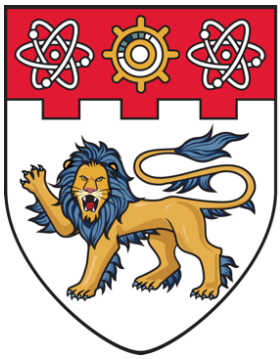

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

AMELIORATING NUTRITIONAL PROPERTIES OF SOYBEAN WASTE (OKARA) THROUGH BIOFERMENTATION USING *RHIZOPUS OLIGOSPORUS*

GUPTA SULAGNA

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

2020

STATEMENT OF ORIGINALITY

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

17th JANUARY 2020

Sulagna Gupta

Gupta Sulagna

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.

17th JANUARY 2020

Professor Chen Wei Ning, William

AUTHORSHIP ATTRIBUTION STATEMENT

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

Chapter 2 has been published as Gupta, S.; Lee, J. J. L. and Chen, W.N. (2018) An analysis of improved nutritional composition of potential functional food (okara) after probiotic solid-state fermentation. *Journal of Agricultural and Food Chemistry.* 66 (21) : 5373-5381.

The contributions of the co-authors are as follows:

- Prof Chen, W.N. designed the research and edited the manuscript.
- I carried out the experiments and wrote the manuscript.
- Lee, J. J. L. commented on the experiments and the manuscript.

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Sulagna Gupta

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To my beloved parents and brother- you were as much a part of this roller-coaster ride as I. WE MADE IT TO THE FINISH!

Dedicated to –

Mum, Blabs, Twa-doom

Chester Bennington

Every novelist/cartoonist whose work I have savoured

&

The city of Singapore

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

$AlCl_3$	Aluminium chloride
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
$C_2H_3KO_2$	Potassium acetate
CO_2	Carbondioxide
CoA	Coenzyme A
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FDA	U.S. Food and Drug administration
$FeCl_3$	Ferric chloride
FRAP	Ferric Reducing Ability of Plasma
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography-Mass Spectrometry

GMO	Genetically Modified Organism
GRAS	Generally Recognized As Safe
HPLC	High Performance Liquid Chromatography
$K_3[Fe(CN)_6]$	Potassium ferricyanide
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography-Mass Spectrometry
MRS	De Man, Rogosa and Sharpe
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass-to-charge ratio
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium chloride
$^{\cdot}NO$	Nitric Oxide
O_2^-	Superoxide
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PDA	Potato Dextrose Agar
Q	Quercetin

QTOF	Quadrupole Time Of Flight
RNA	Ribonucleic acid
RT	Retention Time
SCP	Single-Cell Protein
SFE	Supercritical Fluid Extraction
SmF	Submerged Fermentation
SNP	Sodium nitroprusside
SSF	Solid State Fermentation
TCA	Trichloroacetic acid
TFC	Total Flavonoid Content
TIC	Total Ion Chromatogram
TMCS	Trimethylchlorosilane
TPC	Total Phenolic Content
UAE	Ultrasound Assisted Extraction

THESIS ABSTRACT

Okara is a type of soybean residue, that is generated as a major by-product during the production of tofu and soymilk. It is considered to be one of the prime agri-crop wastes. Given the rise of health consciousness, vegetarianism, veganism and lactose intolerance amongst the world population today, it is to be expected that the generation of this food waste will only increase further. Currently, okara is either disposed in landfills or incinerated. Since not much finances are diverted towards the management of industrial waste, an economical and environment friendly, yet viable solution for the management of such food wastes is the need of the hour for a sustainable approach.

In this thesis, biofermentation has been suggested as a valorization technique to improve the nutritional properties of okara, to gainfully repurpose this food waste. This work was initiated with FDA-approved microbes *Rhizopus oligosporus* and *Lactobacillus plantarum*, but switched to the usage of only the former in the later stages of the study when it yielded better results than the latter.

In the first stage of this study, a GC-MS based metabolomic analysis was conducted to understand the changes occurring due to the microbial biochemical processes. The detected metabolites were subsequently mapped to their respective biochemical pathways, to understand the pathways being triggered during the biofermentation process. A preliminary DPPH test showed that *Rhizopus oligosporus* fermented okara has a radical scavenging activity of 52.81%, as opposed to that 27.44% for raw okara. The results obtained in this stage justified that fermented okara may be a potential functional food.

Next, an attempt was carried out to extricate a clean, green extract from fermented okara using a combination of water and ultrasound sonication. Upon subjecting these extracts to DPPH, FRAP, O_2^- and $^{\cdot}NO$ antioxidant analyses, it was observed that the results were significantly better than those obtained by raw okara extracts. Subsequent erythrocyte lysis assays revealed that fermented okara extract is not only non-toxic to erythrocytes at concentrations as high as 4 mg/mL, but could also prevent AAPH induced haemolysis of erythrocytes at concentrations as low as 500 ng/mL. The study then endeavoured to use the fermented okara extract on cancer cell line HepG2, to check for antiproliferative activities. A parallel was simultaneously run on NIH 3T3 cell line, a non-cancer cell line, to check for toxicity. The experiments revealed that 48 hours incubation yields antiproliferative activities on HepG2 cell line in a dose-dependent manner. After 48 hours incubation, the highest tested concentration (100 mg/mL) of fermented okara extract could inhibit HepG2 cells by 48.47 ± 5.28%, which was significantly different from its effect on NIH 3T3 cells. To understand the compositional differences in the extracts leading to the bioactivities, a GC-MS metabolomics analysis was performed, which showed that the fermented okara extracts contained more amino acids and organic acids, and less sugars. The results obtained in this stage validated the in-vitro antioxidant and antiproliferative properties of fermented okara.

Finally, the study focussed on the improvement of polyphenol content post fermentation, with an emphasis on aglycone isoflavones. TPC and TFC analyses showed that fermented okara extracts yield significantly better results than unfermented okara extracts. An LCQTOF/MS analysis followed by HPLC quantification revealed that ethanolic extracts of fermented samples contained 11.782 ± 0.325 μg/mL and 10.125 ± 1.028 μg/mL of daidzein and genistein respectively. In contrast, extracts of unfermented

okara contained only 6.7 ± 2.42 μg/mL and 4.55 ± 0.316 μg/mL of daidzein and genistein, respectively. The results obtained in this stage established the phenolic enhancement of okara post biofermentation.

Summing up, this thesis demonstrates a method to ameliorate the nutritional properties of okara via biofermentation using *Rhizopus oligosporus*, as a sustainable method of dealing with this agri-industrial food waste.

CHAPTER 1

INTRODUCTION

1.1 Food sustainability

Sustainability refers to meeting the demands of the present generation without compromising on the availability of resources for the future generations. Although there exists no legal definition to it, it encompasses a system that is designed to improve a community's environmental, economic and social well-being. In the same vein, food sustainability entails the supply of food production meeting the demands of the current world population at any given time. Conventionally, food sustainability is governed by inherent factors such as water, land, climate, fertilizers and energy. However, with the world population rising exponentially today, the sustainablilty of the food supply chain will come to rely on choices made by the population, including but not limited to food waste, overconsumption and the involvement of GMO in agriculture (Morawicki and González, 2018).

1.2 Food waste as a type of waste

In broad terms, waste refers to any item that people or industries no longer harbour any use for, and that which they intend to discard or have already disposed. Waste generation is directly proportional to the rate of urbanization and industrialization of a particular geographic location. The environmental impact of wastes may be manifested through air, water and soil pollution, which directly impact human health. Awareness regarding waste generation and its impact on the environment has greatly increased in the recent years at both civil and governmental levels.

1.2.1 Solid waste

Solid waste refers to the waste produced by residential, commercial, industrial and institutional clusters' construction, demolition, municipal processes and day-to-day activities (Ngoc and Schnitzer, 2009). Categorically, food waste is a sub-type of solid waste.

1.2.2 Food Waste

Food waste, as described by the FAO, is "the decrease of food in subsequent stages of the food supply chain intended for human consumption". The cause of this is manifold – food spoilage due to improper handling, harvesting, transport or storage, edible food from leftovers, discarding expired foodstuffs etc. It also includes skins and peels from fruits and vegetables and husks and stalks from grains and legumes. Food waste is generally classified into three types- avoidable, possibly avoidable and unavoidable. The sources of food waste include farms, food manufacturers, food distributors, food retailers, wet markets, supermarkets, hawker centres, restaurants, food courts, caterers and homes. Today, the massive amounts of food wastes produced everyday has become a matter of global concern from the point of view of environmental pollution and food sustainability.

1.3 What is okara?

Soybean (*Glycine max*) is an edible legume that has gained popularity in recent times due to the rise in vegetarianism, veganism and lactose intolerance in the world. It is chiefly used to produce tofu and soymilk. Soy vegetable oil is another important

commercially viable product that is harnessed from soybean. The wastes originating from the harvesting and processing of soybean include soy hulls, soybean meal, okara and soybean straw.

Okara is one of the main agri-industrial wastes generated in the world currently. It is the chief by-product of the tofu and soybean industry (Figure 1). Botanically, it consists of ruptured cotyledon cells and soybean seed coat. It is a white or yellowish fibre-rich residue, insoluble in water and possesses a bland taste. Okara is also known by other names such as soybean residue, soybean dreg, bean curd residue, douzha, tofuzha, tofukasu and bejee. It is one of the agri-wastes that hold immense potential to be harnessed as a functional food.

However, there exist a few limiting factors that hinder the economical implementation of okara in the food chain. Firstly, fresh okara has an extremely high moisture content (~70-80%), making it susceptible to quick putrefaction (Redondo-Cuenca et al., 2008). Drying is an expensive recourse for a waste residue. Hence, industries prefer to either incinerate this by-product or dump it in landfills. These are environmentally harmful options, of which the world is conscious currently. Secondly, high temperatures employed during the cooking step of soymilk/tofu production extensively denatures soybean proteins and makes the residue (okara) insoluble. This, in addition to high fibre content, hampers the direct reuse of okara in the food supply chain (Chan and Ma, 1999). Hence, processing techniques need to be employed prior to re-utilization of this food waste.

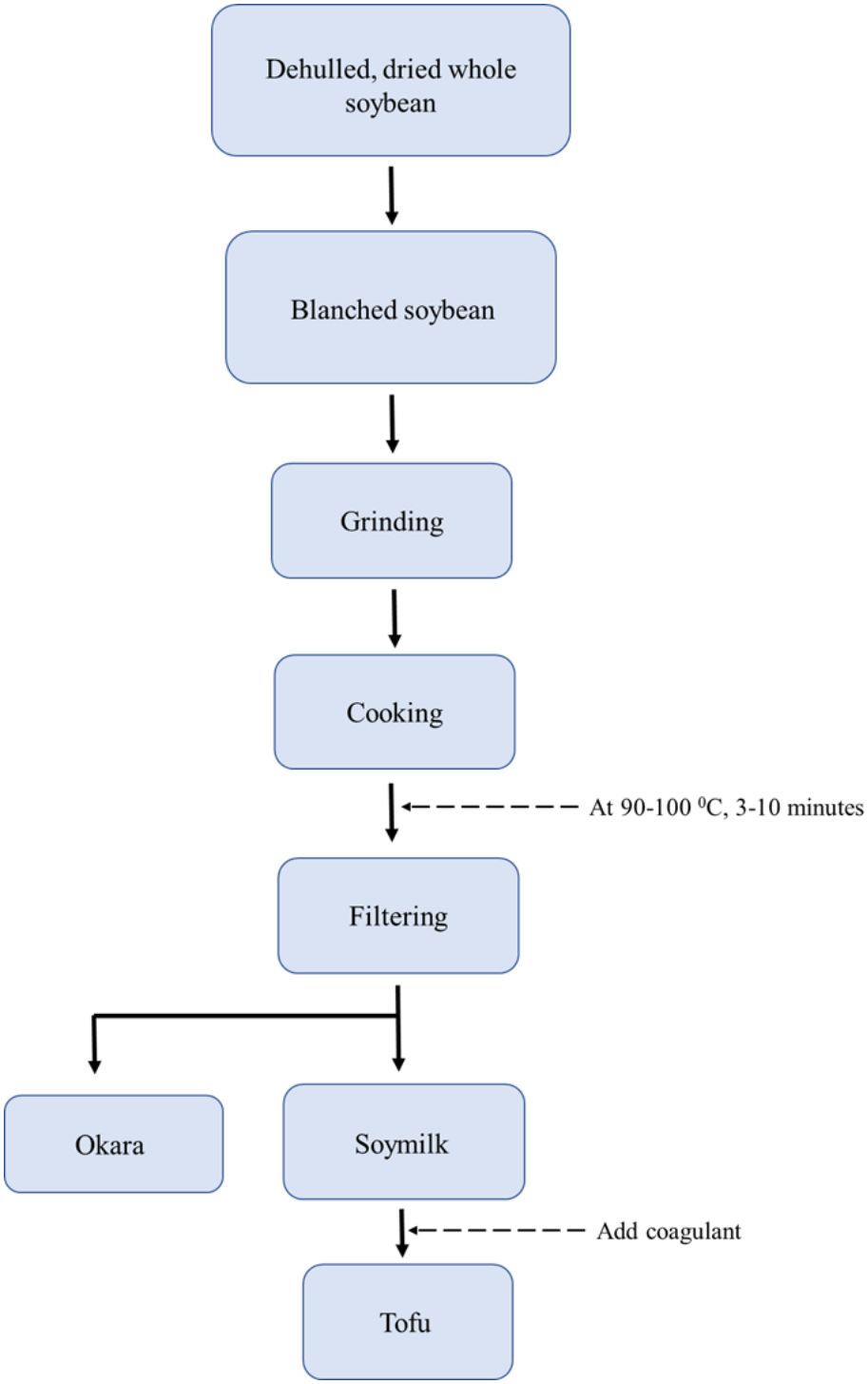


Figure 1 : Production of okara from soymilk/ tofu industry. *Okara is a by-product of these industries and usually discarded as an agri-industrial waste.*

Technavio, a market research company, estimated that the global (APAC, Americas and EMEA countries) compound annual growth rate of tofu would be increasing at a rate of 4% during the period of 2017-2021. Around 1.1kg raw okara is generated for every kilogram of soybean processed into the manufacturing of soymilk or tofu (Khare et al., 1995). Hence, the generation quantities of this waste are already rising exponentially with every passing year, thus making it imperative to discover sustainable methods to tackle this particular food waste.

For a more precise idea, the annual generation of okara in some countries are documented in Table 1.

Table 1 : Annual generation of okara in some countries

Country	Annual production	Reference
Japan	800,000 tonnes	Li et al. (2013)
Singapore	10,000 tonnes	Vong and Liu (2017)
Korea	310,000 tonnes	Ahn et al. (2010)
China	2,800,000 tonnes	Li et al. (2012)
Canada	10,000 tonnes	Vong and Liu (2017)

1.4 Biofermentation

Biofermentation refers to the fermentation of organic wastes such as food waste, agri-industrial crop waste and kitchen-and-garden waste, for the production of various

important products of commercial relevance. The two kinds of biofermentation processes currently in use today are SSF and SmF.

1.4.1 SSF vs SmF

SmF is a type of fermentation that has been conventionally used since generations past. It refers to the process of growth of microbes in a liquid medium. Although it is the most exploited technology for the industrial production of several commercially important metabolites and enzymes, a host of problems encumber SmF. Sterility is the primary requirement for SmF. In case of contamination and in the presence of high-water availability, the contaminating microorganism can quickly outcompete the process organism. This adds to the cost of precision-driven designing of bioreactors. Further, as detailed by Gibbs et al. (2000), the problems associated with SmF using filamentous fungi include: a) using fungal pellets to overcome broth viscosity does not work during the production of extracellular polysaccharide metabolites, as overall fermentation broth rheology exhibits a non-Newtonian behaviour, b) larger reactors need expensive propellers to ensure homogeneity of cell suspension, c) the distribution of sufficient oxygen throughout the reactor during aerobic fermentation processes, d) heat transfer, nutrient and pH levels gradients throughout the reactor interfering with the product production by hindering the activity of the process microbe, e) monitoring biomass concentration and fungal cell morphology to maintain optimum rheology of the fermentation fluid, f) monitoring pellet structure, type and formation in the reactor system, which is specific for the type of compound being produced for extraction.

In contrast, SSF is only recently being researched and explored as a viable option for production of compounds via fermentation. SSF can be defined as “the growth of

microorganisms on (moist) solid substrates in the absence or near-absence of free-water" (Pandey, 1992). Hence, the substrate should possess sufficient moisture in the absorbed form. The substrate is also responsible for supplying nutrients and providing anchorage for microbe propagation. Particle size of substrate should not be too small (leads to substrate aggregation) or too large (lesser surface area) but optimized for a particular microbe or process. Primary focus has been on agri-industrial crop residues for use as substrate in the production of bulk chemicals and value-added fine products (Pandey et al., 2000). Amongst all microbes, filamentous fungi possess the best ability to proliferate in the absence of free water and are the most widely used class of microorganisms employed in SSF. Other factors that control the effectiveness of SSF as a fermentation technique include temperature, moisture, pH, humidity and aeration. It is important to monitor the temperature as heat is generated during microbial fermentation. Low substrate moisture is a necessity, to maintain substrate porosity and facilitate aeration.

SSF has gained popularity over submerged fermentation due to a number of reasons (Sabu et al., 2002). It has an overall simpler set-up, with extremely economical energy requirements and lesser fermentation space requirement. Sterility is not a stringent issue, as in scant water conditions, the active process organism (starter culture) can far outgrow the contaminating microbe. Minimal waste output, easier aeration parameters and absence of foam formation are further advantages. High reproducibility and high product titres make SSF a favourable technique as well. Further, low moisture content in SSF substantially reduces the possibility of contamination. SSF is a useful process for bioremediation and biodegradation.

1.4.2 SSF usage till now

Although SSF has been shown to possess many advantages over SmF, it has not yet reached extensive utilization at industry level. Experiments at laboratory scale have showcased its use for production of a variety of products, including enzymes (ligninase, cellulase, glucoamylase, proteases, lipase, phytase, tannase, xylanase, pectinase etc.), organic acids (citric acid, lactic acid, gallic acid), single cell proteins (SCPs) and bioactive secondary metabolites (gibberellins, alkaloids, flavonoids, antibiotics) (Pandey et al., 2000).

1.5 Microbes used in fermentation of food

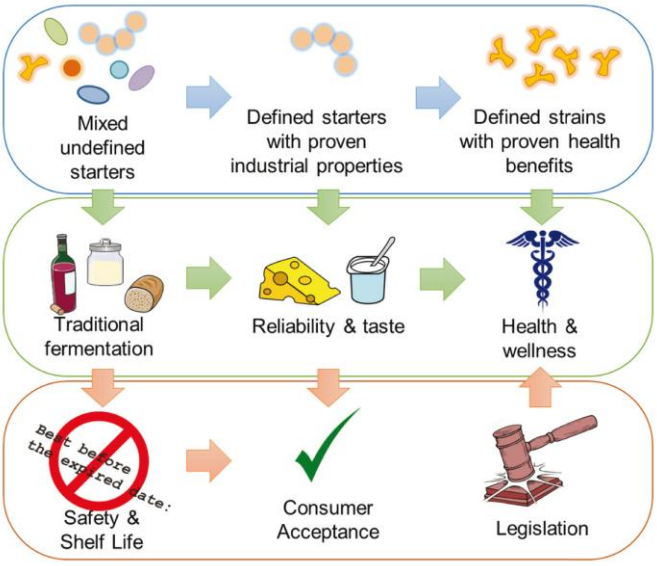


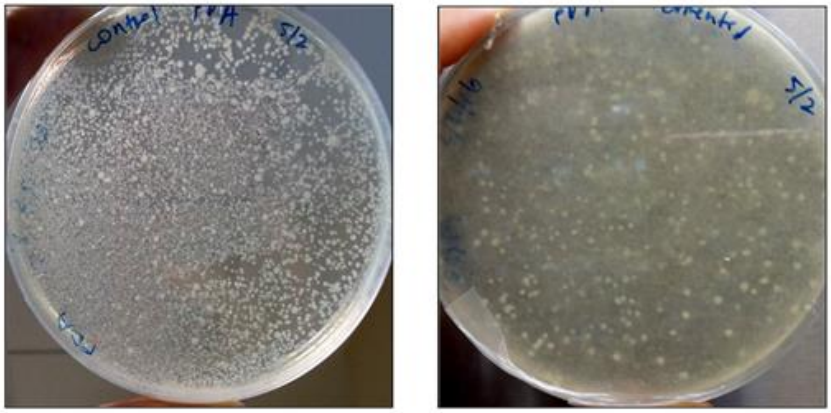
Figure 2 : Fermentation of food over the years. *Employing fermentation in food is an age-old concept, which has slowly broadened its scope in the commercial market. Image extracted from Hill et al. (2017) with permission.*

The concept of employing microbes as a means to improve texture and increase shelf life of food has been around since many generations. Traditional fermentation

techniques were mainly confined to household consumption and therefore used microbial starter cultures of variable origin. This paved the way for commercial starters of defined origin and with proven reliability, that led to the industrialization of fermented products such as butter, pickles, cheese, yogurt, wine etc. (Figure 2).

1.5.1 Microbes used in this study

The FDA-approved, GRAS microbes used as process organisms in this thesis are *Rhizopus oligosporus* and *Lactobacillus plantarum* (Figures 3 and 4).



(a) **(b)**

Figure 3 : Pure colonies used for making culture suspensions for biofermentation (a) *Lactobacillus plantarum,* and (b) *Rhizopus oligosporus.* *Microbial culture suspensions were prepared by harvesting pure colonies from agar plates. The CFU was calculated using serial dilution method, before using these cultures for fermentation.*

Rhizopus oligosporus is a member of the family *Mucoraceae* of fungi. They can grow at relatively high temperatures of 30 ^{0}-40 ^{0}C. These aerobic or microaerophilic fungi (C.W. Hesseltine, 1985) exhibit strong lipolytic and proteolytic activities that

make them desirable for use in fermentation. Further, they also exhibit anti-microbial activity that inhibit the growth of other microbes in the media (Kobayasi et al., 1992, P. Dinesh Babu, 2009). It has been used indigenously in south-east Asian countries for generations and is popularly exploited as a starter culture for tempeh production.

(a) **(b)** **(c)**

Figure 4 : (a) Fresh okara. *Fresh okara contains a high percentage of moisture that leads to its easy spoilage. It also contains a lot of bound nutrients, which makes it a homogenous food waste with high potential for use as a substrate in solid state fermentation.* **(b) Okara fermented with *L. plantarum*.** *Okara fermented with L. plantarum appears crumbly and dry.* **(c) Okara fermented with *R. oligosporus*.** *Okara fermented with R. oligosporus becomes a fluffy patty due to mycelial network formation.*

Lactobacillus plantarum belongs to the genus *Lactobacillus* that includes Gram-positive rod-shaped bacteria. It grows at a temperature range of 15 ^{0}-45 ^{0}C. They are usually facultative anaerobes or miroaerophiles and are acid and bile salt tolerant. *L. plantarum* ferments sugars to produce lactic acid, ethanol or acetic acid. According to the FDA, *L. plantarum* is safe for usage as a food ingredient. Its organoleptic properties

and its ability to produce antimicrobial substances have currently made them one of the most widely used probiotics in the food industry (Rezac et al., 2018).

1.5.2 Fermentation of food across different substrates

Table 2 : Examples of fermented food across different edible substrates

Data taken from Tamang et al. (2015)

Product	Substrate	Substrate classification	Microbe
Yogurt	Milk	Dairy	*LAB species, Bifidobacterium species*
Pumpernickel bread	Rye	Cereal	*LAB, Yeasts*
Pickle	Cucumber	Vegetable	*Leuconostoc mesenteroids, Pediococcus cerevisiae*
Miso	Soybean	Legume	*Aspergillus oryzae, Micrococcus halobius*
Cingwada	Cassava	Tuber	*Species of Corynebacterium, LAB, Micrococcus*
Atchara	Green papaya	Fruit	*Lactobacillus brevis, Lactobacillus plantarum*
Chorizo	Pork	Meat	*Lactobacillus plantarum*
Surstömming	Herring	Fish	*Haloanaerobium praevalens*
Kombucha	Tea liquor	Beverage	*Torulaspora delbrueckii, Candida stellata*

1.6 Natural products

Natural products refer to all organic products that have been synthesized by living organisms. These include biotic materials (for eg.; silk), bio-based materials (for eg:, cornstarch) body fluids (for eg.; milk) and other natural materials (for eg.; coal). Evolution and natural selction have played a role in the high structural diversity and pharmacological properties of natural products. They serve as prime models for the development of synthetic molecules that mimic the bioactivity of these molecules. Presently, natural products are being exploited by the healthcare and food industries for safe, natural and effective options to replace synthetic compounds in use.

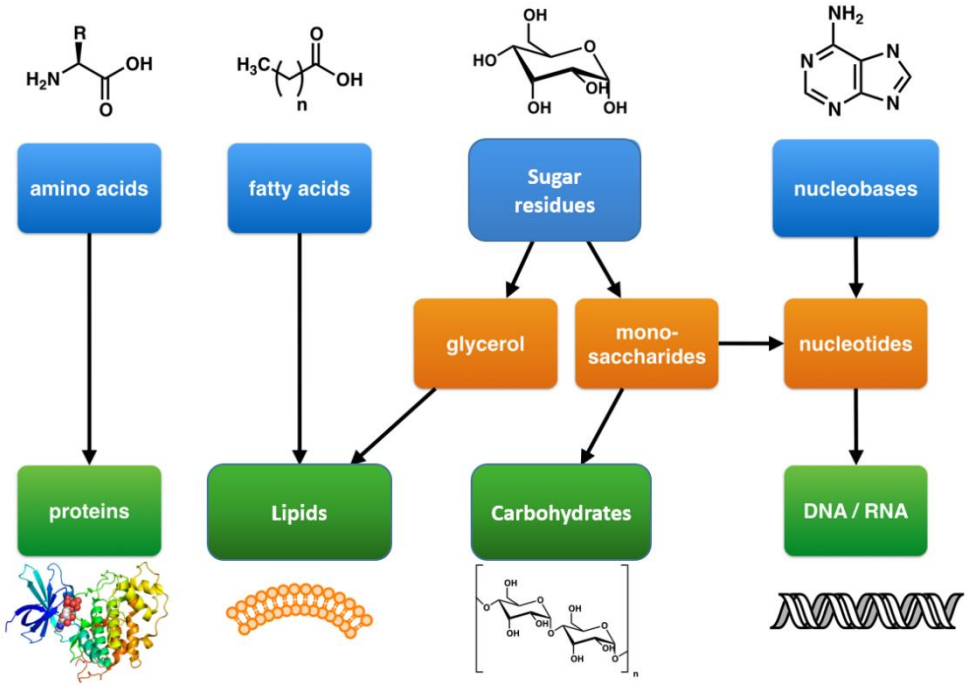


Figure 5: Primary metabolites. *Also known as "building blocks of life", primary metabolites are indispensible to survival. Image extracted from Abozenadah et al. (2017) with permission.*

1.6.1 Primary and secondary natural products

Natural products are broadly divided into two categories – primary metabolites and secondary metabolites. Primary metabolites are organic molecules that are involved in essential for cell survival ans maintenence and encompass cellular activities such as nutrient assimilation, energy production, growth and development (Figure 5). Primary metabolites include amino acids, nucleic acids, sugars, fatty acids, etc. In contrast, secondary metabolites are not essential to cell survival and differ signficantly amongst species.

