyroptophan mainly stem from the compound, PEP. PEP is converted into shikimate with the aid of the enzyme, shikimate dehydrogenase, which then forms chorismate (shikimate pathway). Chorismate would either synthesize tryptophan or L-arogenate, to produce phenylalanine and tyrosine (phenylalanine, tyrosine and tryptophan biosynthesis pathway). Most of the amino acids identified were found to be glucogenic amino acids. Such amino acids could be converted into glucose. On the other hand, a few exclusively ketogenic amino acids, lysine and leucine, were detected as well.

Inferring from the results and hypothesized pathway analyzes, SSF using *B. subtilis* WX-17 on BSG as substrates was able to utilize the respective useful components in BSG. The increase in production of amino acids is similar to the observation in another SSF study of cassava using *Trichoderma pseudokoningii where protein content was increased from 8.4% to 12.5%* (Bayitse, Hou, Laryea, & Bjerre, 2015). *It could be deduced that* SSF would produce useful enzymes and compounds which improved the nutritional content of the food by-product. The increase in amino acids production from the results was also in line with an increase in amino acids observed in SSF of soybeans with *B. subtilis* (Sarkar, Jones, Craven, Somerset, & Palmer, 1997). In terms of amino acids production, SSF has been evaluated on different food by-products and results have shown that it would lead to an upregulation of amino acids.

3.4.3 TCA cycle metabolism analysis

The TCA cycle is an important sequence of enzyme-catalysed reactions which aerobic microorganisms use to generate energy. Metabolites belonging to the TCA cycle were found to increase in the fermented BSG sample, as compared to unfermented BSG. As fermentation progressed, the synthesis of intermediates in TCA cycle will increase as well (H. Song & Lee, 2006). Based on the upregulation of glycolysis, it would lead to an increase in pyruvate which was then converted into acetyl-CoA and entering the TCA cycle. The increased amounts of precursor, acetyl-CoA, into the TCA cycle would subsequently lead to an increase in the TCA cycle intermediates (Figure 14). The analysis showed increased levels of succinic acid and malic acid, which are key components in the TCA cycle. The synthesis of these components are catalysed by the enzymes, succinate dehydrogenase and malate dehydrogenase respectively (Kanehisa et al., 2016).

3.4.4 Fatty acid metabolism analysis

Fatty acids were suggested to be produced from acetyl-CoA and facilitated by the enzyme, fatty acid synthase (fatty acid biosynthesis pathway). Also, *B. subtilis* WX-17 could have possibly produced lipases which helped to hydrolyze lipids present in BSG into fatty acids (Lesuisse, Schanck, & Colson, 1993; Suci, Arbianti, & Hermansyah, 2017). In the presence of fatty acid synthase, various reactions would be catalysed and saturated fatty acids such as stearic acid and palmitic acid were produced through elongation and hydrolysis. Unsaturation would cause the saturated fatty acids to be converted into unsaturated fatty acids such as oleic acid and linoleic acid. With the increase in the precursor, acetyl-CoA due to upregulation of glycolysis observed after fermentation, it was postulated that saturated fatty acids would increase. However, this was not the case as saturated fatty acids were downregulated while unsaturated fatty acids were upregulated. This is possibly due to the rate of unsaturation being higher than the rate of production of unsaturated fatty acids. As can be seen from the results, the results after fermentation gave an increase of 1.643 and 2.366 times for unsaturated fatty acids, linoleic acid and oleic acid respectively. The increase in the fatty acids trend was also observed in another study with soybean fermentation using *B. subtilis*, where increase in unsaturated fatty acids and decrease in saturated fatty acids were observed (Anittaya Kanghae, 2017). Also, the results and pathway analysis suggested similar trend compared to a study that worked on SSF of rice bran using *Mucor rouxiiI,* which had an increase in unsaturated fatty acid such as gamma-linolenic acid (Jangbua, Laoteng, Kitsubun, Nopharatana, & Tongta, 2009). Overall, microbial SSF across these food by-products have displayed an increase in unsaturated fatty acids trend.

3.4.5 Vitamin K_2 MK-7 content analysis

B.subtilis was reported for its uniqueness in production of vitamin K_2 MK-7. fermented BSG was able to provide a unique nutritional component, MK-7 to the host and MK-7 has the ability to reduce the risk of bone fractures and certain cardiovascular disorders.

3.4.6 Antioxidant test analysis

BSG has been reported to be a source of antioxidant phenolic compounds, which could be present in the husk and cell walls (McCarthy, O'Callaghan, Piggott, FitzGerald,

& M O'Brien, 2012; Meneses, Martins, Teixeira, & Mussatto, 2013). Particularly for phenolic acids content in BSG, ferulic acid and *p*-coumaric acid were found to be present in relatively high concentrations (McCarthy et al., 2012).Other phenolic compounds present include flavonoids, proanthocyanidins, amino phenolic compounds. There are various methods such as solid to liquid extraction, acid hydrolysis, saponification to extract phenolic compounds from BSG fermentation (McCarthy et al., 2012; Meneses et al., 2013). It was reported that bioactive phenolic compounds can also be successfully extracted from natural sources using solid state fermentation (S. Martins et al., 2011). In particular, it has been reported that fermentation with *B. subtilis* could produce nattokinase, which is a polypetide that has antioxidant activity (Mani & Ming, 2017; Weng, Yao, Sparks, & Wang, 2017). Hence, both solid state fermentation process and production of nattokinase could account for the increase in antioxidant properties and phenolic content in fermented BSG shown in the results.

3.5. Conclusions

This work demonstrated valorizing BSG through solid state bacterial fermentation using *B. subtilis* WX-17. The increased amount of amino acids, unsaturated fatty acids and antioxidants after fermentation showed the capabilities of *B. Subtilis* WX-17 to degrade the complex macronutrients in BSG into useful components. The increase in the respective useful components suggested that microbial SSF on BSG were able to produce other useful compounds in addition to the other products from SSF such as the enzymes, polyphenols from other studies. The in-depth investigation using GC-MS, statistical analysis and pathway analysis provided the metabolic changes at different time points during fermentation. With the enhanced nutritional content of fermented BSG, future work could involve food related applications or examine more novel fermentation methods for effective valorization of BSG.

Chapter 4. *In vitro* evaluation of enriched brewers' spent grains using *Bacillus subtilis* WX-17 as potential functional food ingredients

Abstract

BSG are nutritious food processing by-products generated in large quantities mainly from the brewing industry. In this study, *in vitro* digestion-fermentation using *Bacillus subtilis* WX-17 was employed to examine fermented BSG as functional food ingredients. Insoluble fibres in BSG were converted into soluble fibres after fermentation, giving an increase from 0.06 ± 4 x 10^{-3} mg/ g BSG to 0.09 ± 5 x 10^{-3} mg/ g BSG. After *in vitro* digestion of unfermented and fermented BSG, various nutritional components were found to be higher in fermented BSG. Components such as amino acids and fatty acids gave concentration of 1.635 ± 0.236 mg/ mL and 6.35 ± 0.65 mg/ mL respectively. Additionally, vitamin K_2 MK7 was detected in fermented BSG with concentration of 1.2 x 10^{-4} ± 5 x 10^{-6} mg/ mL. *Bacillus subtilis* WX-17 was observed to withstand the *in vitro* digestion, which showed availability of probiotics for the host. After *in vitro* fermentation, various short chain fatty acids namely acetic acid, propionic acid and butyric acid were produced at higher amounts for fermented BSG. The concentrations obtained were 124.11 ± 18.72 mM, 13.18 ± 1.38 mM and 46.25 ± 7.57 mM respectively. As for gut microbiota profile, differential genera such as *Bacteroides*, *Ruminococcus* were detected, showing different beneficial and detrimental effects on the intestinal microbiota. This study demonstrates the potential of using microbial fermentation of underutilized BSG to serve as novel functional food ingredients to be incorporated in foods.

4.1. Introduction

Massive amounts of food wastes are generated annually, causing significant environmental, economic, and climatic issues. There are consequently urgent demands to manage food wastes and provide sustainability in the food processing industry. Specific to the brewing industry, BSG represent 85% of the solid wastes generated (Farcas, Socaci, Tofana, Mudura, & Salanta, 2016b). It is the leftover malted barley grain from beer production where approximately 20 kg is obtained for every 100 litres of beer (Combest & Warren, 2019). This substantially amounts to 39 million tonnes of BSG each year (Lynch et al., 2016). Although BSG are currently used as animal feed as well as a fuel source in energy combustion, there is a proportion which is underutilized and eventually disposed in landfills (Luft et al., 2019; S. I. Mussatto et al., 2006). In order to maximize the potential of BSG while minimizing food wastes, one viable solution is valorization of food wastes to extract their nutritional contents (Farcas et al., 2016b).

BSG constitute of barley malt grain husks, pericarp and seed coat layers (Solange I Mussatto, 2014). It possess high nutritive value, being abundant in carbohydrates, dietary fibres (polysaccharides and lignin), lipids, fatty acids, proteins, essential and nonessential amino acids, phenolic compounds as well as polyphenols (Ikram et al., 2017; Niemi et al., 2012; Salihu & Bala, 2011). To obtain such valuable compounds present in BSG, chemical and enzymatic processes have been studied (Behera et al., 2014; Sindhu et al., 2016). The latter is a better approach in the aspect of environmental friendliness (Sindhu et al., 2016). Furthermore, costly commercial enzymes can be replaced with cost effective microbial fermentation to degrade the target substrate.

During microbial fermentation, the bacteria involved would produce metabolites and enzymes, which valorize and degrade BSG respectively. Since there are proteins and nutrients such as phenolic compounds and lipids bound to BSG cell walls (Arola & Linder, 2016; Meneses et al., 2013; Taylor, Thurston, & Kirsop, 1979), degrading the insoluble fibres to soluble fibres would release wall-bound nutrients and increase BSG nutritional content. Among the GRAS bacteria for fermentation, *Bacillus subtilis* (*B. subtilis)* stands out as it can produce many health-benefit and anti-microbial compounds (Y. X. Tan, Mok, Lee, Kim, & Chen, 2019). *B. subtilis* WX-17, probiotics previously isolated from natto, would be used to ferment BSG (Y. X. Tan, Mok, Lee, Kim, & Chen, 2019).

The higher levels of nutritional components in BSG after microbial fermentation would theoretically provide higher quantity and easier absorption of hydrolyzed compounds in humans. Also, the differences between unfermented and fermented BSG would shape the gut microbiota differently. Therefore, to unravel the differences between unfermented and fermented BSG in the gastrointestinal tract after consumption, *in vitro* digestion-fermentation process will be employed. *In vitro* digestion-fermentation process was employed instead in vivo model as in vivo studies are relatively expensive, time-consuming and food materials have to be verified properly before the trials (Sajib et al., 2018). *In vitro* evaluation of the food materials can serve as a cost-effective, simple, rapid and reliable preliminary assessment. Also till date, few studies have been carried out to examine the bioavailability of various nutritional components after *in vitro* small intestinal digestion.

