KHOO SHING CHING

DOCTOR OF PHILOSOPHY

2025

CHARACTERISATION OF PROBIOTIC FERMENTED CABBAGE AND ITS POTENTIAL IN AMELIORATING THE PESTICIDE-INDUCED GUT DYSBIOSIS, AND PHYSIOCHEMICAL ALTERATION

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KHOO SHING CHING

Thesis Submitted in Fulfilment of the Requirement for the Doctor of Philosophy of Science in the Faculty of Science and Marine Environment Universiti Malaysia Terengganu

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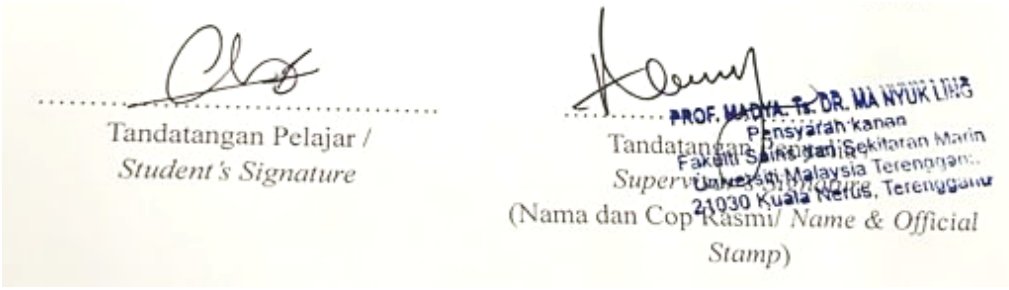

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DEDICATION

Be brave and dedicate in facing every single challenge in life

Self-motivation and many efforts needed to put on in research study

To my supervisor Assoc.Prof. Ts. Dr. Ma Nyuk Ling:

(For your valuable encouragement, guidance and support)

To my beloved boyfriend Lee Chia Hau:

(For endless encouragement and unwavering belief in me fueled this journey)

To my beloved parents and siblings:

(For youreternal love, countless supports and motivations)

To my labmates and friends

(For your helpful supports in my PhD research study)

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfilment of the requirements for the degree of Doctor of Philosophy

CHARACTERISATION OF PROBIOTIC FERMENTED CABBAGE AND ITS POTENTIAL IN AMELIORATING THE PESTICIDE-INDUCED GUT DYSBIOSIS AND PHYSIOCHEMICAL ALTERATIONS

KHOO SHING CHING

APRIL 2025

Main Supervisor	**:**	**Associate Professor Ma Nyuk Ling, Ph.D**
Co-Supervisor 1	**:**	**Professor Mohd Effendy Abd Wahid, Ph.D**
Co-Supervisor 2	**:**	**Associate Professor Vijitra Luang-In, Ph.D**
Faculty	**:**	**Faculty of Science and Marine Environment**

There is growing interest in synbiotic functional food in the development of health-benefit products. However, there is limited knowledge about probiotic-food-gut microbiota interactions, leading to many uncertainties in their development and application. This study aimed to explore the probiotic potential of eight selected strains from fermented products. All probiotics had genetically identified and demonstrated resilience towards all stress factors. Among the probiotic groups, *Enterobacter asburiae* exhibited the highest growth rate with robust tolerance in bile salt, sodium chloride, pH and temperature. *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* exhibited excellent gastric acid resistance with survival rates of 122% and 126%, respectively. All probiotics exhibited different carbon utilisation patterns, demonstrating the functional diversity and adaptivity in the carbon metabolism. This study also focused on the development of synbiotic probiotic fermented cabbage (PFC). The combination of eight selected probiotics under fermentation conditions of 25°C with 3% salt concentration for 48 hours was identified as the optimal condition for PFC production. The optimised formulation exhibited the highest levels of flavonoids (818.6 mg QE/100 g), phenolics (494.62 mg GA/100 g), and glucosinolates (25.097 mg RS/100 g), and demonstrated significantly higher antioxidant and cytotoxic properties compared to raw cabbage. Examination of the simultaneous feeding effect

of PFC and pesticide exposure revealed that rat group C with a single dose of 125 mg PFC/rat kg showed optimal growth after six weeks of treatment, with lower oxidative stress and lower total cholesterol compared to other rat groups. Under pesticide stress, rat group F with a middle dose of 125 mg PFC/rat kg demonstrated better health growth parameters in the renal profile and higher composition of healthy gut microbiota such as Bacteroidota, and Firmicutes and decreased in the abundance of harmful Proteobacteria. This study also provided insight into the interplay of predicted gene functional pathways and enzymatic activities upon PFC and pesticide treatments through 16S rRNA sequencing. All the findings underscored the significant potential of PFC as a functional food, marking a pivotal advancement in the development of synbiotic functional foods and chronic disease prevention. These results demonstrated its versatile applications across the healthcare, food industry, and agriculture sectors.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENCIRIAN KUBIS FERMENTASI PROBIOTIK DAN POTENSI DALAM MENGURANGKAN DISBIOSIS USUS DAN PERUBAHAN FISIOKIMIA YANG DISEBABKAN OLEH RACUN PEROSAK

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Terdapat peningkatan minat terhadap makanan berfungsi sinbiotik dalam pembangunan produk yang memberi manfaat kepada kesihatan. Walau bagaimanapun, pengetahuan mengenai interaksi probiotik-makanan-mikrobiota usus adalah terhad, menyebabkan banyak ketidakpastian dalam pembangunan dan aplikasinya. Kajian ini bertujuan untuk meneroka potensi probiotik daripada lapan strain terpilih yang diperoleh daripada produk fermentasi. Semua probiotik telah dikenalpasti secara genetik dan menunjukkan daya tahan terhadap pelbagai faktor tekanan. Dalam kalangan kumpulan probiotik, *Enterobacter asburiae* menunjukkan kadar pertumbuhan tertinggi dengan toleransi yang kukuh terhadap garam hempedu, natrium klorida, pH dan suhu. *Lactiplantibacillus plantarum* dan *Pediococcus pentosaceus* pula mempamerkan ketahanan asid gastrik yang sangat baik dengan kadar kelangsungan hidup masing-masing sebanyak 122% dan 126%. Kesemua probiotik mempamerkan corak penggunaan karbon yang berbeza, sekaligus menunjukkan kepelbagaian fungsi dan penyesuaian dalam metabolisma karbon. Kajian ini juga memberi tumpuan kepada pembangunan produk fermentasi kubis probiotik sinbiotik (PFC). Kombinasi lapan probiotik terpilih di bawah keadaan fermentasi pada suhu 25°C dengan kepekatan garam 3% selama 48 jam telah dikenalpasti sebagai keadaan optimum untuk penghasilan PFC. Formulasi optimum ini mempamerkan aras

flavonoid tertinggi (818.6 mg QE/ 100 g), fenolik (494.62 mg GA/ 100g), dan glukosinolat (25.097 mg / 100 g), serta menunjukkan sifat antioksidan dan sitotoksik yang jauh lebih tinggi berbanding kubis mentah. Pemerhatian terhadap kesan pemakanan serentak PFC dan pendedahan racun perosak menunjukkan kumpulan tikus C yang menerima dos tunggal PFC 125 mg PFC/kg tikus menunjukkan pertumbuhan optimum selepas rawatan selama enam minggu, dengan tekanan oksidatif dan jumlah kolesterol yang lebih rendah berbanding kumpulan tikus yang lain. Di bawah tekanan pendedahan racun perosak, kumpulan tikus F dengan dos pertengahan PFC pada 125 mg PFC/kg tikus menunjukkan parameter pertumbuhan kesihatan yang lebih baik dalam profil buah pinggang dan komposisi mikrobiota usus yang lebih sihat seperti Bacteroidota dan Firmiculates, serta penurunan ketara Proteobacteria yang berbahaya. Kajian ini juga memberikan gambaran tentang interaksi laluan fungsi gen yang diramalkan dan aktiviti enzimatik hasil rawatan PFC dan racun perosak melalui penjujukan 16S rRNA. Keseluruhan penemuan ini menekankan potensi besar PFC sebagai makanan berfungsi, sekaligus menandakan kemajuan penting dalam pembangunan makanan berfungsi sinbiotik dan pencegahan penyakit kronik. Hasil kajian ini juga menunjukkan aplikasi serba boleh PFC merentasi penjagaan kesihatan, industri makanan dan sektor pertanian.

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APPROVALS

I certify that an Examination Committee has met on **24 April 2025** to conduct the final examination of Khoo Shing Ching on her Doctor of Philosophy thesis entitled **"Characterisation of Probiotic Fermented Cabbage and Its Potential in Ameliorating the Pesticide-induced Gut Dysbiosis and Physiochemical Alteration"** in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded Doctor of Philosophy degree. The members of the Examination Committee are as follows:

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This thesis has been accepted by the Senate of Universiti Malaysia Terengganu in fulfilment of the requirement for the Doctor of Philosophy.

_____

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Date

DECRALATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.

KHOO SHING CHING
Date:

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LIST OF ABBREVIATIONS

NaCl	Sodium chloride
PFC	Probiotic fermented cabbage
QE	Quercetin
GA	Gallic acid
RS	Rapeseed standard
rRNA	Ribosomal ribonucleic acid
h	Hour
GI	Gastrointestinal
GBA	Gut brain axis
ENS	Enteric nervous system
CAGR	Compound annual growth rate
GSLs	Glucosinolates
ITCs	Isothiocyanates
USD	United state dollar
SCFAs	Short chain fatty acid
GRAS	Generally recognised as safe
ATP	Adenosine triphosphate
EMP	Embden Meyerhof Parnas
ISAPP	International Scientific Association for Probiotics and Prebiotics
IBD	Inflammatory bowel diseases
LDL	Low density lipoprotein
LAB	Lactic acid bacteria
EPS	Exopolysacharrides
GABA	Gamma aminobutyric acid
SD	Sprague Dawley
ER+	Estrogen receptor positive
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenultetrazolium bromide
KRAS	Kirsten rat sarcoma viral oncogene homolog
DNA	Deoxyribonucleic acid
CFU	Colony forming unit
LA	Luria Bertani agar
MRSA	De Man Rogosa Sharpe agar
LB	Luria Bertani broth
MRSB	De Man Rogosa Sharpe broth
K_2HPO_4	Dipotassium phosphate
DF	Dilution factor
UV-Vis	Ultraviolet visible
WCD	Well colour development
CSU	Carbon substrate utilisation
ANOVA	One way analysis of variance
PCA	Principle component analysis
PLS-DA	Partial least squares discriminant analysis
SIMCA	Soft independent modelling by class analogy
NCBI	National Center for Biotechnology Information

AC	Accession code
PI	Percentage identity
QC	Query coverage
HSP	High alignment score points
LP	Lag phase
EP	Exponential phase
DP	Death phase
SPSS	Statistical Package for the Social Sciences
mL	Millilitre
µL	Microlitre
mg	Microgram
µg	Microgram
rpm	Revolutions per minutes
Q	Quadrant
PC	Principal component
NAG	N-acetyl-D-glucosamine
M2DH	Mannitol-2-dehyrogenase
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
TFC	Total flavonoid contents
TPC	Total phenolic contents
TGLs	Total glucosinolate contents
RC	Raw cabbage
PC	Positive control
NC	Negative control
K	Potassium
Ca	Calcium
Fe	Iron
Cu	Copper
Mn	Manganese
Zn	Zinc
Al	Aluminium
B	Baron
ICP-OES	Inductively coupled plasma-optimal emission spectrometry
HCl	Hydrochloric acid
HNO_3	Nitric acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
IDRS	Inhibition of DPPH radical scavenging activity
IC_{50}	Half maximal inhibitory concentration
RSM	Response surface modelling methodology
GCMS	Gas chromatography mass spectroscopy
$MgSO_4$	Magnesium sulphate
UMT	Universiti Malaysia Terengganu
DMEM	Dulbecco's Modified Eagle medium
RPMI	Roswell Park Memorial Institute medium
ATCC	American Type Culture Collection
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DHE	Dihydroethidium