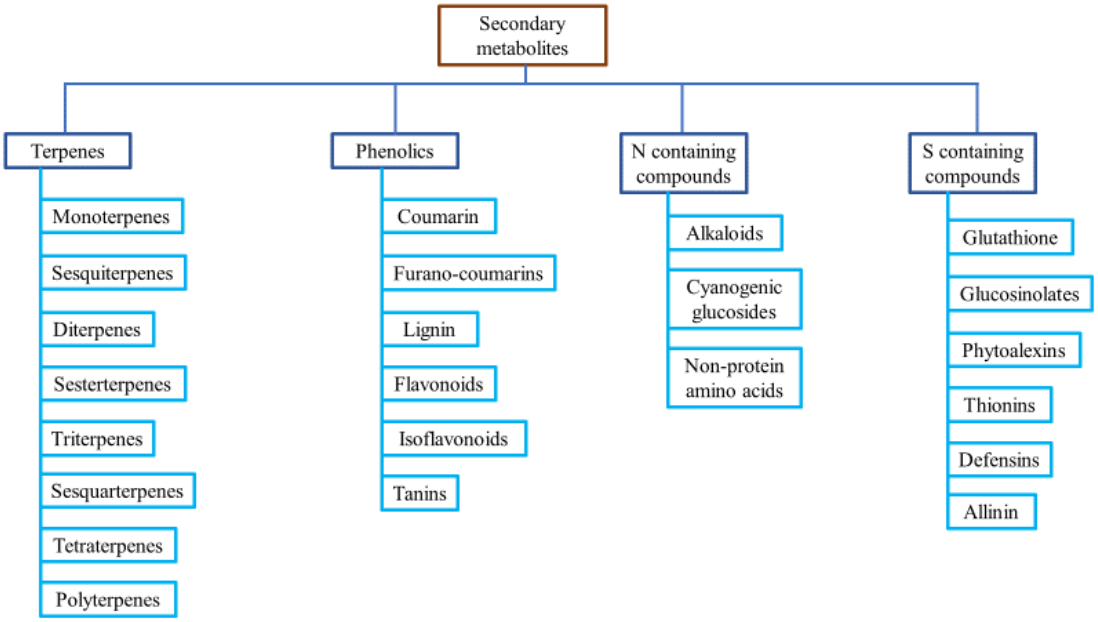


Figure 6 : Classification of secondary metabolites. *Secondary metabolites possess a variety of important benefits such as natural antimirobial and anticancer activities. Image contents extracted from Jamwal et al. (2018) with permission.*

Broadly, secondary metabolites may be defines as organic molecules that elicit an extrinsic response and increase the chances of survival of the producer in the

environment (Figure 6). They have a broad range of pharmacological funcions, and are currently under intense examination for harnessing natural drugs with similar bioactivity as synthetic drugs.

1.6.2 Natural products from SSF

Table 3 : Natural products from SSF of food waste

Natural product	Process microbe	Substrate	Reference
β-carotene	*Blakeslea trispora*	Orange, carrot and papaya peels	Kaur et al. (2019)
Ellagic acid	*Aspergillus niger*	Pomegranate peel	Aguilar et al. (2008)
Cellulase	*Aspergillus niger*	Pea pod waste	Sharma et al. (2015)
Protease	*Streptomyces malaysiensis*	Brewer's spent grain	Nascimento et al. (2011)
Polyunsaturated fatty acids (PUFA)	*Mortierella alpina*	Distiller's dried grains	Yang and Zhang (2016)
Gibberellic acid	*Fusarium moniliforme*	Citric pulp	Rodrigues et al. (2009)
Rifamycin SV	*Amycolatopsis mediterranei*	Ragi bran	Nagavalli et al. (2014)

SSF, as previously mentioned, is prominently used to valorize biomass residues for the production of natural products having antioxidant, antimicrobial and antiproliferative characteristics. It is also used for the amplified production of important enzymes. A few examples of natural products obtained from the SSF of food waste are listed in Table 3.

1.7 Metabolomics as part of the 'omics' family

The term 'omics' is used in refrence to a comprehensive analysis of a molecule with respect to its roles, relationships and activities.

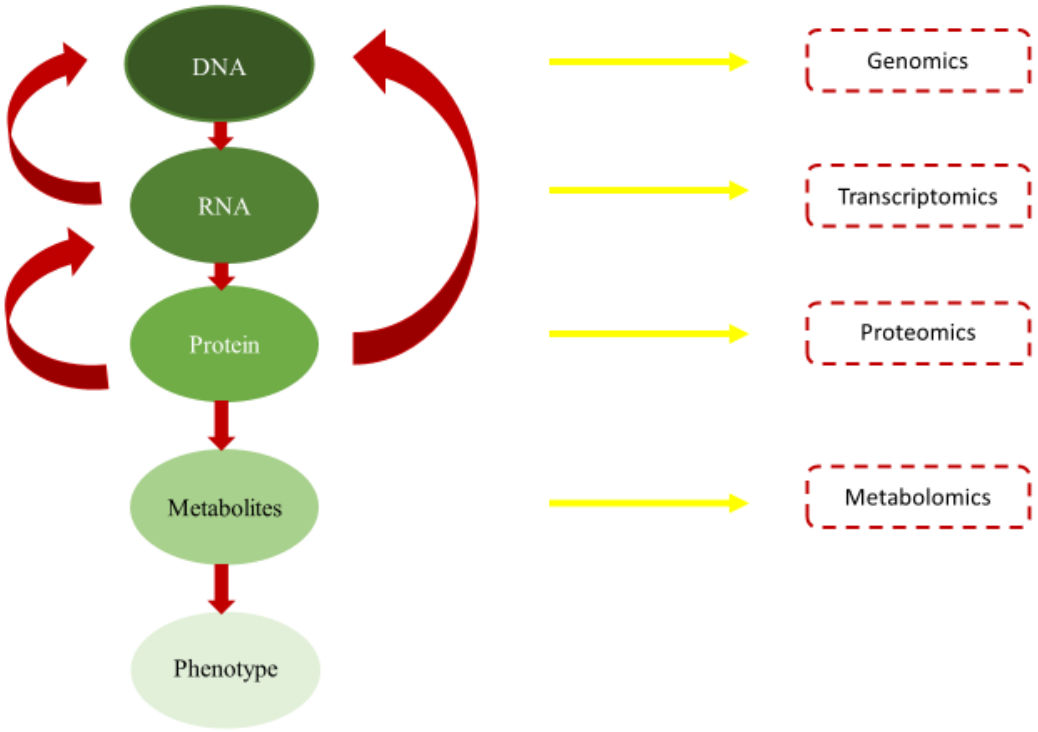


Figure 7 : The 'omics' family. *The 'omics' family refers to the different omics tools that have been created to analyze different compounds of interst from the time of their genetic coding to their final expression in a phenotype.*

The omics flow (Figure 7) begins from the genomic level (‘genomics’), which encompasses the study of the entire genome. It provides an understanding of complex phenotypes and is curerntly being harnessed for the interpreting drug response, patient prognosis and development of personalised medicine. ‘Genomics’ is followed by ‘transcriptomics’, that quantitatively and qualitatively analyzes all the RNA transcripts. This technology has been used for constructing probe-based assays and holds potential to be used for the development of biomarkers or therapeutic targets in disease. ‘Proteomics’ follows next, comprising quantification of proteins and understanding their modifications and interactions. The advent of this technology has allowed researchers to study post-translational modifications, intracellular signalling, protein turnover and transport, etc. which have led to deeper understanding of cellular biochemical activities.

“Metabolomics”, the last member of the omics family, refers to the detection, identification and quantification of small metabolic molecules which are products of various biochemical reactions. For eg; these may be amino acids, carbohydrates, fatty acids, etc. This technology has been extremely useful in studying metabolic flux. Additionally, its use is becoming increasingly favoured by the food and nutrition industry.

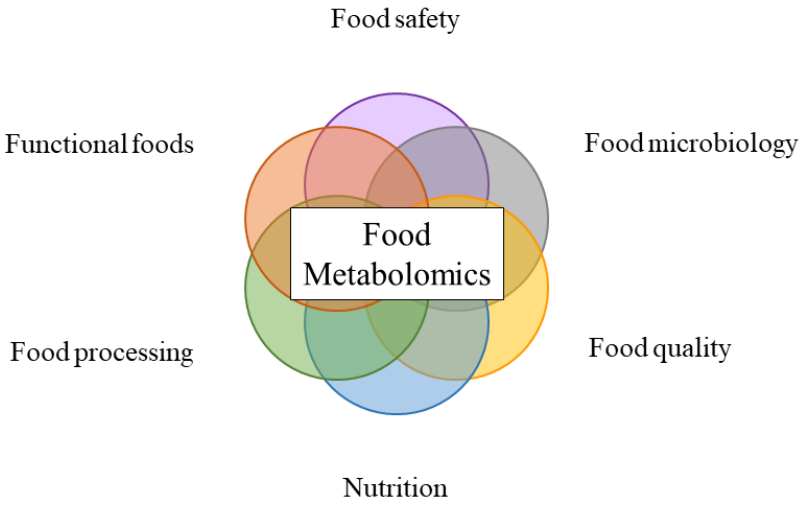


Figure 8 : Applications of food metabolomics. *Food metabolomics is currently finding a plethora of uses in the food and applied healthcare industries, dedicated to the improvement of human health.*

This is because the biochemical profile of food directly governs factors such as shelf life and taste, the information of which would be imperative in monitoring food quality. NMR, GC-MS and LC-MS are the equipment that are favourably employed in metabolomics analyses for detection and characterization of analytes. Food metabolomics is more widely known by its sobriquet “foodomics”, the sub-parts of which have been pictured in Figure 8.

The usual steps of a typical metabolomics assay have been pictured in Figure 9.

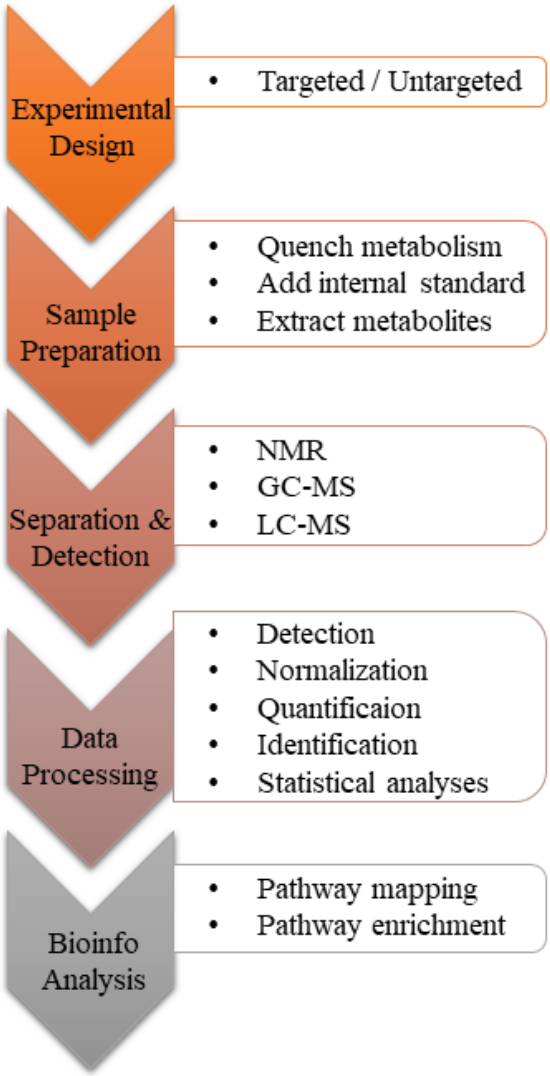


Figure 9 : Steps in a metabolomics analysis. *A metabolomics analysis incorporates these basic steps for separation, detection and identification of compounds of interest from complex mixtures (samples).*

1.7.1 GC-MS

GC-MS works on the principle of separating compounds based on their volatility and detecting them based on their mass-to-charge ratio. It is a highly sensitive and high-throughput platform, which makes it suitable for metabolomics analyses.

An additional procedure to those listed in Figure 9 is added to the sample preparation step whilst performing metabolomic analysis using GC-MS. Labelled 'derivatization', this step is to help volatilize and thermally stabilize the extracted metabolites. The most commonly used derivatization method in untargeted GC-MS analysis is a two-step process involving (i) the methoxymation of the ketone-group containing metabolites into two stable methoximes of constant concentration ratio, and (ii) the silylation of all metabolites into their trimethylsilyl derivatives (Papadimitropoulos et al., 2018).

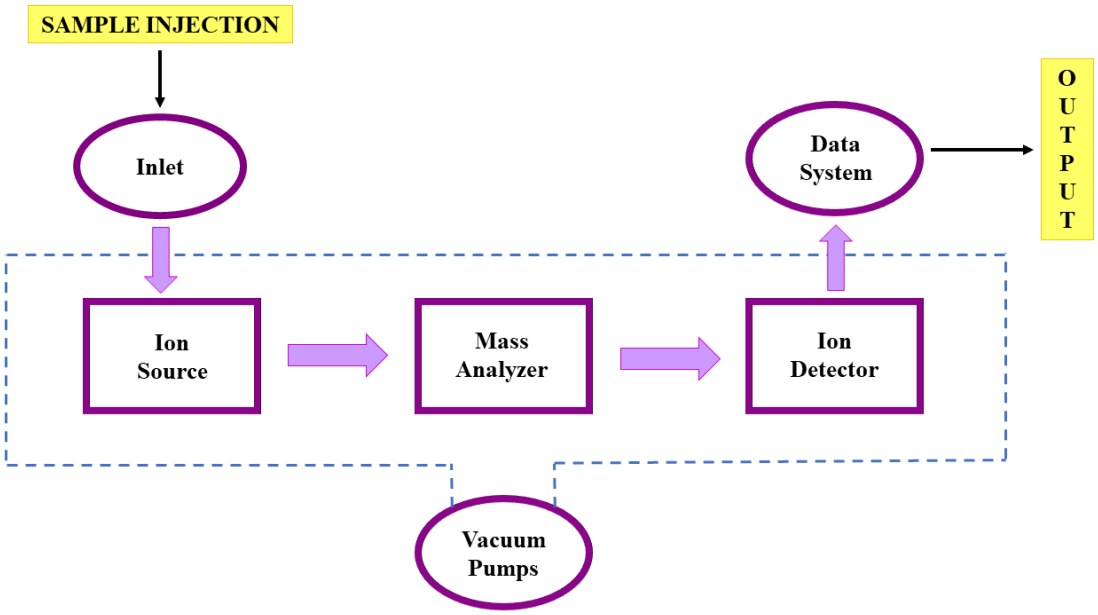


Figure 10 : Schematic of a GC-MS. *A GC-MS separates and detects compounds based of their relative solubility in the stationary (GC-MS column) and mobile (gas) phases.*

The schematic set up of a GC-MS may be seen in Figure 10. Sample is injected through the inlet autosampler and volatilized before entering GC-MS column. The stationary phase is coated on the wall of the GC-MS column, and together with the

gaseous mobile phase helps in chromatographic separation. The compounds are ionized at ion source before they enter the mass analyzer where they are separated according to their mass-to-charge ratios. The ions then enter the detector, where they are transformed to signals via induction of current or simultaneous generation and amplification of secondary ions.

In metabolomics, GC-MS is used for a wide variety of analyses, such as for understanding flavour and aroma profiles (Diez-Simon et al., 2019), for identifying food intake biomarkers in human body fluids (Trimigno et al., 2018), for characterization of compounds attributed to taste (Mabuchi et al., 2019), etc.

1.7.2 LC-MS

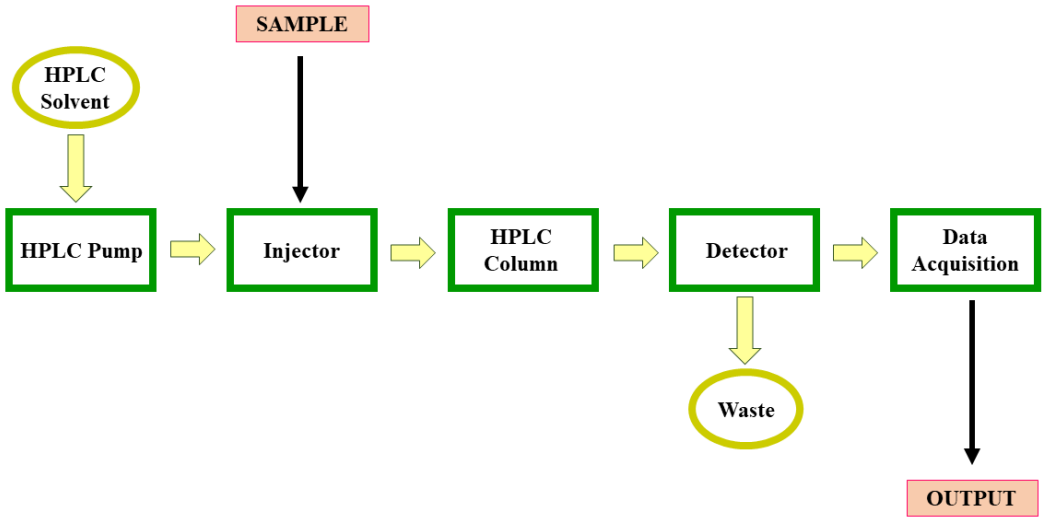


Figure 11 : Schematic of a HPLC. *HPLC is a type of liquid chromatography that separates and detects compounds based on their relative solubility in stationary (HPLC column) and mobile (HPLC solvent) phases.*

LC-MS is a high-throughput analytical tool for the analysis of all detectable compounds in a given sample. It works on the principle of isolation and ionization of individual compounds and then separating them based on their mass-to-charge ratio.

Figure 11 shows the schematic of a typical HPLC. Sample is injected through the injector. Analytes are separated based on their distribution across the mobile phase (HPLC solvent) and stationary phase (HPLC detector). A detection unit (UV detector in HPLC) detects the analytes as they are eluted out with respect to their retention times. The signals from the detector are then converted to chromatograms for analysis via the data aqusition system.

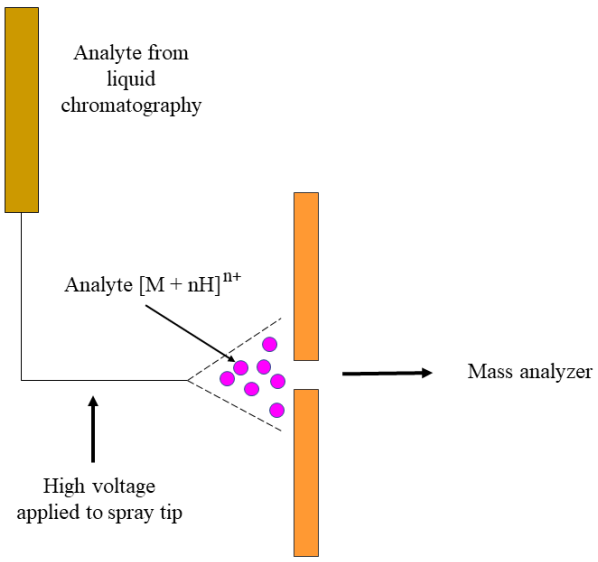


Figure 12 : Schematic of an ESI. *An ESI mode of ionization uses high voltage to assign charges to ions of analyte particles before thery enter the mass analyzer.*

The LCQTOF/MS used for of our studies used an ESI mode of ionization. In this mode, a high voltage is applied, which forms a plume droplet that subsequently

evaporates leaving behind charged ions of the original analytes (Figure 12). A QTOF mass analyzer was used during detection, as it is extremely sensitive and accurate for complex mixtures. Figure 13 displays a schematic of a QTOF analyzer. Quadrupole analyzer 1 (Q1) selects and transits ions of interest in mass filter mode to Quadrupole collision cell (Q2). Q2 fragments the ions from Q1 using collision induced dissociation. Lastly, the TOF mass analyzer separates the fragments from Q2 and remaining precursor ions from Q1 and sends them to the detector for generating an MS/MS spectrum.

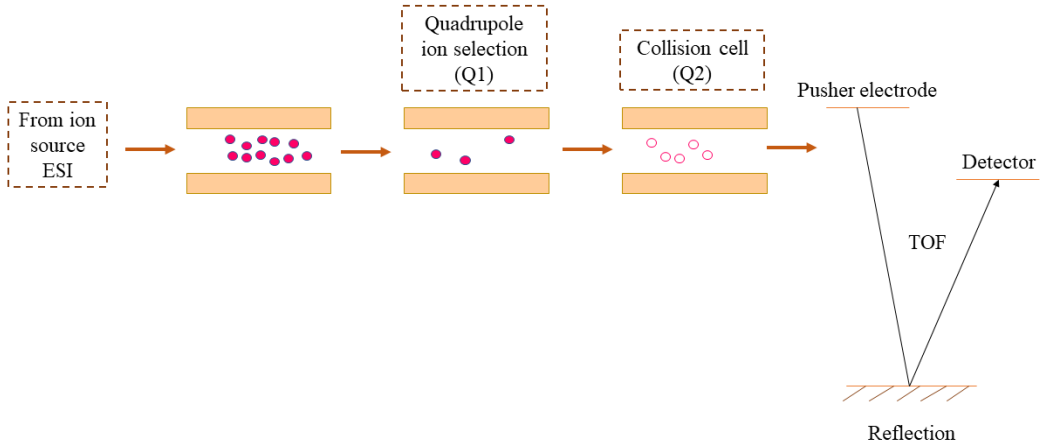


Figure 13 : Schematic of a QTOF analyzer. *A QTOF mass analyzer is an extremely sensitive mass analyzer used in LC-MS analyses for the separation and detection of charged ions from molecules of interest.*

LC-MS is currently being utilized in food science for varied purposes such as detection of adulteration (Campmajo et al., 2019), food profiling (Corte et al., 2015, Nimbalkar et al., 2012) and using plasma metabolite profiles for identification of dietary patterns (Acar et al., 2019).

1.8 Present research status and research gap

1.8.1 Research status in the domain of using okara as a raw material

Current research interest towards understanding the potential value okara has led to a spark in related research, the gist of whose findings are documented in Table 4.

Table 4 : Research conducted using okara as raw material

Area of research	Reference
Composition, utilization and limiting factors	Li et al. (2013)
Composition, nutrition and utilization	Li et al. (2012)
Characteristics and use	O'Toole (1999)
Protein profile	Stanojevic et al. (2012)
As carbon source for developing photothermal nanomaterials	Weng et al. (2019)
As gluten-free flour	Ostermann-Porcel et al. (2017)
Prevention of obesity in diet-induced murine obesity models	Matsumoto et al. (2007)
Hypoglycemic effects	Lu et al. (2013)
Isoflavone extraction	Jankowiak et al. (2014)
Improvement of blood glucose levels in Vietnamese with type II diabetes	Nguyen et al. (2019)

It must be noted that mosr of the available documented research involving okara uses this agri-waste in its raw form. Valorization of its nutrient profile through biofermentation is a relatively new domain that has not been explored to its full potential. Currently, limited research exists in this domain. Biotransformation of raw okara has recently been experimented upon, using different microbes such as *Yarrowia lipolytica* (Vong et al., 2016), *Eurotium cristatum* (Chan et al., 2019) and *Saccharomyces cerevisae* (Santos et al., 2018). The positive results obtained in these works encourage the need for furthering research in this area.

1.8.2 Research gap addressed by this thesis

Although a lot of work has been done to understand the potential of okara as a source of nutrition, there exists a gap when it comes to improving the existing nutrient profile of okara. Further, there is an ever-increasing demand for the manufacturing of natural, green bioactive compounds in lieu of chemically synthesized compounds. This thesis aims to address these gaps, by enhancing the nutritional quality of okara through the process of biofermentation with a food-grade microbe. Whilst the initial work incorporated the usage of two microbes, *Rhizopus oligosporus* and *Lactobacillus plantarum*, only *R. oligosporus* was used in the later stages of the study since it yielded better results in the preliminary stage. Specific target areas of this thesis include a) a metabolomics study before and after fermentation, b) antioxidant analyses on unfermented and fermented okara extracts to gauge functional activity, and c) polyphenolic analyses on unfermented and fermented okara extracts to understand changes post biofermentation.

1.9 Aim and outline of this thesis

Okara is a nutrient-rich agri-industrial by-product contributing to a major chunk of food waste. The chief problems with this food waste are putrefaction and bioactive inavailability of nutrients. As an environment-friendly option of treating okara, valorization using microbial fermentation can make it suitable to be employed as a functional food or nutraceutical. Thus, this thesis is aimed towards economically enhancing the "functionality" of okara through biofermentation using *Rhizopus oligosporus* as the process organism.

In **Chapter 2**, the study explores the possibility of employing biofermentation as a valorization technique for okara. Food-grade FDA approved microbes *Rhizopus oligosporus* and *Lactobacillus plantarum* are used in this work. An extracellular GC-MS metabolomics analysis is carried out to comprehend the biochemical changes ocurring after microbial fermentation. Detected metabolites are tracked back to their metabolic pathways to understand the biochemical reactions triggered during the fermentation process. A premlimary DPPH antioxidant analysis is also carried out, to check the improvement in functionality of fermented okara.

In **Chapter 3**, the study continues with okara fermented with *Rhizopus oligosporus* as the product for analysis, since it yielded better results (as is detailed in Chapter 1). In this stage, a clean, green extract is extricated from fermented okara and its dose-dependent DPPH, FRAP, O_2^- and $^{\cdot}NO$ radical scavenging antioxidant activities observed.The extract is then tested on erthrytocytes to note its toxicity and perceive its capability in inhibiting AAPH induced haemolysis of erthyrocytes. Further, it is tested on cancer cell line HepG2 to assess any antiproliferative activity. Following these, the

study characterizes the key bioactive molecules in the extracts using a GC-MS based analysis.

In **Chapter 4**, the study focusses on phenolic compounds and their examination using TPC and TFC analyses. Targetting isoflavonoids, the extraction process used in Chapter 3 is modified by changing the extraction solvent to suit the current aim. Further, LC-QTOF is employed for the detection of phenolic compounds that significantly increase after fermentation. Subsequently, the compounds of interest are quantified using HPLC.

Lastly, in **Chapter 5**, the major findings in this thesis are recapitulated and followed with the limitations faced during the course of this work. Recommendations for furthering this study are also included here.

CHAPTER 2

AN ANALYSIS OF IMPROVED NUTRITIONAL COMPOSITION OF POTENTIAL FUNCTIONAL FOOD (OKARA) AFTER PROBIOTIC SOLID-STATE FERMENTATION

2.1 Abstract

Okara is a major agro-waste, generated as a by-product from the soymilk and tofu industry. Since okara has a high nutritive value, reusing it as a substrate for solid state biofermentation is an economical and environment friendly option. *Rhizopus oligosporus* and *Lactobacillus plantarum* were the probiotic FDA-approved food-grade cultures used in this study. The study revealed that biofermenting okara improves its nutritional composition. It was found that the metabolomic composition (by GC-MS analysis) and antioxidant activity (by DPPH test) improved after the microbial fermentations. Of the two, okara fermented with *R. oligosporus* showed better results. Further, the metabolites were traced back to their respective biosynthesis pathways, in order to understand the biochemical reactions being triggered during the fermentation processes. The findings of this entire work open up the possibility of employing fermented okara as a potential functional food for animal feed.

2.2 Introduction

Okara, also known as soybean residue, is the insoluble waste by-product from the manufacturing of soybean foods such as soymilk and tofu. Fresh okara is high in moisture content and thus putrefies very fast. Conventionally, it is either dumped into landfills or incinerated. The present awareness and demand for environmentally friendly disposal practices has promoted the recovery and utilization of agricultural wastes. Okara is one of the main agro-wastes generated in East and South-east Asia.