The study aims to assess the potential of fermented BSG as probiotic functional food ingredients. The difference in insoluble and soluble fibre contents would be examined after solid state fermentation using *B. subtilis* WX-17. The bioavailability of nutrients of unfermented and fermented BSG would be compared based on *in vitro* digestion-fermentation and metabolomics study using GC-MS. The difference in gut microbiota would be analyzed by employing 16s rRNA sequencing on the faecal samples. Till date, *in vitro* digestion-fermentation studies have been more focused on SCFAs and phenolic compounds. Limited studies have been carried out to evaluate the various nutrients of fermented food as a food ingredient after *in vitro* digestion-fermentation.

4.2. Materials & Methods

4.2.1 Fermentation of BSG

BSG were fermented as describe in Chapter 3, Section 3.2.2.

4.2.2 B. subtilis WX-17 viability analysis

According to (Y. Tan, Mok, & Chen, 2020) and some minor modifications, ten times dilution of liquid sample was carried out and the samples were diluted for 12 consecutive times. 100 µL of liquid from each tube was plated onto nutrient agar plates and kept at 37 °C overnight. The cell counting in colonies forming units (CFU) was done at the point of inoculation, after fermentation and after *in vitro* digestion.

4.2.3 Dietary fibre analysis

Freeze-dried, defatted unfermented and fermented BSG were analyzed for IDF and SDF following the AOAC 991.43 enzymatic-gravimetric method (AOAC, 2000).

4.2.4 In vitro gastrointestinal digestion setup

In vitro digestion process was carried out according to (S. Pérez-Burillo, Rufián-Henares, & Pastoriza, 2018). The various simulated fluids were prepared accordingly. The first phase is an oral phase, which involves addition alpha-amylase at a concentration of 75 U/ mL and shaken for 2 mins at 37 °C. Next for the gastric phase, pepsin was added at a concentration of 2000 U/ mL, mixture adjusted to pH 3.0 and shaken for 2 hrs at 37 °C and lastly pancreatin was added at a concentration of 13.37 mg/ mL and adjusted pH to 7.0 for the intestinal phase. The mixture was further shaken for 2 hrs at 37 °C. Once intestinal phase is completed, the samples were cooled using ice to stop further digestion reaction. A fraction of supernatant after digestion available for absorption at the small intestine was extracted for testing.

4.2.5 In vitro fermentation setup

According to (S. Pérez-Burillo et al., 2018), *in vitro* fermentation was carried out with faecal samples from three healthy subjects. The subjects were non-smokers aged 25 to 30 years old, had body mass index (BMI in kg/m^2) between 18 to 25, absence of drug, supplement and antibiotic intake in the past 2 months prior to the study. The supernatant and insoluble solid after digestion were separated by centrifugation (6000 rpm for 10 mins). The insoluble solid and 10% of supernatant were used for fermentation (0.5g). After *in vitro* fermentation, fermented supernatant fraction available for absorption and fermented solid residue fraction including faecal samples not available for absorption were obtained for testing.

4.2.6 Derivatization for metabolomics study

According to a previous study (Chen & Chen, 2014) and some minor modifications, 1.5 mL samples were added with 10 µL of 2 mg/ mL ribitol dissolved in ultrapure water as the internal standards. Samples were dried in a heat block at 30 °C overnight. The dried samples were then derivatized with 100 µL of methoxamine hydrochloride in 20 mg/mL pyridine and incubated at 37 °C for 1 h. Then, silylation was carried out by adding 200 µL of MSTFA with 1% TMCS to the precipitate and incubated at 70 °C for 30 min. Each sample was measured in triplicate.

The ion source temperature and injector temperature were set at 230 and 250 °C respectively. The oven temperature was initiated at 75 °C for 4 min, ramped to 280 °C at the rate of 4 °C/min, and lastly held at 280 °C for 2 min. Data were acquired in full scan mode from 35 to 600 m/z with a 0.3-s scan time. The identification of carbohydrates was carried out accordingly by using the NIST08 mass spectral library. Normalization was done using ribitol before comparison.

4.2.7 Analysis of fatty acids using GC-MS

Referring to a method from a previous study (Chen & Chen, 2014), 1.5 mL of samples were prepared by adding 10 µL of 10 mg/ mL heptadecanoic acid dissolved in ethanol as internal standard. The samples were vortexed for 30 secs. Then, 3 mL of chloroform-methanol 2:1 solution was then added before the samples were inverted several times, vortexed vigorously, and centrifuged at 10,000 × g for 10 min at 4 °C. Then, 1 mL of the chloroform layer, which is the bottom layer, 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 heat block set at 95 °C for 20 min. Fatty acid methyl esters (FAMEs) were extracted with the addition of 300 µL saturated NaCl in ultrapure water, then an addition of 300 µL n-hexane. Samples were vortexed for 5 min and centrifuged at 14,800 rpm for 5 min. Then, 200 µL of samples (top layer) were transferred into glass vials for GC-MS analysis. Each sample was measured in triplicate.

The injector temperature was set at 250 °C and MS source temperature was set at 230 °C. The oven was held at a temperature of 80 °C for 1 min, then elevated to 250 °C at a rate of 7 °C/min, and finally maintained at 250 °C for 10 min. Data were acquired in full scan from 35–600 m/z. The identification of fatty acids was carried out by using the NIST08 mass spectral library. Normalization was done by using heptadecanoic acid before comparison.

4.2.8 DPPH free radical-scavenging capacity

Free radical-scavenging activity of the samples was investigated by DPPH with some modifications (Wan et al., 2011). Then, 0.1 mM of DPPH solution was prepared in ethanol. 600 µL of sample were added with 600 µL of 0.1 mM DPPH solution prepared in ethanol. The tubes were then incubated in a dark place for 20 minutes at room temperature. The absorbance of each sample was measured at 515 nm against ethanol as the blank using Thermo Fisher NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.). The antioxidant activity was quantified in signal inhibition percentage. A standard curve was generated by Trolox standards to correlate the weight of Trolox to the signal inhibition (%). The signal inhibition % obtained from each sample was then converted into the weight of Trolox.

4.2.9 Phenolic content analysis

According to a protocol in (Farcas, Socaci, Tofana, Mudura, & Salanta, 2016a), total phenolic content was analyzed and expressed in terms of gallic acids (µg/ g BSG). 100 µL of sample was added with 6 mL of deionized water and 0.5 mL of Folin Ciocalteu's reagent, then shaken for 30 secs. After 5 minutes, 1.5 mL of 7.5 % sodium carbonate dissolved in deionized water was added. The samples were incubated for 2 hours. The absorbance of the mixture was measured at 750 nm using Nanodrop 2000c spectrophotometer, with deionized water as blank.

4.2.10 Vitamin K_2 detection

According to (Sindhu et al., 2016) and some minor modifications, 5 mL of the supernatant collected after *in vitro* digestion was added with 15 mL propan-2-ol and hexane (1:2 v/v) and vigorously shaken for 10 minutes to extract the vitamin K_2 menaquinone-7 (MK-7). Then the mixture was centrifuged at 3000 rpm for 5 minutes and the organic layer was separated and concentrated up to 1 mL. Concentrated sample was then filtered through 0.45 µm membrane and transferred to a glass vial for analysis.

According to (Berenjian et al., 2014), HPLC was carried out using Agilent 1260 Infinity with wavelength detector set at 220 nm was used for measuring the concentration of MK-7 and the HPLC column used was a C18 column (4.6 mm × 150 mm, RESTEK) kept at 40 °C. The mobile phase consisted of only methanol that was used at a flow rate of 1 mL/min. The run time for 1 sample was 45 minutes.

4.2.11 Erythrocyte haemolysis assay

According to (S. Liu & Huang, 2015) with minor modifications, haemolysis assay was employed to analyze the toxicity of unfermented and fermented BSG. Erythrocytes were obtained by centrifugation at 1200 g for 10 min at 4 °C. Next, erythrocytes were washed with PBS thrice. Complete haemolysis setup was carried out by adding 50 µL of the PBS-mixed erythrocytes suspension (20% in PBS) with 950 µL of ultrapure water. Subsequently, an aliquot of 100 µL of the erythrocytes suspension was mixed with 100 µL of PBS, unfermented and fermented BSG at different concentrations (2.5 mg/ mL and 5mg/ mL). The samples were gently mixed for 20 minutes at 37 °C and then incubated at 37 °C for 2 hours. After the incubation for haemolysis reaction, 50 µL of the mixture were added with 950 µL PBS and centrifuged at 1200 g for 10 min at 4 °C. The absorbance of the supernatant was read at 540 nm using Thermo Fisher NanoDrop 2000c spectrophotometer. The percentage of haemolysis inhibition was calculated using absorbance readings.

4.2.12 Short chain fatty acids analysis

SCFAs produced was analyzed according to (Sergio Pérez-Burillo et al., 2019) with few modifications. The analysis was carried out on HPLC (Agilent 1100) equipped with a variable wavelength detector set at 210 nm and C18 column (4.6 mm × 150 mm, RESTEK). The mobile phase used was 0.1M phosphate buffer (pH 2.8) and acetonitrile with a ratio of 99:1 (v/v) at a flow rate of 1.25 mL/ min. The running time for 1 sample is 30 minutes and SCFA standards were prepared in concentrations of 1, 10, 50, 100 mM. 1 mL of the supernatant collected after *in vitro* fermentation were centrifuged to remove solid residues and filtered using a 0.22 µm nylon filter attached to a syringe. The clear supernatant was then transferred to a glass vial for analysis.

4.2.13 16s rRNA genes sequencing and library preparation

The faecal sample was processed using QIAamp Fast DNA Stool Mini Kit to extract the DNA. The concentration of DNA sample was measured using NanoDrop 2000c spectrophotometer. Sterile water was used to dilute the DNA sample to achieve a desired concentration of 1 ng/µL. For detection, primers 341F-806R with the barcode was used to amplify the16S rRNA genes of V3-V4 region. Phusion high fidelity master mix was used to perform the PCR reactions. Purification with Qiagen gel extraction kit was applied on the PCR products. Sequencing was performed by NovogeneAIT (Singapore) on an Illumina paired-end platform and providing 250 bp paired-end reads. Sequencing libraries were based on NEBNext® UltraTM DNA Library Preparation Kit for Illumina.

4.2.14 Statistical analysis

MetaboAnalyst 4.0 was utilized to construct the orthogonal partial least square-discriminant analysis (OPLS-DA) for statistical analysis (Chong et al., 2018). Standard deviation, statistical significant differences of the data were derived based on student’s t-test with $p < 0.05$ significance level.

4.3. Results & Discussion

4.3.1 Dietary fibre

The soluble fibre increased from 0.06 ± 4 x 10^{-3} mg/ g BSG to 0.09 ± 5 x 10^{-3} mg/ g BSG after SSF (Table 7). From this increase, it was inferred that the use of enzymes produced by *B. subtilis* WX-17, such as pectinase and cellulase, degraded the cell walls and converted insoluble fibre (IDF) to soluble fibre (SDF) (Ahlawat et al., 2009; Deka et al., 2013). (Xiros & Christakopoulos, 2012) reported that unfermented BSG has low digestibility and resulting in decreased accessibility to the remaining nutrients present. Hence, the conversion of IDF to SDF through microbial fermentation will help to improve BSG digestibility by decreasing the resistant starch contents and increase the bioavailability of nutrients to be absorbed by the gastrointestinal tract and subsequently into the bloodstream (Reis & Abu-Ghannam, 2014).