RFU	Relative fluorescence unit
HMF	5-Hydroxymethylfurfural
NAC	N-acetyl cysteine
IACUC	Institutional Animal Care and Use Committee
IP	Intraperitoneal
EDTA	Ethylenediaminetetraacetic acid
CAT	Catalase
MDA	Malondialdehyde
SOD	Superoxide dimutase
GSH	Reduced gluthathione
GSSH	Oxidised gluthathione
GPx	Gluthathione peroxidase
LPO	Lipid peroxidation
TBARS	Thiobarbituric acid reactive substances
H_2O_2	Hydrogen peroxide
$O_2^{\bullet-}$	Superoxide radical
•OH	Hydroxyl radical
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
TBA	2-thio-barbituric acid
EPS	Extracellular polymeric substances
LPS	Lipopolysaccharides
JAM	Junctional adhesion molecules
GALT	Gut associated lymphocyte tissues
T_{regs}	Regulatory T cells
T_c	Cytotoxic T cells
T_h	Helper T cells
IL	Interleukin
TNF-β	Tumore necrosis factor-beta
DF	Dilution factor
SIR	SOD inhibition rate
DTNB	5,5'-dithio-bis-2-nitrobenzoic acid
TMTP	1,1,3,3-tetrametoxypropane
PCR	Polymerase chain reaction
ASV	Amplicon sequence variants
KOs	KEGG orthologs
EC	Enzyme commission number
PWK	Assigned metabolic pathway
STAMP	Statistical analysis of metagenomic profiles
RBC	Red blood cells
WBC	White blood cells
Hb	Haemoglobin
PCV	Packed cell volume
MCV	Mean corpuscular volume
MCH	Mean corpuscular haemoglobin
RDW	Red cell distribution width
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphate
TC	Total cholesterol

HDL	High density lipoprotein
TLR4	Toll-like receptor 4
WMS	whole-metagenome sequencing
OECD	Organisation of economic co-operation and development
CLB	Cell lysis buffer
BB	Binding buffer
NsB	Nucleic acid binding silica beads
dNTP	Deoxynucleotides triphosphate
gDNA	Genomic deoxyribo-nucleic acid
BLAST	Basic local alignment search tool
MS	Max score
PI	Percentage identity
QC	Query coverage
v/v	Volume/ volume
mg/kg	Milligram/ kilogram
kPa	kilopascal
kV	kilovoltage
m/z	Mass to charge ratio
nm	Nanometre
mins	Minutes
°	Degree
<	Less than
>	More than
%	Percentage
±	Plus, minus
cm	Centimetre
s	Second

LIST OF FORMULAS

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Exposure to environmental pollutants, such as pesticides, disrupts immune and reproductive health and contributes to diseases like cancer and diabetes (Giambò et al., 2021). Ingestion of food with pesticide residues can also cause acute and chronic health effects, including skin and eye irritation, respiratory problems, and neurological disorders (Al-Ghanim et al., 2020). Gastrointestinal (GI) tract as the first biological and physical barrier towards contaminated food had become the first exposure site to understand on the association of pesticide exposome and gut microbiota (Yuan et al., 2019).

The gut as one of the most vital organ that play significant roles in nutrient absorption, health regulation and immunity defences (Li, J. et al., 2018). Generally, gut microbiota can be defined as a dense groups of bacteria, fungi and viruses residing in the intestinal tract or gut that displayed mutualistic relationships with their host within specific environment (Sender et al., 2016). The imbalance and dysfunction of the gut microbiota can contribute to psychiatric problems, obesity, autoimmunity and chronic GI disorders, to name but a few (Collins et al., 2012; Cryan & Dinan, 2012). Molecular study on the gut microbiome (where the collection of genome of microbe within the specific environment) had been widely studied in animal models such as rat, mice and zebrafish and also human. However, the investigation of diverse and numerous microbiome dataset is complicated. High biological knowledge backgrounds and skills on analysis tools are required for accurate prediction on the

influence factors of microbe-microbe interaction network in association with health risk and diseases outcomes (Belizário & Faintuch, 2018). In addition, pesticide exposomes can disrupt the gut-brain axis (GBA) by altering the gut microbiota and causing neurotoxicity, potentially leading to neurodegenerative disorders over time. This research focuses on exploring the effects of probiotic and synbiotic functional foods in restoring gut microbiota balance and mitigating these disruptions.

A global habit shift towards healthy lifestyle had emphasised the needs for more healthy food and supplement (Alizadeh Behbahani et al., 2024). The introduction of synbiotic functional food had tackled emerging research interests in fulfilling demand of healthy product (Yadav et al., 2024). Synbiotic functional food is derived from the probiotic (live microorganism with health benefit) and prebiotic (dietary fibre that stimulate growth of gut bacteria). The global functional foods market is expected to reach $275.77 billion by 2025, growing at a compound annual growth rate (CAGR) of 7.9% from 2018 to 2025 (Grand View Research, 2022.). Functional foods can take various forms, including fortified beverages, probiotic yogurts, and snack bars with added vitamins and minerals. They exhibited various beneficial bioactive compounds like antioxidants or phytochemicals and known for the improvement of gut health and overall well-being (Damián et al., 2022).

The high demand for synbiotic functional foods is closely associated with the wide application of probiotics in the food and health industry (Akram et al., 2024). Probiotics are live microorganisms that offer health benefits when consumed in adequate amounts (Vinderola & Reinheimer, 2003). Probiotics, known for their potential health-promoting properties, have gained attention for their ability to modulate the gut microbiota and enhance the antioxidant defense system in the host (Akram et al., 2024). Probiotic had been recognised as alternative unconventionally approaches with numerous advantageous features including feed utilisation, survival, disease resistance and immunity enhancement and activity improvement of gastrointestinal microbiota (De et al., 2014). The study of intestinal microbiota with probiotic based functional food is crucial to maintain the immune homeostasis, and trigger sufficient biological defence response towards infection and diseases (Han et al., 2015).

Green cabbage (*Brassica oleracea*) is a unique cruciferous vegetable from Brassiacae family. Cabbage can act as potent good prebiotic source because they consist of a group of thioglucoside (also known as glucosinolate) that can be hydrolysed by myrosinase enzymes forming biologically active secondary metabolites such as isothiocyanates (ITCs), sulforaphane and indole (Oloyede et al., 2021).High level of glucosinolates (GSLs) in cabbage can be converted into ITCs. These breakdown by-products had been reported with good health-promoting values such as antioxidant, anti-inflammation, anti-bacterial properties (Gaafar et al., 2014; Chun et al., 2004; Drozdowska et al., 2020). In addition, cabbage demonstrated several benefits over other vegetables, including low cost, high availability, and long shelf life. They possess minimal processing and higher versatility in its consumption compared to other functional food that requiring lengthy processing procedure. However, natural GSLs always destroyed by the cooking process. Nevertheless, the exogenous interaction of bacterial community has demonstrated the ability to produce ITCs. Therefore, this study aimed to use selected probiotics to enhance the production of ITCs by hydrolysis process.

Fermentation is the best food-processing method to preserve fresh foods and effectively extend the shelf-life of foods (Godbey., 2022). During fermentation, endogenous enzymes and autochthonous microorganisms cause reactions that alter the bioactivity profile and bioactive compounds in cabbages. For instance, fermentative microbes produce peptides, organic acids, amino acids, and antioxidant compounds (flavonoid and phenolic) and change the physiochemical aspects of the fermentation products (Zhang et al., 2021). Additionally, GSLs and phenolic compounds in cabbages have been reported to be transformed into active molecules during fermentation, in which the fermented cabbage with an increased biological value that has anti-cancer properties to health (Šamec et al., 2016). Both previous studies found that active compounds from fermented cabbage can potentially be implemented in functional foods as bio-therapeutics with anticancer properties against cancer cells. The coupled effect of synbiotic with fermentation contributed to chemical changes and flavour development, and was found to improve the overall nutrient profile and bioactive compounds (Dahiya & Nigam, 2022a).

In summary, this study aimed to investigate the characterisation of selected probiotic, and utilise the probiotic in the development of synbiotic product PFC that can yield high bioactive compounds with great health benefit. To fortify our understanding about the beneficial properties of synbiotic PFC and probiotic feeding in rat, the gut microbiota and physiological responses of rat in the present of pesticide treatment are crucially important to be discovered. The manipulation of gut microbiota function at the onset of stress may offer a potential avenue for the prevention and treatment for mental disorder and other neurodegenerative illness.

1.2 Problem statement

The selection of suitable probiotic is often challenging as their efficacy depends heavily on the specific strain used, from the production, storage to consumption process (Hathi et al., 2021). In addition, there is a lack of global standardization and unclear regulation in probiotic strain selection, which causing significant variation in product quality and consumer trust (Bustos et al., 2024). Therefore, known stress tolerant probiotic is vital for maximal survival performance with enhanced product stability and maintained cell viability across various stress during the manufacture, storage and delivery process (Wendel, 2022).

The growing market in the synbiotic functional food also encountered significant hurdles due to the complexity of prebiotic-probiotic interactions. Cost is another challenge, as functional foods are often more expensive than traditional foods, making it difficult for companies to reach a wider audience (Niamh Huny, 2022). Many "functional" products are not backed by scientific evidence, leading to increased scrutiny from regulatory bodies (Niamh Huny, 2022). Additionally, the information about the functional properties of probiotics fermented cabbage that produced from combination of various probiotic strains is still remaining scarce. Thus, it is needy to develop cheap and scientific proven synbiotic functional food from known probiotic sources.

The study on the gut microbiota in rat is promising to provide intrinsic fundamental information about the roles of probiotic fermented cabbage (PFC) on the nutrient supply in rat, yet it is still in pioneer research stage. Besides that, exposure to agricultural pesticides such as organophosphate, organochlorine, pyrethroids and carbamate has been linked to cancer development and associated with the alteration of gut microbiome. Pesticide exposure can lead to gut dysbiosis through reduction of beneficial gut microbiome and promotion of pathogenic microbes. The pesticide-induced gut microbiome changes can may indirectly promote carcinogenesis, highlighting a need for further research on this interplay. Therefore, the synbiotic fermented food may offer a promising avenue to counteract the oxidative damage induced by pesticide exposure. However, despite the theoretical basis, there is a lack of comprehensive research exploring the potential of PFC in mitigating oxidative stress induced by pesticide treatments.

To address aforementioned problems, this study explored the probiotic potential of selected strains from fermented food sources. Native Brassica vegetables (cabbages) were used to explore the potential of bioactive compounds and functional properties like antioxidant and cytotoxicity properties by fermentation of probiotics consortium. This study also aimed to observe the physiological changes and gut microbiota of rat *in-vivo* under pesticide treatment. The health beneficial properties of PFC had been investigated in rat that feed with pesticide contaminated diets. Therefore, the objectives of this study had been designed to solve or minimise all the problems encountered in this study.

1.3 Significance of study

This study is significant to provide reliable and precise genetic information in the DNA level about the selected probiotic strains. Accurate species identification can ensure the right probiotic strains validation, support accurate health benefits claims, and enabl detailed characterisation of their potential interactions in the scientific study. Besides that, detailed evaluations on the selected functional probiotic strains including their growth patterns and various stress tolerance tests can ensure the survivability and

viability of selected strains under unfavorable fermented environment and acidic gastrointestinal environment. The carbon metabolism profiling is crucial to explore the carbon source utilisation pattern and provide insight on the prebiotic utilisation efficiency. With the efforts of various probiotic characterisation analysis, this study greatly aided in the development of functional food and health benefit product.

This study provides comparative insight on the individual strain specificity and multi-strains synergy effect for the optimal yield of bioactive compounds and antioxidant properties. The optimisation modelling performed with higher yields allow cost-effective production and broader health impacts. This study also contributed to the development cost-effective synbiotic PFC, which offers promising potential to enhance nutritional value, reduce the incidence of cancer, and advance future medical applications.

Besides that, this study can provide a clear picture on the effect of PFC and pesticide on the oxidative stress and gut microbiota changes in rat. The synbiotic fermented food may offer a promising avenue to counteract the oxidative damage induced by pesticide exposure. In overall, the whole study contributed great efforts to promote development of cost effective synbiotic functional food that possess great yield of health benefit bioactive compounds and seeks to contribute valuable insights into the potential of functional foods to alleviate the health risks associated with pesticide exposure and unlock the knowledge gap between the interplay between probiotics, fermentation, and pesticide-induced gut dysbiosis.

1.4 Research questions

The research questions are:

1. How do the selected probiotic stains perform in term of growth behavior, stress tolerance and carbon metabolism under simulated production and storage conditions?
2. What are the optimal fermentation conditions for production of PFC with high yield of bioactive compounds and enhanced functional properties?