Table 5 (a): Micronutrient composition of okara

S. No.	Composition	Quantity (per 100g dry matter)	Reference
1.	**Minerals**		Riet et al. (1989)
	Potassium (K)	1046-1233 mg	
	Sodium (Na)	16.2-19.1 mg	
	Calcium (Ca)	260-428 mg	
	Magnesium (Mg)	158-165 mg	
	Iron (Fe)	6.2-8.2 mg	
	Copper (Cu)	1.1-1.2 mg	
	Manganese (Mn)	2.3-3.1 mg	
	Zinc (Zn)	3.5-6.4 mg	
	Phosphorus (P)	396-444 mg	
2.	**Vitamins**		
	Thiamine	0.48-0.59 mg	
	Riboflavin	0.03-0.04 mg	
	Nicotinic acid	0.82-1.04 mg	
3.	**Isoflavones**	0.14 g	Stanojevic et al. (2014)
	Daidzin	76 µM	Li et al. (2012)
	Glycitin	16 µM	
	Genistin	81 µM	
	Daidzein	88 µM	
	Glycitein	18 µM	
	Genistein	60 µM	

Raw okara possesses a high nutritive value (Table 5), which makes it potentially suitable for production of functional foods. However, since most of the nutrients are present in an insoluble form, pre-treatment is required before employing it for feed purposes.

SSF is an economically viable option to biotransform crop residues for nutritional enrichment. Crop residues that have been successfully used in SSF so far include corn grits, soybean waste, wheat bran, cassava, sugar beet pulp, bagasse, etc. Soybean residue is one of the leading agro-wastes that has been engaged in studying SSF and its benefits. Enzymes secreted during the fermentation cause several desirable changes, leading to improved nutrition, texture, flavour and aroma of the final product. Complex macromolecules (fats, proteins, carbohydrates) are hydrolysed to smaller nutrients and anti-nutritional compounds (e.g., trypsin inhibitors, lectins, tannins) are considerably decreased after SSF (Parades-Lopez and Harry, 1989, Wagenknecht et al., 1961). Fermentation releases phytases (Pandey et al., 2001) that act upon phytic acid (an antinutrient) and increase bioavailable iron (Moeljopawiro et al., 1987), zinc (Moeljopawiro et al., 1988), calcium and inorganic phosphorus (Sutardi and Buckle, 1986) contents. Unbound forms of antioxidants are also released during SSF, adding to the nutritional value of the final product. These changes make fermented okara easier to digest than raw okara, which in turn makes it a potential contender to be employed as livestock feed.

Table 5 (b): Macronutrient composition of okara

S. No.	Composition	Quantity (per 100g dry matter)	Reference
1.	**Protein**	25.4-28.4 g	Li (2012)
	Aspartic acid	117 (mg/g protein)	Chan and Ma (1999)
	Threonine	41 (mg/g protein)	
	Serine	50 (mg/g protein)	
	Glutamic acid	195 (mg/g protein)	
	Glycine	46 (mg/g protein)	
	Alanine	46 (mg/g protein)	
	Cysteine + Methionine	26 (mg/g protein)	
	Valine	51 (mg/g protein)	
	Isoleucine	51 (mg/g protein)	
	Leucine	81 (mg/g protein)	
	Tyrosine + Phenylalanine	95 (mg/g protein)	
	Lysine	65 (mg/g protein)	
	Histidine	28 (mg/g protein)	
	Arginine	75 (mg/g protein)	
	Proline	36 (mg/g protein)	
2.	**Fat**	9.3-10.9 g	Li et al. (2012)
3.	**Dietary fibres**	52.8-58.1 g	
	Insoluble dietary fibre	40.2-43.6 g	
	Soluble dietary fibre	12.6-14.6 g	
4.	**Carbohydrates**	3.8-5.3 g	
	Rhamnose	0.85 g	O'Toole (1999)
	Fucose	0.45 g	
	Arabinose	6.35 g	
	Xylose	5.14 g	
	Mannose	1.26 g	
	Galactose	10.83 g	
	Glucose	15 g	
	Uronic acids	5.03 g	
	Stachyose	140 g	
	Raffinose	30 g	
	Sucrose	230 g	
	Starch	59 g	
5.	**Energy value**	300 kcal	Stanojevic et al. (2014)

In the recent years, SSF has been chiefly exploited for the production of value-added products such as biologically active primary and secondary metabolites from a waste by-product. Several industries are based on the commercialization of SSF-derived products such as hydrolytic enzymes, bioactive compounds, organic acids and other miscellaneous compounds such as pigments, carotenoids, vitamins and biopesticides (Pandey et al., 2000).

Industrial SSF processes usually employ pure cultures in order to control substrate utilization and end product formation. If the fermented product is to be enlisted for consumption, it is imperative that the microbes used in the SSF process are of GRAS status. *Rhizopus* and *Lactobacillus* are the most prominent fungi and bacteria genera respectively, used by food industries for fermentation. These microaerophilic microbes can sustain relatively high temperatures of 30 ^{0}C - 40 ^{0}C and have been used as starter cultures since centuries.

Although a lot of fermentation-based work has been carried out on cooked soybean substrates (e.g., tempeh), a gap exists in utilization of raw soybean wastes (e.g., okara). This paper has detailed the investigation of improved nutritional composition of okara after probiotic biofermentation with *Rhizopus oligosporus* (*R. oligosporus*) and *Lactobacillus plantarum* (*L. plantarum*), thereby proposing its potential use as a functional food.

2.3 Materials and methods

2.3.1 Chemicals and microorganisms

Fresh okara was sourced from Unicurd Food Company Pte Ltd (Singapore) and stored in air-tight plastic bags at -80 ^{0}C, until used. All chemicals were purchased from

Sigma-Aldrich (St. Louis, MO, USA). Dehydrated MRS and PDA media were bought from Becton, Dickinson and Company (BD Difco, USA) and reconstituted as per supplied instructions. Fungal strain *Rhizopus oligosporus* (DSM 1964), obtained from the Leibniz Institute DSMZ German Collection of Microorganism and Cell Cultures, was grown on Potato Dextrose Agar (PDA) plates at 30 ^{0}C for 48 hours. Bacterial strain *Lactobacillus plantarum* (DSM 12028), obtained from the Leibniz Institute DSMZ German Collection of Microorganism and Cell Cultures, was grown on De Man, Rogosa and Sharpe (MRS) agar plates at 37 ^{0}C for 48 hours.

2.3.2 Fermentation

For fungal fermentation, 10g of fresh okara was inoculated with a culture inoculum of 10^5 CFU *Rhizopus oligosporus* and incubated at 30 ^{0}C for 48 hours. For bacterial fermentation, 10g of fresh okara was inoculated with a culture inoculum of 10^7 CFU *Lactobacillus plantarum* and incubated at 30 ^{0}C for 48 hours.

2.3.3 Extracellular metabolite analysis

2.3.3.1 Sample preparation

Extracellular metabolite extraction was carried out using the method followed by Sasidharan et al. (2012) with minor modifications. Firstly, 5 mL of chilled saline (0.9% NaCl) solution was added to 10g of fermented sample to quench the microbial cells. 30 mL ice-cold methanol was then added to the samples as the extraction solvent for extracellular metabolites. The mixture was spiked with 600 µL ribitol (2 mg/mL in water; internal standard) and centrifuged at 1400 rpm for 20 minutes at 4 ^{0}C. This was

followed by centrifugation at 16,100 g for 5 minutes at 4 ^{0}C. The supernatant was used as crude extract for further steps. The residual debris was discarded. 1mL of crude extract was kept at 30 ^{0}C on heat block until completely dry.

Sample preparation for GC-MS (gas chromatography-mass spectrometry) was done using the method followed by Chen and Chen (2014). Firstly, 50 µL of 20 mg/mL methoxyamine hydrochloride in pyridine was added to the dried sample for carbonyls protection. The mixture was then vortexed for 1 minute and subsequently incubated at 37 ^{0}C for 1 hour. Following this, 100 µL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was added to the sample for silylation. Samples were incubated at 70 ^{0}C for 30 minutes and finally centrifuged at 14,000 rpm for 1 hour at room temperature before being transferred to glass vials for GC-MS analysis.

2.3.3.2 GC-MS conditions and analysis

All samples for GC-MS were analysed within 12 hours of preparation. Chromatography was performed using 5975C Inert MSD with Triple-axis Detector from Agilent Technologies. The capillary column was 0.25 µm thick and had dimensions of 30 m X 0.250 mm. Hexane was used as the stationary phase. 1 µL sample was injected in splitless mode; carrier gas helium was maintained at a purge flow of 50 mL per minute. The inlet was sustained at an isothermal temperature of 230 ^{0}C. The GC oven was programmed to initiate at 75 ^{0}C (4 minutes hold), and ramped to 280 ^{0}C at 4 ^{0}C per minute, with a final hold time of 2 minutes at 280 ^{0}C. Injection needle was cleaned with hexane thrice before starting sample run and twice after every measurement. Data was acquired in full scan from 30 to 800 *m/z*. Metabolites were identified using NIST08 mass

spectral library. The chromatographic peaks were normalized according to the internal standard (ribitol) before being subjected to statistical analyses.

2.3.4 Antioxidant activity

The antioxidant activity of the samples was measured as a test of their free-radical scavenging capacity. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used for assessment, following the method used by Wan et al. (2011) with minor modifications. Firstly, 0.1g of sample was taken in an Eppendorf tube to which a small quantity of glass beads was added. The volume was made to 1.5 mL with ethanol and the tube was then spun on FastPrep-24 (MP Biomedicals) for 5 cycles of 20 seconds each following which centrifugation was carried out at 21,000 g for 5 minutes. To 500 µL of supernatant, 500 µL of DPPH solution (0.6 mM in ethanol) and 4mL ethanol was added and the mixture incubated in the dark at room temperature for 30 minutes. 1 mL of this solution was then measured for absorbance at 515 nm using Nanodrop 2000c spectrophotometer (Thermo Scientific). Ethanol was used as blank, 4.5 mL ethanol with 500µL DPPH was used as control and quercetin (0.1mg/mL in ethanol) was used as a positive control.

The antioxidant activity was calculated according to the formula:

$$\textbf{DPPH scavenging activity (\%)} = [A_C - A_S]/A_C \times 100$$

where, A_C is the absorbance value of the control and A_S is the absorbance value of the test samples

2.3.5 Statistical analyses

All data have been represented as an average of three replicates. Multivariate analysis was performed using OriginPro 2017. Clustering heatmap was generated using group-average clustering and Euclidean distance calculation. Principal component analysis was carried out and represented as a two-dimensional biplot, to confirm the findings acquired by the cluster dendogram.

2.4 Results and discussion

2.4.1 Extracellular metabolite analysis using GC-MS

The GC-MS metabolomic analysis performed on unfermented and fermented okara samples were examined to observe noticeable variations and similarities. Figure 14 displays the spectra obtained after the (extracellular) metabolomic analysis of unfermented okara (control), okara fermented with *R. oligosporus* and okara fermented with *L. plantarum.*

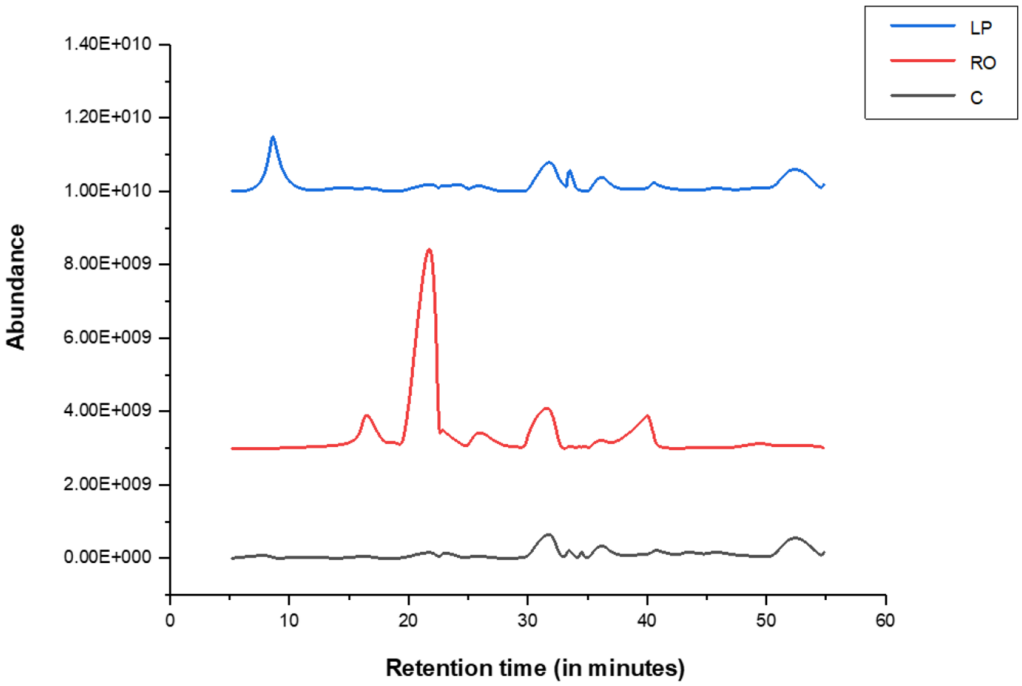


Figure 14 : GC-MS chromatogram spectra of unfermented (control) and fermented okara samples. *Key: Unfermented okara control (C); okara fermented with R. oligosporus (RO); okara fermented with L. plantarum (LP).*

A comparative study of the metabolites detected in okara before and after fermentation was carried out. A total of 48 metabolites (excluding internal standard ribitol) were identified. The metabolites identified via GC-MS could be grouped into seven parent categories (Table 6). The parent categories include organic acids, amino acids, inorganic acids, fatty acids, carbohydrates, alcohols and a miscellaneous group. As shown in Table 6, our results indicated that microbial fermentation of okara by both *R. oligosporus* and *L. plantarum* yielded greater number of metabolites. This is expected due to the action of microbial hydrolytic enzymes that break down complex molecules in okara to their constituent simpler units.

Table 6 : Metabolites detected via GC-MS analysis of unfermented and fermented okara samples with their relative abundances (x10^7)

Metabolite	Control (unfermented okara	Okara feremented with *R. oligosporus*	Okara fermented with *L. plantarum*
Organic acids			
Lactic acid	N.D.	N.D.	222.64
Propanedioic acid	4.92	4.78	2.92
Butanedioic acid	4.73	116.88	10.58
2-Butenedioic acid	N.D.	43.21	5.83
Malic acid	21.89	813.81	25.71
Propanoic acid	18.39	13.92	11.19
Pentanedioic acid	N.D.	7.69	22.64
α-Aminoadipic acid	N.D.	4.01	N.D.
1-Propene-1,2,3-tricarboxylic acid	N.D.	3.03	N.D.
Ribonic acid	2.86	N.D.	N.D.
Tartaric acid	2.85	N.D.	N.D.
Galactonic acid	2.24	N.D.	N.D.
Benzoic acid	N.D.	7.07	N.D.
Gluconic acid	1.98	3.99	3.18
Galactaric acid	1.29	N.D.	N.D.
Inorganic acids			
Phosphoric acid	3.36	51.61	3.46
Amino acids			
Valine	N.D.	5.65	3.38
Glycine	5.15	25.71	2.44
Serine	N.D.	10.38	N.D.
Threonine	N.D.	16.71	N.D.
Alanine	1.72	4.86	2.18
Proline	9.53	58.78	18.03
Glutamine	4.76	59.15	21.62
Asparagine	N.D.	14.03	N.D.
Tyrosine	2.98	N.D.	25.46
Fatty acids			
Hexadecanoic acid	49.99	30.91	56.49

Linoleic acid	11.76	79.19	8.89
trans-13-Octadecenoic acid	6.69	101.23	6.76
Octadecanoic acid	19.22	31.89	29.47
Palmitic acid	1.88	N.D.	1.89
Decanoic acid	4.47	N.D.	N.D.
Carbohydrates			
Erythro-pentose	N.D.	N.D.	2.97
Galactose	28.19	7.58	84.83
Mannose	17.40	N.D.	N.D.
Glucopyranuronic acid	16.68	N.D.	15.33
Heptulose	14.02	N.D.	4.19
Turanose	5.08	1.56	N.D.
α-D- Glucopyranoside	90.52	17.03	13.02
Xylose	23.39	513.69	N.D.
Maltose	58.65	7.00	63.97
Talose	54.98	6.33	59.32
Xylo-hexos-5-ulose	N.D.	5.45	N.D.
α-D-Xylopyranose	17.09	N.D.	19.43
Alcohols			
Glycerol	1.27	N.D.	N.D.
Inositol	95.28	147.75	109.77
Glucitol	N.D.	4.60	4.39
Mannitol	25.66	1.42	N.D.
Oleyl alcohol	20.20	N.D.	14.76
2-Monopalmitin	1.46	4.24	5.26
Others			
Urea	N.D.	9.48	13.00
Pyrimidine	N.D.	10.22	N.D.

*** N.D.: Not detected**

2.4.2 Statistical analyses

It is difficult to gauge how similar or unsimilar the fermented samples are to the control based on a visual scrutiny of Figure 14. Hence, statistical analysis tools were employed to make sense of the data generated by GC-MS.

To validate the metabolic changes before and after fermentation, a hierarchical agglomerative clustering heatmap was generated (Figure 15). The heatmap indicated the abundance levels of the metabolites that were detected in unfermented and fermented okara samples. As evidenced by Figure 15, the cluster dendogram uncovered that *L. plantarum* fermented okara was in closer proximation to control (unfermented okara) than to *R. oligosporus* fermented okara, in terms of extracellular metabolite composition. The minimum and maximum values of the Euclidean distance used in our calculations were 0 and $10*10^9$, respectively. The columns denoting okara fermented with *L. plantarum* and control (unfermented okara) converged at a Euclidean distance of $2.50*10^9$. This cluster then converged with the column representing *R. oligosporus* fermented okara at a Euclidean distance of $8.37*10^9$. Hence, we can reason that at a Euclidean distance below $2.50*10^9$, all three columns are significantly unsimilar in their metabolite pattern distribution. Between Euclidean distances $2.50*10^9$ and $8.37*10^9$, it can be concluded that okara fermented with *R. oligosporus* and the cluster (composed of control and *L. plantarum* fermented okara) were significantly different from each other, with respect to their extracellular metabolic composition.

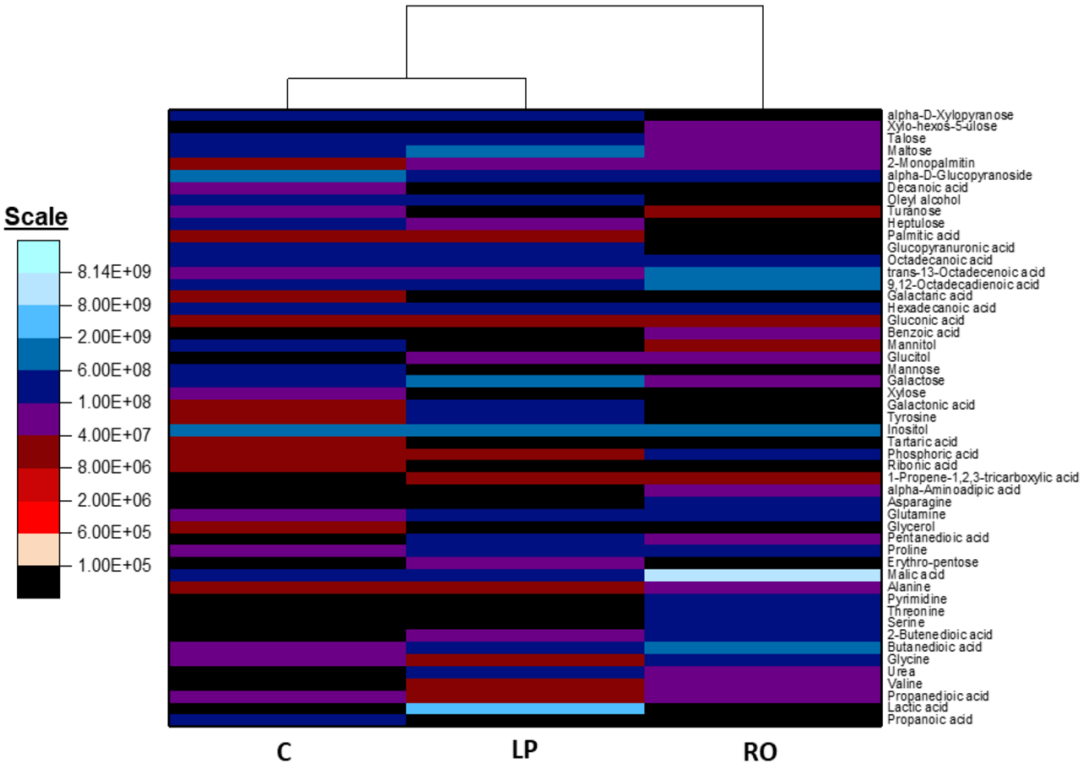


Figure 15 : Heatmap dendogram of metabolites detected via GC-MS analysis in unfermented (control) and fermented okara samples. *The scale indicates the relative abundance of each metabolite. Key: Unfermented okara control (C); okara fermented with R. oligosporus (RO); okara fermented with L. plantarum (LP).*

Additionally, a PCA biplot was developed (Figure 16) to highlight the variance in distribution of the three groups- unfermented okara, okara fermented with *R. oligosporus* and okara fermented with *L. plantarum*- across the metabolites, with respect to the principal components. The GC-MS data was analysed using a correlation matrix with listwise exclusion. The first two principal components (PC), PC1 and PC2, attributed to 50.63% and 22.74% of variance respectively, and thus cumulatively accounted for 73.37% of the total variance.

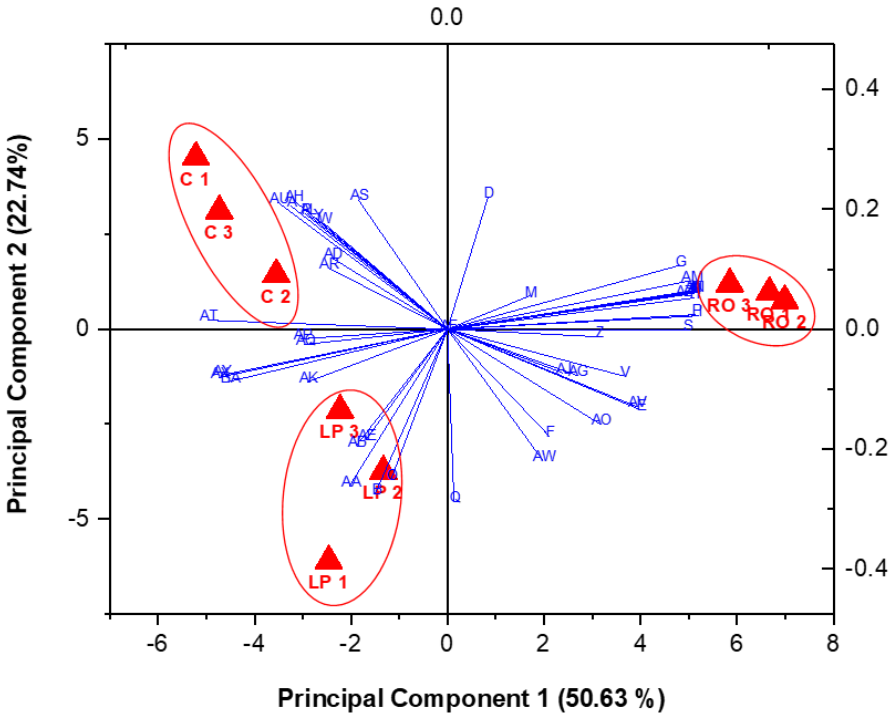


Figure 16 : PCA biplot derived from GC-MS data for unfermented (control) and fermented okara samples. *Key: Unfermented okara control (C); okara fermented with R. oligosporus (RO); okara fermented with L. plantarum (LP).*

According to PC1, it can be clearly seen that *L. plantarum* fermented okara samples and control (unfermented) samples are similarly distributed, which differed from the distribution of *R. oligosporus* fermented okara. On the other hand, as seen in Figure 16, the control samples and okara fermented with *R. oligosporus* exhibited similar patterns of distribution across PC2, which differed from that of okara fermented with *L. plantarum*. PC1 being the larger principal component, it can be interpreted that *L. plantarum* fermented okara and control (unfermented okara) are relatively similar, with respect to extracellular metabolite composition. The distribution across PC2, the smaller component, validates that a smaller degree of similarity exists between the

control (unfermented okara) and okara fermented with *R. oligosporus*. The result interpreted from the PCA biplot study was in accordance with the prior implication made by the cluster dendogram.

The results established by this GC-MS metabolomic analysis and multivariate statistical studies can be used for an in-depth investigation of the metabolic mechanism of microbial fermentation.

2.4.3 Tracing detected metabolites to their biosynthesis pathways

By analysis of the extracellular metabolites released before and after fungal (*R. oligosporus*) and bacterial (*L. plantarum*) biofermentation, a hypothesis can be drawn to understand the various metabolic pathways occurring during the process. Figures 17 and 18 display the link between the various biochemical pathways that were believed to be triggered during the microbial fermentation of okara.

It has been well established that both fungi and bacteria release lipases during fermentation (Esteban-Torres et al., 2015, Mahapatra et al., 2010). Lipid hydrolysis could explain the increase in the levels of saturated medium-chain fatty acids (Table 6) such as hexadecenoic acid (palmitic acid), trans-13-octadadecenoic acid, 9,12-octadecanoic acid (linolenic acid), octadecanoic acid (stearic acid) etc. after fermentation. Cleavage of bonds in long-chain lipids also led to the increase in the levels of fatty acid derivatives such as 2-monopalmitin. On the other hand, derivatives like oleyl alcohol decreased as they entered lipid metabolism. Levels of decanoic acid (capric acid), a saturated medium-chain triglyceride, decreased after fermentation as well; this may be due to the lipolytic enzymes cleaving the saturated bonds in the compound.

Glucitol (sorbitol), mannitol, myo-inositol and glycerol were the alcohols detected during our metabolomics analysis (Figures 17 and 18). Sorbitol and mannitol are sugar alcohols derived from sugars such as glucose and fructose by reversible reactions; this may explain the increase in sorbitol levels and decrease in mannitol levels after fermentation. Mannitol then enters glycolysis, which further explains its decreased level. Glycerophospholipid hydrolysis generates glycerol and fatty acids; the former is converted to dihydroxyacetone phosphate (DHAP) before entering glycolysis (which may account for its decreased levels after fermentation), and the latter undergoes fatty acid hydrolysis to finally enter the Krebs cycle as acetyl CoA. Further, both *R. oligosporus* and *L. plantarum* release microbial phytases during fermentation (Sabu et al., 2002, Zamudio et al., 2001). Phytases are enzymes that hydrolyze phytic acid, an anti-nutrient present in soy. The hydrolysis yields myo-inositol and phosphoric acid (Figures 17 and 18). This can explain the increased levels of phosphoric acid and myo-inositol after fermentation.