According to (Stojceska, 2011), dietary fibre has been reported to provide physiological effects in humans and linked to prevention of diseases. Insoluble fibre has the benefits of reducing the potency of cytotoxic materials in the large intestine, increasing fecal bulk and excreting bile acids (Mudgil, 2017). Whereas soluble fibre can reduce cholesterol levels and easily fermentable by colonic bacteria to produce SCFAs (Mudgil, 2017; Pangestuti & Kim, 2014). Based on the results, the increase in soluble fibre would further enhance BSG as a potential source of dietary fibre and other beneficial nutrients released.

4.3.2 Amino and fatty acids

Total amount of *in vitro* digested amino acids detected from unfermented BSG was 0.376 ± 0.018 mg/ mL whereas there were a total of 1.635 ± 0.236 mg/ mL amino acids detected in fermented BSG. Fermented BSG had various statistically higher amino acids ($p < 0.05$) such as glycine, alanine, valine, proline, methionine, aspartic acid, lysine, tyrosine, isoleucine and threonine (Table 7). It was found that polysaccharide-protein complexes can be very resistant to enzymatic degradation (Reis & Abu-Ghannam, 2014). The results suggested that the degradation of cell walls could result in recovering higher amount of proteins to be hydrolyzed by *B. subtilis* WX-17 as proteins are bonded to the cellulose. Apart from the additional proteins obtained after fermenting BSG, the digestive enzymes from the gastric would assist in breaking down proteins available in the unfermented BSG as well.

In general, amino acids are the building blocks of proteins, other amino acid roles include functioning as precursors to sugar and fatty acids to produce NADH and ATP (Cole & Kramer, 2016). A total of 6 upregulated essential amino acids were detected after *in vitro* digestion which include leucine, valine, methionine, threonine, isoleucine and lysine. Essential amino acids are nutritional components that cannot be produced by the human body and have to be supplied through food or supplement (Akal, 2017). According to (Chatterjee, Sarkar, & Boland, 2014), essential amino acids stimulate muscle anabolism and decreases protein breakdown. In particular, leucine was reported to be uniquely important as a signalling molecule and building block for muscle. The results suggest that digested-fermented BSG offers a better source of essential amino acids for absorption to the body compared to the unfermented BSG.

Total fatty acids available after *in vitro* digestion from fermented BSG were 6.35 ± 0.65 mg/ mL whereas unfermented BSG only had 2.54 ± 0.29 mg/ mL of digested fatty acids. The increase was in both saturated and unsaturated fatty acids. The unsaturated fatty acids detected were linoleic acid and oleic acid and the saturated fatty acids detected were palmitic acid and stearic acid. Increase in unsaturated fatty acids would provide more positive health effects such as reduction in the risk of certain forms of cardiovascular disease and inflammatory diseases (Finley & Shahidi, 2001). In particular, essential unsaturated fatty acid, linoleic acid was speculated to provide cardioprotective benefit and has to be provided through food or supplements as humans are unable to synthesize essential fatty acids (Kapalka, 2010). Hence, it is inferred that the increased levels of unsaturated fatty acids available supported the hypothesis of higher nutritional value in fermented BSG. However, *in vitro* digestion of fermented BSG resulted in increase in palmitic acid that could possibly elevate the LDL cholesterol level, which is undesirable and linked to heart disease (Grundy, 2003).

As for the *in vitro* digested vitamin, vitamin K_2 MK-7 was detected after *in vitro* digestion of fermented BSG. It was statistically higher ($p < 0.05$) with concentration of $1.2 \times 10^{-4} \pm 5 \times 10^{-6}$ mg/ mL while unfermented BSG did not have any amount of MK-7 detected (Table 7). MK-7 was found to be produced by employing *Bacillus* species for fermentation and low amounts are readily present in food products such as cheese and meat (J. Song et al., 2014). From the results, fermented BSG was able to provide a unique nutritional component, MK-7 to the host and MK-7 has the ability to reduce the risk of bone fractures and certain cardiovascular disorders.

Table 7: Nutritional components including fatty acids, amino acids, vitamin expressed in (mg/ mL) after *in vitro* digestion for both unfermented and fermented BSG

	Unfermented BSG	**Fermented BSG**
Insoluble fibres (g/ g BSG)	0.56 ± 0.04	0.43 ± 0.02
Soluble fibres (g/ g BSG)	0.06 ± 4 x 10^{-3}	0.09 ± 5 x 10^{-3}
Fatty acids (mg/ mL)		
Palmatic acid*	0.58 ± 0.07	1.65 ± 0.19
Linoeleic acid*	1.18 ± 0.15	2.29 ± 0.21
Oleic acid*	0.40 ± 0.04	1.11 ± 0.09
Stearic acid*	0.38 ± 0.03	1.30 ± 0.16
Total fatty acids	2.54 ± 0.29	6.35 ± 0.65
Amino acids (mg/ mL)		
Glycine*	0.022 ± 1 x 10^{-3}	0.056 ± 0.01
Alanine*	0.186 ± 0.056	0.912 ± 0.135
Valine*	0.018 ± 1 x 10^{-3}	0.046 ± 0.011
Proline*	0.091 ± 0.281	0.418 ± 0.054
Leucine	0.009 ± 1 x 10^{-3}	0.013 ± 2 x 10^{-4}
Methionine*	0.005 ± 3 x 10^{-3}	0.027 ± 6 x 10^{-3}
Aspartic acid*	0.015 ± 0.002	0.047 ± 4 x 10^{-4}
Lysine*	4 x 10^{-4} ± 2 x 10^{-5}	5 x 10^{-3} ± 4 x 10^{-4}
Tyrosine*	2 x 10^{-3} ± 4 x 10^{-4}	0.023 ± 3 x 10^{-3}

Isoleucine*	$9 \times 10^{-3} \pm 1 \times 10^{-3}$	$0.03 \pm 6 \times 10^{-3}$
Threonine*	$0.019 \pm 2 \times 10^{-4}$	$0.059 \pm 5 \times 10^{-3}$
Total amino acids	0.376 ± 0.018	1.635 ± 0.236
*Vitamin K_2 MK-7 (mg/ mL)**	N.D.	$1.2 \times 10^{-4} \pm 5 \times 10^{-6}$

* denotes component with significant difference ($p < 0.05$) between unfermented and fermented samples. N.D. = not detected (detection limit: 2×10^{-7} mg/ mL).

4.3.3 DPPH and Folin-Ciocalteu assays

A small portion of inhaled oxygen in humans would naturally transform into free radicals, which can cause damage to the body. Antioxidant plays a vital role being the phytochemicals that can prevent degenerative diseases and scavenge free radicals to protect host from oxidative damages (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011). Fermented BSG had approximately 2 times increase in terms of trolox equivalent after *in vitro* digestion compared to unfermented BSG, from $0.02 \pm 3.1 \times 10^{-4}$ mg/ mL to $0.04 \pm 3.6 \times 10^{-4}$ mg/ mL (Table 8). As for after *in vitro* fermentation, fermented BSG also had about 2 times increase in trolox equivalent from 0.11 ± 0.012 mg/ ml to 0.21 ± 0.016 mg/ mL. Generally, digested fermented BSG had statistically more antioxidant activity ($p< 0.05$) than unfermented BSG. The presence of *in vitro* antioxidant activity after *in vitro* digestion was in line with a study stating that the stomach and small intestine would absorb the antioxidants (Halliwell, Zhao, & Whiteman, 2000). It was inferred from the results of having increased antioxidant activity could be due to the degradation of insoluble to soluble fibres that led to the release of wall bound antioxidants, such as ferulic and *p*-coumaric acids in BSG (Maillard & Berset, 1995). In terms gallic acid equivalent, fermented BSG had 1.69

times more gallic acid equivalent of 2.94 ± 0.10 mg/ mL and 1.72 more gallic acid equivalent of 14.99 ± 0.81 mg/ mL compared to unfermented BSG after *in vitro* digestion and *in vitro* fermentation respectively (Table 8). Similar to DPPH assay results, the assay showed that the digested fermented BSG had statistically more phenolic compounds ($p < 0.05$) than unfermented BSG. It was observed that fermented BSG had approximately 5 times increase in both antioxidant activities and phenolic compounds after *in vitro* fermentation compared to *in vitro* digestion. This observation of less phenolic compounds identified after *in vitro* digestion was in agreement with (Quirós-Sauceda et al., 2014), where accessible phenolic compounds in foods are partially absorbed in the small intestine. The results of having higher amounts of phenolic compounds and antioxidant activities after *in vitro* fermentation could be highly due to the remaining non-absorbable phenolic compounds, associated with food matrix, that can reach the large intestine and exhibit antioxidant characteristics (Rocchetti, Giuberti, & Lucini, 2018).

Table 8: Weight of Trolox and GAE quantifications for antioxidant capacity after fermentation

Samples	Weight of Trolox (mg/ mL)	Gallic Acid Equivalent (mg/ mL)
After in vitro digestion		
Unfermented BSG	$0.02 \pm 3.1 \times 10^{-4d}$	1.74 ± 0.02^{e}
Fermented BSG	$0.04 \pm 3.6 \times 10^{-4d}$	2.94 ± 0.10^{e}
After in vitro fermentation		
Unfermented BSG	0.11 ± 0.012^{f}	8.71 ± 0.55^{g}
Fermented BSG	0.21 ± 0.016^{f}	14.99 ± 0.81^{g}

Mean ± standard deviation values with similar lowercase within the same column are significantly different ($p<0.05$)

4.3.4 B. subtilis WX-17 viability

From inoculation to after fermentation stage, approximately 6.18 log CFU/ mL of *B. subtilis WX-17* grew to 10.7 log CFU/ mL of *B. subtilis WX-17* (Figure 15), which was put through the *in vitro* digestion. After *in vitro* digestion, *B. subtilis WX-17* cell count dropped to 10.3 log CFU/ mL. Although there was a decrease in *B. subtilis WX-17* viability, it can still be inferred that *B. subtilis WX-17* was able to survive the digestion process. The survival of *B. subtilis WX-17* was in line with the features of *B. subtilis* reported, which include the ability to form spores, biofilms, to be advantageous that it possessed to survive in the gastrointestinal tract (Hong et al., 2009). The survival of probiotics would help to enhance the nutritional value of BSG. The bioavailability of various nutritional components results align with a previous study that fermentation can disrupt the cell walls, which degraded the nutrient-matrix complexes and improved the bioavailability of the nutrients (Parada & Aguilera, 2007).