3. How does PFC supplementation mitigate pesticide-induced gut dysbiosis and physiological alteration in rats?

1.5 Objective of study

The objectives of this study are:

1. To characterise the selected probiotic strains by evaluating their growth behavior, stress tolerance, and carbon metabolism.
2. To optimise the fermentation process for probiotic-fermented cabbage (PFC) and identify the yield of bioactive compounds with enhanced antioxidant and cytotoxicity properties.
3. To assess the impact of PFC supplementation on pesticide-induced physiological changes, oxidative stress, and gut microbiota composition *in-vivo*.

1.6 Research scope

The scopes of this study are:

1. Identification of the selected probiotic strains using Sanger DNA sequencing.
2. Assessment on the growth behaviour, stress tolerance profiles and carbon metabolism profile to characterise the selected functional probiotic strains.
3. Investigation of the single and multiple probiotic consortium effect on the PFC production.
4. Optimisations on the fermentation conditions such as pH, temperature, gastric acid, bile salt and fermentation period to maximise bioactive compound yields in PFC.
5. In-vivo assessment of PFC efficacy to evaluate the physiological alterations and gut microbiota changes following pesticide exposure and PFC feeding.

1.7 Hypotheses

The hypotheses for this study are:

1. Probiotic strains exhibit high stress tolerance and carbon metabolism, making them suitable for functional food production due to their specific metabolic pathway and great capability in maintaining viability under stress.
2. Fermentation with probiotics significantly enhances cabbage's bioactive compound, antioxidant and cytotoxicity properties as compared to raw cabbage.
3. PFC supplementation mitigates pesticide-induced gut damage and improves oxidative stress markers due to production of various bioactive compounds that enhanced the overall functional properties.

For better understanding of the overall project's scope, an overview of the experiment flow in this study had summarised in Figure 1.1.

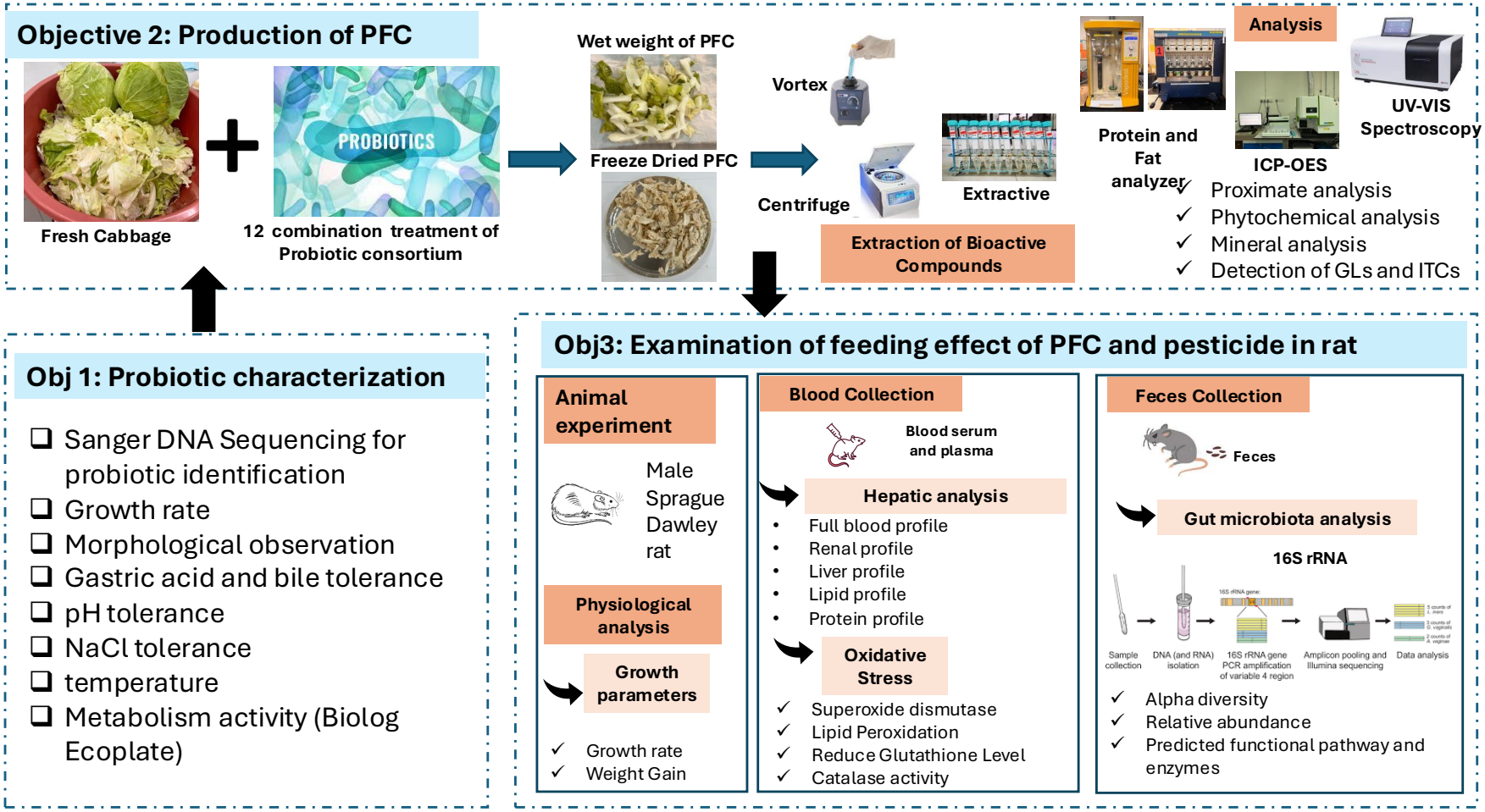


Figure 1.1 Overview of the experimental design, including probiotic characterisation, PFC fermentation, and rat model evaluation

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

Probiotics have garnered increasing attention due to their ability to regulate gut health, improve immune function and overall wellness. Probiotics are combination of good bacteria microorganism that confer health benefit and eliminate harmful bacteria within the host (Nambiar et al., 2023). The global market value of probiotic had been reported about 77.12 billion USD in 2022 and expected to strike up about 14% of compound annual growth rate in 2030 (Grand View Research, 2023). The probiotic can interact with broad diversity of gut microbiome in the host to maintain the balance of intestinal gut microbiota. It is hence contributing significant benefits towards overall gut health, including improve gut barrier protection, improve lactose intolerance and secret specific enzyme to degrade enteric antigens (Suez et al., 2019).

Probiotics play a pivotal role in human health due to their high digestibility and efficacy in degrading of complex compounds such as carbohydrates and large complex molecules from food (Dahiya & Nigam, 2022b). Through enzymatic metabolism, probiotics can produce various useful metabolites, such as oligosaccharides and short chain fatty acids (SCFAs). The production of SCFAs like acetate, propionate, and butyrate can acts signalling molecules between the host and gut microbiota, act as a key regulator in the peripheral and intermediary metabolism and promote cell apoptosis in cancer cells (Chin et al., 2024; Besten et al., 2013;

Morrison & Preston, 2016). Probiotics can promote the protein digestion with secretion of more functional amino acids, in the same increase the absorption of amino acids and thus leading to an overall improvement of the nutrient bio-availability (Jäger et al., 2020; Walden et al., 2022).

Intake of probiotics had significantly improved antioxidant activities and reduced the oxidative damage within the host (Wang, Y. et al., 2017). Probiotic can increase total oxygen availability and alter the overall pH within the GI tract to mimic the growth of the harmful pathogen. A study showed that multiple probiotic strains (namely SLAB51) can improve oxygenation in the patient with in respiratory distress syndrome (Baldassarre et al., 2023).

Probiotics are unique as they possess several functional mechanisms such as elimination of harmful organism through antagonism, specific binding interaction with host, improve gut barrier with strengthening the epithelial and also production of antimicrobial agents against the pathogen (Javanshir et al., 2021). Hutt et al. (2006) demonstrated the antagonistic properties of Bifidobacterium and Lactobacillus against pathogen through growth suppression of urinary and enteric pathogens. The beneficial role of probiotic in gut can be further seen from the recovery of antibiotic therapy treatment to restore and improve the gut microbiome (Dudek-Wicher et al., 2020). Chin et al. (2024) reviewed that probiotic from genera *Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus* and *Streptococcus* showed improvement of gut health through several mechanisms such as production of antimicrobial peptides production of free radical like hydrogen peroxide to inhibit growth of harmful microorganism.

In addition, probiotic acts as key immunomodulators in stimulation of various immunological effect such as induce the production of cytokine and stimulate cell-mediated response towards external stress (Fong et al., 2022). Probiotic can create immune suppression, stimulate immunogenicity to eliminate pathogens, strengthen immune system to fight against many harmful disease (Topol et al., 2023). There are emerging clinical and epidemiological studies supported the benefits of probiotics are significant associations with improvement of health and reduction of disease incidence (Garcia-Gonzalez et al., 2021). Therefore, probiotic bacteria had been used to combat various diseases, including type 2 diabetes (Li, G. et al., 2023), inflammatory bowel

disease (Han, M. et al., 2024; Roy & Dhaneshwar, 2023; Xia, B. et al., 2023), obesity (Calcaterra et al., 2023; DiMattia et al., 2024), enterocolitis (Lin & Wu, 2023; Sajankila et al., 2023), diarrhoea (Amiri Khosroshahi et al., 2023; Dahiya & Nigam, 2022b), colon cancer (Deng et al., 2023; Kumar et al., 2024), and allergic disease (Forouhandeh et al., 2024; Yang, J. et al., 2023).

2.1.1 Selection criteria of probiotic

As mentioned early in the problem statement, the selection of right strain of probiotics is challenging. There are 5 aspects needed to consider a microorganism as "probiotic". Firstly, the probiotic must be taxonomically recognised and prioritised on the strain-specific functionality (de Melo et al., 2018). Secondly, probiotics must be present in the product in sufficient numbers to meet the viability standard, and ensure its compatibility with the product matrix, as well as the storage and processing conditions to preserve desired properties and accurately label (Khushboo et al., 2023). For instance, probiotic must show capability to survive under gastrointestinal environment, including includes withstanding the acidic stomach (low pH) and the presence of bile salts in the intestine (Tegegne & Kebede, 2022). Thirdly, probiotic required to have competitive exclusive of pathogen, withstand the gastrointestinal condition, and capable to adhere to the mucosa of intestine in order to be recognised as probiotic (Pramanik et al., 2023). Next, safety to use and recognised as Generally Recognised as Safe (GRAS). Lastly being evidenced with at least one positive clinical trials that confer health benefit to humans (Rodríguez-Sojo et al., 2021). In short, the selection of probiotic is critically important in term of its safety, functionality and technological usability .

As the utilisation and variety of probiotic products increased, the right choice of the probiotics has become challenging. The selection of suitable probiotic is critically important for maximising the overall health benefits. The target probiotic strains need to meet specific criteria for success. Strain specific and the disease specificity are predominantly important based on its effectiveness towards target health purpose (McFarland et al., 2018). The multiple combination of probiotic strains

like *Lactobacillus rhamnosus, Lactobacillus casei,* and *Lactobacillus acidophilus* showed specific efficacy on the prevention of antibiotic associated diarrhea (McFarland et al., 2018). The mixture of *L. lactis* Bb12 and *L. rhamnosus* GG was proven to be more effective than single strain of *L. rhamnos*us alone in the prevention of *Helicobacter pylori* infection (McFarland, 2021).

Amongst vast diversity of probiotic strains, lactic acid bacteria from genera *Lactobacillus* and *Bifidobacter* were the most commonly used probiotic bacteria for human consumption (González-Herrera et al., 2021). These two genera showed specific characterisations like free from lipo-polysaccharides that might trigger inflammation, and release specific active metabolites to maintain the balance in GI tract (Dudek-Wicher et al., 2020). The Lactobacilli are non-spore forming Gram Positive bacteria with generally in the rod shape (Kullar et al., 2023). Due to vast diversity and functionality of this genera, reclassification of this genera had been updated by the scientist and further classified into 25 new genera based on the genetic differences and functional role (Oberg et al., 2022; Todorov et al., 2023). These popular new genera such as *Levilactobacillus, Limosilactobacillus, Lentilactobacillus, Lactoplantibacillus* and so on (Todorov et al., 2023).