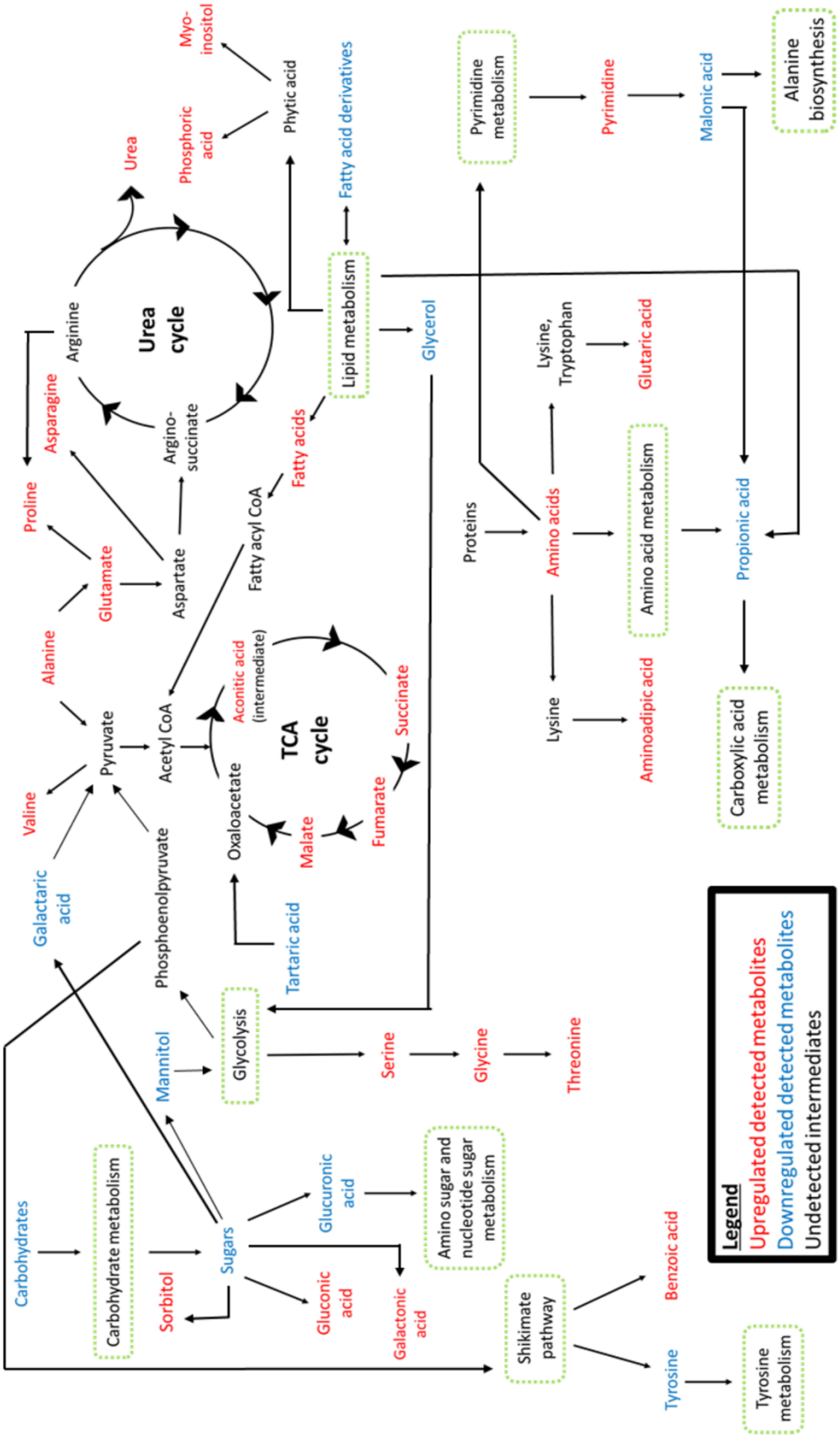


Figure 17 : Interlinkage of metabolic pathways occurring during biofermentation of okara by *R. oligosporus*

Microbial proteases released by both bacteria and fungi during fermentation (Ikasari and Mitchell, 1994, Khalid and Marth, 1990) aid in breakdown of complex proteins into their constituent amino acids. Serine, glycine and threonine are synthesized via glycolysis (Figure 17) over a series of catalysing reactions (serine, glycine and threonine metabolism pathway). Reversible degradation of alanine yields pyruvate and glutamine; the former is involved in the synthesis of valine, an essential amino acid (Figures 17 and 18). Proline is produced from glutamine (alanine, aspartate and glutamate metabolism pathway) and arginine (arginine and proline metabolism pathway). This pathway is also responsible for the catalysis of asparagine from aspartate (Figure 17). Alanine is synthesized from malonate after pyrimidine metabolism (Figures 17 and 18). However, tyrosine levels decreased after fermentation, indicating a triggering of the tyrosine metabolism pathway in which it was utilized to finally enter the Krebs cycle. An interesting observation is that all the amino acids detected during the analysis belonged to the glucogenic category ie, they are capable of being converted into glucose via gluconeogenesis. This may justify their overall low abundance levels. Arginine either proceeds to undergo multi-step reactions to be converted to proline or is enzymatically converted to urea (Figures 17 and 18). This would account for the increase in urea content in the fermented samples as compared to the control (unfermented sample).

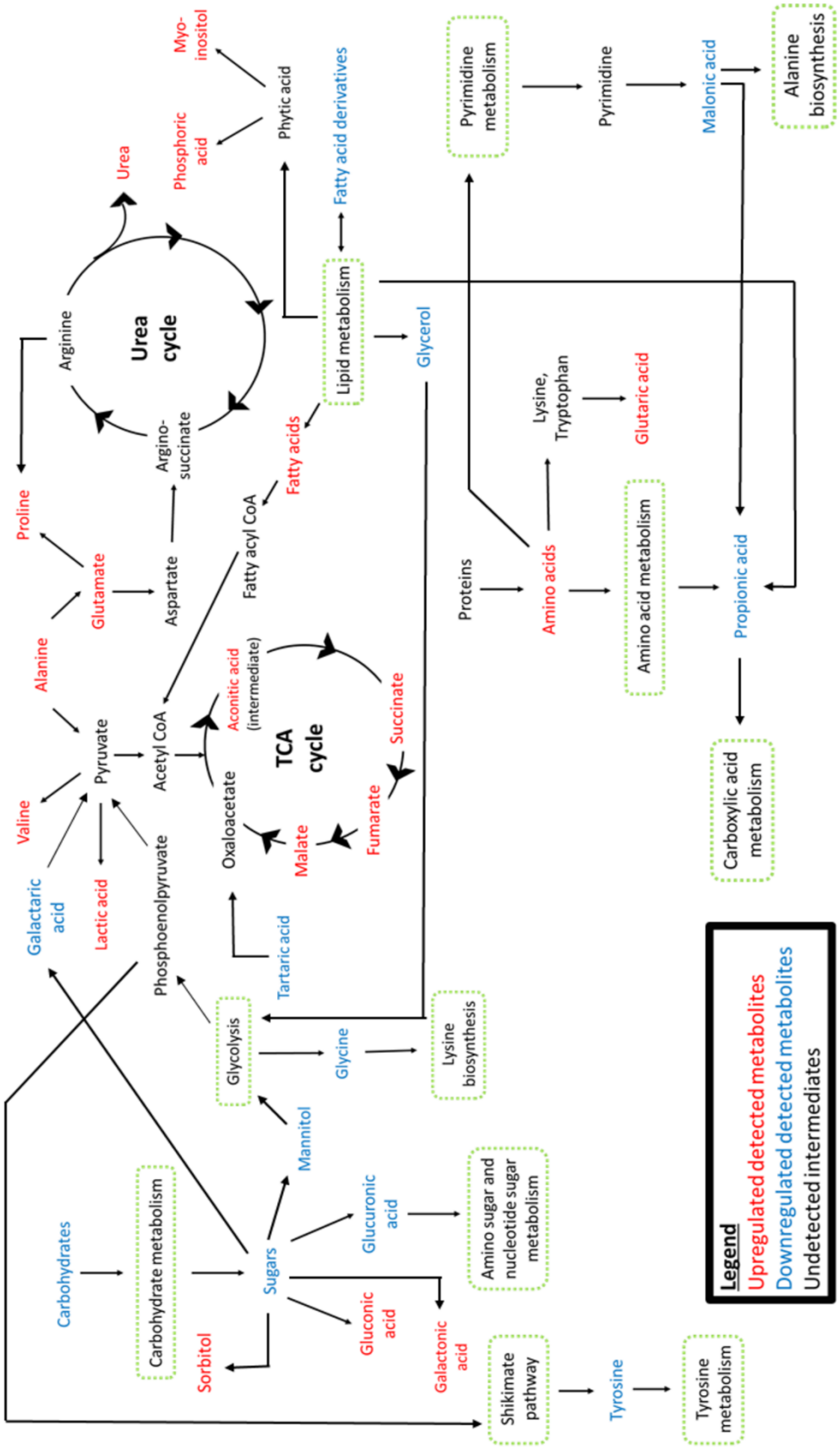


Figure 18 : Interlinkage of metabolic pathways occurring during biofermentation of okara by *L. plantarum*

As seen in Table 6, several sugars such as xylose, galactose, mannose, turanose, maltose etc. were detected by the GC-MS analysis. It was noticed that the majority of the monomeric sugars decreased after fermentation; this could be explained by hypothesizing that they may have been interconverted to glucose and entered the glycolysis pathway or may have been converted to sugar alcohols such as sorbitol and mannitol. The other half of the detected carbohydrates increased after fermentation, supporting the fact that microbial enzymes such as amylases (Giraud et al., 1993, Muhammad Irfan, 2012), cellulases (Parajó et al., 1997), pectinases (Handa et al., 2016) etc. were released during the bioprocess, thereby breaking down long-chain polymeric carbohydrates to more simpler forms.

Benzoic acid is synthesised over multiple catalysed steps in the shikimate pathway by fungi (Figure 17). Phenylalanine is the precursor for the compound. Amino acids lysine and tryptophan may account for production of glutaric acid, thus leading to elevated levels of glutaric acid in both *R. oligosporus* and *L. plantarum* fermented okara (Figures 17 and 18). As was expected, the levels of the aforementioned detected metabolites were higher in microbial fermented samples than the control (Table 6). The levels of tartaric acid dropped after fermentation. It may be reasoned that tartaric acid was converted to oxaloacetate via a two-way reaction and subsequently entered the Krebs cycle. Aminoadipic acid was detected in okara sample fermented by *R. oligosporus*. Aminoadipic acid is an intermediate in the lysine metabolism via the α-aminoadipate pathway. The α-aminoadipate pathway is unique to fungi; this is evidenced by our results. Malonic acid levels increase after fermentation by both *R. oligosporus* and *L. plantarum*, which may be attributed to pyrimidine metabolism by the microbes.

Propanoic acid (propionic acid) is a short-chain saturated triglyceride that is majorly formed as a product of amino acid (cysteine, isoleucine and methionine) metabolism, odd-chain fatty acid metabolism and from malonate after pyrimidine metabolism (Figures 17 and 18). Decreased levels of propionic acid after fermentation (Table 6) was in accordance with the expectation that microbes convert propanoic acid to propionyl-CoA and utilize it for carboxylic acid (organic acid) metabolism. Gluconic acid, glucuronic acid (a precursor of ascorbic acid), galactonic acid and galactaric acid are sugar acids derived from glucose. The levels of gluconic acid and galactonic acid increase after fermentation, signifying oxidation of glucose and activation of the pentose phosphate pathway and pentose and glucuronate interconversion pathway, respectively. On the other hand, the levels of glucuronic acid and galactaric acid declined after fermentation. This decrease may be attributed to glucuronic acid being utilized for amino sugar and nucleotide sugar metabolism, and galactaric acid being converted to pyruvate and entering the Krebs cycle (Figures 17 and 18).

The Krebs cycle, being the chief energy generating source of aerobic microbes, is greatly activated during fermentation process. Many of the metabolites detected from the samples are the by-products of the Krebs cycle (Figures 17 and 18). This accounts for the increased abundance of metabolites in the fermented samples. Butanedioic acid (succinic acid), 2-butenedioic acid (fumaric acid) and malic acid are key intermediates in the cycle. Malic acid also enters the Krebs cycle through its aspartate-arginosuccinate shunt. This can explain the remarkably elevated levels of malic acid in the fermented samples. Prop-1-ene-1,2,3-tricarboxylic acid (aconitic acid) is a minor intermediate in the conversion of citrate to isocitrate.

It was expected that lactic acid be detected in okara fermented with *L. plantarum* (Table 6). Plenty of studies conducted earlier vouch that Lactobacillus species produce

lactic acid during fermentation (Liu, 2003, A.Sheeladevi and N.Ramanathan, 2011). Lactic acid is synthesized from the catabolism of pyruvate (Figure 18). Pyruvate is produced chiefly from the degradation of carbohydrates and amino acids, and is primarily converted to acetyl CoA before entering the Krebs cycle for yielding energy. Hence, lactic acid is one of the prominently abundant metabolites detected in okara fermented by *L. plantarum*. Since Rhizopus species are not known for their lactic acid producing capabilities, it is in tandem with the obtained results.

Thus, it may be summarized that bioactive compounds in fermented okara samples showed an overall positive trend, when compared to the levels in raw okara.

2.4.4 DPPH Radical Scavenging Activity

The scavenging activity of samples to reduce the DPPH free radical was calculated (Table 7) from the DPPH assay. The results indicated that okara fermented with *R. oligosporus* had the greatest radical scavenging activity (% RSA) of 52.81%. Okara fermented with *L. plantarum* had a slightly better RSA of 29.10% than the unfermented control (27.44%). Quercetin, the positive control, displayed an RSA of 94.33%.

It has been documented that the main isoflavones present in crude okara are daidzein, glycetein and genistein (aglycones), daidzin, glycitin and genistin (β-glucosides) and malonyl-daidzin, malonyl-glycitin and malonyl-genistin (malonyl-glucosides) (Jankowiak, 2014). The increase in antioxidant activity in okara after fermentation may be attributed to an increase in phenolics content.

Table 7 : DPPH antioxidant activity of extracted samples

Sample	% RSA
Control (unfermented okara)	27.44
Okara feremented with *R. oligosporus*	52.81
Okara fermented with *L. plantarum*	29.10
Quercetin (0.1 mg/mL)	94.33

2.4.5 Fermented okara as potential functional food in animal feed

Functional foods may be described as "those whole, fortified, enriched or enhanced foods that provide health benefits beyond the provision of essential nutrients (e.g., vitamins and minerals), when they are consumed at efficacious levels as part of a varied diet on a regular basis" (Hasler, 2002).

From Table 6, it may be observed that the nutrient profile of okara improved after fermentation. The quantities of organic acids, amino acids and fatty acids were found to have increased on an average. It may also be noted here that fermentation with *R. oligosporus* yielded better results than fermentation with *L. plantarum*. From the aforementioned table, it is clear that the levels of organic acids favourably increased after the biofermentation process. According to Koninklijke DSM N.V., a Dutch multinational company dealing with health, nutrition and sustainable living, organic acids are immensely important supplements in animal feed. Nowadays, organic acids are gaining attention from researchers, as there is growing mass reluctance to consume

products from antibiotic-dosed animals. Organic acids have been found to be associated with improving gut health in swine by reducing gastric pH, thereby converting inactive pepsinogen to active pepsin for protein hydrolysis (Suiryanrayna and Ramana, 2015). Picking an example of an organic acid from Table 6, it may be seen that the levels of succinic acid rose post fermentation. Commercially, BioAmber Inc., a renewable materials company, demonstrated that dietary bio-succinic acid in nursery pigs contributed to growth performance and gut health.

Dietary amino acids play a vital role in growth, development and reproduction of farm animals (Li et al., 2011). As documented in Table 6, the amino acid amounts in okara increased after fermentation, of which valine and threonine were essential amino acids. According to Kemin Industries, a global nutritional ingredient company based in the USA, lack of optimum amino acids concentrations in animal feed is associated with disease, low body weights and subpar production. Citing a specific case in point, it may be seen from Table 6 that levels of proline increased by more than six times after fermentation with *R. oligosporus*. In the study conducted by Brunton et al. (2012), it was noted that supplementing proline in parenteral diet resulted in an accelerated rate of protein synthesis in the muscles, skin and small intestines in neonatal Yucatan miniature piglets.

Currently, there is a lot of interest encircling the importance of fatty acids in the diets of farm animals. This is because the dietary fatty acid profile directly affects the fatty acid composition of the tissues of the animals fed with this diet. Hence, this is correlative to the productive performance of cattle, pigs and poultry, as the effect affects meat, milk, eggs and embryo development (CetİngülandYardimci, 2008). In this study, the quantities of fatty acids were amplified after biofermentation of okara (Table 6), thus enhancing the nutritional quality of okara. To include a specific example, the levels of

linoleic acid increased after fermentation of okara with *R. oligosporus*. The FAO considers linoleic acid to be an essential fatty acid for poultry. This is crucial in case of breeder hens, as linoleic acid is vital to embryonic health, viability and chick hatchability (Cherian, 2015).

According to the International Food Information Council, antioxidants may be added to feed not only to preserve freshness, flavour and shelf life, but also to supplement nutritional benefits. It has been widely documented by many researchers that dietary antioxidants confer health benefits (Gordon, 2012, Huang, 2018). Therefore, from the results obtained in Table 7, it may be postulated that okara fermented with *R. oligosporus* may be favourable as animal feed.

Hence, it may be concluded that biofermentation of okara led to the generation of an enhanced end-product, which may be considered a potential functional food.

2.5 Conclusion

In conclusion, it can be summarized that this work holds substantial potential. With the growth in popularity of soy-based products, the quantity of okara being produced worldwide is on the rise. This work attempted to reuse okara, a food waste, as a potential functional food. The study revealed that biofermenting okara with food-grade probiotic microbes *R. oligosporus* and *L. plantarum* enhanced its nutrient profile. The GC-MS extracellular metabolite analysis showed that the fermentation processes released microbial hydrolases that degraded the complex macromolecules in okara into simple sugars, amino acids and short-chain fatty acids, that are easier to digest. The levels of beneficial organic acids also increased after biofermentation, while the abundance of phytic acid, an anti-nutrient present in raw okara, decreased. Further, the

antioxidant activity increased post fermentation, indicating an improvement in phenolics content. Our future target plan involves investigating the microbial enzymes released during biofermentation, as well as examining the phenolics of fermented okara.

CHAPTER 3

CHARACTERIZATION AND IN-VITRO BIOACTIVITY OF GREEN EXTRACT FROM FERMENTED SOYBEAN WASTE

3.1 Abstract

Extracts were extricated from raw okara and okara fermented with *Rhizopus oligosporus* using a clean, green protocol; water was used as the extraction solvent and coupled with ultrasound assistance for enhanced extraction. In-vitro antioxidant analyses for antioxidant potential and capacity, superoxide scavenging activity and nitric oxide scavenging activity validated that fermented okara yielded superior bioactive performance compared to raw okara. Fermented okara extracts showed no toxicity to erythrocytes and successfully prevented induced haemolysis. After 48 hours incubation at the highest tested concentration (100 mg/mL), fermented okara extracts could inhibit HepG2 cells by 48.47 ± 5.28%, which was significantly different from their effect on NIH 3T3 cells. GC-MS characterization of extracts validated amino acids to be the chief fraction responsible for the detected bioactivity of the fermented okara extract. The results derived in this study open up the possibility that biofermented okara extract may be a potential novel sustainable nutraceutical.

3.2 Introduction

Okara, a prime agri-waste, is the insoluble waste residue generated by soybean processing industries. As it is highly perishable due to its high moisture content, its standard discard methods include incineration and disposal in landfills. However, there exists a potential for recycling and reusing fresh okara as it has been proven to encompass a variety of nutrients. According to Li et al. (2012), per 100g dry matter raw okara possesses a protein content of 25.4-28.4g, fat content of 9.3-10.9g, dietary fibre content of 52.8-58.1g and carbohydrate content of 3.8-5.3g. Riet et al. (1989) observed that okara also contains several important minerals and vitamins as well. Further, raw okara also has a low calorific value (Stanojevic et al., 2013).

In recent times, solid state fermentation (SSF) has garnered a lot of attention for its ability to economically bulk produce enzymes, organic acids and bioactive secondary metabolites using industrial agro-wastes as substrates. It is also a viable valorization treatment technique to enrich the nutritional content of the substrate through microbial activity (Parades-Lopez and Harry, 1989, Wagenknecht et al., 1961, Moeljopawiro et al., 1987, Moeljopawiro et al., 1988). Currently, there is a growing awareness to curb the usage of petrochemical solvents for extraction purposes. This is because most of the organic solvents are toxic, volatile, flammable and contribute to environmental pollution and greenhouse effect. Water, being polar, is suitable for the extraction of natural water-soluble products such as flavonoids. An added benefit is that easy manipulation of its dielectric constant is possible through modulating temperature and pressure. This allows water to even be used as an extraction solvent for compounds possessing low polarity (Teo et al., 2010). Ultrasound is a clean, green method for extraction of compounds of commercial importance, including but not limited to polysaccharides, proteins and bioactive molecules. Various physical and chemical phenomena including agitation,

vibration, pressure, shock waves, shear forces, microjets, compression and rarefaction, acoustic streaming, cavitation and radical formation are responsible for ultrasonic effect that causes rupturing of biological cell walls, thereby allowing for the release of bioactive compounds into the solvent system (Tiwari, 2015). Ultrasound assisted extraction (UAE) is preferred to conventional methods of extraction because it has certain advantages such as being inexpensive, requiring low volumes of solvent, decreased matrix interferences, being a quicker process, increase in yield and purity of final extract, and being versatile (can customize solvents according to compounds of interest being extracted) (Mandal et al., 2015, Duarte et al., 2014). These advantages make it a popular economic option in the quest for sustainable green technology extraction method.

Liver cancer may be triggered by several factors, notable of which include chronic hepatitis B and C infection and exposure to aflatoxins. Hepatocellular carcinoma poses a major challenge to the healthcare sector; there is a persistent quest for finding a safe, efficient economic medication, with little or negligible side effects.

Today, there is a lot of focus on discovering natural healthcare products in order to avoid the various side-effects caused by the consumption of synthetic antioxidants. This paper has detailed the investigation of in-vitro antioxidant and antiproliferative assays carried out using an extract sourced from okara fermented with *Rhizopus oligosporus*, an FDA approved, GRAS (Generally Recognized As Safe) food-grade fungus using a clean, green method.

3.3 Materials and methods

3.3.1 Chemicals and microorganisms

Fresh okara and *Rhizopus oligosporus* were acquired and maintained as outlined in Chapter 2, Section 2.3.1. Cell lines HepG2 and NIH 3T3 were purchased from ATCC. NaCl, methoxyamine hydrochloride, pyridine, MSTFA, TMCS, DPPH, quercetin, $K_3[Fe(CN)_6]$, TCA , $FeCl_3$, NBT , EDTA, riboflavin, PBS, ascorbic acid, SNP, Greiss reagent, DMEM, FBS, penicillin, streptomycin, MTT, DMSO and hexane were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Dehydrated MRS and PDA media were bought from Becton, Dickinson and Company (BD Difco, USA) and reconstituted as per supplied instructions.

3.3.2 Fermentation and extract preparation

Okara was fermented as described in Chapter 2, Section 2.3.2. For extraction of phytocompounds, 1g of fermented sample (procedure repeated for unfermented sample) was added to 7 mL extraction solvent (water), macerated and ultrasonicated (200 watts, 20 kHz; at a cycle of 0.7, 70% amplitude percentage) for 10 minutes in an ice-bath. The crude mixture was then incubated at 40 ^{0}C in shaking condition for 4 hours, following which, it was centrifuged at 10,000 g for 15 minutes. The supernatant was used as extract for all antioxidant assays. For antiproliferative assays, the supernatant was freeze-dried, re-dissolved in PBS and filtered using 0.45 µM filter prior to treating the cell lines.

3.3.3 DPPH radical scavenging assay

The dose-dependent DPPH radical scavenging activities of the extracts at different concentrations were procured using the method followed in Chapter 2, Section 2.3.4.

3.3.4 Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP was calculated using the methodology employed by Jemli et al. (2016), with minor modifications. 2.5 mL PBS and 2.5mL 1% $K_3[Fe(CN)_6]$ were added to 1 mL extract (at different concentrations), and the mixture incubated at 50 ^{0}C for 20 minutes. Subsequently, 2.5 mL 10% TCA was added, and the mixture centrifuged at 3000 rpm for 10 minutes. To 2.5 mL of the supernatant, 2.5 mL distilled water and 2.5 mL 0.1% $FeCl_3$ were added and the absorbance measured at 700 nm. Quercetin was used as the standard.

3.3.5 Superoxide radical (O_2^-) scavenging activity

The O_2^- scavenging activity of the extracts were obtained using the procedure followed by Parimala and Selvan (2017), with minor modifications. To 100 µL of extract (at different concentrations), 0.1 mL 1.5 mM NBT, 0.2 mL 0.1 M EDTA, 0.05 mL 0.12 mM riboflavin and 2.55 mL 0.067 M PBS were added, and the mixture illuminated for 30 minutes. Absorbance was then measured at 590 nm. Quercetin was used as the standard.

3.3.6 Nitric oxide radical (·NO) scavenging activity

The ·NO scavenging activity of the extracts were estimated using the methodology employed by Parimala and Selvan (2017). 3 mL 5 mM SNP was added to 200 µL of extract (at different concentrations) and the mixture incubated at 25 ^{0}C for 150 minutes. 1mL of this mixture was then removed and added to 1 mL Griess reagent. Absorbance was measured at 546 nm. Quercetin was used as the standard.

3.3.7 Erythrocyte toxicity and haemolysis inhibition assays

Erythrocyte toxicity and haemolysis inhibition assays were performed as described by Cheung et al. (2003), with minor modifications.

For the erythrocyte toxicity assay, 100 µL fresh erythrocytes were added to an Eppendorf tube containing 300 µL PBS and 100 µL sample extracts at different concentrations. The mixtures were allowed to incubate for 3 hours at room temperature in a shaking condition at 200 rpm. Following this, each tube was diluted with 8 mL PBS and centrifuges at 1041g. The supernatant was subsequently read at 540 nm. PBS was used as blank. PBS and sterile distilled water were used in place of samples, in the two control tubes respectively.

The toxicity was calculated according to the formula:

$$\textbf{Toxicity (\%)} = [A_S - A_P]/[A_W - A_P] \times 100$$

where, A_S is the absorbance value of the sample, A_P is the absorbance value of PBS control and A_W is the absorbance value of the water control

The erythrocyte haemolysis inhibition assay was carried out in the same way as the above, except for a minor modification in the experimental set-up. 100 µL fresh

RBCs were added to an Eppendorf tube containing 200 µL PBS, 100 µL 0.5 M AAPH and 100 µL sample extracts at different concentrations. 100 µL of 0.125 mg/mL Vitamin C was added in place of sample in the control tube.

The haemolysis inhibition was calculated according to the formula:

$$\textbf{Haemolysis inhibition (\%)} = [1-(A_S-A_P)/(A_W-A_P)] \times 100$$

where, A_S is the absorbance value of the sample, A_P is the absorbance value of PBS control and A_W is the absorbance value of the water control

3.3.8 Cell culture, treatment and MTT cell viability assay

Human liver cancer cell line HepG2 and mouse embryo fibroblast cell line NIH 3T3 were grown on DMEM medium with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin at 37 ^{0}C, 5% CO_2 till 75-80% confluency was reached. Cells were then seeded into 96-well microplates, with each well containing 10^4 cells.

When the wells achieved a confluency of ~ 70%, 10 µL of extract was added to each well (except control wells) and the microplates incubated for 24 hours and 48 hours. On completion of their respective incubation periods, old medium was removed, and an equivalent quantity of fresh medium was added to each well. 10 µL of 12mM MTT solution was added next, followed by incubating the microplate at 37 ^{0}C, 5% CO_2 for 2 hours. Subsequently, 25 µL of solution was allowed to remain and the rest carefully removed without disturbing the cell layer at the bottom of the well. 100 µL DMSO was then added to each well and mixed thoroughly to dissolve the formazan crystals. The microplate was then again incubated for 10 minutes at 37 ^{0}C, 5% CO_2 and finally read at 570 nm.

3.3.9 GC-MS analysis of extracts

Sample preparation for GC-MS was done using the method followed in Chapter 2, Section 2.3.3.1. GC-MS analysis was carried out using the same conditions as described in Chapter 2, Section 2.3.3.2.