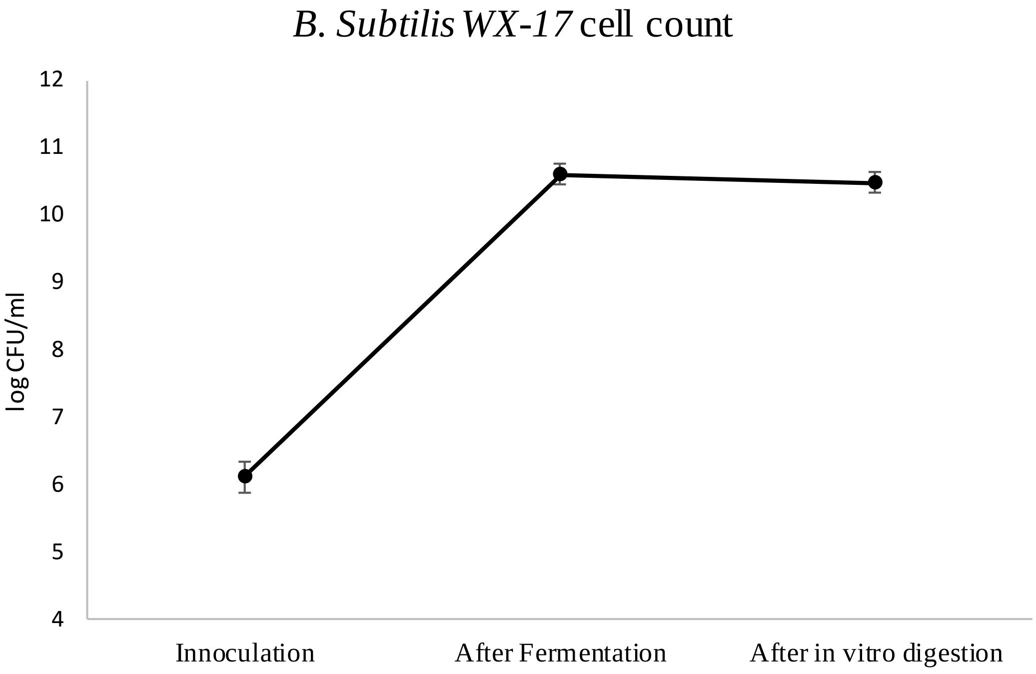


Figure 15: Changes in cell count of *B. subitilis* WX-17 reported in log CFU/ mL after inoculation of *B. subitilis* WX-17 in media, after SSF was done on BSG and after *in vitro* digestion. *The error bars represent the standard deviation of measurements for 3 replicates.*

4.3.5 In vitro erythrocyte haemolysis assay

To serve as a functional food ingredient, the safety of the product would have to be assessed and the toxicity of fermented BSG was analyzed. Haemolysis assay using red blood cells demonstrated at with fermented BSG compounds at a concentration of 5 mg/ mL, there was no haemolysis (Figure 16). Hence, this result suggested that the components in fermented BSG entering the blood stream are relatively safe.

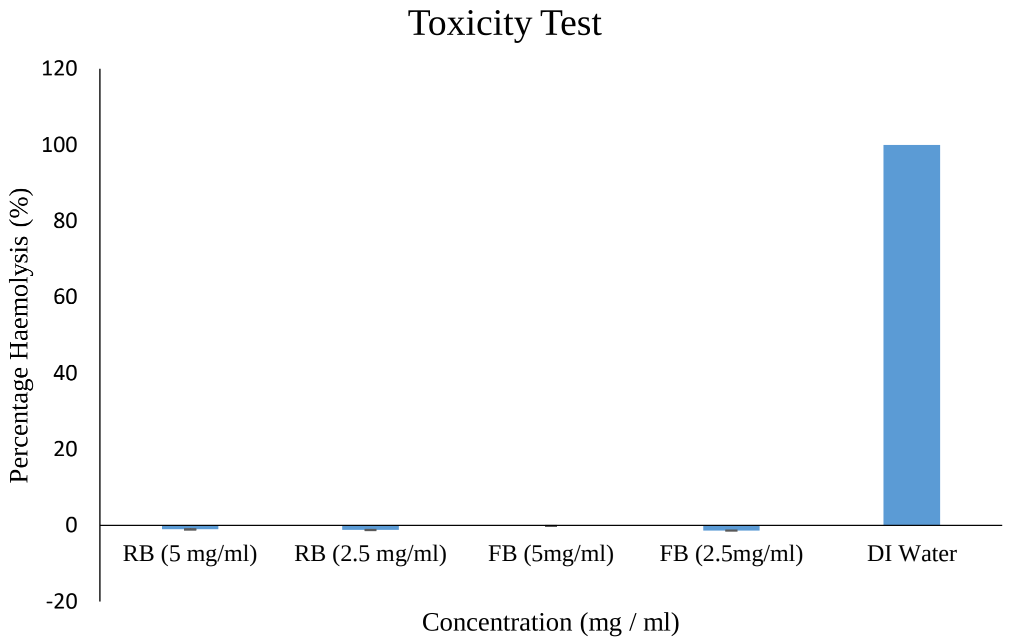


Figure 16: Toxicity of fermented BSG based on percentage of haemolysis.

4.3.6 Short chain fatty acids analysis

SCFAs, namely acetic acid, propionic acid and butyric acid, were quantified and fermented BSG were found to produce higher amounts of all three SCFAs. An increase of 1.4-fold, 1.8-fold and 2-fold for acetic acid, propionic acid and butyric acid respectively (Table 9) compared to *in vitro* fermentation of unfermented BSG. According to (J. Tan et al., 2014), the reason for higher amounts SCFAs is due to the soluble fibre content in fermented BSG, which would provide more fermentable carbohydrates that can be easily fermented by the intestinal microbiota. On the other hand, insoluble fibre has little fermentability and might not have significantly contribute to the production of SCFAs (Mudgil, 2017). Hence, having more soluble fibre after SSF that are prone to fermentation by colonic bacteria, it will result in production of SCFAs after *in vitro* fermentation. The detected amount of acetic acid, propionic acid and butyric acid from fermented BSG after *in vitro* fermentation were 124.11 ± 18.72 mM, 13.18 ± 1.38 mM and 46.25 ± 7.57 mM respectively (Table 9). The quantities obtained were slightly higher than a previous study which carried out *in vitro* evaluation on BSG treated with starch extraction and autohydrolysis (Gómez, Míguez, Veiga, Parajó, & Alonso, 2015). It can be deduced that fermenting BSG can achieve similar effects compared to other means of processing BSG to produce more SCFAs for health benefits. SCFAs were reported to play important role in maintenance of health and deficiency of SCFAs may affect the development of a diverse range of diseases (J. Tan et al., 2014). SCFAs act as fuel source for intestinal epithelial cells, modulate different processes in gastrointestinal tract, immune tissues, leukocyte development, survival, and function (Vieira & Vinolo, 2019; Vinolo, Rodrigues, Nachbar, & Curi, 2013).

Table 9: SCFAs produced on fermented BSG with B. subitilis WX-17 and unfermented BSG after 24 h of *in vitro* fermentation

Organic Acids (mM)	Unfermented BSG	Fermented BSG
Acetic Acid	89.11 ± 5.93	124.11 ± 18.72
Propionic Acid^	7.15 ± 1.12	13.18 ± 1.38
Butyric Acid^	23.18 ± 4.68	46.25 ± 7.57

^ denotes components with significant difference between unfermented and fermented BSG ($p < 0.05$)

The effects of unfermented and fermented BSG on the intestinal microbiota were analyzed after *in vitro* fermentation with subjects' faecal microbiota. The more abundant genera in the intestinal microbiota after *in vitro* fermentation were *Bacteroides*, *Parabacteroides* and *Escherichia-Shigella* among the top 10 genera (Figure 17). Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was able to separate the groups of samples with eight identified discriminating genera (Figure 18). Good discrimination was justified by the OPLS-DA model having an explained variance, R^2 value of 0.828 and relatively modest predictive ability, Q^2 value of 0.582.

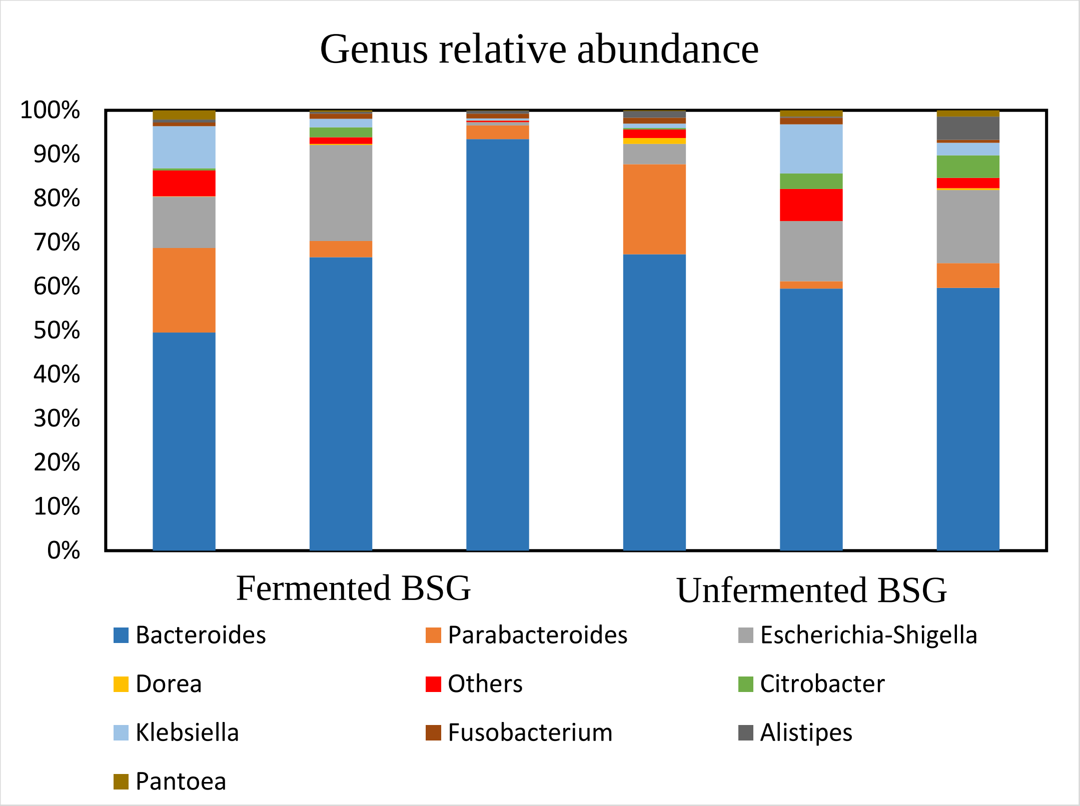
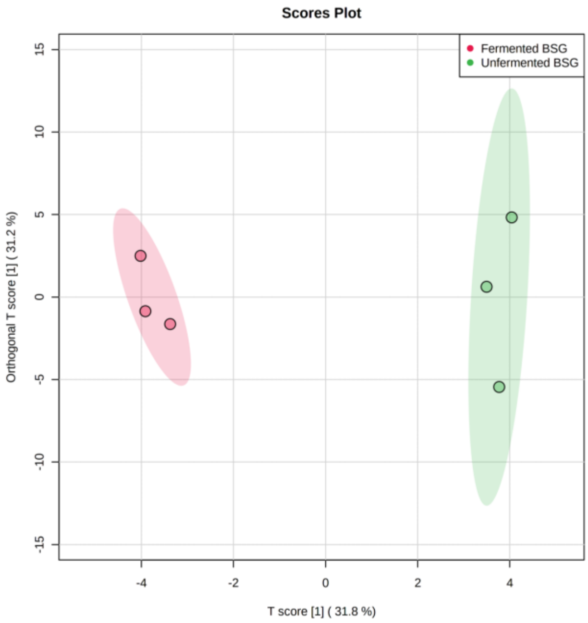