2.1.2 Probiotic metabolism

Probiotic can be also divided into two group based on their metabolism activities, such as homofermentative metabolism and heterofermentative metabolism. Homofermentative metabolism involved in the production of primary end product of lactic acid up to 85-90% of the total production of fementation yield with yields more energy up to 2 ATP where this process is termed as glycolysis (EMP pathway) while heterofermentative metabolism is the fermentation that resulted in the production of end products consisting the mix of lactic acid, acetic acid, carbon dioxide using phosphoketolase pathway (Prückler et al., 2015). Homofermentative probiotics owned advantage with higher lactic acid production, which leading to lower pH that are beneficial in killing pathogenic bacteria, better preserving food, and enhance the colonization of probiotic within the gut (Krieger-Weber et al., 2020; Wang, T. et al.,

2020).The examples of homofermentative bacteria are *L. acidophilus* (Cunha et al., 2021), *L.lactis (Kondrotiene et al., 2023), Streptococcus thermophilus* (Han, M. et al., 2022), *Pediococcus acidilactici* (Bansal et al., 2022) and *Pediococcus pentosaceus* (Adesulu-Dahunsi et al., 2021) whereas heterofermentative bacteria are like *L.fermentum* (Capra et al., 2023), *Levilactobaccilus. brevis (Cataldo et al., 2020)*, and *Limosilactobacillus reuteri (Selvamani et al., 2022)*. *L. fermentum* showed its heterofermentative metabolism through the ability to metabolise different carbon source and the production of acetic acid during the exponential growth phase, and then accumulate metabolic products during the cell proliferation (Capra et al., 2023; Hossain, 2022). Table 2.1 summarises the metabolism type, health benefit and applications of the selected probiotic used in the recent literature studies.

Table 2.1 Metabolism type, health benefit and applications of selected probiotic used in this study.

Probiotic	Metabolism type	Health benefit and applications	References
Lactiplantibacillus plantarum	HMF	-antifungal and anti-mycotoxic activities	(Li, Q. et al., 2023)
L. plantarum	HMF	-great ability in the adhesion and absorption in the GI tract	(Garcia-Gonzalez et al., 2021)
L. plantarum 2-33	HMF	-regulate energy metabolism -improve diversity of the intestinal flora	(Bao, W. et al., 2022)
L. plantarum Z-1	HMF	-antioxidant properties -anti-inflammatory activity	(Xu et al., 2024)
Lactococcus lactis	HMF	-exhibit antimicrobial peptide against MCF-7 cancer cell	
L. lactis NK34	HMF	-anticancer properties -reduce production of nitric oxide -reduce proinflammatory cytokines	(Han et al., 2015)
L. lactis subsp. *lactis*	HMF	-produce exopolysaccharides that exhibit good antioxidant activity	(Pan & Mei, 2010)
L. lactis NCDO 2118	HMF	-exhibit potent anti-inflammatory effect	(Luerce et al., 2014)
L.lactis subsp. *lactis* JCM5805	HMF	- improved intestinal health -enhance immune status	(Xia, Y. et al., 2018)
Limosilactobacillus fermentum	HTF	-improve cardiometabolic disorders -enhance gut microbiota impairment	(De Araújo Henriques Ferreira et al., 2022)
L. fermentum	HTF	-antidiabetic properties	(Lacerda et al., 2022)
L. fermentum WXZ 2-1	HTF	- exhibited high antioxidant capacity - improved the texture characteristics in milk fermentation	(Liu et al., 2024)
L. fermentum U-21	HTF	-potential postbiotic -demonstrated significant antioxidant potential	(Grishina et al., 2023)
L. fermentum 664	HTF	-anti-inflammatory effect	(Hao et al., 2024)
Levilactobacillus brevis KU15151	HTF	- high antioxidant effects -high adhesion activity to HT-29 cells	(Yang, S. et al., 2020)

L. brevis KU15147	HTF	-induced nitric oxide synthase -express tumour necrosis factors -high adhesion activity to HT-29 cell	(Kim et al., 2021)
L. brevis Lb13H	HTF	- inhibit *Listeria monocytogenes's* adhesion to intestinal cells -antimicrobial activity	(Rahmati-Joneidabad et al., 2024)
L. brevis 47f	HTF	-improve production of SCFAs -associated with antidepressant and antistress effect	(Olekhnovich et al., 2021)
Pediococcus pentosaceus	HMF	-high antioxidant activity in fermented gold milk -enhance production of fatty acid	(Balakrishnan & Agrawal, 2014)
P. pentosaceus 1101	HMF	- high resistance to acidity with 87% survival rate -resistant to bile salt with 99% survival rate	(Escobar-Sánchez et al., 2022)
P. pentosaceus NB-17	HMF	- stimulate immune activities -suppressed IL-4 productions -induce IL-12 and IFN -γ	(Jonganurakkun et al., 2008)
Enterobacter asburiae E7		-stimulate immune response -resistance to *Aeromonas veronii* infection	(Li, J. et al., 2023)
E. asburiae ES1	MAF	-produce laccase -exhibit high dephenolisation activity	(Edoamodu & Nwodo, 2022)
E. asburiae D2	MAF	-resistance to high salt -act as plant growth promoter	(Ning et al., 2024)

Notes: HMF= homofermentative metabolism, HTF=heterofermentative metabolism, MAF=mixed acid fermentation, IL= interleukin, IFN= interferon

2.2 Prebiotic

Prebiotic can be defined as the non-digestible fibre that support the growth and metabolite production of probiotic (Mishra et al., 2018). Experts from International Scientific Association for Probiotics and Prebiotics (ISAPP) had redefined the term "prebiotics" as substrates that are utilised by beneficial microorganisms to provide health benefits (González-Herrera et al., 2021). These benefits are especially the role play in the inhibition of GI tract, improvement in mental health and reduction of cardio metabolism (González-Herrera et al., 2021).

The selection of prebiotic is critically important in promoting the growth of probiotic in the development of synbiotic. Several criteria needed to be concerned on the selection of prebiotic was reported by Markowiak & Śliżewska, (2017), including ability to remain unabsorbed in the upper GI tract, poorly fermented by bacteria either beneficial or pathogenic in the oral cavity and bowel, readily fermented by intestinal microbiota, and possess great resistance in adverse acidic gastrointestinal environment. The intake of prebiotic is usually from the natural plant source such as grain, vegetables and fruits. These categories of plant are generally enriched with flavonoid and phenolic compounds which can contribute to significant health benefit (Yadav et al., 2024). These plant sources had been reported as good prebiotic candidate, such as microalgae (Patel et al., 2021), oat (Zhang, Y. et al., 2023), cabbage and curry tree leave (Bhatt et al., 2020).

Non-digestible carbohydrates such as inulin and its hydrolysis byproducts that extracted from wide range of plant sources like garlic, onion, chicory roots, and banana had been explored and reviewed in depth by many researchers (Bhanja et al., 2022; Hughes et al., 2022; Teferra, 2021). The prebiotic effect of inulin can cause upregulation of the bifidobacteria population in the gut. Torres-Maravilla et al. (2022) had reported on the interplay roles of prebiotic in supporting the growth of probiotics, including triggers changes in the production of different metabolites such as alter changes in the stool mass, colonic pH, the regulation in systematic health and modulation of intestinal flora. Prebiotic effect of dietary polyphenols like anthocyanin, proanthocyanidins and catechins had been proven in the preclinical studies(Alves-

Santos et al., 2020). Anthocyanin had been recognised as good prebiotic source and being reviewed in depth by Wang et al. (2022).

2.2.1 Brassicae vegetable cabbage

Among various prebiotic sources, Brassica vegetables like cabbage are unique due to their glucosinolate content, which can be transformed into bioactive compounds during fermentation. Cabbage contain important compound called glucosinolate that composed of thio-glucoside that is linked to sulfonated oxime group and amino acid derived side chain (Mitreiter & Gigolashvili, 2021). Glucosinolate can be hydrolysed by myrosinase enzymes forming biologically active secondary metabolites such as isothiocyanates, sulforaphane and indole (Oloyede et al., 2021). These breakdown by-products had been reported with good health-promoting values such as antioxidant, anti-inflammation, anti-bacterial properties (Gaafar et al., 2014; Chun et al., 2004; Drozdowska et al., 2020). Glucosinolate and its derivative compounds had been widely acknowledged for their distinctive benefits to plant defence and human nutrition (Grubb & Abel, 2006). The glucosinolate-myrosinase activity is greatly reduced by high heat due to degradation of enzymes.

Cabbage (*Brassica oleracea*) is important cruciferous vegetable from Brassicaceae family that are growing globally especially in the Asia countries. They are widely available in different colour and size and being cooked in a variety type of cuisine. The annual production of cabbage in Asia countries is estimated more than 1,000,00 tons, accounted for about 70% of the total global yield (Guo, 2022). Cabbage demonstrated several benefits over other vegetables, including low cost, high availability, and long shelf life (Song et al., 2023). They possess minimal processing and higher versatility in its consumption compared to other functional food that requiring lengthy processing procedure. However, these beneficial natural molecules are usually destroyed during the cooking process.

Fermentation is one of the good option for serving cabbage food. Fermented cabbage is generally a traditional product produced worldwide and goes by various

regional names, including *sauerkraut* in Europe and *pao cai* in China (Wang and Shao., 2018), *kimchi* in Korea, and *ka-lum-plee-dong* in Thailand, fermented turnips and pickle in different countries. It is popular in various culinary dishes (Bousquet et al., 2021). In modern food industry, fermented cabbage has gained popularity in the market as the development of synbiotic functional food has grown.

2.3 Synbiotic functional food

In recent decade, most of the people have a shift of food preference for the functional food, opt for better food quality products with health benefit. The shifting of eating habit had increased the demand on the functional food. Functional food can be defined as the food product with specific constituents that confer health benefit (Barros et al., 2022). Some claimed that functional food is the food with enriched probiotics strain that can improve gut health and overall well-being (Damián et al., 2022). The definition of functional food can be varied due to the complexness and inaccurate claims from the commons. In latest updates, the definition of functional food is food compound with one or more beneficial effect that can improve the viability of probiotic with new technological improvements (Nambiar et al., 2023).

The emerging of the concept of synbiotic functional food begin when scientists found out that the simultaneous effect on the incorporation of probiotic and prebiotic can significantly improve the overall health compared to single effect of probiotic and prebiotic. The term “synbiotic” is firstly recognised by Gibson & Roberfroid (1995), which revealed the combination of probiotic and prebiotic can enhanced the viability and survival of probiotic strains, and promote their colonization within GI tract.

There are two forms of synbiotics including complementary and synergistic. Complementary symbiotic products are usually comprise of both probiotic and prebiotic, but they work separately to gain one or more functional health benefits. One of the example of complementary synbiotic can be seen in the case of prevention of colibacillosis in the pigs, where the use of lactulose (prebiotic) did not support the growth of probiotic *Lactiplantibacillus plantarum* (Guerra-Ordaz et al., 2014).

Contrarily, synergistic synbiotic means both probiotic and prebiotic worked together to increase the overall health function (Chin et al., 2024). Synbiotic products offer synergistically improvement of metabolism activity and improve modulation activities of gut ecosystem (Nambiar et al., 2019; Yadav & Chauhan, 2022). The synergistic mechanism includes improve the survival of gut bacteria, enhance the gut barrier function, improve colonization of bacteria within the gut epithelial cells (Chin et al., 2024; Nambiar et al., 2023).

Since the emerging of synbiotic concept and the increase recognition of the beneficial effects of synbiotic to human gut, many synbiotic functional products had been introduced and applied in multiple industries including food and beverage industry, pharmaceutical and disease prevention, nutrition, agriculture and aquaculture, and health care. Synbiotic products had been predominantly targeted and commercialised in various forms, including fermented-based products like , process food like dairy product, supplement and medicine pills (Zavišić et al., 2023).

Synbiotic plays unprecedented roles in the food and beverage industry. Amongst of all food type, various dairy products such as infant milk powder, yogurt, ice cream, cheese, and fermented skim milk had predominantly contributed to the development of synbiotic function food (González-Herrera et al., 2021). For instance, the incorporation of lyophilised probiotic with prebiotic maltodextrin in the milk formulation had improved the overall bioavailability of nutrients profile (Pérez-Conesa et al., 2006). The synbiotic drink derived from probiotic *Lactobacillus helveticus* with prebiotic polydextrose had been examined for the prevention of irritable bowel syndrome (Bahrudin et al., 2020). In addition, different technologies had been investigated for the development of synbiotic functional food. For instance, co-capsulation and micro-capsulation technology had been developed for probiotic and prebiotic in many dairy product (Rashidinejad et al., 2022) and freeze dried technology for synbiotic yogurts and ice cream (Jouki et al., 2021; Maleki et al., 2023).