3.3.10 Statistical analyses

All analyses were carried out in triplicate. Data has been represented as an average of the three trials. Antioxidant and cell culture assays were subjected to t-test, with $p < 0.05$ being considered significant. GC-MS data was analysed by one way-ANOVA and post-hoc t-test.

3.4 Results and discussion

3.4.1 Antioxidant analyses

Oxidative stress is responsible for a plethora of serious health concerns. Biofermentation of okara using *Rhizopus oligosporus*, an FDA-approved, food-grade GRAS microbe, was expected to contribute positive nutritional enhancements in conjecture with the work carried out by Gupta et al. (2018). Natural antioxidants as evaluated in our study, have the advantage of not having the side effects associated with commercial synthetic antioxidants.

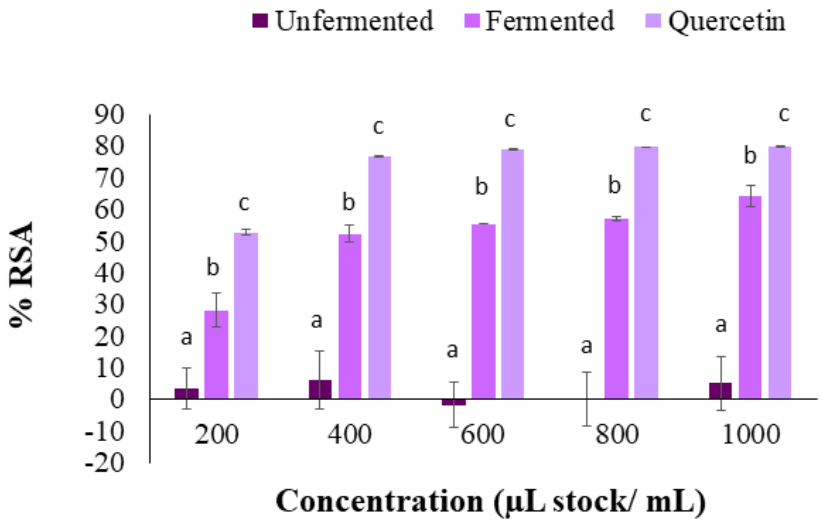


Figure 19 : DPPH radical scavenging potential of raw and fermented okara extracts. *n = 3; alphabets with different letters represent significant difference between groups at a particular concentration.*

DPPH assay is one of the most commonly used assays for testing radical scavenging activities (RSA) of samples. Antioxidants are able to quench the stable DPPH radical (dark purple) to its non-radical stable form (colourless) as a measure of their free radical scavenging potential. Figure 19 demonstrates the antioxidant potential of dose-dependent fermented and unfermented okara extracts against a positive control of quercetin. As is evident from the results obtained, fermented okara extracts possess significantly higher scavenging activities as compared to unfermented okara extracts at same concentrations. However, the activities of fermented okara extracts were lesser than the activities of quercetin at the respective concentrations. The results of this experiment warranted a deeper investigation of the antioxidant potential of the fermented okara extract using different antioxidant tests (FRAP, O_2^- and $^{\cdot}NO$.)

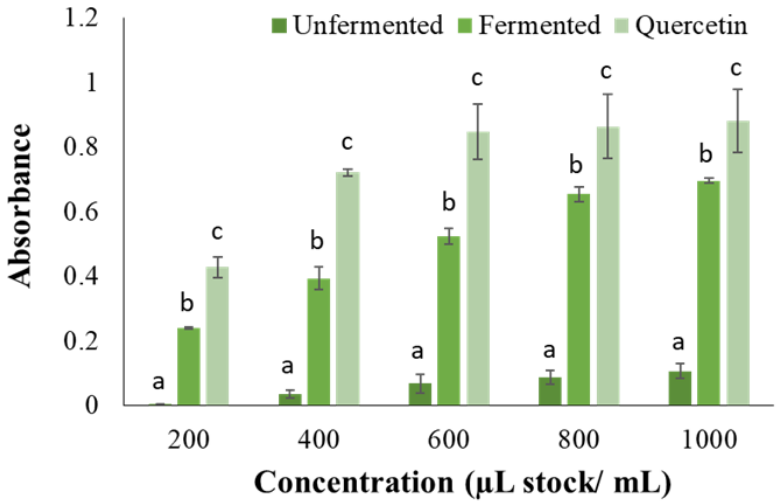


Figure 20 : FRAP potential of raw and fermented okara. *n = 3; alphabets with different letters represent significant difference between groups at a particular concentration.*

The results of FRAP assay to estimate antioxidant capacity of fermented okara have been illustrated in Figure 20. The reducing power of the extracts were studied as a function of their concentration. The mechanism of FRAP is based solely on electron transfer; the ability of the extracts to reduce Fe^{3+} to Fe^{2+} by electron donation was taken as a representation of the potential of the corresponding compound to reduce free radicals in plasma and tissues, thereby modulating redox state. As seen in Figure 20, the antioxidant capacities of fermented okara extracts far surpassed those of unfermented okara extracts at their respective equivalent concentrations. However, fermented okara extracts remained less efficient when compared with standard antioxidant quercetin.

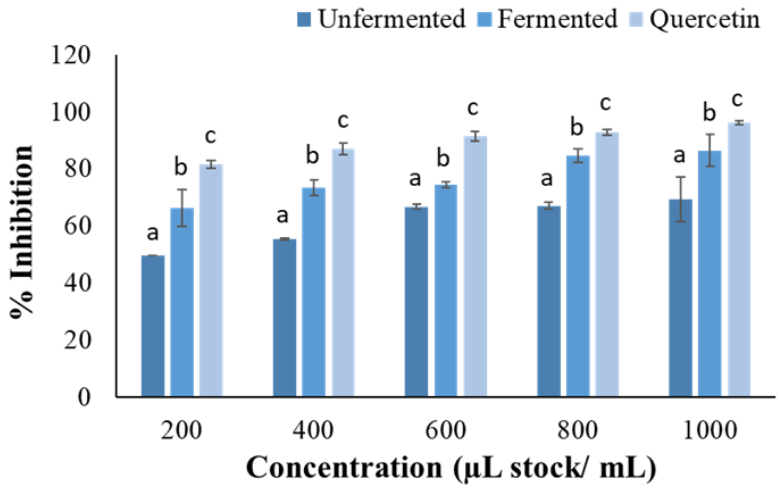


Figure 21 : Superoxide radical scavenging activity of raw and fermented okara. *n = 3; alphabets with different letters represent significant difference between groups at a particular concentration.*

Superoxide anions generated from dissolved oxygen in a riboflavin-light-NBT system can reduce NBT in the system. In absence of any antioxidant activity, superoxides can reduce the yellow dye (NBT^{2+}) to produce formazan. Antioxidants can prevent this activity. As perceptible from Figure 21, the superoxide inhibitory activity of extracts rose with increase in concentration of extracts. Fermented okara extracts yielded better results than raw okara extracts. Superoxide scavenging potential is widely relevant to the biological system as superoxides are capable of reducing certain iron complexes (eg; cytochrome c), causing peroxidation of lipids and generating singlet oxygen and hydroxyl radicals that react with biomacromolecules and thereby induce tissue damage (Zargar et al., 2014).

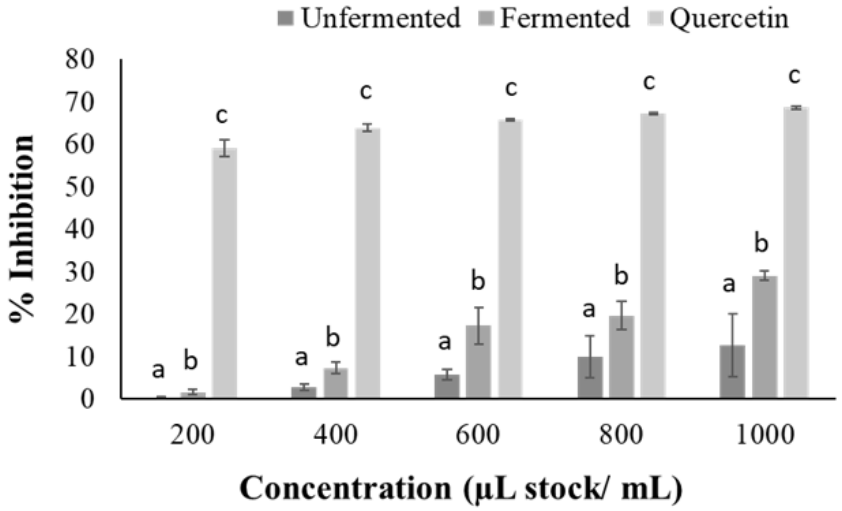


Figure 22 : Nitric oxide radical scavenging activity of raw and fermented okara. *n = 3; alphabets with different letters represent significant difference between groups at a particular concentration.*

In the reaction system used for evaluating nitric oxide scavenging activity, nitric oxide is generated from SNP and reacts with oxygen to form nitrite. The nitrite ions subsequently react with the components of Greiss reagent by diazotizing sulphanilamide and coupling with napthylethelediamine. These reactions produce a pink color, the formation of which is inhibited when antioxidants scavenge the free radicals. As conspicuous from Figure 22, the nitric oxide inhibitory activity of extracts intensified with increase in concentration of extracts. Once again, fermented okara extracts yielded better results than raw okara extracts. Nitric oxide scavenging activity is of immense importance to the healthcare industry. Biologically, chronic exposure to nitric oxide has been linked to various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Boora et al., 2014). Nitric

oxide toxicity increases exponentially when it reacts with superoxide radicals to form the highly reactive peroxynitrite anion ($ONOO^-$).

Table 8 depicts the correlation between the different antioxidant analyses for fermented okara extracts. As is evident from the high correlation coefficients ($R^2 > 0.5$), there exists a strong correlation between the different assays performed for evaluation of antioxidant capacities.

Table 8 : Correlation chart of the different antioxidant analyses performed using fermented okara extract

	O_2^-	·NO
FRAP	0.9401	0.9342
·NO	0.8501	-

3.4.2 Erythrocyte lysis assays

The erythrocyte toxicity assay (Figure 23) proved that unfermented okara extracts may be considered as toxic at concentrations higher than 2 mg/mL. However, fermented okara extracts were observed to be non-toxic to erythrocytes at concentrations even as high as 4 mg/mL. Hence, haemolysis inhibition assay was carried out with the fermented okara extract, to further study its effect on erythrocyte cells.

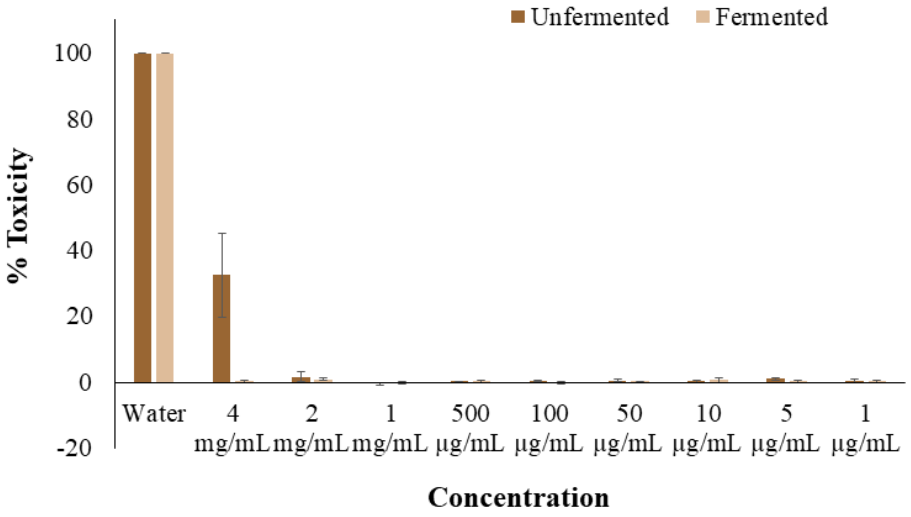


Figure 23 : Erythrocyte toxicity assay using unfermented and fermented okara extracts. *Fermented okara displayed no toxicity even at the highest concentration tested; n = 3.*

AAPH generates free radicals and attacks erythrocytes to induce chain oxidation of proteins and lipids, ultimately leading to haemolysis.

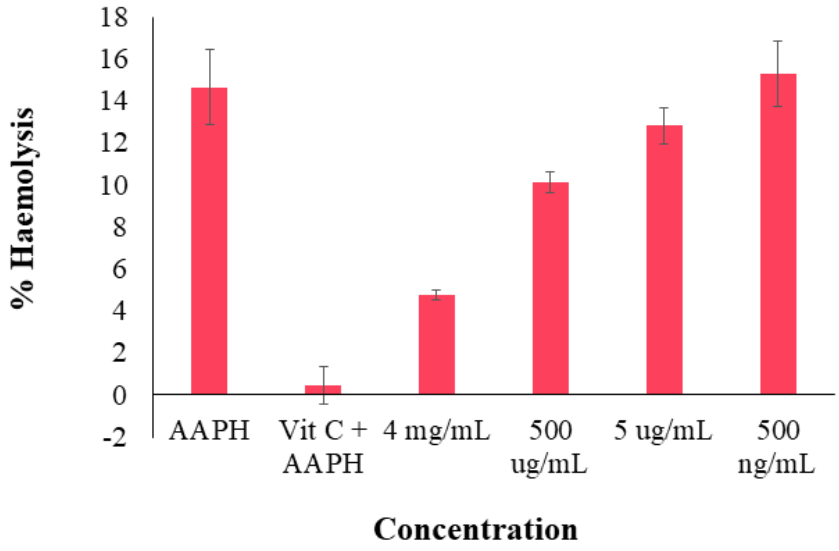


Figure 24 (a) : Percentage haemolysis of erythrocytes in presence of fermented okara extracts. *Haemolysis was induced by AAPH; n = 3.*

As seen from Figure 24, fermented okara extracts were able to prevent AAPH induced haemolysis of erythrocyte cells in a dose-dependent manner. On carrying out a t-test, no significant differences were observed between the percentage haemolysis inhibitions at the highest and lowest concentrations of tested fermented okara extract. This attests that even at concentrations as low as 500 ng/mL in PBS, fermented okara extract is able to prevent AAPH induced lysis of red blood cells.

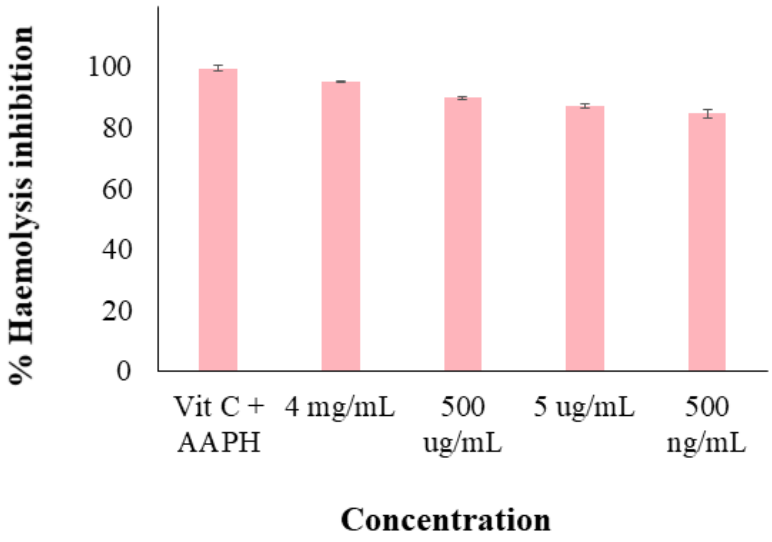
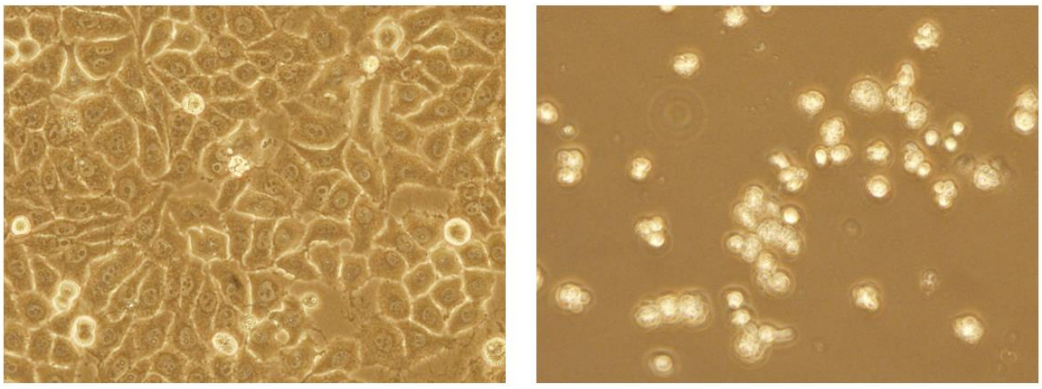


Figure 24 (b) : Percentage of inhibition of haemolysis of erythrocytes in presence of fermented okara extracts. *Fermented okara extracts were able to prevent haemolysis of erythrocytes. Haemolysis was induced by AAPH; n = 3.*

3.4.3 Antiproliferative assay

Cancer is believed to be a result of reactive oxygen species inducing oxidative damage to biomolecules such as lipids, proteins, carbohydrates and DNA. According to Sun (1990), free radicals are involved in both the establishment and propagation of multistage carcinogenesis. Antioxidants scavenge free radicals and are therefore considered to be potential anticarcinogens. The results obtained from our antioxidant analyses (Figures 19-24) gave rise to a strong hypothesis that fermented okara extracts would be able to exhibit antiproliferative activities in cancer cell lines. Recent research has been focussed on identification of anticancer agents from soybean.



(a) **(b)**

Figure 25 : (a) : Live HepG2 cells (before treatment); (b) : Dead HepG2 cells (after treatment). *HepG2 cells were observed to be sensitive to fermented okara extracts.*

Figures 25 (a) and (b) exhibit a section of HepG2 cells before and after extract treatment, respectively. To comparatively ascertain the effects of fermented and unfermented okara extract treatments on HepG2 and NIH 3T3 cells, contour plots were created [Figure 26 (a) and (b)] that illustrate the range of viability changes that occurred in both HepG2 and NIH 3T3 cell lines as a function of extract concentration and time.

In both figures, it could be seen that at lower concentrations, the extracts were able to induce cell proliferation, thereby resulting in a viability above control cells. This may be explained by the hypothesis that at low concentrations, some drugs are capable of upregulating the viability of cancer cells by accelerating cell proliferation and decreasing apoptosis (Wen et al., 2018).

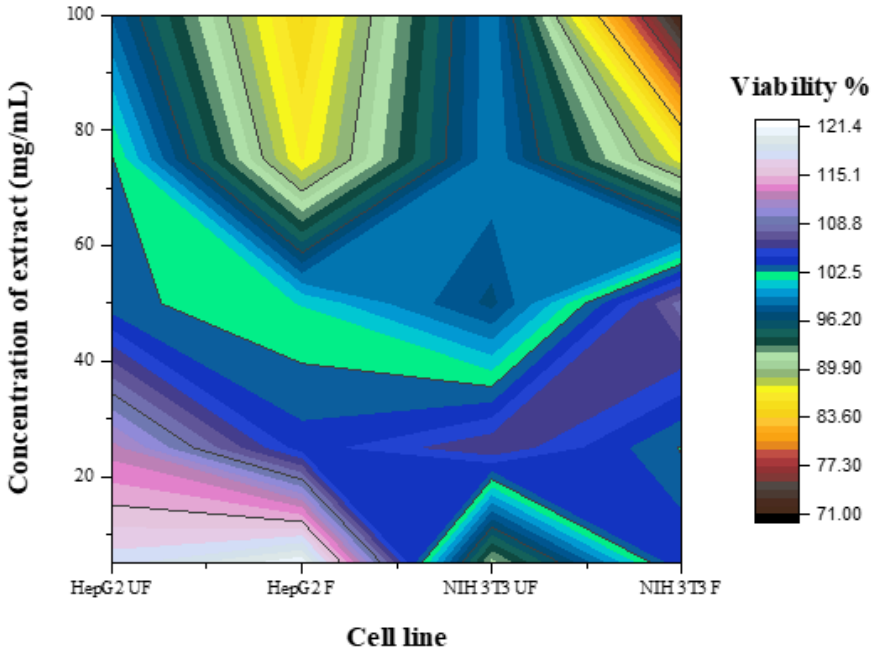


Figure 26 : (a) Contour plot representing viability of cell lines after 24 hours extract treatment. *Key: HepG2 treated with unfermented okara extract (HepG2 UF); HepG2 treated with fermented okara extract (HepG2 F); NIH 3T3 treated with unfermented okara extract (3T3 UF); NIH 3T3 treated with fermented okara extract (3T3 F).*

It is clear from Figure 26 that fermented okara extracts induced greater loss of viability as opposed to unfermented okara extracts. As may be observed from Figure 26 (a), there was not a significant decrease in viability in HepG2 cells after treatment for 24 hours. Treatment for 48 hours showed a much more favourable response, as

evidenced by Figure 26 (b); viability of HepG2 steadily decreased with analogous increase in concentration of fermented extract. It may be postulated from this study that the viability losses were due to apoptosis and necrosis mechanisms, triggered by the extracts.

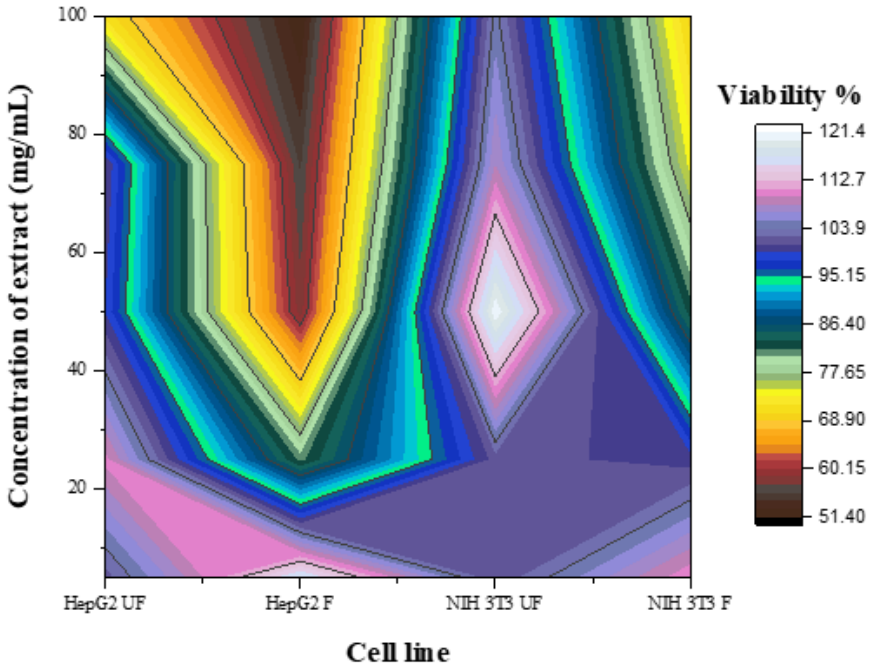


Figure 26 : (b) Contour plot representing viability of cell lines after 48 hours extract treatment. *Key: HepG2 treated with unfermented okara extract (HepG2 UF); HepG2 treated with fermented okara extract (HepG2 F); NIH 3T3 treated with unfermented okara extract (3T3 UF); NIH 3T3 treated with fermented okara extract (3T3 F).*

Although from Figure 26 (b) it was evident that the viability differed significantly across the cell lines with respect to the extract treatment, statistical analyses tools were employed to gauge the levels of similarity or unsimilarity. A paired t-test analysis was thus carried out (Table 9).

Table 9 : Paired t-test results as a function of HepG2 F versus HepG2 UF and HepG2 F versus NIH 3T3 F (F: fermented okara extract, UF: unfermented okara extract)

	Concentration (mg/mL)	HepG2 UF	NIH 3T3 F
HepG2 F	5	0.098848	0.256113
	25	0.031167*	0.011401*
	50	0.001777**	0.040201*
	75	0.009315**	0.042723*
	100	0.005864**	0.041499*

n = 3; * = p < 0.05, ** = p < 0.01

As may be observed, the results validate the implications generated by Figure 26 (b). Since higher concentration of fermented okara extract yielded desirable inhibition of viability in HepG2 cell line after 48 hours incubation, a comparative analysis was made to justify the usage of the same. It was noted that at corresponding concentrations, a significant difference existed between HepG2 cells treated with fermented extracts versus treated with unfermented extracts. Except for the lowest concentration tested, at all other extract concentrations the p-values were lesser than 0.05. A similar trend was observed on comparing the effects of parallel concentrations of fermented extract on HepG2 cells versus NIH 3T3 cells; except for the lowest concentration tested, at all other extract concentrations the p-values were lesser than 0.05, thereby indicating significant difference in the viability percentages (Table 9). Thus, it may be stated from the results obtained that treatment with fermented okara extracts could induce a better reduction in viability of cancer cell line HepG2 as opposed to treatment with

unfermented extracts but had not as great an effect on normal cells NIH 3T3 at analogous concentrations, thereby implying its non-toxicity on normal cells.