Figure 17: *In vitro* fermentation of fermented and unfermented BSG by human faecal microbiota resulted in different community profiles. *Each column represents a community structure derived from an independent in vitro fermentation of fermented and unfermented BSG sample. Relative abundances of the top nine most abundant genera detected were shown and the sum of the other genera relative abundances were labelled as "others".*

The different bacterial genus was categorized as upregulated or downregulated genus. The upregulated genera after fermentation are *Bacteroides* and *Odoribacter* whereas the downregulated genera are *Ruminococcus*, *Blautia*, *Lactonifactor*, *Lachnoclostridium*, *Dorea* and *Citrobacter* (Figure 18). The respective genus has different health effects and studied previously. According to (Wexler, 2007), *Bacteroides* thrive in the gastrointestinal tract, aid to break down complex carbohydrates providing energy to host and develop mature immune system but *Bacteroides* can also be associated with infections or disease. Upregulated *Odoribacter* are considered a main producer for SCFAs, butyrate (Gomez-Arango et al., 2016). For the downregulated genera detected, *Ruminococcus* have been linked to Crohn's disease, an inflammatory bowel disease and *Blautia* were associated with non-alcoholic fatty liver disease (Bastian, Hasan, Lesmana, Rinaldi, & Gani, 2019; Henke et al., 2019). *Lactonifactor* were reported to increase in patients with disease, myalgic encephalomyelitis/chronic fatigue syndrome (Fremont, Coomans, Massart, & De Meirleir, 2013). *Lachnoclostridium* served as marker for colorectal adenoma (Liang et al., 2019). *Dorea* and *Citrobacter* were related to irritable bowel syndrome (Ganji et al., 2016; Rajilic-Stojanovic et al., 2011). Based on the differential genera and their health effects, it was postulated that fermented BSG provided a more beneficial intestinal microbiota profile.



Top differential genera (+)	
Bacteroides	0.59
Odoribacter	0.50

Top differential genera (-)	
Ruminococcus	-0.66
Blautia	-0.65
Lactonifactor	-0.59
Lachnoclostridium	-0.55
Dorea	-0.52
Citrobacter	-0.49

Figure 18: The orthogonal partial least square-discriminant analysis (OPLS-DA) plot of the genus abundance in the samples. *Each coloured dot represents each community structure derived from in vitro fermentation of fermented and unfermented BSG sample. Table listed with the highest discriminating genera.*

4.4. Conclusion

This work examined the effects of *in vitro* digestion-fermentation of fermented BSG using *B. subtilis* WX-17 and unfermented BSG. The main areas covered include the bioavailability of vitamin K_2 MK-7, amino acids, fatty acids, *B. subtilis* WX-17, phenolic compounds, antioxidant activity, SCFAs production and intestinal microbiota. After *in vitro* digestion, fermented BSG showed higher amounts of vitamin K_2, amino acids, fatty acids, antioxidant capacity, phenolic compounds and presence of probiotic *B. subtilis* WX-17 compared to unfermented BSG. SCFAs production were also higher for fermented BSG after *in vitro* fermentation, which could be due to higher soluble fibre content in fermented BSG. Differential genera were found after *in vitro* fermentation of fermented and unfermented BSG, showing different beneficial and detrimental effects on the intestinal microbiota. With preliminary results using *in vitro* method, more studies would be needed to explore the potential of using fermented BSG as functional food ingredients and investigate more compounds that would affect human health.

Chapter 5. Potential novel nutritional beverage using submerged fermentation with *Bacillus subtilis* WX-17 on brewers' spent grains

Abstract

Food processing generates side streams that are not fully utilized and typically treated as waste materials. One of such food by-product, BSG are disposed in huge quantities from the beer industry annually. Submerged fermentation of BSG using Bacillus subtilis WX-17, without supplementary components, is herein employed. The fermentation products were extracted in the liquid phase, resulting in a potential novel nutritional beverage containing Bacillus subtilis WX-17. Bacillus subtilis WX-17, was still viable after a period of 6 weeks with a final cell count of 9.86 log CFU/ mL. GC-MS was employed for identification of the metabolites produced from the growth of Bacillus subtilis WX-17. Seven essential amino acids and TCA intermediates were found to have increased significantly ($p < 0.05$) whereas all carbohydrates decreased significantly ($p < 0.05$) in the beverage after submerged fermentation. Additionally, antioxidant activity quantified using DPPH radical scavenging activity, increased by 2.08-fold while total phenolic content increased from 125.7 ± 0.74 μg/ mL to 446.74 ± 1.26 μg/ mL. The results proved the potential of employing submerged fermentation of BSG using Bacillus subtilis WX-17 to produce a novel and highly nutritious beverage.

5.1. Introduction

BSG are generated as waste side streams during beer production process, where global yield is estimated at up to 38.6 x 10^6 tonnes per year (S. I. Mussatto, 2014). Currently, they are commonly used as either animal feed (e.g. for cattle) (Lynch et al., 2016; S. I. Mussatto et al., 2006) or disposed in landfills (Buffington, 2014b). In parallel, BSG have been reported to contain proteins, fibres and phenolic compounds in abundant amounts (Ikram et al., 2017; S. I. Mussatto, 2014). It is therefore worth exploring the development of high-value BSG-based products by harvesting their remaining nutrients, especially from the perspective of food security, economics as well as environment sustainability. Fermentation is a promising approach to increase nutrient content, thereby achieving a high-value BSG-based products. At the same time, this technique would enhance the utilization of BSG.

Microbial fermentation provides a feasible way to re-utilize or add value to bio-waste/ food-products. *Bacillus subtilis (B. subtilis)* is one of the most noticeable bacterial workhorse for fermentation, since it can produce many health benefits and anti-microbial compounds (Caulier et al., 2019). Specifically in food processing, *B. subtilis* has been used for the production of traditional soya-based natto for centuries (Hsu, Lee, Wang, Lee, & Chen, 2009). Although fermentation using BSG has been proven to be viable for the cultivation of several microorganisms (Charalampopoulos, Pandiella, & Webb, 2002; H. M. Patel, Wang, Chandrashekar, Pandiella, & Webb, 2004), studies are rare on submerged fermenting of *B. subtilis* without additional supplementation. It is well established that *B. subtilis* can secrete abundant amounts of extracellular enzymes, including amylases and cellulases (Schallmey et al., 2004). As such, *B. subtilis* is able to release the nutrients of BSG by breaking down the material, which is rich in protein and cellulose, in turn releasing or producing antioxidants such as phenols. Considering that no submerged fermentation of *B. subtilis* with BSG and *B. subtilis* drink have been reported till date, development of a nutritional *B. subtilis* beverage from BSG will be intriguing and worth exploring. According to (Cheng, Jiang, & Hu, 2019), *B. subtilis* is a common species present in health beneficial products that is widely used as supplements and improving human health.

This work studies the possibility of utilizing BSG as substrates in submerged fermentation to enable the growth of *Bacillus subtilis* WX-17 *(B. subtilis* WX-17*)* without additional supplements yet producing nutritional components in the liquid. The microorganism involved is *B. subtilis* WX-17, which was isolated previously from natto. Antioxidant assays and metabolomics using GC-MS, coupled with testing of *B. subtilis* WX-17 viability across time would be employed to evaluate the fermented liquid product as a nutritional beverage.

5.2. Methods and Materials

5.2.1 Submerged fermentation of B. subtilis WX-17 with BSG

Fresh BSG and *B. subtilis* WX-17 were acquired as described in Chapter 3, Section 3.2.1 and Section 3.2.2. With minor modifications from (Zdanowska et al., 2019), BSG were grinded into 5 mm mesh size using ceramic mortar and pestle. Ten grams of autoclaved, grinded BSG were inoculated with *B. subtilis* WX-17 (10^6 CFU/g) and 50 mL sterile water in an Erlenmeyer flask. Submerged fermentation was carried out for 72 hours at 37 °C, 200 rpm. Samplings of both unfermented and fermented BSG were carried out at 0 h and 72 h. The samples were filtered using vacuum filtration with filter paper of 40 µm particle retention size. The supernatant collected was stored in -20 °C until further analysis.

5.2.2 Metabolomics analysis

Metabolomics analysis was conducted according to the method in (Chen & Chen, 2014) with some minor modifications. 200 µL of methoxamine hydrochloride in pyridine (20 mg/ mL) was added for derivatizating1 mL of freeze dried samples and incubated for 1 hour at 37 °C. Next, 400 µL of MSTFA with 1% TMCS silylation reagent was added to the samples and incubated at 70 °C for 30 min. 100 µL was drawn from each sample and measured thrice. The column specifications and GC-MS method were used according to (Chen & Chen, 2014).

5.2.3 Phenolic content analysis

According to a protocol in (Kamtekar, Keer, & Patil, 2014), total phenolic content was analyzed and expressed in terms of GAE. 1 mL of sample was added to 5 mL of purified water and 0.5 mL of Folin-Ciocalteu reagent before being vortexed. Samples were left to sit for 5 minutes. 1.5 mL of 20% sodium carbonate was added to each sample and tubes were filled up to 10 mL using purified water. The samples were incubated at 20 °C in dark condition for 2 hours. Subsequently, the optical density was measured using Thermo Fisher NanoDrop 2000c spectrophotometer at wavelength of 750 nm. Deionized water was used as blank.

5.2.4 Antioxidant activity analysis

DPPH free radical-scavenging capacity was investigated according to (Wan et al., 2011) with some modifications. 600 µL of sample was added to 600 µL of 0.1 mM DPPH solution prepared in ethanol. The samples in tubes were afterwards incubated for 20 minutes in the dark condition at 20 °C. Samples were measured at a wavelength of 515 nm using spectrophotometer. The antioxidant activity was obtained from the conversion of absorbance readings and quantification in terms of weight of Trolox.

5.2.5 Evaluation of B. subtilis WX-17 viability

Ten times dilution was carried out by adding liquid sample to sterile water in an Eppendorf tube. The dilution process was performed 11 times consecutively. 100 µL from each tube was plated onto nutrient agar petri dishes and incubated for 24 hours at 37 °C. Thereafter, the cell counts in CFU were recorded. This is repeated weekly for a duration of 6 weeks.

5.2.6 Statistical analysis

Samples were statistically analyzed by partial least square-discriminant analysis (PLS-DA) and heatmap using MetaboAnalyst 4.0 (Chong et al., 2018). Heatmap was generated with Euclidean distance calculation together with ward clustering algorithm. Statistical differences between samples were deduced based on Student’s t-test with $p < 0.05$ as the benchmark for statistical significance.