The development of synbiotic is greatly associated with the development of therapeutic and pharmaceutical products. The wide application of synbiotic had been reported with anticancer, anti-inflammation, anti-diabetic, anti-diarrhoea, anti-microbial and anti-oxidant activities in the following section. For anticancer, synbiotic

product with probiotic *Lactobacillus acidophilus* and prebiotic *Chenopodium formosanum* in the downregulation activity of cancer-express genes (Lee et al., 2019). The use of probiotic *Lactobacillus casei* and prebiotic *Smallanthus sonchifolius* in reduction of tumor multiplicity in gastric cancer (Hwang et al., 2013).

Synbiotic was proven with improve immune system and growth of rainbow trout through the application of probiotic *Enterococcus faecalis* and prebiotic mannan oligosaccharide (Salek et al., 2023). In term of anti-inflammatory action, the use of probiotic *Bifidobacterium breve* and *Lactobacillus casei* with prebiotic 4G-β-Galactosyl-sucrose in reduction of inflammation through downregulate inflammatory-genes (Ruiz et al., 2017). The synergistic effect of probiotic *Bacillus coagulans* and prebiotic fibres in sugar cane displayed anti-inflammation activities against inflammatory bowel disease (IBD) (Shinde et al., 2019).

Co-association of probiotic *Lactobacillus paracasei* and prebiotic fructo-oligosaccharide and arabinogalactan had significant improved the insulin resistance through stimulation peroxisome proliferator-activated receptors gene expression and down-regulation of inflammatory markers (Mattace Raso et al., 2014). The use of synbiotic (*Lactobacillus plantarum* and inulin) had greatly increased gut dysbiosis, antioxidant capacity, and also oxidative stress status (Morshedi et al., 2020). Evidence of antidiabetic activities can be seen from the study of probiotic *Lactobacillus acidophilus* and prebiotic fructo-oligosaccharide & isomalt-oligosaccharide in synbiotic fermented milk (Shafi et al., 2019). In addition, probiotics such as *Lactobacillus kefiranofaciens and Saccharomyces boulradii* worked with prebiotic inulin demonstrated good antibacterial activity against pathogens that causing diarrhoea (Sheela & Suganya, 2012).

Besides combating disease and being use for therapeutical purpose, extensive researches on synbiotic were devoted to improve personal health care and nutrition values. The prebiotic inulin had been co-administrated with several probiotics such as *L. acidophilus, L. casei, L. plantarum* and *Bifidobacterium bifidum* in controlling the blood glucose level (Tajabadi-Ebrahimi et al., 2017) and reducing the triacylglycerol (Wong et al., 2013). Liong et al. (2007) reported that co-administration of probiotic *Lactobacillus acidophilus* and prebiotic like fructo-oligosaccharide, mannitol, inulin

can reduce the total cholesterol level and low-density lipoprotein (LDL) cholesterol. Tang et al. (2020) reduced the lipid accumulation and improve the lipid metabolism within the high diet fed mice with probiotic *Lactobacillus plantarum* and prebiotic hull-less barley β-glucan. The use of probiotic *Bacillus coagulans* and prebiotic inulin to improve the overall glutathione level (Pandey et al., 2015).

The indispensable role of synbiotic had been observed in the aquaculture and agricultural industry. There are many literature evidences reviewed on the current knowledge of synbiotic use in the aquaculture and marine application (Amenyogbe et al., 2020; Huynh et al., 2017; Jose Meseguer, 2011). All these review articles had highlighted the importance and use of synbiotic across various type of aquatic or marine organisms against various challenges encountered in the aquaculture field. For instance, the probiotic *Lactobacillus plantarum* and prebiotic Cacao pod husk pectin was used in the improvement of immune system and growth in shrimp *Litopenaeus vannamei (Kuo et al., 2021).* The use of probiotic *Bacillus sp.* and prebiotic β-gluco-oligosaccharides to promote growth and immune system in fishes against infection and disease (Hasan et al., 2018). Synbiotic was proven to promote health benefit in the growth and survival of several fish species such as Atlantic salmon (Abid et al., 2013) , yellow croaker (Ai et al., 2011), and cobia (Geng et al., 2011).

The promising synbiotic also plays key role in the development of biofilm that can be used as food coating and packaging of food products (Seyedzade-Hashemi et al., 2022) and nanocoating against diseases like inflammatory bowel disease (IBD). Study of Seyedzade Hashemi et al. (2022) demonstrated the biofilm produced from the incorporation of probiotics with prebiotic such as inulin, starch, fructo-oligosaccharide, starch, polydextrose and inulin emerged as new bioactive packaging. Bio-coating techniques had been established using probiotic *Bacillus subtilis* (Kimelman & Shemesh, 2019), and combination strains of *Lactobacillus helveticus, Lacticaseibacillus paracasei* in the Gouda cheese packaging (Aleksandrovas et al., 2024).

In summary, synbiotics have emerged as novel approach in promoting health benefit and overall wellness. This study focuses on synbiotic functional foods,

combining probiotics with cabbage-derived prebiotics to enhance gut health and mitigate oxidative stress. Nevertheless, the optimal dosage of probiotics and prebiotics to achieve their full potential remains a topic of debate. Key challenges include development of technology for better stability production and processing of synbiotics to meet market demand. It is thus imperative to conduct a comprehensive examination of the various technologies that are used to create synbiotics in food products. Additionally, *in-vitro* and *in-vivo* clinical studies are necessary to evaluate the impact of synbiotic consumption on individuals with a variety of health conditions. This evidence could establish a solid foundation for the optimal use of probiotics in treatment.

2.4 Advantages of synbiotic fermentation

Fermentation has a long history rooted as food preservation to maintain the shelf life of food product. It is a biochemical process where microorganisms break down glucose, producing ethanol, acid, and carbon dioxide. Fermentation is the best food-processing method to preserve fresh foods and effectively extend the shelf-life of foods (Godbey, 2022). A variety of food such as vegetables, fruits, meat, seafood, dairy, cereals and legumes were fermented to create various tasty foods and beverages (Bousquet et al., 2021). Fermentation can occur spontaneously through starter cultures, selected beneficial microorganisms obtained in raw food, small amounts of previously fermented batches, or commercial starters (Fijan et al., 2024). The starter culture can be taken from food likes natto, kombucha, water kefir while the commercial starter obtained from beer, cheese and yogurts.

Lactic acid fermentation can enhance the bioactivity of raw materials by increasing the nutrient bioavailability likes polyphenols, flavonoids, dietary fibre, vitamins, minerals, and functional phytochemicals (including glucosinolate and their breakdown derivatives) (Fijan et al., 2024). Lactic acid bacteria (LAB) are primarily employed in the fermentation processes because they can undergo carbohydrates metabolism in the production of lactic acid. Furthermore, LAB is widely known due to their non-harmful characterisation to ensure safety and functionality

(Vougiouklaelimki et al., 2023). The primary LAB used in the fermentation of cruciferous vegetables include *Levilactobacillus brevis*, *Latilactobacillus sakei*, *Latilactobacillus curvatus*, *Lactiplantibacillus pentosus* and *Lactiplantibacillus plantarum* (Fijan et al., 2024). Predominantly, many LAB from phylum Firmicutes had been widely used in the development of fermented functional food product . There are wide range of product being synthesied by LAB under fermentation condition, including organic acid, flavonoid, phenolic, vitamin, bacteriocins, exopolysaccharides (EPS), gamma aminobutyric acid (GABA) and so on, in which all these compounds contributed to significant health promoting activities (Abdul Hakim et al., 2023).

The synbiotic fermentation demonstrated more advantages over natural plant sources through the coupling benefits of fermentation process and the incorporation advantages of probiotic and prebiotics. The advantages of synbiotic fermentation process includes food quality improvement where the pathogenic bacteria being eliminated under high salt environment and competitive exclusion by the beneficial bacteria within the synbiotic functional food (Park et al., 2014). Fermentation of synbiotic have significantly enhanced the overall nutrient profile with higher production of vitamin, bioactive peptide, enzymes, flavonoids, phenolics, saponin and so on (Sharma et al., 2020). Fermentation process improved the metabolism of fermentable substrates such as proteins and carbohydrates, contributing to the production of many bioactive secondary compounds (Gaudioso et al., 2022). SCFAs is one of the most important metabolites produced during the fermentation process. It regulates gut health activities through providing energy to cell metabolism and maintaining the intestinal homeostasis, including promotion of anti-oxidative, anti-inflammatory, mediation of intestinal motility and stimulation of signaling molecule pathway (Martin-Gallausiaux et al., 2021). The study of Turkmen et al. (2019) revealed that synbiotic fermented cabbage capable to increase antioxidant activity about 3-4 fold as compared to the raw cabbage.

There are several factors needed to be considered in the fermentation process, including fermentation period, fermentation temperature, salt concentration, pH, type of probiotic. Fermentation temperature had great impacts on the microbial growth as they can only survives under specific optimal range (Khan et al., 2018). A extreme high temperature that is deviated from optimal range can inhibit the microbial growth,

causing denaturation of proteins and enzymes, and drastically impair fermentation process whereas a lower temperature can cause the microbial cell to be inactive, exert slow growth rate (Auesukaree, 2017). Besides temperature, pH can significant influence the fermentation activity, especially in the ethanol fermentation. A study revealed that pH 4-5 is ideal for maximal fermentation efficiency, the incubation time for maximum ethanol concentration would be longer when the pH is lower than 4, while when the pH is exceeding 5, the quantity of ethanol production was significantly decreased (Mengesha et al., 2022). Fermentation period is another crucial influencing factor in fermentation process. There are different fermentation time ranges being used in the traditional fermentation process. Theoretically, longer fermentation time ensure complete microbial metabolism and high efficiency of substrate breakdown into more desirable and nutritional bioactive compounds (Martinez-Villaluenga et al., 2009). However, fermentation with efficient amount of substrate and starter can significantly shorter the fermentation period. Therefore, fermentation time is greatly associated with the substrate and starter concentration.

The concentration of substrate like salt and sugar used in the fermentation is greatly impact on the final yield of the fermentation quality (Mengesha et al., 2022). There are various salt concentrations reported in the cabbage fermentation such as 6-8% salt in Türkiye (Erdoğan & Ertekin Filiz, 2023), 4% salt in Chinese sauerkraut (T. Xiong et al., 2016), 2% salt for cabbage fermentation under starter *Leuconostoc mesenteroides* (Johanningsmeier et al., 2007). According to Yang et al. (2020), the microbial growth is greatly influence by the salt concentration with statistically report 2-5% salt concentration increase the overall LAB population. Starter concentration like yeast and beneficial microorganism is vital for the fermentation. The microbiological metabolism showed significant influence on the fermentation process with various enzymatic breakdown.

2.5 Animal study

In this study , animal studies including *in-vitro* cell line research and *in-vivo* rat experiments were conducted to examine the effect of PFC on the cytotoxicity properties, oxidative stress and gut microbiota changes. The studies are critically important in exploring the biological effects of PFC in the cell line level and also assessing their therapeutical potential and safety of PFC in the target animal for instance Sprague Dawley (SD) rat (*Rattus norgegicus*) in this study.

2.5.1 *In-vitro* cell culture analysis

In-vitro animal cell culture is an important tool in the biotechnology and biomedicine field. In-vitro cell culture has to conduct in a controlled laboratory environment with the use of biosafety cabinet (Chandra et al., 2022). Cell culture technique allowed researcher to study the behaviour and interaction of animal cell under various conditions, in addition explore various cellular and molecular mechanisms such as cytotoxicity effect, signalling pathway and gene expression (S. Wu et al., 2020). For instance, researchers can observe the growth inhibition, change in cell structure, apoptosis of cell line when treated with a given drug to determine for its anticancer properties (Neophytou et al., 2021). Besides that, this culture technique can be applied in the discovery of drug, manufacture of biological product like therapeutic protein and vaccine (Cid & Bolívar, 2021). In this study, there are two cancerous cell lines, namely HCT-7 and HCT-116 and one non-cancerous cell line called 3T3-L1 had been used to examine the cytotoxicity properties of PFC.