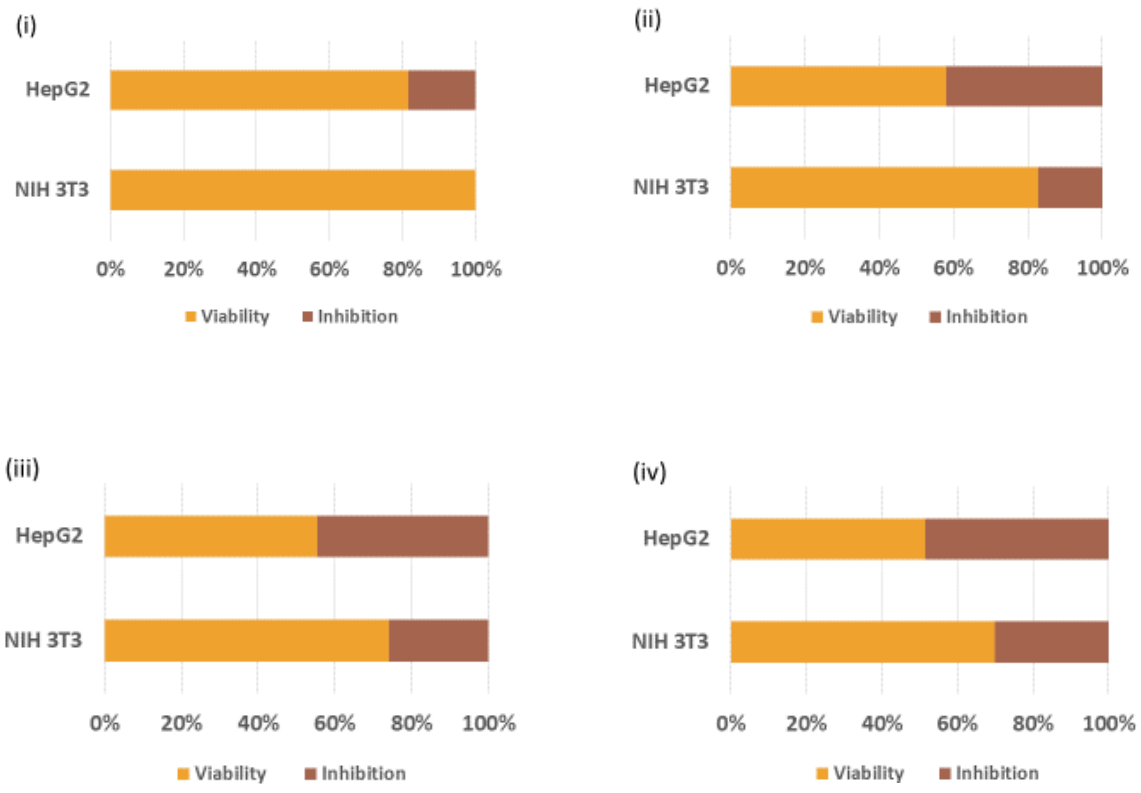


Figure 27 : Increased inhibition of HepG2 cell line after 48 hours treatment with rise in concentration fermented okara extract. *Key- (i): 25 mg/mL extract; (ii) : 50 mg/mL extract; (iii) : 75 mg/mL extract; (iv) : 100 mg/mL extract.*

Figure 27 displays a stacked column analysis of the variation in inhibition across the four highest concentrations of fermented okara extracts tested on HepG2 and NIH 3T3 cell lines after 48 hours incubation. As may be observed from Figures 27 (i), (ii), (iii) and (iv), the percentages of inhibition rise with increase in concentrations of extract used for treatment. There was an inhibition of 18.49 ± 3.43%, 42.07 ± 3.35%, 44.47 ±

5.6% and 48.47 ± 5.28% in HepG2 viability, on treating cells with a dose of 25mg/mL 50mg/mL, 75mg/mL and 100mg/mL respectively. Conversely, NIH 3T3 cells remained less affected by extract treatment; loss of viability was 0 ± 7.49%, 17.28 ± 7.59%, 25.81 ± 2.69% and 30.11 ± 2.73% for treatment doses of 25 mg/mL, 50 mg/mL, 75 mg/mL and 100 mg/mL respectively. The results obtained heighten the possibility that increasing the incubation time would elicit a greater inhibitory response against the tested cancer cell line, at parallel concentrations of extract treatment. NIH 3T3 is a murine non-cancer cell line that is often used as a comparative standard against cancer cell lines, for cytotoxicity analyses (Bisht et al., 2016, Tomankova et al., 2015, Ahmad et al., 2016, Tajudin et al., 2012, Danihelová et al., 2013). Obtaining a positive result on NIH 3T3 cells signified a positive preliminary testing, which paves the path for further advanced toxicity testing. Reactive species are produced in minute quantities by normal cells for signal transduction processes, before being eliminated by the biological metabolic system. However, in case of cancer cells, reactive species are produced at an advanced rate to maintain their accelerated rate of proliferation (Liou and Storz, 2010). Superoxide anions and nitric oxide are the most commonly generated ROS and RNS radicals, respectively. From the antioxidant analyses detailed in this study (Figures 20-24), it may be noted that the principle was based on the radical scavenging abilities of the okara extracts. Hence, it may be said that in tandem with our present results, it would be a valid hypothesis that corresponding treatment would be non-toxic on normal cell lines that do not overproduce reactive species on which the scavenging activities would have an effect upon.

3.4.4 Characterization of extracts using GC-MS

A GC-MS metabolomics approach was used to characterize the extracts used in the antioxidant and antiproliferative analyses, to investigate the bioactive components responsible for the activities.

Table 10 : Significantly different ($p < 0.05$) metabolites detected via GC-MS analysis of unfermented and fermented okara samples with their relative abundances ($x10^9$)

Metabolite	Unfermented okara	Fermented okara
Amino acids		
Alanine	N.D.	7.41 ± 0.05
Valine	N.D.	10.49 ± 0.32
Leucine	N.D.	0.41 ± 0.05
Glycine	N.D.	1.64 ± 0.92
Serine	N.D.	1.12 ± 0.03
Threonine	N.D.	1.71 ± 0.12
Proline	N.D.	1.46 ± 1.39
Tryptophan	N.D.	0.68 ± 0.03
Organic acids		
Butanedioic acid	N.D.	1.42 ± 0.17
Mannonic acid	N.D.	0.84 ± 0.07
Sugars		
D-Glucose	0.34 ± 0.07	2.68 ± 0.34
α-D-pyranoside	0.37 ± 0.08	N.D.
D-Ribose	0.29 ± 0.05	N.D.

*** N.D.: Not detected**

Clearly separated compounds with high abundance have been indicated in Figures 28 (a) and (b). The compounds identified were grouped into three parent groups: amino acids, organic acids and sugars (Table 10). Four essential (valine, tryptophan, leucine, threonine) and four non-essential (alanine, glycine, serine, proline) amino acids were detected.

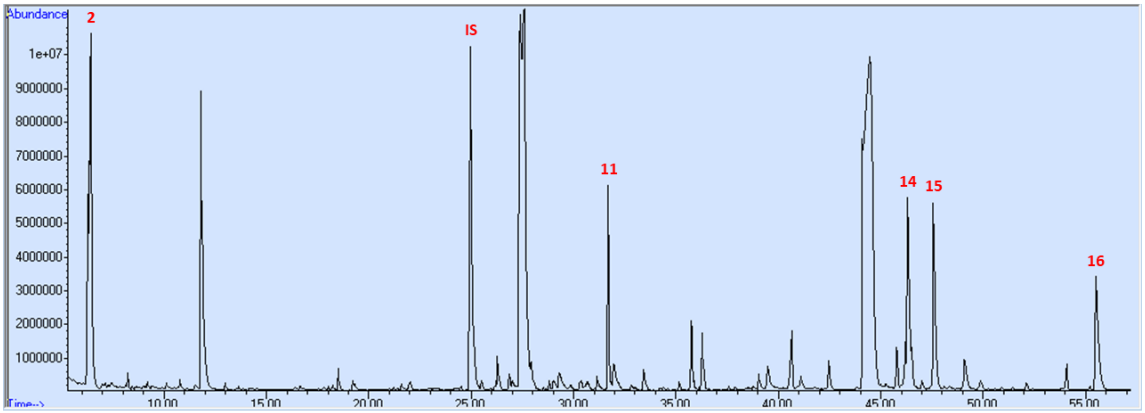


Figure 28 : (a) GC-MS chromatogram of unfermented okara extract. *Key: 2= propanoic acid; 11= hexadecenoic acid; 14= α-D-galactopyranoside; 15= D-glucose; 16= D-ribose; IS= internal standard (ribitol).*

Further, as seen from Figures 26 (a) and (b), there exists a marked difference in the metabolomic output between fermented and unfermented okara extracts.

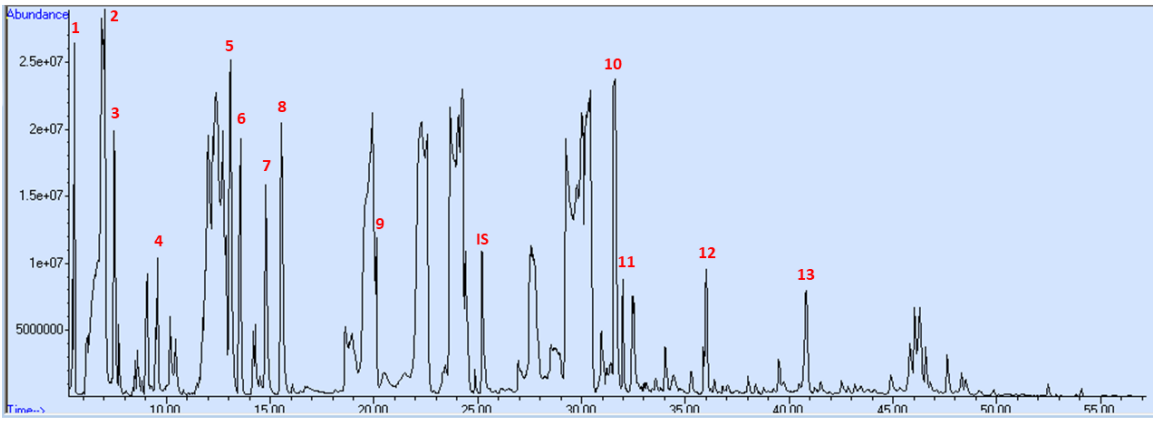


Figure 28 : (b) GC-MS chromatogram of feremented okara extract. *Key: 1= alanine; 2= propanoic acid; 3= valine; 4= leucine; 5= glycine; 6= butanedioic acid; 7= serine; 8= threonine; 9= proline; 10= D-glucose; 11= hexadecanoic acid; 12= tryptophan; 13= mannonic acid; IS= internal standard (ribitol).*

Hence, a PCA biplot (Figure 29) was generated to validate the variance between the unfermented and fermented samples. The GC-MS data was analysed using a correlation matrix with listwise exclusion. The first two principal components (PC), PC1 and PC2, attributed to 91.45% and 6.82% of variance respectively, and thus cumulatively accounted for 98.27% of the total variance. PC1 being the larger principal component, it may be interpreted that the distribution pattern of metabolites detected in unfermented and fermented extracts were significantly different.

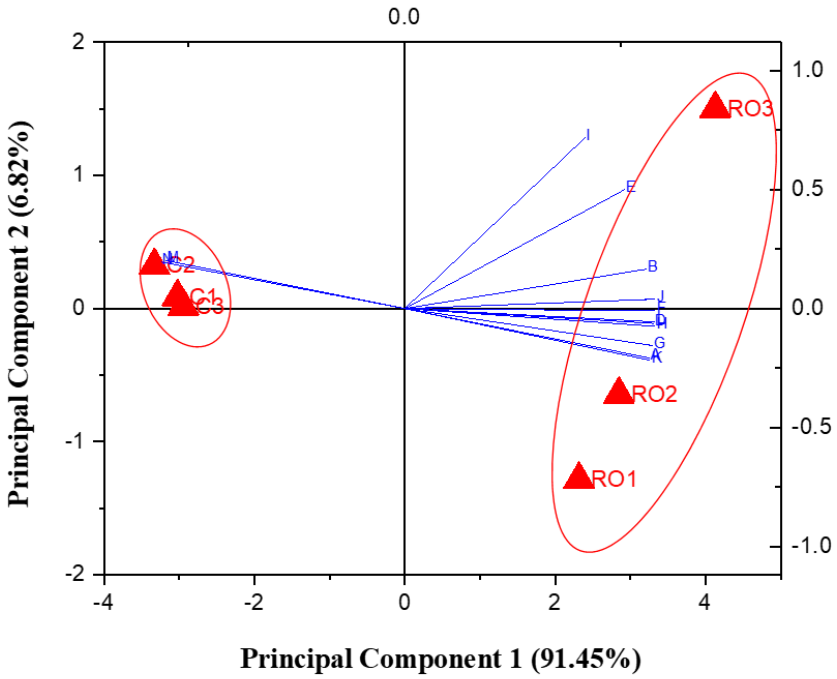


Figure 29 : PCA biplot derived from GC-MS data for unfermented (control) and fermented okara samples. *Key: Unfermented okara control (C); okara fermented with R. oligosporus (RO).*

The fold-change of characterized compounds after fermentation has been documented in Figure 30. As may be observed, fermented okara extracts contained significantly increased levels of amino acids and organic acids. On the other hand, sugar concentration seemed to chiefly decrease after fermentation. This may be attributed to microbial bioactivity breaking down complex proteins into their constituent amino acids, while using basic sugars as nutrition.

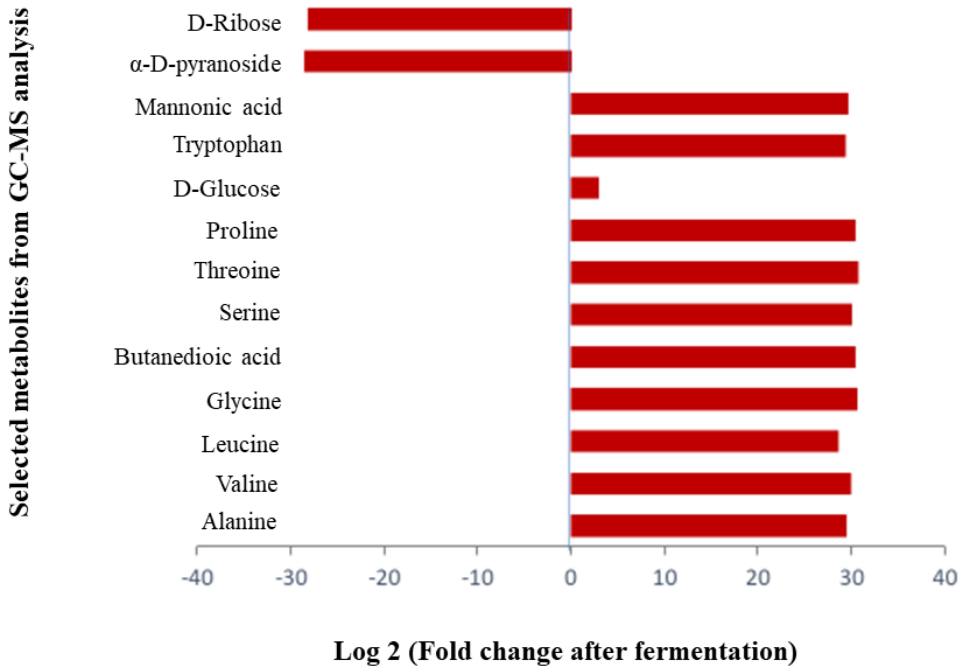


Figure 30 : Fold change in selected metabolites after fermentation of okara with *R. oligosporus.* *Amino acids and organics acids increased after fermentation while majority of sugars decreased.*

3.4.5 The link of characterized metabolites with bioactivity

High levels of sugars are associated with metastasis of malignant cancer. Glucose formed by glycolysis, along with glutamine is able to generate the carbon skeletons, NADPH and ATP to serve as the building blocks for new cancer cells. These cancer cells are able to survive in hypoxial conditions, and subsequently modify the metabolic pathways for cell growth and survival, thus leading to malignancy (Dang, 2012).

Butanedioic acid, commonly known as succinic acid, has been studied to have an effect on antioxidant capacities and has been suggested in its implementation as an

effective preventive antioxidant (Zarubina et al., 2012). Further, it has been observed to increase the activities of several enzymes including superoxide dismutase, catalase and peroxidase (Kolupaev et al., 2011), all of which are vital plasma antioxidant enzymes.

Amino acids play an essential role in the maintenance of cancer redox homeostasis. Proline is a well-known stress adaptor molecule. Its metabolism influences various cell signalling pathways, thereby playing a crucial role in triggering tumour suppression and cell survival in animals (Liang et al., 2013). Further, work carried out by Vaughan et al. (2014) documented that alanine is capable of supressing tumour cells. Dietary threonine has been documented to improve levels of superoxide dismutase, catalase, glutathione peroxidase and complement components C3 and C4 (Habte-Tsion et al., 2016). Studies indicate that branched chain amino acids, including valine and leucine, elicit $^{\cdot}NO$ scavenging biofunctional responses and inhibit lipid peroxidation (Jin et al., 2015). Nayak and Buttar (2016) observed that tryptophan too possesses antioxidant and antiproliferative properties. The bioactivity of serine was observed by Maralani et al. (2012) through the elevation of several antioxidant factors. Oral administration of glycine has been shown to reduce oxidative stress (Wang et al., 2019).

3.5 Conclusion

In conclusion, it may be summarized that this work holds substantial potential for employing biofermented okara as a sustainable functional food. Previous studies carried out (Gupta et al., 2018) documented that biofermentation proved to not only increase the quantity of several important compounds (amino acids, fatty acids, tastants etc.), but also generated an increase in antioxidant activity. Our study established this indication qualitatively, through DPPH, FRAP**,** O_2^{-} and $^{\cdot}NO$ radical scavenging

activities. Moreover, fermented okara extracts were not only found to be non-toxic on erythrocytes but were also capable of inhibiting AAPH induced haemolysis of erythrocytes. Further, the effect of the extracts on HepG2 and NIH 3T3 cell lines revealed the possibility of utilization of the fermented okara extract as a potent nutraceutical, as with increased incubation time and at particular concentrations, a greater, more significant loss of viability was noticed in HepG2 cells as compared to NIH 3T3 cells. An important criterion of our study was to propose the extraction of bioactive fractions using green technology. Lastly, qualitative characterization of the extracts revealed amino acids to be the chief group of bioactive compounds in the fermented okara extract. This is in tandem with our bioactivity studies as Marcuse (1960) suggested that amino acids have antioxidant activities and Bonfili et al. (2017) observed that essential amino acids can activate apoptosis in cancer cells.

CHAPTER 4

AN ANALYSIS OF POLYPHENOLIC CONTENT OF OKARA AFTER FERMENTATION WITH *R. OLIGOSPORUS*, WITH A FOCUS ON AGLYCONE ISOFLAVONES

4.1 Abstract

Extracts of unfermented okara and okara fermented with *Rhizopus oligosporus* were obtained using ethanol as extraction solvent, coupled with ultrasound sonication for enhanced extraction. TPC and TFC assays were performed as phenolics analyses; fermented okara extracts were found to yield significantly better results as compared to raw okara extracts in both the tests. A qualitative LCQTOF/MS analysis revealed that unfermented okara extract contained the glycosidic forms, whereas fermented okara extract contained the aglycone forms of the same isoflavones (daidzein and genistein). Since the aglycone forms have been associated with a host of health benefits, a qualitative HPLC analysis was carried out using standards of the same. Fermented okara extracts had daidzein and genistein concentrations of 11.782 ± 0.325 μg/mL and 10.125 ± 1.028 μg/mL, as opposed to that of 6.7 ± 2.42 μg/mL and 4.55 ± 0.316 μg/mL in raw okara extracts respectively. The results obtained propose that the fermented okara extracts should be examined further for specific functional activities.

4.2 Introduction

Okara is an agri-industrial waste generated by soybean processing industries during the production of tofu and soymilk. Its high moisture content and unavailable bioactive components keep it from being harnessed as a food source. SSF is an economically viable process that is employed for the utilization of crop residues as substrates. Moreover, since many generations, microbial fermentation has been observed to add nutritional enhancements to the substrate through the enzymes released during the biochemical process.

Although there exists no chemical definition for it, polyphenols are broadly described as the plant compounds that serve as beneficial micronutrients to human diet upon ingestion. Lately, many polyphenols have been associated with a litany of therapeutic effects (Williamson, 2017) that have increased an interest in the effective and economical procurement of these natural bioactive molecules. In 1999, the FDA officially approved the consumption of soy and soy-derived products with the alleviation of cardiovascular diseases (Tepavcevic et al., 2010).

Isoflavones, a class of polyphenols, primarily is found in legumes and beans. Isoflavones typically possess phytoestrogenic and antioxidant properties (Wang et al., 2013). According to Tepavcevic et al. (2010) who studied the distribution over several different cultivars of soy, the most consistently occurring soy isoflavones are daidzein, daidzin, acetyl-daidzin, malonyl-daidzin, glycitin, glycitein, malonyl-glycitin, acetyl-glycitin, genistin, genistein, acetyl-genistin and malonyl-genistin.

Thus, the aim of this study was to do a metabolomics analysis of the polyphenolic changes occurring in okara after fermentation with *Rhizopus oligosporus*.

4.3 Materials and methods

4.3.1 Chemicals and microorganisms

Fresh okara and microbes were acquired as described in Chapter 1, Section 2.1. All chemicals and media were sourced from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2 Fermentation and extract preparation

Fermentation was carried out as detailed in Chapter 1, Section 2.1. Sample preparation was carried out as described in Chapter 2, Section 2.2, with minor modifications. Ethanol and methanol were the extraction solvents used. The final extracts were filtered through 0.2 µm syringe filters prior to the LC-MS and HPLC analyses.

4.3.3 Total Phenolic Content (TPC)

The TPC was estimated using the protocol followed by Puranik et al. (2017), with slight changes. 200 µL of extract was added to 1.5 mL freshly diluted (10x) Folin-Ciocalteau reagent and incubated in dark for 5-10 minutes. 1.5 mL of 7.5% aqueous Na_2CO_3 was then added and the mixture incubated at 37 ^{0}C in dark for 2 hours. Absorbance was measured at 765 nm. Gallic acid was used as the standard. Experiments were carried out in triplicate.

4.3.4 Total Flavonoid Content (TFC)

The TFC was estimated using the protocol applied by Parimala and Selvan (2017). 0.1 mL $AlCl_3$ (10%), 0.1 mL $C_2H_3KO_2$ (1 M) and 4.3 mL alcohol (80%) were added to 0.5 mL extract in the order stated. The mixture was incubated at room temperature for 40 minutes. Absorbance was measured at 415 nm. Quercetin was used as the standard. Experiments were carried out in triplicate.

4.3.5 LCQTOF/MS conditions

Polyphenolic compounds were detected by Agilent 6550 iFunnel Q-TOF LC/MS with Agilent Jet Stream ESI in negative mode, with a scanning range from m/z 100-900. The column used was Agilent Eclipse Plus C18 (5m, 4.6 x 250mm). Flow rate was kept at 0.3 mL/min. Eluant A and B were water + 0.1% formic acid and methanol + 0.1% formic acid, respectively. The mobile phase was set as follows: 90% A (v/v) from 0-15 mins, 90-75% A (v/v) from 15-30 mins, 75-25% A (v/v) from 30-45 mins, 25-0% A (v/v) from 45-75 mins, 0-90% A (v/v) from 75-76 mins and 90% A (v/v) from 76-90 mins. 10 μL of sample was injected per run. The drying gas temperature was set at 200^0C, drying gas flow at 14 L/min, nebulizer at 35 psig, sheath gas temperature at 350^0C, sheath gas flow at 11 L/min, Vcap at 3500 V, nozzle voltage at 1000 V, and fragmentor voltage at 175 V. METLIN Metabolomics Database was used to identify the metabolites in the samples.

4.3.6 HPLC conditions

HPLC was used to quantitate the two isoflavone aglycones that were prominently detected via LC-QTOF analysis. The same column was used for HPLC analysis as was used in LC-QTOF analysis. The mobile phase was a gradient of water and methanol; gradient parameters were the same as used in the LC-QTOF analysis. Detection wavelength used was 260 nm and a photodiode array detector was used to generate the spectra.

Calibration curves for standards of daidzein and genistein were prepared in ethanol, with concentrations 40-0 µg/mL (Figure S1). Concentration of these two compounds in the unfermented and fermented okara samples were calculated using their corresponding standard calibration curves. Experiments were carried out in triplicate.

4.4 Results and discussion

4.4.1 Polyphenol analyses

Biofermentation of okara using *Rhizopus oligosporus*, an FDA-approved, food-grade GRAS microbe, was expected to contribute positive nutritional enhancements in conjecture with the work carried out by Gupta et al. (2018). TPC and TFC assays were performed to analyse changes in polyphenol levels after fermentation.

As evidenced by Figure 31, the levels of phenolic contents increased after fermentation of okara with *R. oligosporus*. TPC analysis was carried out using methanol and ethanol as extraction solvents. As may be observed from Figure 31, both extraction solvents extracted nearly the same quantities of phenolic compounds from the samples. Fermented okara extracts had TPCs of 786.63 ± 3.40 mg GAE/g okara sample (methanol

extraction) and 709.33 ± 2.92 mg GAE/g okara sample (ethanol extraction). In comparison, unfermented okara extracts had TPCs of 322.07 ± 9.57 mg GAE/g okara sample (methanol extraction) and 402.11 ± 9.16 mg GAE/g okara sample (ethanol extraction). It was inferred from this analysis that fermented okara extracts had significantly better TPCs than unfermented okara extracts.

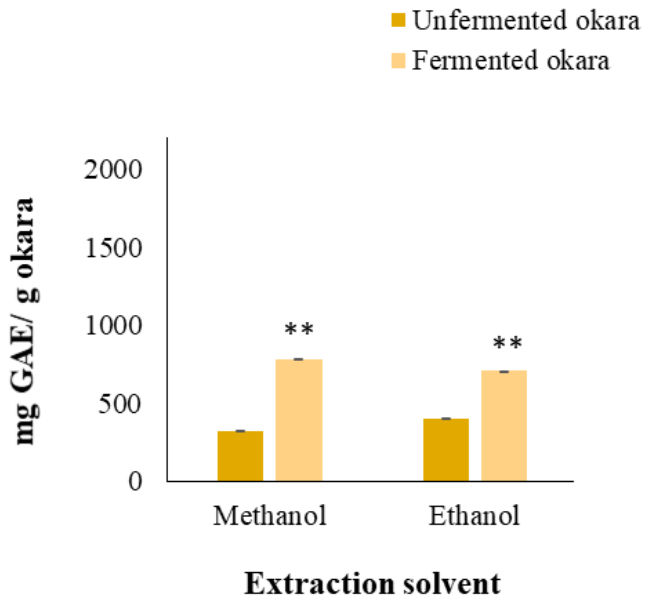


Figure 31 : Total Phenolic Content of raw and fermented okara. *Different extraction solvents were used (n = 3; ** = p < 0.01).*

Experiments conducted verified that the flavonoid content also increased post fungal biofermenting okara with *R. oligosporus* (Figure 32). TFC analysis was carried out using methanol and ethanol as extraction solvents. As may be observed from Figure 32, methanol yielded better results than ethanol. However, since our aim was to propose sustainable methods without adding to further environment pollution, we have chosen to work with the ethanol extracts for all further experiments since ethanol is considered as a green solvent (Chemat et al., 2012). Fermented okara had TFCs of 63.43 ± 10.34

mg Q/g okara sample (methanol extraction) and 35.1 ± 4.97 mg Q/g okara sample (ethanol extraction). In comparison, unfermented okara had TFCs of 20.43 ± 3.86 mg Q/g okara sample (methanol extraction) and 10.77 ± 1.25 mg Q/g okara sample (ethanol extraction). It was inferred from this analysis that fermented okara extracts had significantly better TFCs than unfermented okara extracts.

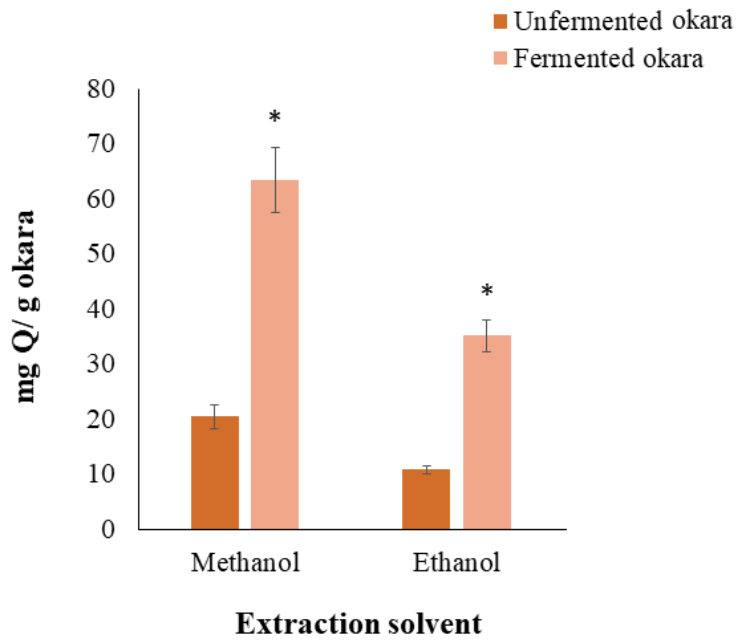


Figure 32 : Total Flavonoid Content of raw and fermented okara. *Different extraction solvents were used (n = 3; * = p < 0.05).*

4.4.2 LC-QTOF analysis

An untargeted LC-QTOF/MS analysis revealed that microbial fermentation exerted a selective effect on the polyphenols present in okara. For instance, the abundance of soyasaponin I remained relatively unchanged before and after fermentation. On the contrary, the levels of isoflavone glycosides daidzin and gensitin

were found to decrease upon fermentation, to give rise to their aglycone isoflavone forms which thus increase in abundance post fermentation (Figure 33).