5.3. Results & Discussion

5.3.1 Evaluation of B. subtilis WX-17 viability

The changes in *B. subtilis* WX-17 cell count in colonies forming units/ mL (CFU/ mL) were recorded over 6 weeks at 4°C. The count decreased slightly across 6 weeks without much variations, with an initial cell count of 10.48 log CFU/ mL in week 0 to a final cell count of 9.86 log CFU/mL in week 6 (Figure 19). The relatively high survival rate of *B. subtilis* WX-17 after 6 weeks could be due to the formation of endospores by *Bacillus* species, which have been found to be metabolically dormant life forms and extremely resistant to various external conditions such as gamma radiation, UV as well as dessication (Casula & Cutting, 2002; Coleman, Chen, Li, Cowan, & Setlow, 2007). The viability cell count amount of *B. subtilis* WX-17 obtained is relatively higher or similar to other common beneficial microorganisms in beverages such as *Bifidobacterium adolescentis and Lactobacillus casei,* which are reported to be around 9 log CFU/ mL and 8.72 log CFU/ mL respectively (Shori, 2016). *B. subtilis* was evaluated to be potentially beneficial for humans and biotherapeutic (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017; Permpoonpattana, Hong, Khaneja, & Cutting, 2012). Hence, it can be inferred that the viability of *B. subtilis* WX-17 in the fermented beverage after 6 weeks would provide beneficial properties. In addition, the fermented liquid with *B. subtilis* WX-17 can possibly serve as a feed supplement for domestic animals. According to (Jenny, Vandijk, & Collins, 1991), the presence of *B. subtilis* tends to have a positive effect on feed efficiency of Holstein calves.

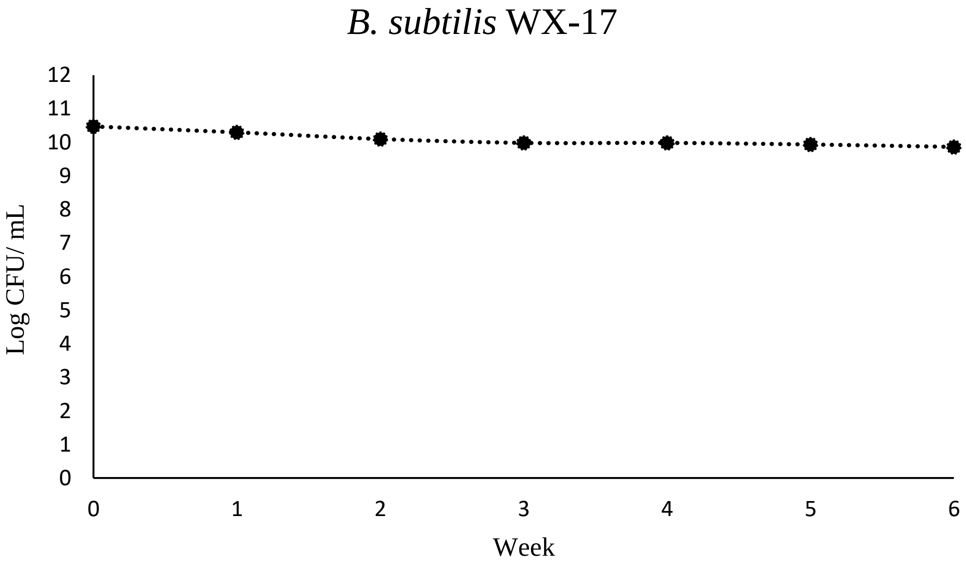


Figure 19: Changes in cell count of *B. subitilis* WX-17 grown in BSG media stored at 4°C over 6 weeks.

5.3.2 Statistical analysis on detected metabolites

An untargeted metabolomics analysis was employed to gain insights into the submerged fermentation process. A total of 23 significantly different metabolites ($p < 0.05$) were observed. Using a statistical analysis, PLS-DA, a distinct separation between the metabolites in fermented and unfermented BSG was obtained (Figure 20). The variances of the principal components namely, PC1 and PC2 on the axes were 94.3% and 2.9% respectively. The clear difference between the samples is mainly due to the first principal component. The PLS-DA plot can be evaluated using R^2 value and Q^2 value, which represents the explained variance and the predictive capability of the model respectively (X. Kong et al., 2015). With a R^2 value and Q^2 value of 0.99, it can be considered to be significantly substantial (Henseler et al., 2009).

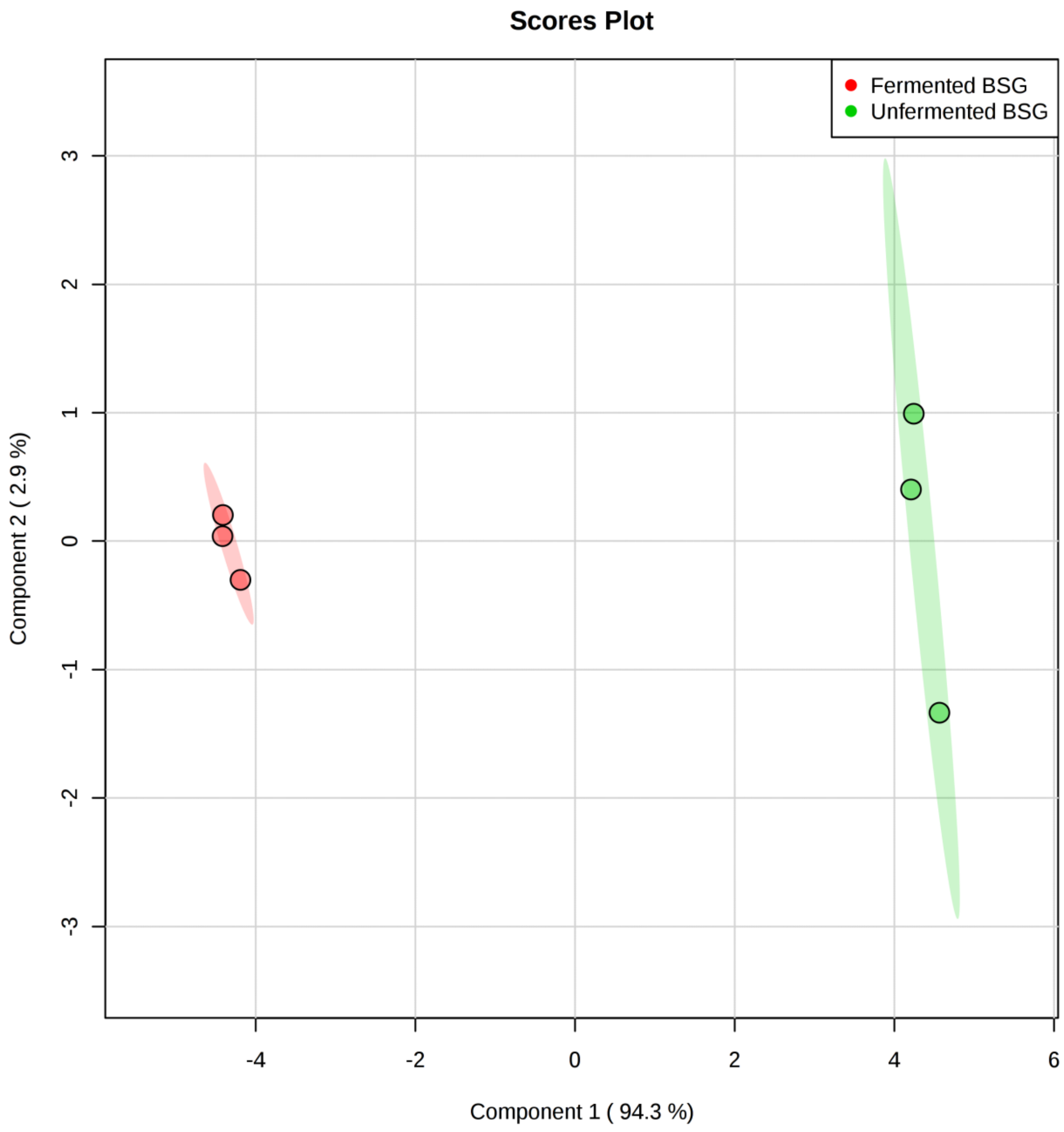


Figure 20: A partial least square-discriminant analysis (PLS-DA) 2D plot for different metabolites detected by GC-MS. *Control (unfermented BSG) samples in green and fermented BSG samples in red as shown in the legend. Each cluster was presented with 95% confidence interval and explained variances are indicated in the brackets. Each dot represents metabolites in each replicate.*

The detected metabolites were mapped onto a clutstering heatmap and can be mainly classified into types of amino acids, carbohydrates, and TCA cycle intermediates (Figure 21). Heatmap provides a visual overview and uses a color gradient to indicate the changes in the metabolites after fermentation (Metsalu & Vilo, 2015). There were a total of 13 amino acids detected by GC-MS that were upregulated after fermentation of BSG. Increased levels of amino acids would enhance the nutritional value of the fermented beverage. During the fermentation process, *B. subtilis* could possibly produce enzymes such as proteases, which allows proteolysis to take place and produce various amino acids. This hydrolysis of protein complexes present in BSG into simple amino acids is in line with an observation in another fermented food study (Lee et al., 2016).

Amino acids are required nutrients for human functions, growth and health (Wu et al., 2013). As proteins are made up of amino acids, the increased levels of amino acids would also provide better health benefits such as improved skeletal muscle protein synthesis (Ha & Zemel, 2003). The various amino acids can be further categorized into essential and non-essential amino acids. Essential amino acids are non-producible by the human body and only can be obtained from dietary intake (Galili & Amir, 2013). The essential amino acids detected in this study include lysine, threonine, methionine, valine, leucine, tyrosine, phenylalanine (Tessari, Lante, & Mosca, 2016). The increase in the concentrations of 7 essential amino acids would improve the nutritional value of the beverage as a supplement for humans (Table 10).

Table 10: Essential Amino acids results (μg/ mL) before and after submerged fermentation

Essential amino acids	Unfermented BSG	Fermented BSG
Leucine	N.D.	4.11 ± 0.74
Valine	N.D.	0.82 ± 0.02
Threonine	N.D.	0.69 ± 0.09
Phenylalanine	N.D.	31.2 ± 2.87
Methionine	N.D.	5.21 ± 0.27
Lysine	$0.01 \pm 2 \times 10^{-4}$	44.92 ± 4.28
Tyrosine	N.D.	28.11 ± 1.89

N.D. – Not detected (detection limit: 1×10^{-5} μg/ mL).