2.5.1.1 Breast cancer cell line MCF-7

MCF-7 cell line is a breast cancer cell line that origin from human patient with breast adenocarcinoma. MCF-7 cells exhibited epithelial-like morphology with cobblestone-like shape and tightly packed to form a monolayer during cell proliferation (Sweeney et al., 2018). Breast cancer is the top 2 most common cancer

cell with about 2.3 millions new cases reported globally in year 2022 (World Cancer Research Fund, 2024). Among the reported new cases of cancer in US, breast cancer had accounted 30% among all cancer cell types in 2024 (Giaquinto et al., 2024). This cell line is one of the most common cancer cell line being used in wide application of animal cell culture. MCF-7 exhibited good cell proliferation in response to the hormone oestrogen (Park, et al., 2020). Therefore, it had been recognised as ideal model to be used in the study of hormone dependent breast cancer with its oestrogen-receptor-positive (ER+) properties (Ejaz et al., 2022). Researchers utilised MCF-7 cell line to study the anticancer properties of functional food via in-vitro MTT assays. For instance, several literature studies reported high anticancer properties against MCF-7 cell from Chinese kale with enriched selenium (Luang-In et al., 2020a), *Brassica napus* extract , pickled cabbage extracts (Luang-In et al., 2020b). Anti-proliferation of cell, anti-migration properties and apoptosis induction analysis had been examined to indicate the anticancer properties.

2.5.1.2 Colon cancer cell line HCT-116

HCT-166 cell line is a colorectal carcinoma cell line derived from colon cancer male patient. This cell line is popular with its high tumorigenic properties, which suggesting it as novel cancer cell line in the colorectal cancer research and drug development (Butler et al., 2017). Besides that, HCT-116 cell line is highly sensitivity in microsatellite instability which enable accurate defect detection of DNA mismatch repair (Huang, W. et al., 2023). Moreover, HCT-116 cell line played crucial role in the genetic modification, gene function and gene therapy research as it possessed KRAS mutation gene that often contributed to genetic alteration in the colorectal cancer (Varshavi et al., 2021). Surprisingly, there is dramatically raise in the death cases reported from colon cancer, which occupied the top one and top two leading cause in male adult and female adult under 50 years old in 2023 from the 4th leading cause in year 1990 (Siegel et al., 2024). Various literatures reported with the use of HCT-116 cell line in the anticancer activities of Brassica vegetables such as *Brassica rapa* (Kim et al., 2022), *Brassica juncea* leave (Kwak et al., 2016), broccoli and radish (Pocasap & Weerapreeyakul, 2016).

2.5.1.3 Fibroblast cell line 3T3-L1

The 3T3-L1 cell line is a fibroblastnon -canceorouse cell line that derived from the embryos of Swiss albino mouse. This cell line is well established in the fat adipocyte research as it exhibited excellent adipocyte differentiation ability, displayed fibroblast-like morphology, and play vital roles in the energy exchange, gene expression and lipid accumulation characteristics. The cells tends to look like spindle spindle-shape with elongated processes in the preadipocyte stage, while it turn into round shape upon occurrence of adipocyte differentiation during lipid droplet accumulation. In addition, the adherent properties with fibroblastic appearance and high cell proliferation of 3T3-L1 had further strengthened its role to be one of the most common used non-cancerous cell line in the cancer research. The efficacy of 3T3-L1 had been tested in a variety of toxicological and biological researches, for instance reduction of reactive oxygen species (ROS) (Li, Y. et al., 2023), insulin resistance (Chen, C. et al., 2020), anti-obesity from broccoli extract (Ranaweera et al., 2022), anti-diabetic (Majeed et al., 2024) tissue regeneration (Contessi Negrini et al., 2020) and also wound healing (Luo, Z. et al., 2024; Pan et al., 2021).

2.5.2 *In-vivo* rat model analysis

In-vivo studies using rat models had been widely used in the long history of animal research. Sprague Dawley (SD) rat (*Rattus norgegicus*) had been used as host in this study. Sprague Dawley rats provide abundance of genetic resources and is ideal for safety and efficacy testing, aging, behaviour, reproduction and surgical modifications (Zhao et al., 2023). They are widely used in laboratory animal testing are due to their calmness behaviours and ease of handling compared to other species of rat or mice (Brandt et al., 2016). In addition, SD rats have high adaptability to diverse diets, hence it is best match to our objective study for oral feeding testing.

Detailed and vast information available on the genomic, proteomics and metabolomics related studies and also clinical related experiments on the strain of SD rat can be further used as the supportive material to explain the research findings

in gut microbiota in this study (Letertre et al., 2020; Mesnage et al., 2021; Wang, J. et al., 2018). Most importantly, high similarities of genetic makeup to human DNA can further fortify our understanding on the potential toxicity effect of pesticide and the recovery potential of probiotic fermented cabbage towards human.

In this study, the *in-vivo* rat study is conducted in male animal. Due to consideration of large variation in female rat such as the hormone change throughout female cycle might cause female rat to react differently to the same stimuli (Pestana & Graham, 2024). In addition, there are some cases reported that hormonal molecules may interacted with the tested samples and potentially contributed to confounding findings (Pestana & Graham, 2024). Hormonal fluctuation occurred during the oestrous cycle in female rat may significantly impact on the experimental outcome, and complicate the data analysis and interpretation process (Kundakovic & Rocks, 2022). Hence, male rats had been selected for the whole experiment for more consistency and simplified study design.

2.6 Oxidative stress, reactive oxygen species and antioxidant mechanisms

In this study, the oxidative stress (OS) level in rat had been observed through the measurement of reactive oxygen species (ROS) reactive radicals and antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), antioxidant agent like the total glutathione level (GSH) and lipid peroxidation (LPO) had been performed to indicate the OS level. Pesticide exposure induced OS which subsequently causing overproduction of ROS that can damage cellular components. This imbalance of ROS and antioxidant agents could potentially contributed to organ dysfunction, neurotoxicity, inflammation. Synbiotic PFC on the other hand may offer a promising intervention to counter oxidative damage, as it is enriched with high bioactive compounds with high antioxidant capacity. Additionally, probiotics can mitigate OS by scavenging free radicals of ROS, improving antioxidant activity, and potentially restoring gut microbiota balance.

2.6.1 Reactive oxygen species (ROS)

ROS represented a group of high reactive molecules that containing oxygen such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2) (Demirci-Çekiç et al., 2022). ROS are created by all living organisms in a variety of cellular compartments, including mitochondrial membrane, plasma membrane, peroxisomes and cytosol (Schieber & Chandel, 2014). They are by-product of the normal oxygen metabolism and present at stationary or low level to maintain cellular functional. A steady balance is maintained between ROS and the cellular antioxidant systems in the normal condition. However, excessive ROS production may occur in intracellular or extracellular spaces due to exposure to xenobiotics and environmental factors, potentially leading to cellular dysfunction and apoptosis. The highly reactive $O_2^{\bullet -}$ can be produced in the electron transport chain reaction in the mitochondria and also mediated by NADPH oxidase while the formation of H_2O_2 is derived from the catalytic reaction by xanthine oxidase of hypoxanthine and xanthine in the presence of water and oxygen (Liu, T. et al., 2022). The cellular origin and mechanisms involved in the production of high reactive $O_2^{\bullet -}$ and H_2O_2 within the cells had been showed in Figure 2.1.

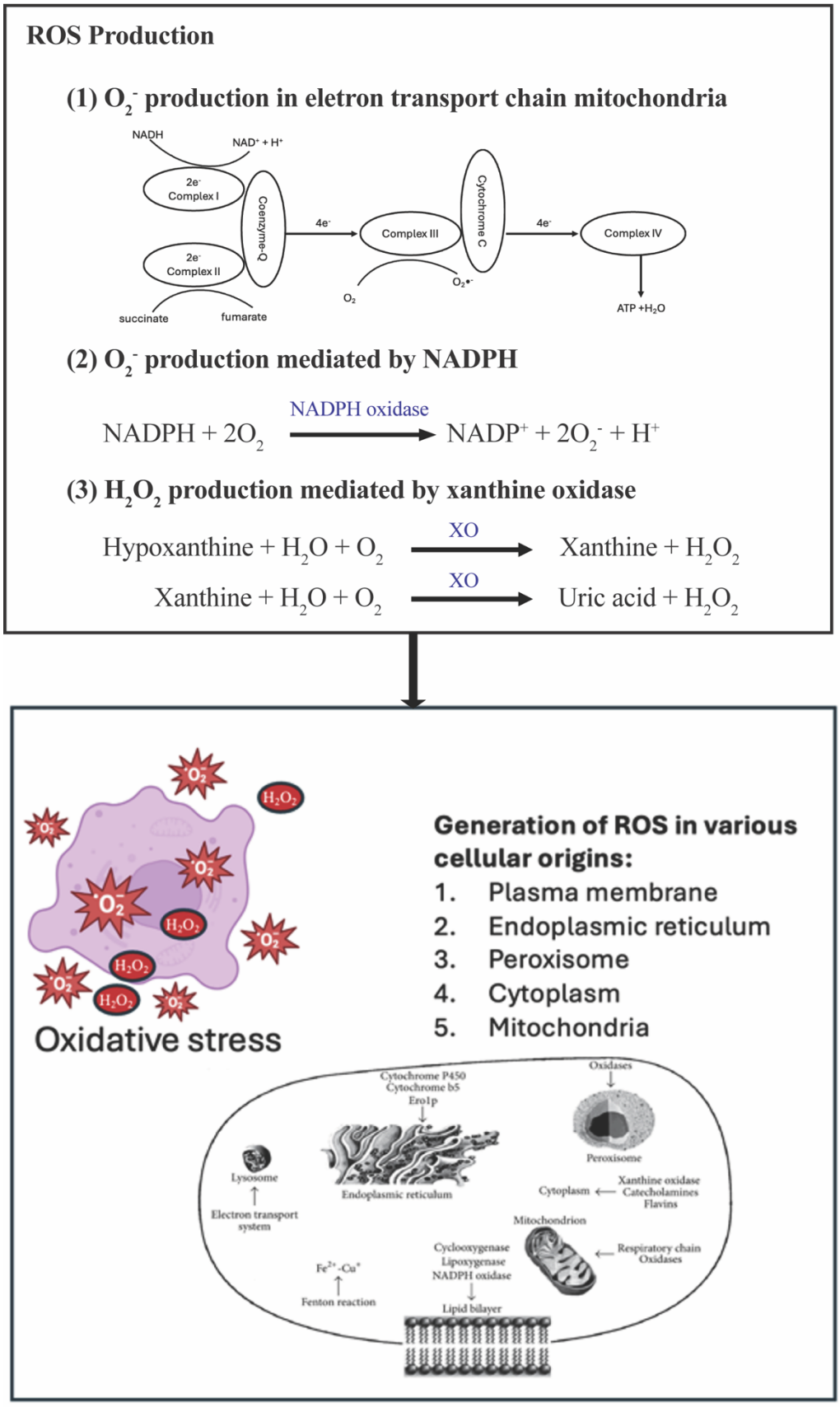


Figure 2.1 The cellular origin of ROS, production of $O_2^{\bullet-}$ and H_2O_2 in the mitochondria and cells and contributed to oxidative stress.

ROS is often associated with the stimulation of inflammation signalling pathway, and the oxidation of biomolecules within the body such as proteins, lipids, and nucleic acid (Schieber & Chandel, 2014). When the body experienced high ROS level, it promoted the activation of signalling molecules to produce various signalling cascade response and promote the gene expression of pro-inflammatory molecules (Aleksandrova et al., 2021). These phenomenon will further cause the dysregulation of protein and gene expression and ultimately resulting in the abnormal growth of cancer cell and tumour initiation (Bardelčíková et al., 2023). In addition, increased ROS production can boost the production of cytokines and expression of adhesive molecules, which further caused the endothelial dysfunction and cardiovascular disease (Maniaci et al., 2021).

2.6.2 Oxidative stress (OS)

Oxidative stress (OS) can be defined when there is an increase of intracellular oxygen radicals that causing damages towards DNA, protein and lipids (Wang et al., 2017). This condition occurred when there is imbalance interplay between cell's antioxidant capacity and the production of ROS (Zhao, F. et al., 2021).