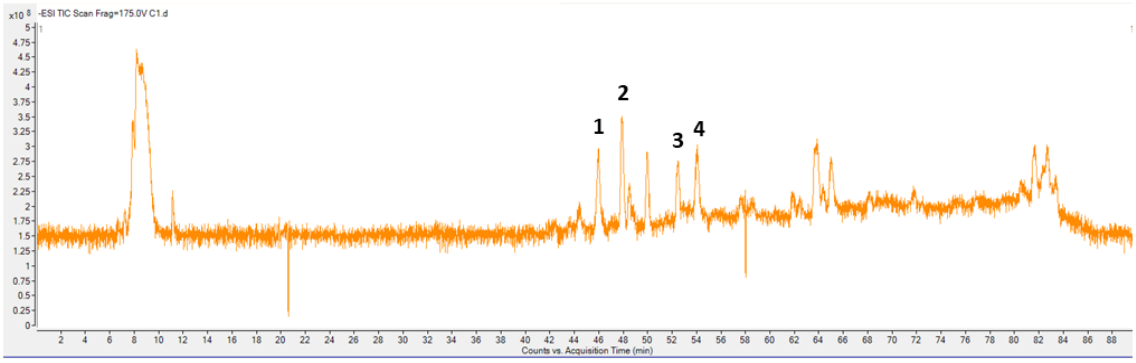


Figure 33 : (a) TIC of unfermented okara extract. *Key: 1 = daidzin; 2 = genistin; 3 = daidzein; 4 = genistein.*

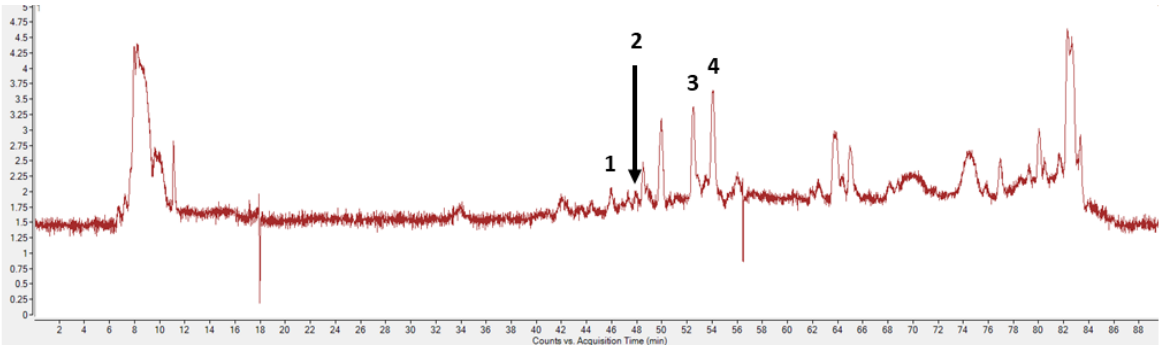


Figure 33 : (b) TIC of fermented okara extract. *Key: 1 = daidzin; 2 = genistin; 3 = daidzein; 4 = genistein.*

The m/z spectra of detected daidzein and genistein are displayed in Figure S2 and S3, respectively. Standards of these two aglycone isoflavones were also run, in order to confirm the presence of these two compounds (Figure 34).

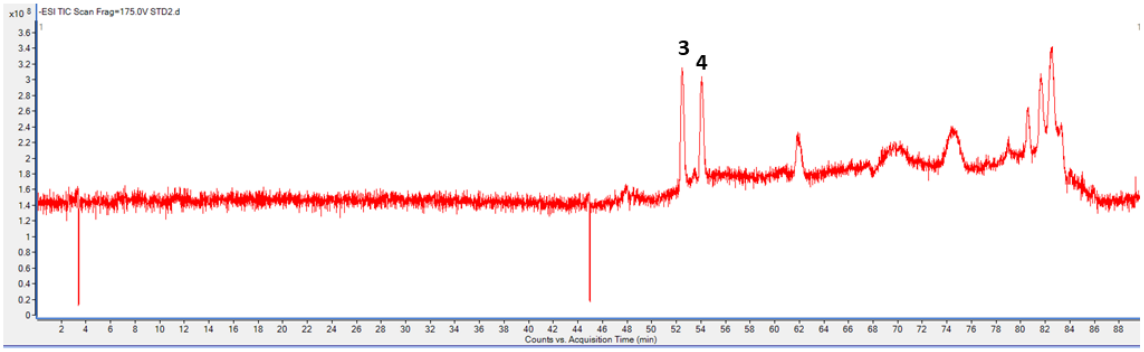


Figure 34 : TIC of daidzein and genistein standard. *Key: 3 = daidzein; 4 = genistein.*

Table 11 displays isoflavones of interest positively confirmed through LCQTOF/MS analysis of unfermented and fermented okara extracts, corresponding to the peaks in Figures 33 and 34.

Table 11 : Isoflavones detected through LCQTOF/MS analysis of okara extracts

Peak number	RT	m/z	Ionization	Phenolic identification
1	45.969	461.1086	[M+HCOO]-	Daidzin
2	47.893	477.1037	[M+HCOO]-	Genistin
3	52.482	253.0521	[M-H]-	Daidzein
4	54.074	269.0456	[M-H]-	Genistein

4.4.3 HPLC analysis

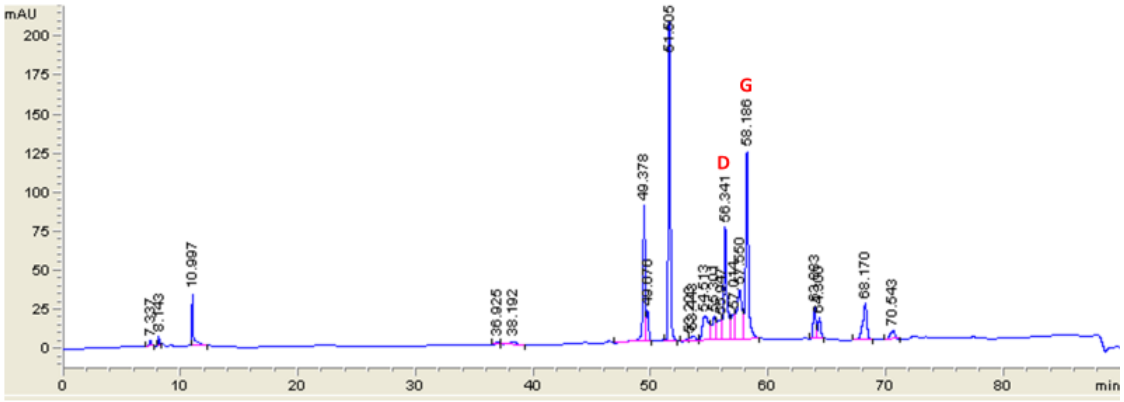


Figure 35 : (a) HPLC characterization of unfermented okara extract. *Key: D = Daidzein; G = Genistein.*

Ethanol extract of okara fermented with *R. oligosporus* yielded higher abundances of daidzein and genistein, when compared with ethanol extract of unfermented okara (Figure 35).

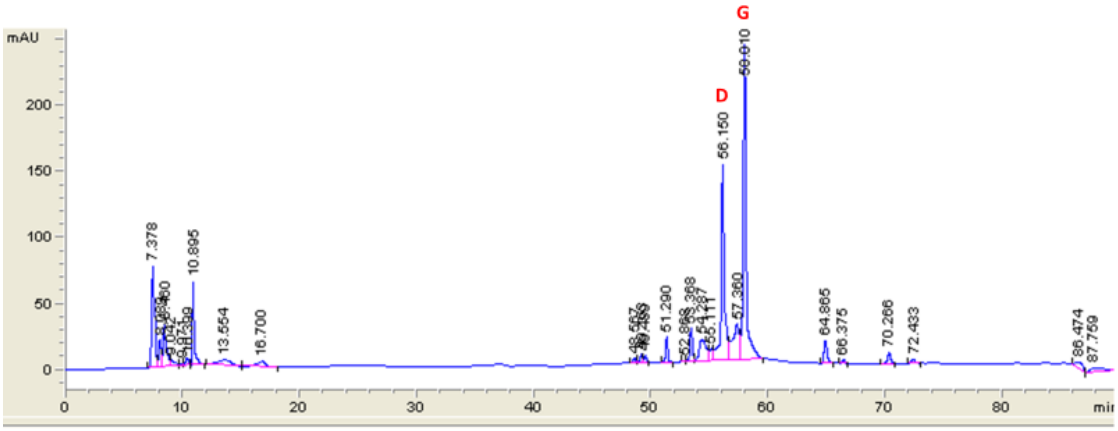


Figure 35 : (b) HPLC characterization of fermented okara extract. *Key: D = Daidzein; G = Genistein.*

Quantification of these two isoflavones against their respective standards (Figure 36) was performed in order to determine their concentration in the extracts (Table 12).

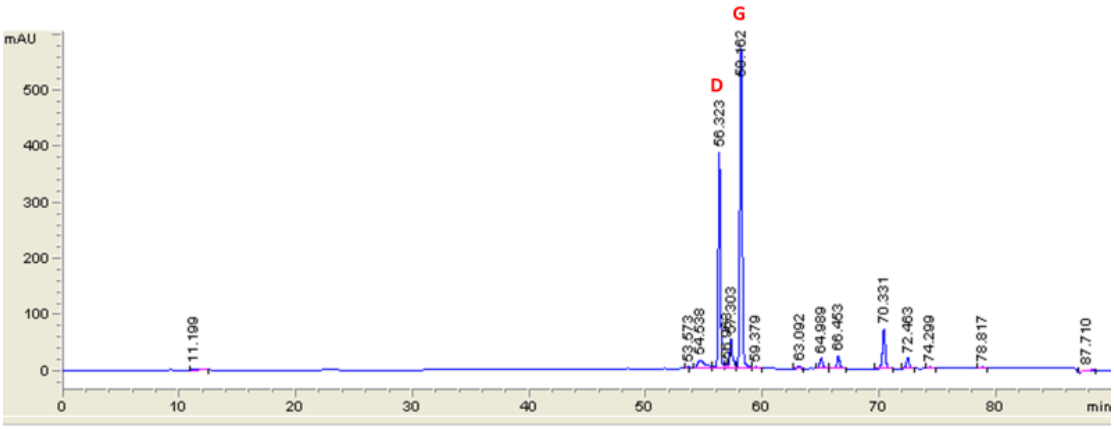


Figure 36 : HPLC characterization of standard solution of isoflavones. *Key: D = Daidzein; G = Genistein.*

Although the concentration of both genistein and daidzein was found to be higher in fermented okara extracts as compared to unfermented okara extracts, the increase may be considered significant with respect to the ANOVA test only in the case of genistein.

Table 12 : Concentration of isoflavones daidzein and genistein in fermented and unfermented okara extracts (in µg/mL)

	Unfermented okara	**Fermented okara**
Daidzein	6.7 ± 2.42	11.782 ± 0.325
Genistein	4.55 ± 0.316	10.125 ± 1.028*

*** = p < 0.05**

To validate the results of the HPLC analysis using a statistical tool other than ANOVA, a hierarchical agglomerative clustering heatmap dendogram heatmap was obtained (Figure 37). The abundance of genistein and daidzein in the unfermented and fermented okara extracts are denoted in the heatmap. The minimum and maximum values of the Euclidean distance used in our calculations were 0 and 8, respectively. The columns denoting extracts of okara fermented with *R. oligosporus* and control (unfermented okara) converged at a Euclidean distance of 7.5437. Hence, it can be elucidated that at a Euclidean distance below 7.5437, both columns were significantly unsimilar to each other.

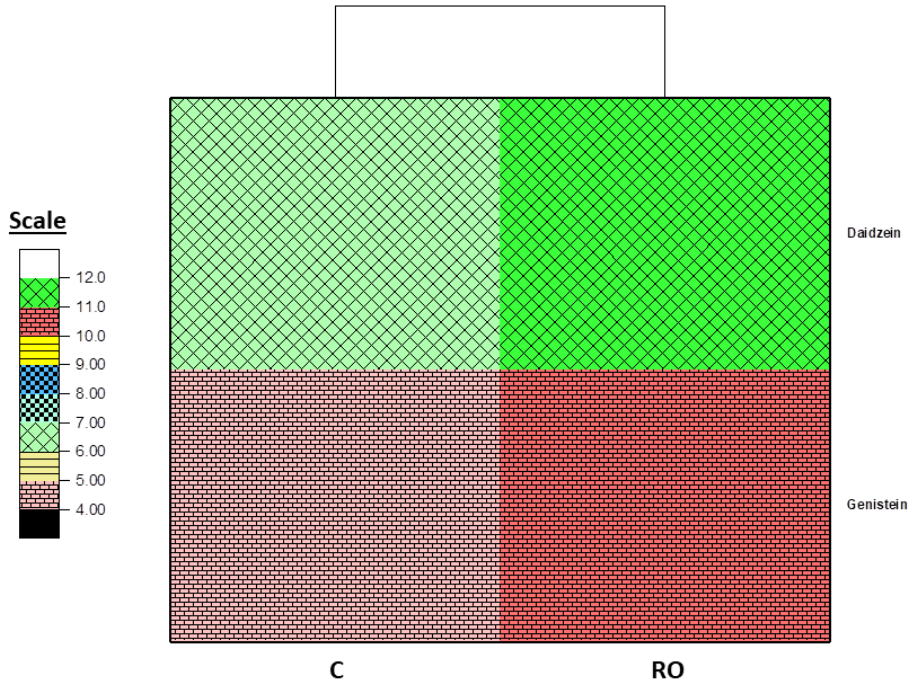


Figure 37 : Heatmap dendogram of isoflavones of interest quantified via HPLC analysis. *The scale indicates the concentration (in µg/mL) of each compound. Key: Extract of unfermented okara [control] (C); Extract of okara fermented with R. oligosporus (RO).*

On comparing concentrations of genistein and daidzein in unfermented and fermented okara extracts (Table 12), percentage increases were calculated to be 122.52% and 75.85% respectively. Figure 38 displays the fold change of these two isoflavones after fermentation.

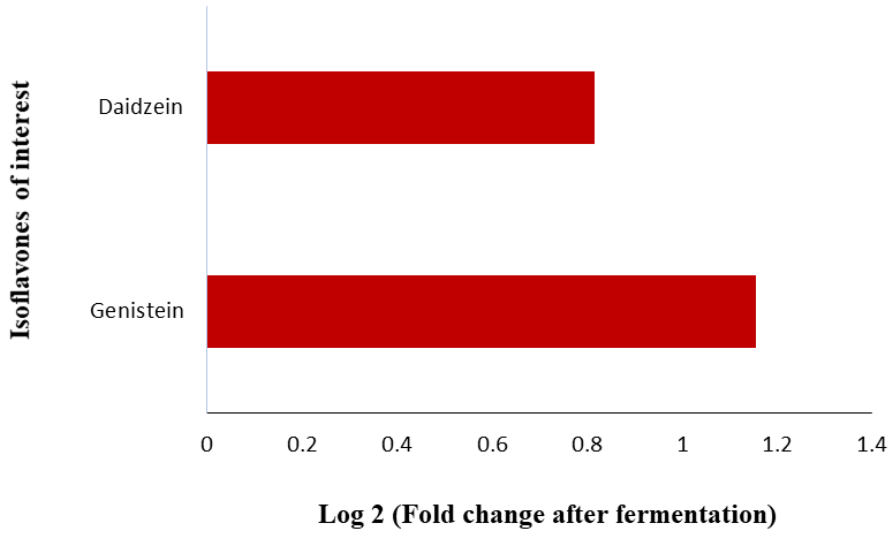


Figure 38 : Fold change in isoflavones of interest after fermentation of okara with *R. oligosporus.* *The concentrations of daidzein and genistein were found to have increased after the biofermentation process.*

4.4.4 Current uses of daidzein and genistein

Phytoestrogens (Figure 39) are polyphenolic non-steroidal compounds that are structurally similar to mammalian estrogens and hence, functionally, bind competitively to estrogen receptors. Intake of phytoestrogens has recently been attributed with a plethora of health benefits, including but not limited to, the lowered risks of heart diseases, oseteoporosis, type 2 diabetes, brain function disorders, breast cancer and menopausal symptoms. However, because the absence of dietary phytoestrogens is not

ascribed to deficiency syndromes and because they are not linked with any essential biological function, phytoestrogens are not officially considered as nutrients.

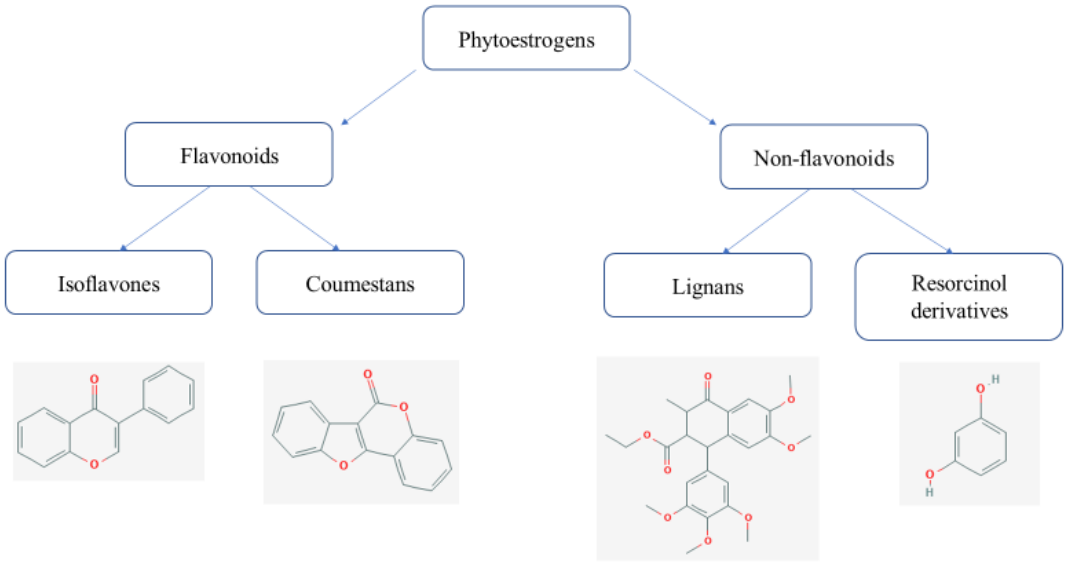


Figure 39 : Classification of phytoestrogens. *Natural phytoestrogens have been discovered to possess a variety of health benefits, leading to a commercial interest in their economical production. Structures extracted from PubChem with permission.*

Isoflavones are the group of phytoestrogens that primarily occur in soy. The major issue with this subgroup is that they typically occur in their glycoside form (ie, attached to a glucose or carbohydrate moiety) which remains biologically inactive post consumption. Mammalian beta-glucosidases are capable of deconjugating some glycosidic flavonoids to their aglycone forms, but not all (Lambert et al., 1999). Hence, the challenge is to convert the conjugated isoflavones to their respective unconjugated, bioavailable aglycone forms (Figure 40).

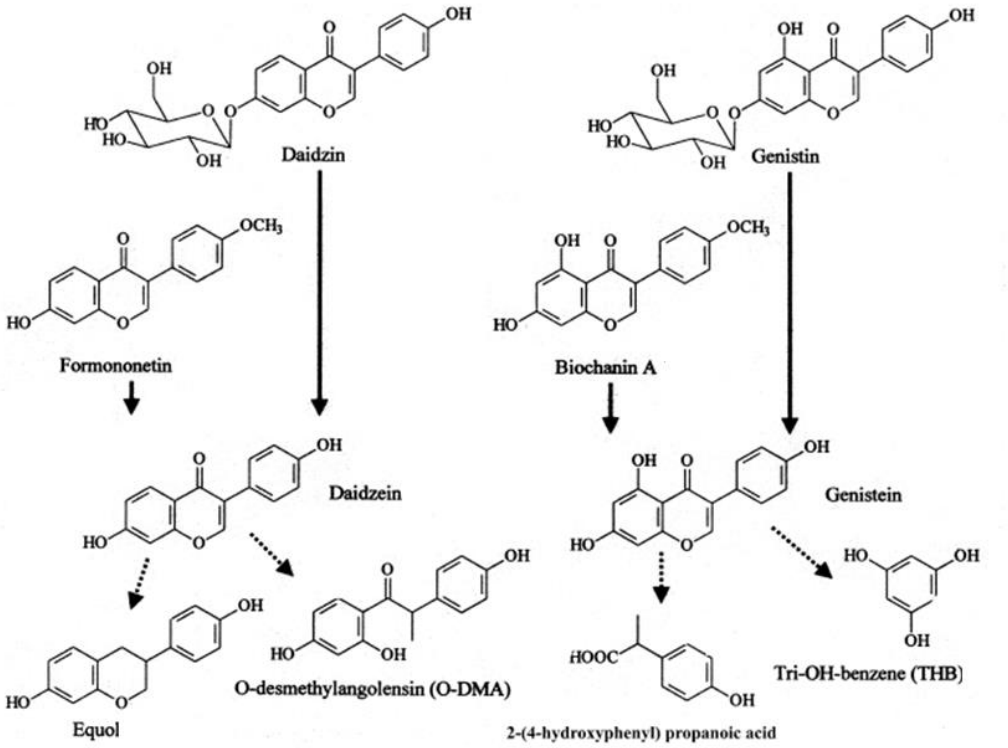


Figure 40 : Biotransformation of daidzin and genistin. *Daidzin and genistin are enzymatically converted to their respective bioavailable forms (daidzein and genistein) in vivo. Image extracted from Cos et al. (2003) with permission.*

It has been observed that fermented soy foods such as miso and tempeh contain a larger percentage of aglycone isoflavones than glycosidic isoflavones. The results obtained in our biofermentation study are in conjecture with this trend. The dosage recommendation of commercially available isoflavones differs amongst manufacturers, but typically lies within the range of 20-80 mg/day. With the health benefits of isoflavones being a current 'hot topic' in research, it is conceivable that they may be classified as nutrients in the future.

Genistein has been proven to be an inhibitor of transthyretin tetramer dissociation and amyloidogenesis, which are associated with amyloid diseases such as familial amyloid polyneuropathy, senile systemic amyloidosis and familial amyloid cardiomyopathy (Green et al., 2005). It has also been discovered that genistein displays therapeutic effects on a wide range of cancer (liver, gastric, lung, colorectal and breast cancers) types by altering apoptosis, the cell cycle, and angiogenesis and inhibiting metastasis. According to Spagnuolo et al. (2015), targeting caspases, B cell lymphoma 2 (Bcl-2)–associated X protein (Bax), Bcl-2, kinesin-like protein 20A (KIF20A), extracellular signal-regulated kinase 1/2 (ERK1/2), nuclear transcription factor κB (NF-κB), mitogen-activated protein kinase (MAPK), inhibitor of NF-κB (IκB), Wingless and integration 1 β-catenin (Wnt/β-catenin), and phosphoinositide 3 kinase/Akt (PI3K/Akt) signalling pathways may act as the molecular mechanisms of the anticancer, therapeutic effects of genistein. Potent anticancer drugs such as adriamycin, docetaxel, tamoxifen have been observed to have a synergistic effect when taken in combination with genistein. Furthermore, dietary administration of 250 ppm genistein in SENCAR mice was found to significantly increase the activities of catalase in small intestine, liver, and kidney, the activities of superoxide dismutase and glutathione peroxidase in skin, and the activity of glutathione reductase in skin and small intestine, which may account for the chemotherapeutic properties of this isoflavone (Cai and Wei, 1996).

Daidzein is slightly less potent than genistein, in its therapeutic capabilities. Nevertheless, studies have confirmed that it confers some pharmacological benefits. Yu et al. (1999) observed that daidzein is capable of inhibiting the growth of human colon tumour cells. The mechanism of action was postulated to be via reduction of density of cell surface charge and increase in the level of membrane protein conformation. Further, peripubertal exposure to daidzein resulted in protection against decrease of bone mineral

density in Sprague-Dawley rats (Tousen et al., 2015). Experiments conducted by Fischer et al. (2012) have shown that daidzein mediates an increased resistance of *Caenorhabditis elegans* versus pathogenic bacteria *Photorhabdus luminescens* and heat. The relevance of this study lies in the fact that vitellogenins, invertebrate egg yolk proteins with a high grade of homology to mammalian ApoB-100, have emerged as potential determinants of longevity in humans and in nematodes. Daidzein has also been accredited with carcinogenic estrogen replacement therapy, for aiding improvement of hippocampal neural cell viability and proliferation, possibly mediated by the BDNF-Trk pathway (Pan et al., 2012). Hippocampal neural cells are imperative in learning and memory function, which raises the possibility of daidzein being utilized for the prevention of Alzheimer's disease.

4.5 Conclusion

Summing up, it may be concluded that the polyphenol profile of okara was enhanced post fermentation. This work furthered our previous studies (Gupta et al., 2018, Gupta and Chen, 2019) carried out to prove the nutritional enrichment of okara through biofermentation. The TPC and TFC were observed to have improved after fermentation. Qualitative LC-QTOF/MS analysis of polyphenols in unfermented and fermented extracts revealed amplified polyphenols of interest to be genistein and daidzein, and the extracts were subsequently subjected to a quantitative HPLC analysis. Although the levels of both genistein and daidzein increased after fermentation, the increase was significant in case of genistein. A dendogram heatmap and fold change graph were generated to validate this result. Polyphenols are one of the major classes of phytochemicals. Of late, their bioactivity and health benefits for alleviating several diseases has received much scrutiny by researchers. Interest in harvesting naturally occurring antioxidants is increasing

exponentially, with a view to correct the imbalance created between free radicals and the natural antioxidant capacity of the body when the latter succumbs to diseases. Yao et al. (2004) and Kozłowska and Szostak-Węgierek (2018) have documented the promising healthcare benefits of flavonoids sourced from food, included but not limited to anti-inflammatory, antidiabetic, anticancer and cardioprotective activities.

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS

5.1 Conclusions

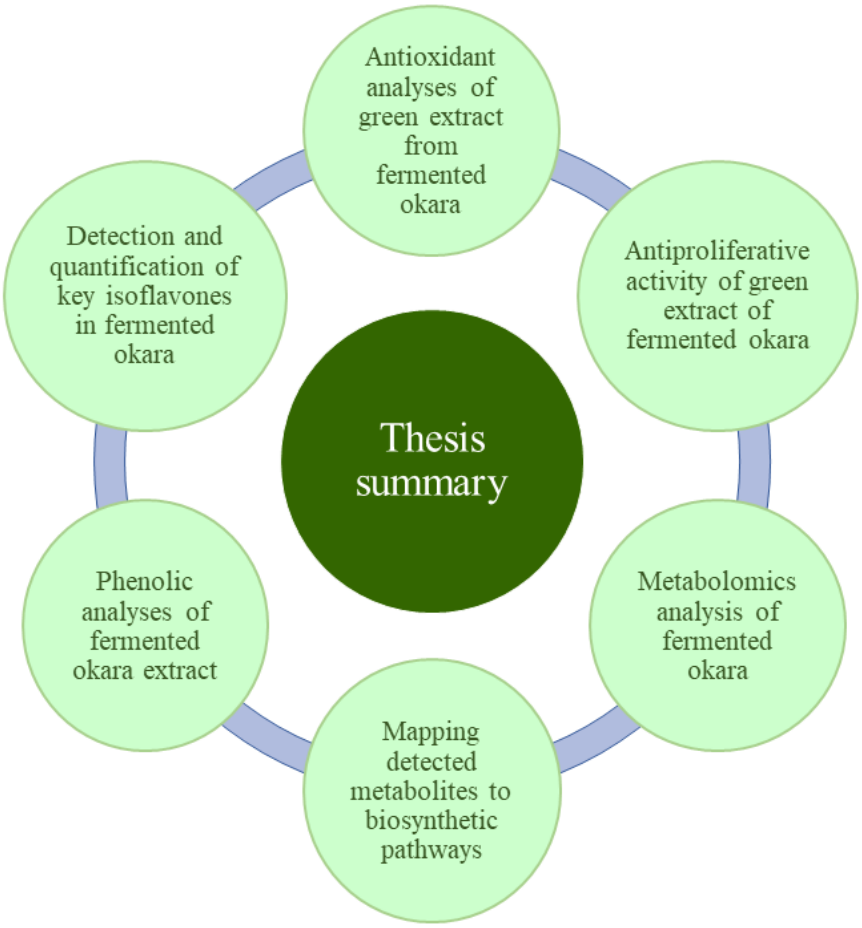


Figure 41 : Summary of this thesis. *The key findings of this thesis have been consolidated in this figure, as a quick recapitulation of the results obtained in the course of this work.*

The focus of this thesis was to ameliorate the nutritional composition of okara, a major agri-industrial soybean by-product that is also considered as food waste, through biofermentation using *Rhizopus oligosporus*. The approach was aimed towards a sustainable reuse of this food waste, drawing inspiration from the system of circular economy instead of the conventional linear economy. Based on the results obtained from

the studies, the key findings (Figure 41) in this thesis can be summarized into the following points:

1. Fermenting okara with *R. oligosporus* yielded more nutritional enhancements such as free amino acids, fatty acids, tastants, improved antioxidant capacity, etc. as compared to fresh okara or okara fermented with *L. plantarum*. Metabolomics analysis with pathway tracking helped in further understanding of the biochemical reactions occurring during the process of microbial biofermentation.