Based on the heatmap, various carbohydrates detected substantially decreased after submerged fermentation (Figure 21). It can be inferred that remaining carbohydrates present in BSG served as carbon sources for *B. subtilis* WX-17 growth. It is postulated that the fermentation using *B. subtilis* WX-17 produced various types of microbial enzymes that hydrolyze polysaccharides into simpler carbohydrates (Ahlawat et al., 2009; Lincoln & More, 2017b; Raul et al., 2014). Metabolites categorized under the TCA cycle intermediates, which are succinic acid and malic acid, were observed to have increased after submerged fermentation (Figure 21). In the growth of microorganisms, the TCA cycle is a series of chemical reactions that generate energy to aerobic microorganisms. The upregulation in TCA cycle intermediates is in line with previous studies (Clements, Streips, & Miller, 2002; H. Song & Lee, 2006), which showed that increase in TCA cycle metabolites stimulate growth of microorganisms. Overall, in both heatmap and PLS-DA, several significantly different metabolites ($p < 0.05$) were obtained after submerged fermentation.

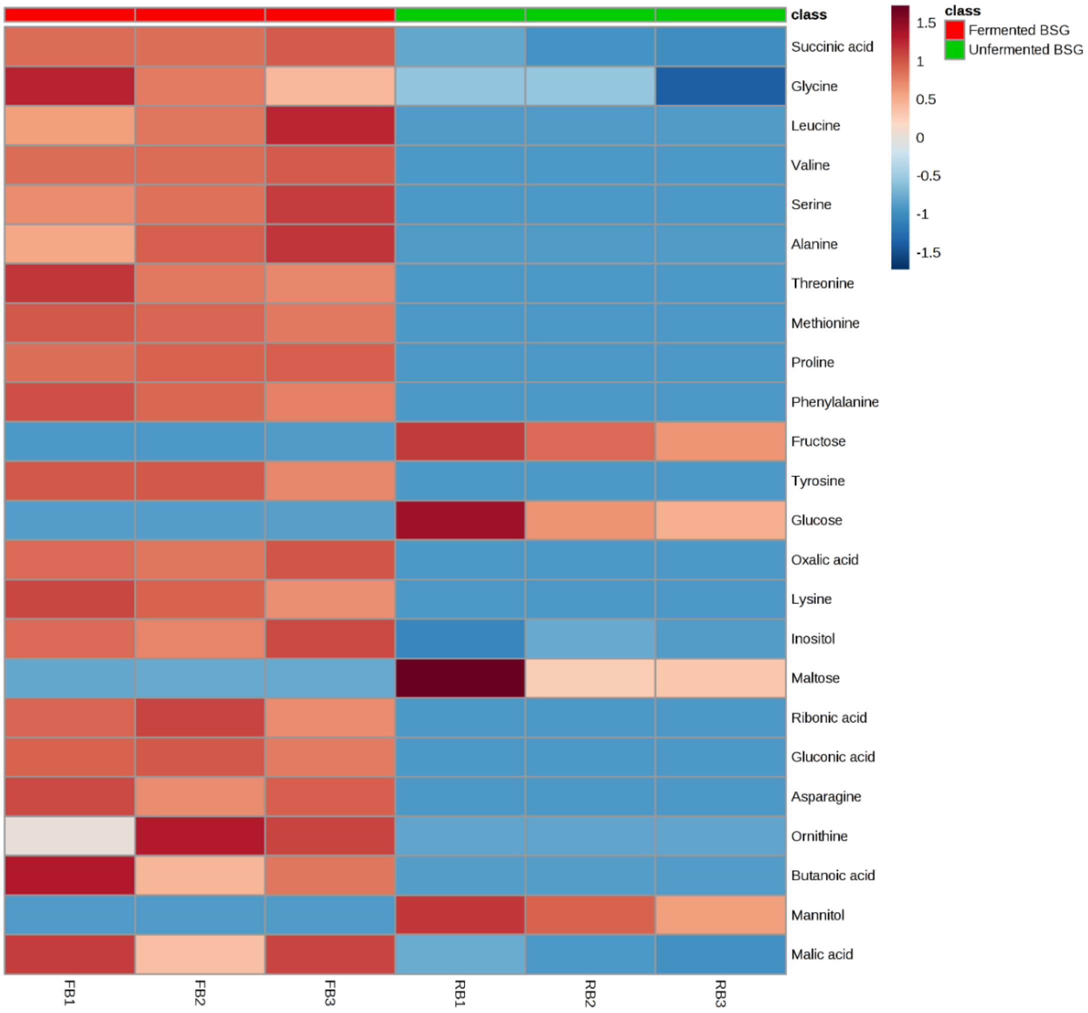


Figure 21: A heatmap analysis of metabolites detected by GC-MS in both unfermented and submerged fermented BSG. *The submerged fermented BSG samples, in triplicate, are displayed from the left, followed by the unfermented BSG samples, in triplicate. The areas shaded in red indicate a higher amount of the specific metabolites, whereas the areas shaded in blue indicate a lower amount of the specific metabolites. FB1, FB2, FB3 represent fermented samples whereas RB1, RB2, RB3 represent unfermented samples.*

5.3.3 Antioxidant and phenolic content assay

Based on DPPH radical scavenging activity results from this study, submerged fermented BSG produced approximately 2.08 times more antioxidants in terms of Trolox equivalent compared to the unfermented BSG (Table 11). A small portion of inhaled oxygen in humans would naturally transform into free radicals, which might cause damage to the body. As long as there is a balance between free radicals transformation and eradication, these free radicals will not cause any harm (Hu et al., 2004). However, external conditions such as stress and smoking could lead to an increase in levels of free radicals and might result in several human diseases (Bandyopadhyay, Das, & Banerjee, 1999). Adequate amounts of antioxidant intake would be able to control the excessive amounts of free radical production (Álvarez, Alvarado, Mathieu, Jiménez, & De la Fuente, 2006). With an increase in DPPH radical scavenging activity after submerged fermentation, the antioxidant compounds present would further enhance the nutritional value of the beverage.

In this study, there was an increase in phenolic content from 125.7 ± 0.74 µg GAE/ mL to 446.74 ± 1.26 µg GAE/ mL after submerged fermentation, which is in agreement with a study that reported microbial fermentation serving as a powerful method for extracting phenolic compounds (Table 11) (Bhanja Dey, Chakraborty, Jain, Sharma, & Kuhad, 2016). BSG had been found to contain phenolic compounds that are bounded to the cell wall (McCarthy et al., 2012; Meneses et al., 2013). Cell wall degrading enzymes would be required to extract the components (Bhanja Dey et al., 2016). Hence, it is postulated that *B. subtilis* WX-17 was able to produce enzymes such as cellulases to degrade the cell wall and extract the phenolic compounds through submerged fermentation (Deka et al., 2013).

Table 11: Trolox quantification for antioxidant activity before and after fermentation

Samples	Signal Inhibition %	Weight of Trolox (μg/ mL)	Gallic Acid Equivalent (μg/ mL)
Unfermented BSG (Control)	29.96 ± 1.03	3.51 ± 0.15	125.7 ± 0.74
Fermented BSG (Day 3)	66.39 ± 1.45	7.31 ± 0.11	446.74 ± 1.26

5.4. Conclusion

This work demonstrates that submerged fermentation with BSG as the sole substrate was feasible to grow *B. subtilis* WX-17 on BSG. Growth of *B. subtilis* WX-17, in the beverage had a final cell count of 9.86 log CFU/ mL after a period of 6 weeks when stored at 4 °C. It produced a nutritious beverage, tested to contain an increased amount of amino acids, total phenolic content and antioxidant activity. The combination of higher nutritional content together with presence of viable *B. subtilis* in the liquid would justify the potential of using the liquid as a novel nutritional beverage for human health. Further investigations on consumer acceptance, flavour of the beverage or different nutritional component analysis can be executed.

Chapter 6. Conclusion and future directions

6.1 Conclusion

This thesis explored the biological methodologies to recover remaining nutrients and enrich underutilized BSG for food applications. By focusing on the application of potential functional food ingredients using BSG, this approach aimed at achieving a zero waste processing, sustainable way of recycling BSG. Also, this approach of reusing BSG, it will enhance food security. Bacterial fermentation was performed on BSG and untargeted metabolomics was employed to provide insights of the fermentation process by analyzing all the metabolites produced. To understand and assess the health effects of fermented BSG, *in vitro* digestion-fermentation method was applied. The summary of this thesis work is shown in figure 22. The summary of key work and findings in thesis is as follows,

- Solid state fermentation on BSG for improved nutritional profile
- Untargeted metabolomics analysis of fermented BSG
- Pathway analysis of metabolites during fermentation
- *In vitro* evaluation of fermented BSG as functional food ingredients
- Health effects of fermented BSG based on gut microbiota
- Potential novel nutritional beverage using submerged fermentation

The motivation behind this study is the large amount of nutritive, unutilized BSG treated as waste materials and being disposed. The amount of residual materials generated over time are leading to serious environmental issues which in turn threatens the food security. In addition, more resources have to be allocated to dispose the wastes either in the landfill or manage the wastes. BSG still contain valuable compounds such as dietary fibres, fatty acids and proteins that can be utilized for more useful applications.

In Singapore's context, food security relies heavily on import of food from overseas. Moving forward, it is important for Singapore to adopt innovative solutions to produce own food to strength food security. By employing GRAS grade bacteria to ferment BSG, this can possibly provide a processing technology solution to improve food security and as well as reducing amount of waste disposal in the landfill. SSF was applied on BSG described in chapter 3. The various components formed after fermentation were examined by metabolomics, which was able to provide a comprehensive molecular scale analysis. The results showed by using SSF on BSG with *B. subtilis* WX-17, it contains higher amount of amino acids, essential fatty acids, antioxidants and additionally produced vitamin K_2 MK-7 compared to raw BSG. Metabolic pathway analysis helped to reveal the reactions taking place during fermentation. These enhanced components serve as promising properties for functional food ingredients.

Chapter 4 assessed the health effects of valorized BSG as potential functional food ingredients by using an *in vitro* digestion-fermentation model. The assessment showed that with a higher soluble fibre content, fermented BSG produced higher amount of fatty acids, amino acids, antioxidants, vitamin K_2 MK-7, SCFAs available for absorption after *in vitro* digestion and fermentation. From the results of intestinal microbiota through 16s rRNA sequencing, it was found that fermented BSG provided a more beneficial intestinal microbiota profile. Hence, the findings obtained from the evaluation support using fermented BSG as potential functional food ingredients.

The findings from chapter 5 showed that using submerged fermentation on BSG as the sole substrate was feasible to grow *B. subtilis* WX-17 for food application. The probiotics, *B. subtilis* WX-17 was still viable after a period of storage. It produced a potential functional beverage, tested to contain a higher amount of amino acids and phenolic compounds. The combination of higher nutritional content together with presence of viable *B. subtilis* in the liquid would justify the potential of using the liquid as a potential novel functional beverage for improving human health. The overall workflow and findings are summarized in figure 23.