The impact of OS on the organism can be seen on various factors, including the availability of oxidant, the efficacy of antioxidant, the ROS intensity, the origin of its generation, and the activation of repair mechanisms (Demirci-Çekiç et al., 2022). Long term effect of OS promotes the onset of various chronic diseases such as neurodegenerative disease, cardiovascular disease and cancer (De Almeida et al., 2020; Rojas-Gutierrez et al., 2017). Chronic OS can be observed through various immune-modulatory responses such as activation of inflammatory biomarkers, and cell damage caused by oxidation of biomolecules (Lira & De Sousa Rodrigues, 2016). DNA damage increases the risk of mutagenesis, where some important genes being mutated and lead to malfunction of certain cell function (Bardelčíková et al., 2023). Besides that, when there is an homeostasis imbalance happened in GI tract, it can ruin the normal functionality of GI tract cells, including membrane dysfunction, protein aggregation and DNA damage (Zińczuk et al., 2021). In

addition, homeostasis disruption can further effect on the cell integrity and compromised defensive in the gut mucosal membrane, and subsequently provoke the invasion of harmful microorganisms (Bardelčíková et al., 2023).

2.6.3 Antioxidant mechanisms against oxidative stress

An antioxidant is a molecule can minimise cellular or tissue damage resulted from reactive free radical within the body. Under OS, antioxidant agent can slow down or block the oxidation of biomolecules compounds to defence body system from oxidative damage through reacting with the free radical (Awang Daud et al., 2022; Y. Wang et al., 2017). Antioxidants mitigate the cellular damages caused by OS through by interacting with ROS and reactive nitrogen species (RNS), neutralizing or halting chain reactions before critical molecules in the body are harmed. Antioxidant enzymes like CAT and SOD and non-enzymatic antioxidant molecules like GSH and LPO demonstrated complementary insights in the ROS removal to maintain the OS homeostasis and cellular redox balance (Aleksandrova et al., 2021).

2.6.3.1 Superoxide dismutase (SOD)

SOD is an important antioxidant enzyme that can be found in various organelles such as mitochondria and cytoplasm. This enzyme serves as the first line defence against high reactive superoxide radicals (a major ROS generated by metabolic processes and mitochondrial respiration) in the regulation of antioxidant mechanism and reduction of OS (Zheng et al., 2023). SOD enzyme can catalyse the superoxide radical anion to hydrogen peroxide and oxygen, where the reaction happened as followed:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(Equ 2.1)}$$

where O_2^-= superoxide radicals, H+= hydrogen ion, H_2O_2 = hydrogen peroxide, and O_2= oxygen molecules.

2.6.3.2 Catalase (CAT)

CAT is one of the key antioxidant enzymes for biological defence system that had been widely in all living organisms. This enzymes are widely detected in the peroxisomes located next to mitochondria where it regulate the body defence when there is detection of high level of H_2O_2 molecules in the body (Goyal & Basak, 2010). CAT is an enzyme that breaks down hydrogen peroxide, a harmful byproduct of oxidative stress. This enzyme can scavenges the H_2O_2 molecules from the body to prevent the accumulation of hydroxyl radicals (one of the primary ROS) through catalysing the decomposition of H_2O_2 into water (H_2O) and O_2 molecules (Menzel et al., 2021), where the catalysing reaction happened as follow:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2. \qquad \text{(Equ 2.2)}$$

where H_2O_2 = hydrogen peroxide, H_2O = water molecule, and O_2= oxygen molecules.

2.6.3.3 Total glutathione (GSH)

Glutathione is antioxidant tripeptide compounds that made up of glycine, cysteine, and glutamate. They are naturally existed in two forms, namely: reduced glutathione (GSH) and oxidise glutathione (GSSG). Under normal physiological, reduction of GSSH to GSH occurs under the catalysis reaction of enzyme glutathione reductase in conjunction with glutathione peroxidase (GPx) (Menzel et al., 2021). GSH as one of the most abundant intracellular antioxidant, plays a crucial role in protecting cells from oxidative stress. GSH molecules present as the main form within the body, especially in the mitochondria of the cells. GSH is vital in the physiological functions, includes electrophile elimination, antioxidation, and free radical scavenging (Khan et al., 2012). Moreover, GSH is a vital component of cellular metabolism, playing key roles such as regenerating vitamins C and E, removing mercury from cells, participating in phase II enzymatic reactions, and neutralizing singlet oxygen and free radicals (Menzel et al., 2021). Excessive ROS may react with GSH disrupt the balance of ROS/GSH, which later give impact on the structure modification and malfunction of biomacromolecules, deleterious oxidation, and inhibition of cell proliferation. GSH molecules play vital roles in the maintaining the cell homeostasis and withstanding the

OS through the regulation of disulfide bonds within protein molecules and the removal of electrophiles and oxidants. The catalysing reaction happened as follow:

$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ (Equ. 2.3)

where GSH= reduced glutathione, H_2O_2= hydrogen peroxide, GSSG = oxidise glutathione and H_2O = water molecules

2.6.4 Lipid peroxidation (LPO)

Lipid peroxidation can be described as the oxidative degradation of lipids and had been used as key biomarker of oxidative stress . This process occurs when the free radicals react with the lipid molecules, primarily polyunsaturated fatty acid (PUFA) within the cell membrane (Gaschler & Stockwell, 2017). Lipids is critical for maintaining the functionality of cellular membrane function (Schneider, 2009). The oxidative degradation of lipid can occurs through a series of radical chain reaction, which is categorised into three phases, namely initiation, propagation, and termination phases (Valgimigli, 2023). The process was initiated when ROS attract the hydrogen atom to generate fatty acyl radicals, followed by rapid oxidation of these radicals with oxygen to form peroxyl radicals in the propagation phase (Hajieva et al., 2023). In this phase, hydroxyl radicals (OH^-) can initiate reactions with all PUFA, and O_2^- radicals begin to react with the activated fatty acids. Termination happens when two radicals combine to form a non-radical product or react with antioxidants, yielding stable radicals (Valgimigli, 2023). LPO and its associated derivatives produce a diverse array of reactive molecules like aldehydes, ketones, and alkanes, many of which had been reported to show harmful physiological effects such as genotoxicity and can serve as critical markers of LPO .

Malondialdehyde (MDA) is formed during LPO when the ROS react with PUFA. The thio-barbituric acid-reactive substances (TBARS) method had been adopted to measure the MDA level that indicated for the oxidative stress condition within a biological samples (Tsikas, 2017). The measurement involved the formation of brown red complex from the reaction between MDA and 2-thio-

barbituric acid (TBA), which can be detected by ultraviolet absorbance or fluorescent intensity. The elevation of MDA is often associated with various chronic conditions, including cardiovascular diseases like atherosclerosis (Gianazza et al., 2021), neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Angelova et al., 2021; Rojas-Gutierrez et al., 2017), and age-related macular degeneration (Zhao, T. et al., 2021). Excessive free radical of ROS can attack PUFA and trigger the LPO within cell and biological fluids (Yin et al., 2011).

2.6.5 Interaction of ROS, antioxidant mechanism under pesticide exposure

There are various scientific evidences proved that pesticide exposure increased the ROS level and reduce the capability of defence against OS (Teng et al., 2019). Various type of pesticides had well reported to induce oxidative stress in different in-vitro and in-vivo animal studies. Pesticide-induced OS is highly associated with the level of ROS and reactive nitrogen species (RNS) and become the one of lead cause contributed to various diseases like cardiovascular disease (Zago et al., 2022), neurologic dysfunction, Parkinson’s diseases (Islam et al., 2021), Alzheimer’s disease (Li, Y. et al., 2021), and chronic kidney disease (Jacobson et al., 2021). Pesticides from organophosphate and organochlorine were recognised as the most associated classes with cancer (Varghese et al., 2021). Different studies showed the toxicity of pesticides that contributed to the incurrence of cancers including brain (Gatto et al., 2021), colon (Matich et al., 2021), breast (Da Silva et al., 2022), lymphoma (Kim et al., 2023) and leukemia (Karalexi et al., 2021).

The toxicity effect of carbamate and organophosphate can result in the accumulation of acetylcholine via inhibition of acetylcholinesterase enzymatic reaction (Tsagkaris et al., 2020). Acetylcholine is one of the important components in the nicotinic receptors (nAChRs) and muscarinic receptors (mAChRs), which play vital roles in modulating intracellular signalling cascades activities (Kawashima et al., 2012). The substantial outcome of the signalling activities had further resulted in high NADPH oxidation and mitochondrial dysfunction which ultimately contributed to the raise of ROS level and OS. This oxidative stress is associated with the increased lipid

peroxidation and reduced antioxidant capacity (Sule et al., 2022). Furthermore, the exposure of pesticide demonstrate proteosome (a protein complex responsible for maintain protein quality through degrading the damaged and unuse proteins and control cellular processes) inhibition in neuroblastoma cells (Islam et al., 2021). Exposure of pesticides from chlorpyrifos, trifluralin, pendimethalin, metalaxyl, ethylene bromine had been reported to increase the health risk index for acute myocardial infraction (Zago et al., 2022). The increase of blood pressure is associated with the exposure of pesticides from malathion, fenitrothion (Zago et al., 2022).

The elevation of ROS in pesticide-exposed animals is well-documented in many *in-vivo* animal study models, such chronic toxicity of flutolanil in zebrafish (Teng et al., 2019), toxicity effect of fluindapyr in earth worm (Ji et al., 2023), toxicity effect of buprofezin towards tilapia fish (Azouz et al., 2021) and mice (Bibi & Qureshi, 2019). Besides that, there are various studies reported about pesticide cytotoxicity effect in *in-vitro* cell lines such as in human keratinocyte cell line (Abhishek, Amar et al., 2014), rat pheochromocytoma cell PC12 (Heusinkveld & Westerink, 2017), human peripheral blood lymphocytes and HepG2 cell line. Many studies revealed the exposure of pesticides is greatly contributed to an increase in lipid peroxidation, which can be due to a combination of ROS buildup, including SO_2-and H_2O_2, as well as resulting in the decreased activity in two antioxidant enzymes (e.g. SOD and CAT) (Sule et al., 2022).

2.7 Gut microbiome modulation

Gut microbiota is a diverse array of microorganisms inhabiting the digestive tract forming a mutualistic relationship with their host and contributing to a wide range of physiological functions (Xiong et al., 2017a; Xiong et al., 2017b). The human gut is home to close to 100 trillion bacteria, with local concentrations in the colon ranging from 10^{11}-10^{12} bacteria per gram (Anglin et al., 2015; Kelly et al., 2016). Although different between individuals, the composition of the adult gut microbiota can be partially genotyped and broadly categorised into three enterotypes: *Bacteroides* spp. and *Prevotella* spp. from phylum Bacteroidota and *Ruminococcus* spp. from phylum

Firmicute (Arumugam et al., 2011). The gut is important for nutrient absorption, health regulation, and immune defence mechanisms (Li et al., 2018). The microorganism in gut showed mutualism relationship with the host organism (Xiong et al., 2017b). Recent studies highlight the close association of gut microbiota with factors such as rearing conditions, health status, diet, and developmental stages, particularly in mice models (Xiong et al., 2017b). This underscores the importance of investigating gut microbiota as a critical determinant of host health. This section reviews gut microbiota modulation in two major contexts: (1) gut dysbiosis resulting from pesticide exposure, (2) gut modulation by probiotic and synbiotic."

2.7.1 Pesticide pollution induced gut microbiome toxicity

The gut microbiota plays a crucial role in protecting against the toxic properties of chemicals through a dynamic bidirectional relationship. This mutual interplay is of significant toxicological importance, as environmental chemicals have been shown to impact the composition and biodiversity of the intestinal microbiota, as well as modulated metabolic pathways (Giambò et al., 2021). These changes may play a role in shaping an individual's micro-biotype (Xia, J. et al., 2017; Liu, L. et al., 2022).

Pesticide pollution may directly impacts on gut microbiota composition. and it is often termed as gut dysbiosis when there is imbalance of gut microbiota composition and functionality interruption.The gut dysbiosis is characterised by the imbalance between beneficial and pathogenic microorganism within the gut, this changes is subsequently leading to serious diseases, including obesity (Hou et al., 2017), chronic kidney disease (Kanbay et al., 2018), diabetes (Blandino et al., 2016; Sedighi et al., 2017), cancer (Oke & Martin, 2017), cardiovascular disease (Kitai and Tang, 2017) and and even neurological disorders like Alzheimer's disease and depression (Das & Nair, 2019; Yamashiro, 2017).