2. Aqueous extracts from okara fermented with *R. oligosporus* have enhanced in-vitro antioxidant scavenging activities (DPPH, FRAP, O_2^- and $^{\cdot}NO$) in a dose dependent manner, when compared to similar extracts from fresh okara. Further, fermented okara extracts were found to be non-toxic to erythrocytes at concentrations as high as 4 mg/mL and were also capable of inhibiting AAPH induced haemolysis of erythrocytes as a dose dependent response. Fermented okara extracts also displayed higher antiproliferative activities against HepG2 cell line, as compared to unfermented okara extracts. A metabolomics analysis helped to determine the different metabolites in each extract and offered an insight into the bioactive molecules responsible for the functional properties.

3. Phenolics assays (TPC and TFC) yielded better results with ethanol extracts from okara fermented with *R. oligosporus*, as compared to unfermented okara extracts. Untargeted LC-QTOF analysis performed showed an increase in

aglycone isoflavones after fermentation. Upon quantification using HPLC, genistein and daidzein were found to be in higher quantities in fermented okara extracts, as opposed to unfermented okara extracts.

Hence, the overall findings in this thesis reveal the functional potential of okara after valorization of its nutritional properties through biofermentation. In the quest for economic sustainable solutions to combat food waste, the results obtained in this thesis open up the possibility to enhance this residual soybean waste and employ it as a potential nutraceutical.

The skeletal structure of this thesis has been outlined in Figure 42, which entails the cascade of the workflow followed and a brief display of the results obtained.

5.2 Limiting factors in this study

5.2.1 Untargeted metabolomics

In the metabolomics studies using GC-MS and LC-QTOF, it must be noted that both the cases comprised untargeted approaches. Hence, the compounds identified were qualitatively matched only against those in the available libraries. For accurate, quantitative measurements, standards of the selected compounds of interest need to be run at various concentrations, to verify retention times and calculate the amounts of the same. Since there exists no standardized protocol for pre-analytical, analytical and post-analytical methods, quality control across the globe remains one of the main concerns of this tool.

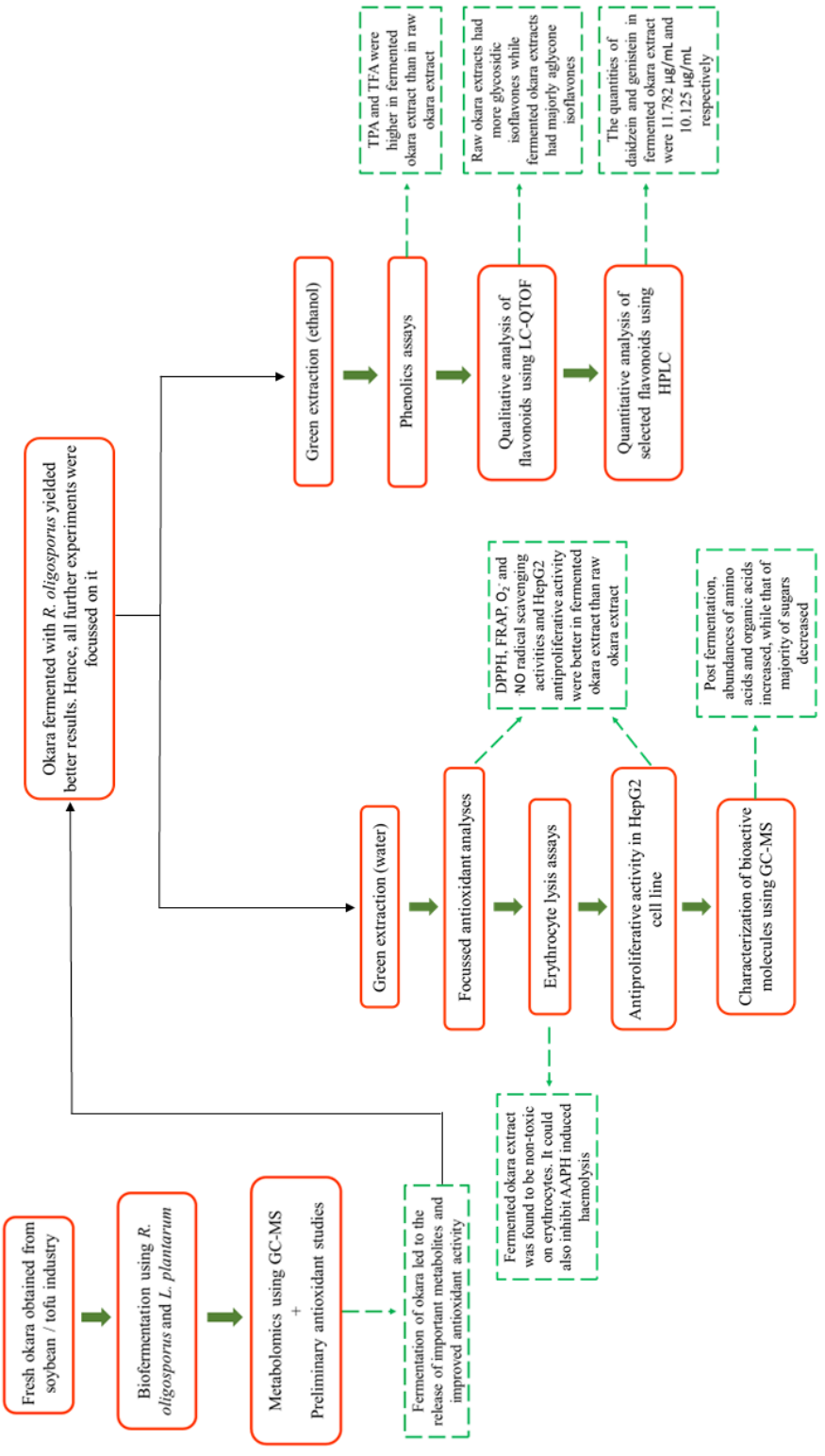


Figure 42 : Overall workflow and main findings of this thesis

5.2.2. Use of pure extraction solvents

Extracts of fermented and unfermented okara were prepared in pure ethanol and ultrapure distilled water during the course of this study. It would add a more realistic dimension to the results obtained if the work can be repeated using the same approach, but with the bio-solvents obtained from industries. This would help in gauging if the purity levels of green solvents hinder the functional abilities of the bioactive components obtained from the green extraction of fermented okara.

5.2.3 Stability of the compounds of interest

This study dealt with the detection and identification of a number of bioactive polyphenolic metabolites. However, a key concern might be the stability of these compounds over time. Repeating these experiments with a particular sample set over a wide range of time period may help understand their deterioration pattern and thus calculate their shelf life.

5.2.4 Primary cells

Primary cells provide a high-quality contextual model for the study of normal cell signalling. In this study, experiments for checking the antiproliferative activities of fermented and unfermented okara extracts on human liver carcinoma cell line HepG2 used non-cancer cell line NIH 3T3 as a parallel control. Keeping testing parameters consistent, it would yield more validity to the results were primary cells from the same tissue (liver) and testing species (human) be employed instead of the generic non-cancer model.

5.3 Recommendations for future research

5.3.1 Testing ethanol extracts on cell lines

Isoflavone aglycones, such as the ones detected and quantified in fermented okara ethanol extracts in this study, are practically insoluble in water. As studied by Yu et al. (1999) and Spagnuolo et al. (2015), isoflavones daidzein and genistein have a strong potential for exhibiting antiproliferative activities against cancer cell lines. Hence, there is a need to test the ethanol extracts on the cell lines in an experimental model parallel to that used for aqueous extracts, in order to observe if there is any significant improvement in the results obtained.

5.3.2 Optimization of ultrasonication parameters

Numerous variables are involved in governing the extraction efficiency and yield of UAE. Some of such parameters include operation frequency, amplitude, sample preparation, solvent, temperature, sample:solvent ratio and extraction time. As noted by Medina-Torres et al. (2017), it is the sum-total of all these parameters that affects the final output; therefore, simultaneous effects of variables must be studied using Response Surface Methodology (RSM), to generate the optimum conditions. Of the different methods of RSM, it was observed that the one most frequently employed by researchers is the design by Box-Behnken, followed by the central composite and face-centred cubic experimental designs

5.3.3 Supercritical Fluid Extraction

In the future, supercritical fluid extraction (SFE) may be considered for an alternative green method to extract bioactive fractions from the samples described in this thesis. As exemplified by Farías-Campomanes et al. (2015) and S.Malaman et al. (2011), SFE has already been proven to successfully extract polyphenols from lees and cherries, respectively.

SFE is a technology that "uses the properties of gases above their critical points to extract selective soluble compounds from a raw material" (Cavero et al., 2006). CO_2 is widely used as the extraction solvent, as it is regarded as safe for the extraction of commercially relevant biomolecules and fine chemicals. This is because CO_2 is non-toxic, non-explosive, cheap and easy to remove from the extracted products. The chief advantage of SFE technology is that higher yields of compounds can be obtained with no residual solvent contamination. Further, this process usually employs low temperatures which leads to protection of bioactive compounds from deterioration due to higher temperatures. The only minor inconvenience of this technology is that extraction of polar compounds requires the addition of small quantities of organic modifiers to aid extraction.

5.3.4 Upscale fermentation for industry

The main goal of this thesis was to suggest a method towards reducing food waste, thus playing a part in reducing environmental pollution. The methods suggested in this thesis, although promising, have been performed only at laboratory scale. SSF at industrial scale has not yet been explored to the depths of SmF. This is because upscaling SSF needs the concerted efforts of microbiologists, engineers, organic chemists and

enzymologists for modulating the entire process to match that of the laboratory scale set-up.

According to Mitchell et al. (2006), the complications associated with large scaling SSF arise from the following parameters: a) variations in biomass formed, b) development of large scale inoculum, c) aeration, d) agitation, e) heat removal, f) moisture content of substrate, g) sterility and contamination control, h) pH control, i) heterogeneity, j) downstream processing, k) waste stream management, and l) handling of solids. Thus, a well-founded scale-up system would need to address all these criteria. Current scale up approaches for setting up industrial level bioreactor plants include the trial-and-error approach, geometric similarity approach, scale down approach and maintenance of constant heat and water approach (Lonsane et al., 1992).

5.3.5 Extension to invivo trials

The correlation of invitro cancer models with invivo experimental systems is the key to furthering anticancer research, especially for monitoring the bioavailability and stability of such compounds of interest. Several studies have already been conducted in this direction, which points towards a feasible transition from cell lines to animal models. For instance, work carried out by Longato et al. (2011) showed that neolignans of *Piper regnellii* were potent against prostate, ovary, kidney and breast cancer cell lines in vitro and validated by its activity against Ehrlich solid tumor in vivo. Similarly, Akindele et al. (2015) researched that an extract of *Sansevieria liberica* strongly inhibited HeLa cells invitro and demonstrated strong antitumor activity in invivo Sarcoma-180 ascites and L1210 lymphoid leukaemia models.

5.3.6 Working towards a circular economy

Society, by default, follows the linear system of economy [Figure 43 (a)] whereby raw materials are used for the manufacturing process and all wastes arising from the process discarded. Conversely, a circular economy [Figure 43 (b)] is a type of economic system that advocates the complete utilization of raw materials with an output of minimal waste. This approach of phasing out waste and pollution is gaining widespread popularity, especially in the field of industrial ecology, production economics and operations research (Lahti et al., 2018).

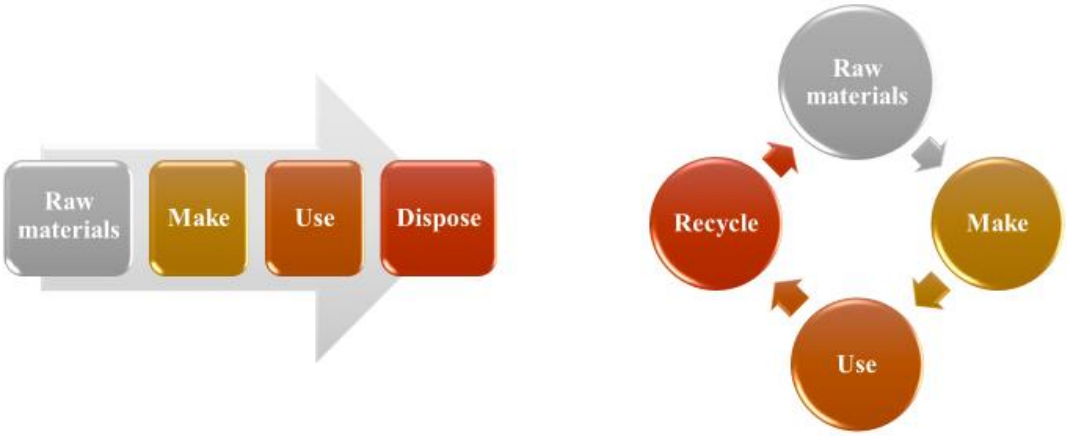


Figure 43 : (a) Linear economy, (b) Circular economy. *Today, more and more researches are geared towards incorporating a circular economy as a sustainable means to prevent generation of waste. This approach may be gainfully employed by food industries, as agricultural wastes hold a lot of potential to be reused and recycled.*

According to the concept of circular economy, growth and prosperity are dissociated from the consumption of natural resources and degradation of the ecosystem. This is designed to inspire a healthy economy that is in balance with nature and its resources. Aimed towards sustainability, this idea chiefly implements the following

policies: a) continuous regeneration of renewable resources, b) increased lifespan of manufactured products, c) linked value chains towards a 'zero waste' system, and d) markets designed for optimal access and use of manufactured products.

In context of this study, a linear economy entails the current handling of soybean; after the processing of the legume for the production of tofu, soymilk and soy oil, the residual waste parts are incinerated and/or dumped in landfills. This thesis attempts to suggest a method to change this linear economy to a circular one by proposing biofermentation as a valid amelioration technique to recycle okara. Further fibrous biomass generated from this work may be reused for making biodegradable hydrogels (Sannino et al., 2009) and plastics (Nawrath et al., 1995), thus giving rise to a 'zero waste' food processing system.

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LIST OF PUBLICATIONS

1. Gupta S., Lee J. J. L., Chen W.N., (2018). An analysis of improved nutritional composition of potential functional food (okara) after probiotic solid-state fermentation. *Journal of Agricultural and Food Chemistry*. 66 (21) : 5373-81.

2. Gupta S., Chen W.N., (2019). Characterization and in-vitro bioactivity of green extract from fermented soybean waste. *ACS Omega*. 4 (26) : 21675-83.

APPENDIX

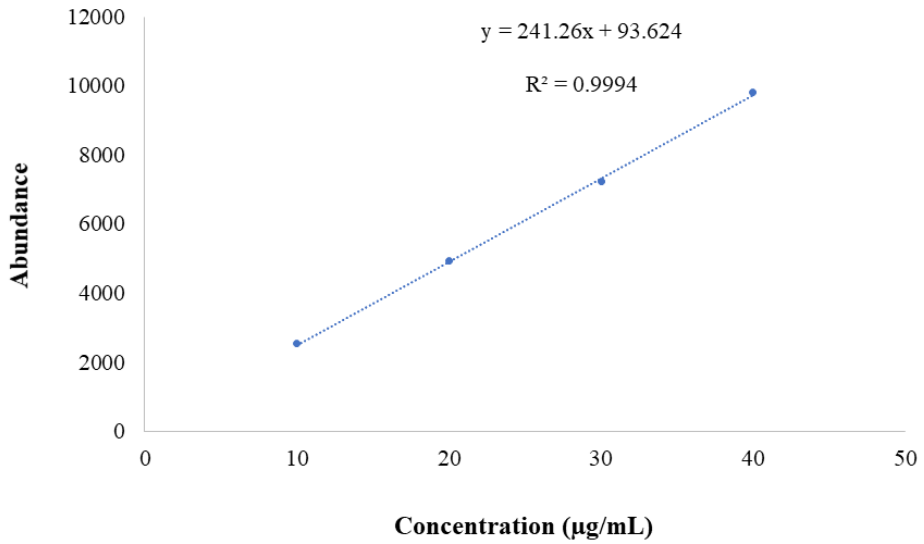

Figure S1 : (a) Standard curve of daidzein. *Concentration range: 10-40 µg/mL*

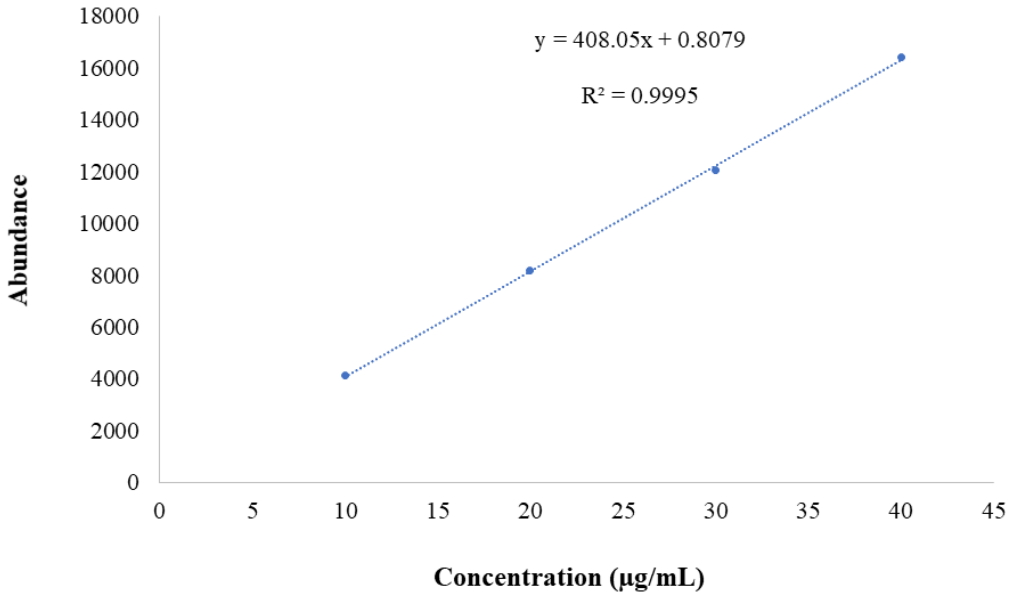

Figure S1 : (b) Standard curve of genistein. *Concentration range: 10-40 µg/mL.*

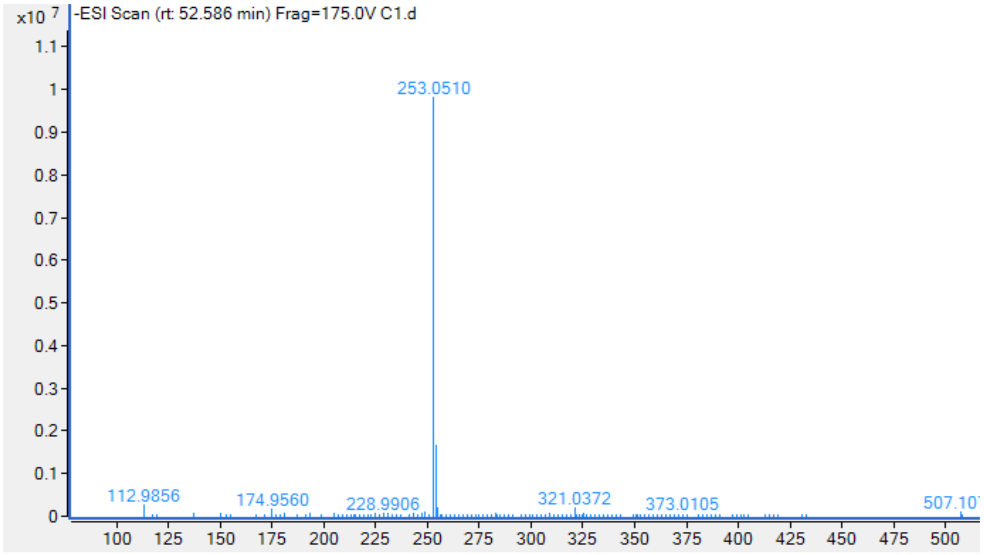

Figure S2 : (a) m/z spectrum of daidzein in unfermented okara extract. *m/z of daidzein is 253.05.*

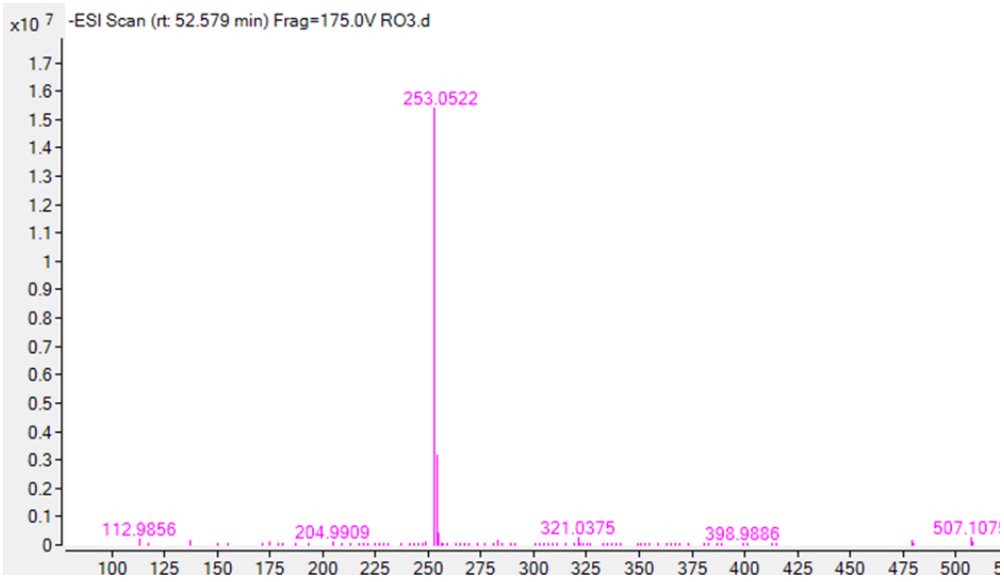

Figure S2 : (b) m/z spectrum of daidzein in fermented okara extract. *m/z of daidzein is 253.05*

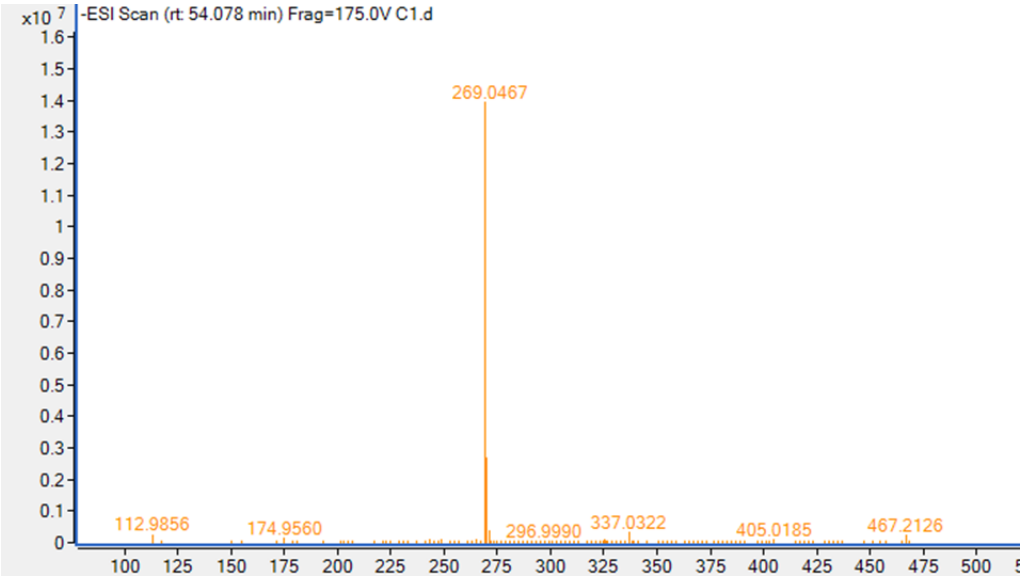

Figure S3 : (a) m/z spectrum of gensitein in unfermented okara extract. *m/z of genistein is 269.04.*

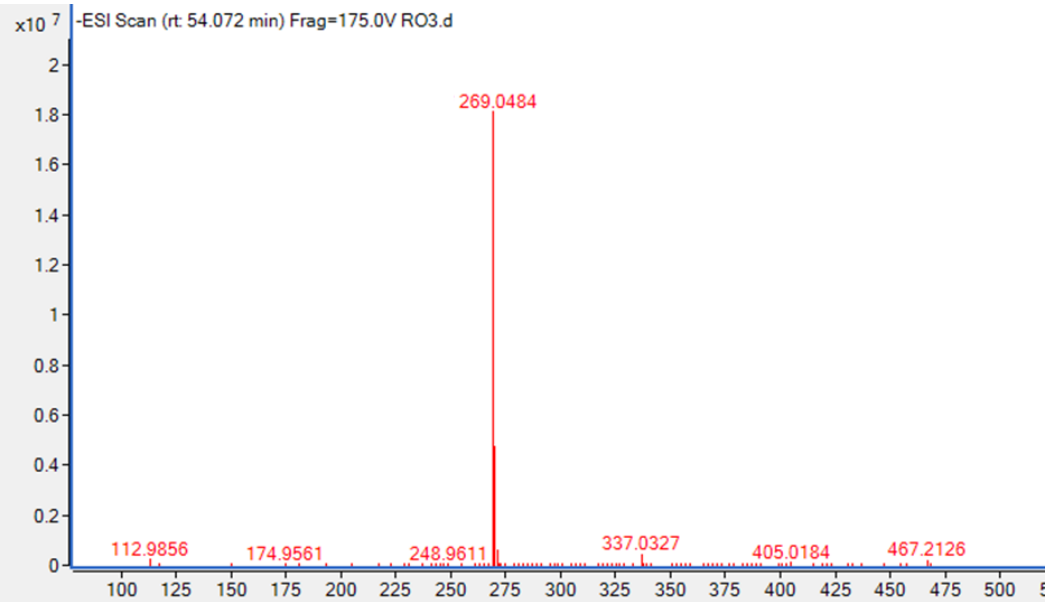

Figure S3 : (b) m/z spectrum of genistein in fermented okara extract. *m/z of genistein is 269.04.*