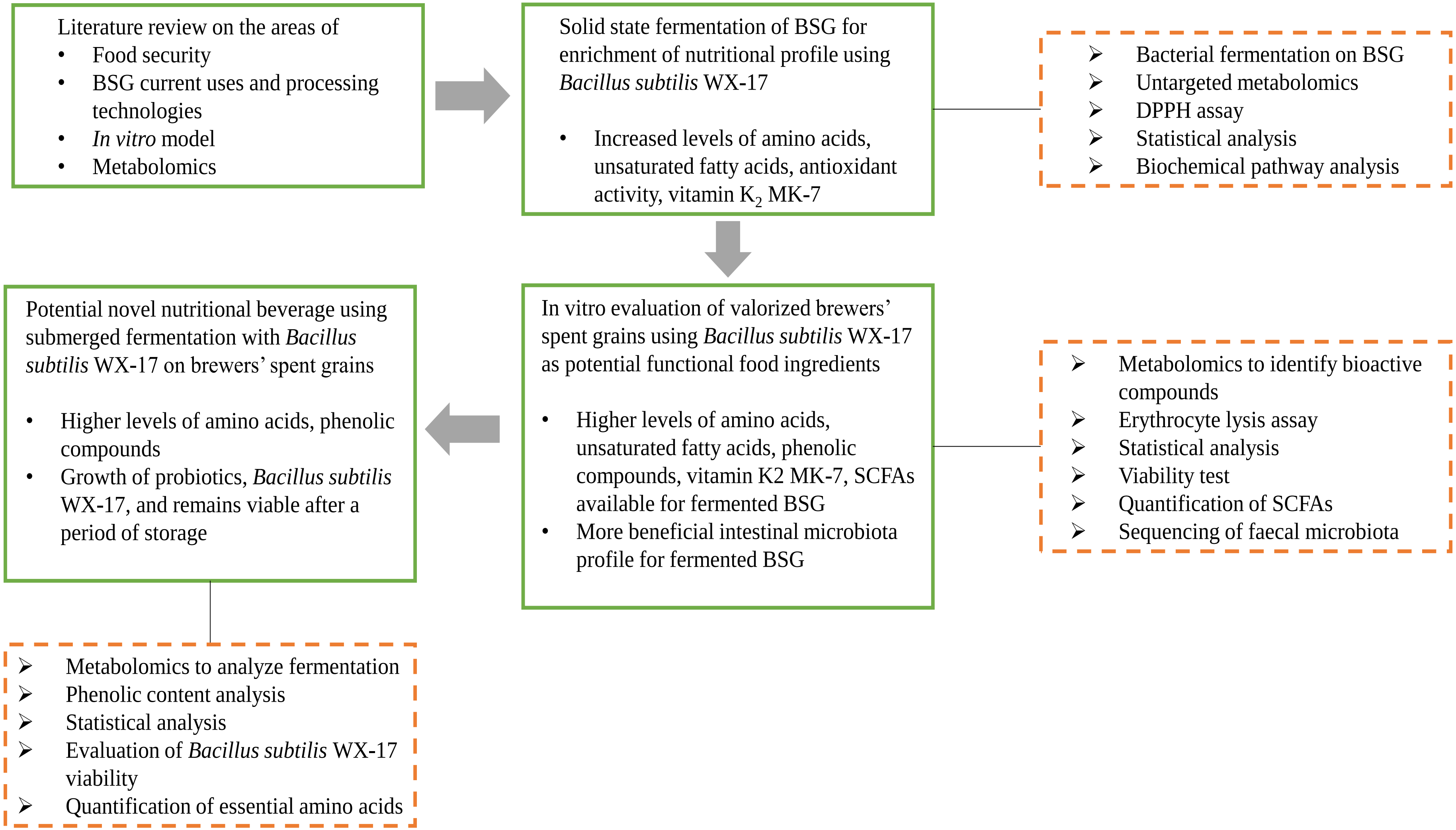


Figure 22: Overall overview of thesis workflow to employ biofermentation on BSG to achieve zero waste processing.

This study has demonstrated that fermenting BSG is able to utilize, recover the remaining beneficial nutrients and creating a loop process of re using resources to provide a zero waste solution. The conventional way of processing waste is following the traditional linear economy system, where raw materials were used for production and the wastes were disposed. Alternatively, a circular economy approach is a system where unavoidable wastes resources are continuously valorized, recirculated for the longest time possible and resulting in minimal wastes. The approach in this study is based on the concept of circular economy system (Figure 24).

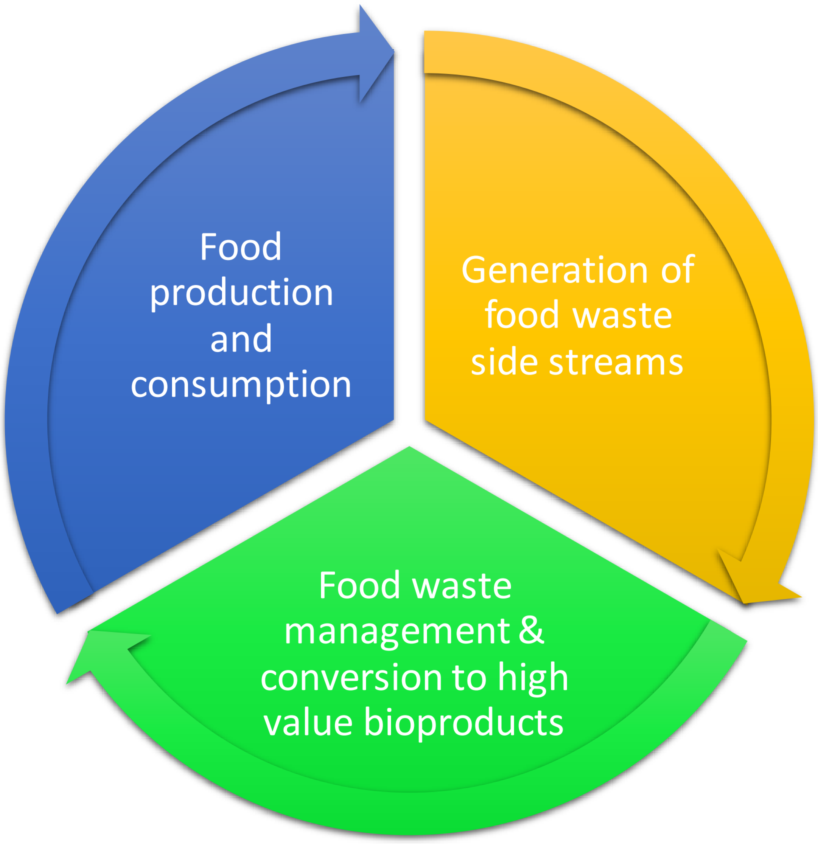


Figure 23: Circular economy for food waste processing. *This approach of reusing resources and preserving its value to longest time possible will provide a sustainable means of managing unavoidable wastes produced from manufacturing processes.*

6.2 Future directions

6.2.1 Solid state fermentation upscale

Despite the potential that the research work can possibly provide, there are several limitations and future work that can be proposed. Firstly, one of the challenges lies in the scaling up process of SSF into a large scale bioprocess. According to Manan and Webb (2017), there are several engineering problems to operate large scale SSF are mainly due to temperature building up, difficulties in optimal oxygen transfer, substrate, moisture gradients, pH control, uneven distribution of nutrients, moisture content and aeration. A more comprehensive system design will be required to overcome the issues in order to successful upscale SSF operation. Nonetheless, SSF has advantages such as simplicity in operation, higher fermentation productivity, absence of foam formation, less energy requirement and smaller reactor volume (Nighojkar, Patidar, & Nighojkar, 2019).

On the other hand, Manan and Webb (2017) reported that scaling up submerged fermentation operation has been more common and established in the fermentation industry. Submerged fermentation has advantages such as control over environmental parameters and lower space requirements. However, submerged fermentation on a larger scale would also face some disadvantages such as lower product yield, longer fermentation process. In addition, excessive foam can be produced after long period of submerged fermentation and prevent optimum amount of oxygen transfer. Therefore, to enable the scaling up of submerged fermentation of BSG, further engineering solutions and work will also be required as well.

6.2.2 Metabolomics

The untargeted metabolomics approach to evaluate the fermentation process presents a few limitations. Firstly, fermentation kinetics can be possibly applied to obtain more information on the processes. The current study employed GC-MS to detected and identify the range of metabolites. To further more explore a broader range of metabolites produced after fermentation, other analytical tools such as Q-TOF MS or TQMS. Such spectrometers can provide higher levels of sensitivity and additional information to this study on the detection of metabolites (Gowda & Djukovic, 2014). With untargeted metabolomics data identified based on few library databases from the study, future targeted metabolomics can be applied to further accurately examine the more important compounds for specific requirements of a desired application based on the retention time. By running pure standards at various concentration for the compound of interest, accurate quantification of the targeted compound can be achieved.

6.2.3 Appearance and consumer acceptability

The colour and aroma of the fermented product will be a concern and pose challenges to incorporate fermented BSG into foods (Johnson, Paliwal, & Cenkowski, 2010). The presence of dark brown colour in fermented BSG will limit its area of application, for example it will not be applicable in desired off-white products. The amount of BSG that can be incorporated into food product is also limited to the processing conditions. In cases where heat treatment is required for the food product, the presence of BSG would affect the consumer acceptability. Apart from the colour and aroma issues, the flavour and texture of the fermented BSG will also pose challenges for consumer acceptability.

According to (Combest & Warren, 2018), subjects reported lingering fibre particles and aftertaste for foods made with BSG. Having aftertaste being a major sensory attribute in foods, it will be the most important barrier to overcome in the future work. Foods incorporating BSG also resulted in a ‘tough’ product texture. Texture would contribute to the sensory properties that consumer will prefer. Lastly, the lack of awareness in BSG as an ingredient in foods would prevent them from purchasing such foods. Nonetheless, there were positive response from some of the subjects who perceive the smell from BSG foods as pleasant.

6.2.4 In vivo study

Apart from the advantages of simplicity, low cost, easy to operate and suited for initial investigation on gut microbiota and metabolic activities, *in vitro* model possesses a few limitations that should be noted. According to (Pham & Mohajeri, 2018), an *in vitro* digestion-fermentation model has an absence of host cells and lack of dialysis for evaluation. One of the next possible extensions to the *in vitro* study can be incorporating a dialysis system to simulate the absorption of various nutritional components through intestinal walls. The extension study will allow better understanding of the concentration of the nutrients entering the blood stream. With more findings, further extension can be carried out by conducting *in vivo* trials in rats to ascertain and correlate the effects of fermented BSG evaluated by *in vitro* model. For an instance, a study was conducted by Ibe et al. (2009) to investigate the antihypertensive effects of traditional Japanese food natto in spontaneously hypertensive rats.

6.2.5 Alternative uses of BSG

In summary, this thesis work attempts to propose methodologies employing biofermentation, evaluate the fermented product through *in vitro* model to recycle BSG and create a circular economy loop with zero waste generated. There are other alternatives uses of enriched BSG and one of such possible alternative application is enhanced animal feed. Currently, untreated BSG are used as animal feed and by having enriched BSG as animal feed, it is possible to further improve milk production by cows and meat quality of livestock (Bolwig, Mark, Happel, & Brekke, 2019). Other approach to achieve the same objective of no residual waste includes reusing the fibrous food waste, BSG in varying percentages with other material to produce packaging material (Ferreira, Martins, Carvalho, & Magalhães, 2019).

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