Chronic exposure to pesticides has been shown to induce gut microbial alteration, leading to dysbiosis, which is associated with gastrointestinal disorders and diseases affecting other organs, such as the blood-brain barrier (Abou Diwan et

al., 2023). For instance, pesticide exposure of chlorpyrifos can promote obesity and insulin resistance through influencing gut and gut microbiota of mice (Yuan et al., 2019). When a broad-spectrum benzimidazole fungicide like carbendazim enters the food chain, alteration of gut microbiota composition and decreases serum lipoprotein lipase levels, resulting in metabolic problems were observed (Jin et al., 2018). Studies have also focused on the ecological dysbiosis of the gut microbiota as an inducing factor of the toxic effects of pesticides (Meng, Z. et al., 2021). Dysbiosis resulting from pesticides has been associated with various diseases, including autoimmune diseases, hypertension, chronic kidney disease, and respiratory diseases, highlighting the systemic effects of gut dysbiosis (Khan & Wang, 2020; Lima et al., 2022). Detail literature studies on the gut microbiota dysbiosis upon pesticide exposure and health risk condition on their host had been summarised and published in the Khoo et al. (2024).

Besides that, studies on the long-term systemic health effects of exposure to pesticides had resulted in a variety of respiratory diseases as well as stress and cognition (Kaikai et al., 2023; Ratanachina et al., 2022; Ye et al., 2017). The toxins also interact with the biological activity of human gut microbiota and affect the microbiome and global homeostasis, with destructive changes to the host. As these pollutant chemicals have the ability to disturb the functions of the gut microbiota, potentially disrupting the balance of the host's internal stability (Defois et al., 2018). Hence, it is critical to emphasise the vital role of gut microbiota in maintaining the integrity of the intestinal barrier, with its composition, structure, and function being crucial for ecotoxicological assessments. Various contaminants, including microplastics, antibiotics, heavy metals, organic pollutant and pesticides have been shown to harm the intestinal barrier by affecting the gut microbiota.

There are increasing studies revealed that gut microbiota can be altered when exposed to pesticides (Feng et al., 2019; Luo, J. et al., 2017). Pesticide exposure has been linked to dysmetabolism and microbiota alterations, such as reduced in the phylum *Bacteroidetes* and increased in phylum *Firmicutes*, which are associated with dysmetabolism risk factors (Djekkoun et al., 2021). Bacterial diversity in the gut of green turtles decreased when exposure to glyphosate herbicide had been reported (Kittle et al., 2018). Rueda-Ruzafa et al. (2019) reported that beneficial intestinal

bacteria such as *Bacillus badius, Bacillus cereus, Enterococcus faecalis* and *Entercoccus faecium* were sensitive to glyphosate based herbicide whereas *Clostridia sp, Salmonella sp,* and *Escherichia coli* could potentially developed resistance against glyphosate.

In sum, the impact of pesticide residue leading to gut dysbiosis is crucial due to the significant consequences on metabolic regulation and microbiota. The existing literatures underscores the importance of understanding the mechanisms linking pesticide exposure, dysmetabolism, microbiota and gut brain axis to assess the impact on overall health. Hence, this impact of pesticide had selected as the cornerstone of study to further emphasise the negative impacts resulting from pesticide residue, highlighting the need for comprehensive research review on the interplay between pesticides, microbiota and gut brain function in upcoming discussion.

2.7.2 Gut modulation by synbiotic functional food

Exploring the gut microbiota in rats provides valuable insights into the impact of functional foods, such as probiotic-fermented cabbage (PFC) on the nutrient metabolism, gut health, and overall physiological well-being. Such studies offer a foundation for understanding the potential of PFC and similar functional foods in promoting health and mitigating adverse effects in animal models and, potentially, in humans.

Gut microbial modulate inflammatory and immune system process via increasing antimicrobial peptide gene expression, controlling the build-up of fat, thus control the development of obesity related diseases via absorption and fermentation process of dietary polysaccharides (Blandino et al., 2016; Paris et al., 2020). The gut microflora produce biofilm within the intestinal epithelium, which actively contributes to the degradation of toxic compounds, thus forming a protective barrier against pathogens (Vásquez et al., 2012). The regulation of the adaptive immune system, as well as the promotion of food digestion and xenobiotic metabolism, which trigger the

host's defensive response, are crucial functions of gut microbiomes (Belizário & Faintuch, 2018).

Gut microbiota modulation typically aims to restore balance in the microbiome, as opposed to the gut dysbiosis that reflected for the imbalance interplay between beneficial and harmful microbes within the GI tract (Li, E. et al., 2018). Emerging evidences highlighted the importance roles of gut microbiota in the regulating the efficacy and toxicity of anti-cancer treatments (Kakihana et al., 2016). The gut microbiome is essential in regulating immune response and maintaining homeostasis balance.]

There are several mechanisms being reported for effective modulation of the gut microbiota. There are 4 major mechanisms including (1) introduced of beneficial healthy microbes, (2) elimination of pathogenic and harmful microbes, (3)strength the GI barrier integrity, and (4) regulation of immune functions (Fakharian et al., 2023; Safadi et al., 2022). Figure 2.2 showed that mechanisms involved in the modulation of gut microbiota.

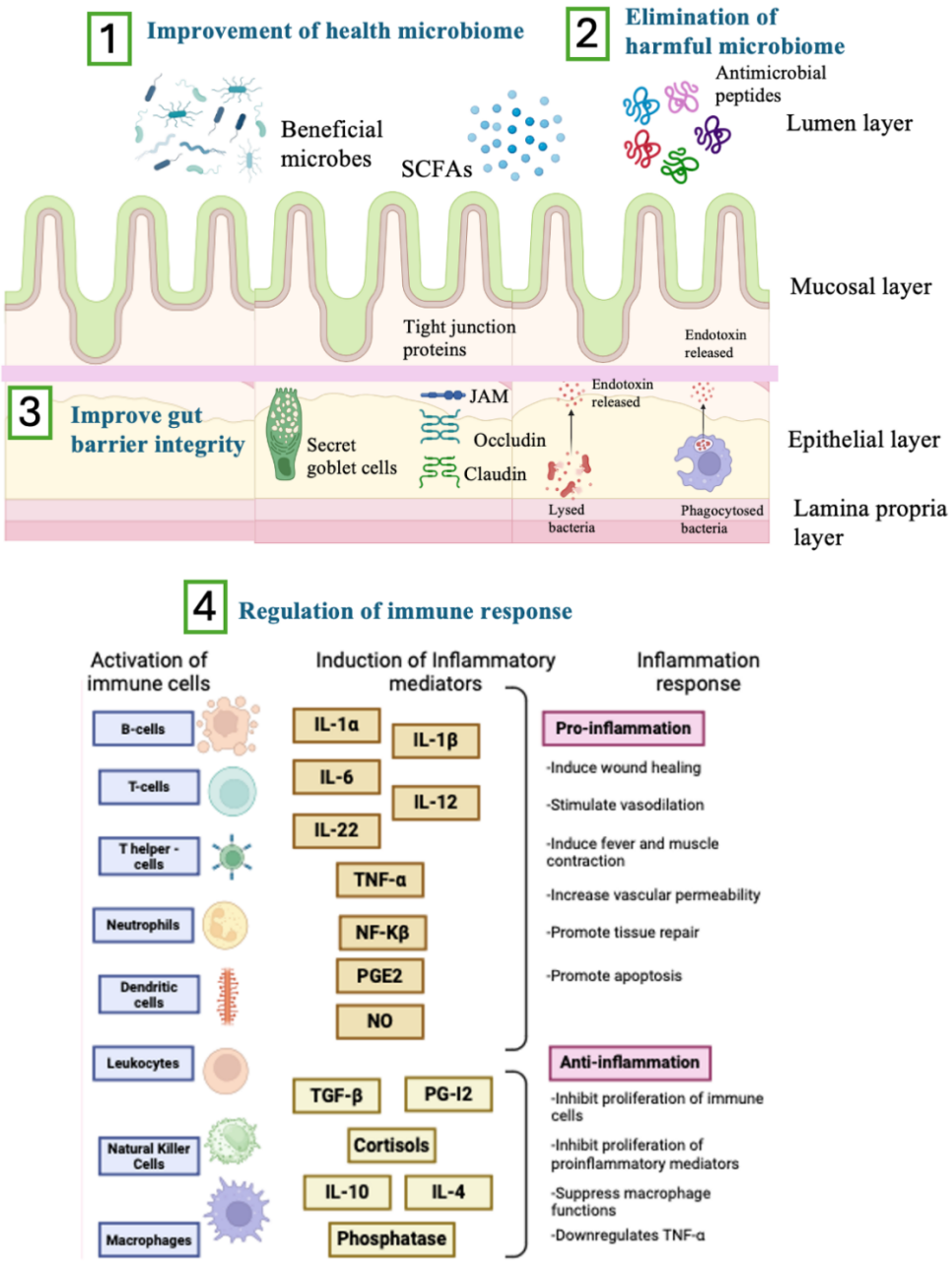


Figure 2.2 The mechanisms involved in the modulation of gut microbiota.

The improvement of healthy gut microbiome can be modulated by the consumption of dietary fibre, probiotic, postbiotic and synbiotic products (Delzenne et al., 2024). Diet as one of the key modulators in the gut microbiota composition. Intriguingly, the gut microbiota modulations can alter the functionality or composition of gut microorganisms. The introduction of high-fibre diets promoted the growth of healthy microbiome like Bacteroidetes and Firmicutes and improved secretion of SCFAs (Cronin et al., 2021). Various bioactive metabolites produced from the healthy

microbiome demonstrated vital role in maintaining homeostasis balance in the GI tract (Cao et al., 2024). For instance, the production of SCFAs, bacteriocin, extracellular polymeric substances (EPS) and antimicrobial peptides were proven to eliminate pathogenic bacteria by apoptosis induction and growth inhibition of cancer cells (Thoda & Touraki, 2023). The enriched microbiome from dietary food sources also promote rapid degradation and elimination of carcinogens via various beneficial hydrolytic enzymes (Ejike & Liman, 2022).

Contrarily, diets with high-fat or low-fibre had reported with increase harmful microbiome and reduced microbial diversity (Chen, J. et al., 2023). Excessive consumption of artificial additives, refined sugars, and processed foods can negatively impact on the composition of gut microbiota (Baspakova et al., 2023). Taking Proteobacteria as example, this microbial group existed as minor component in a normal healthy gut microbiota. However, it is spotted that the abundance of Proteobacteria increased when exposed to pathogen or chemical infections (Meng et al., 2021). Proteobacteria can induce pro-inflammatory factors and stimulate the production of endotoxin lipopolysaccharide (LPS) production (Vasques-Monteiro et al., 2021). The gut-derived endotoxin LPS have been demonstrated to facilitate the exacerbation of pathogenic metabolic perturbations (Vasques-Monteiro et al., 2021).

The GI tract exhibited unique anatomy to maintain neutrality to gut commensals and exert wall of defences against external stimuli and pathogens. Four important layers such as lumen, mucosal, epithelia, and the lamina propria made up the intestinal gut barrier (Rosendo-Silva et al., 2023). The improvement of gut barrier integrity can be observed from the secretion of goblet cells, activation of tight junction proteins such as junctional adhesion molecules (JAM), occludins, and claudins, stimulation protein receptors for the activation of cytokines and innate lymphoid cells (Alizadeh et al., 2022; Górecka et al., 2024). The gut associated lymphocyte tissues (GALT) also supported the gut integrity via proliferation of epithelial cell and induction of proteins expression of defensins, claudins, and mucin (Fakharian et al., 2023). The gut microbiota supported the innate immunity through regulation of the microbial communities with host immune cells such as macrophages, dendritic cells, and epithelial cells within GALT.

Immune cell like dendritic cells (DC) play a vital role in communication by delivering antigens to T cells and initiating the adaptive immune response (Mbongue et al., 2014). Adaptive immune response began with the activation of B cell, T cells and also the antibody production. Immune cells such B cells, T cells, and T helper (Th) cells interplay their roles within each other in the resolution of inflammation via regulation of pro-inflammation and anti-inflammation activities. These cells work together in strengthening the immune response, promoting tissue healing, stimulating phagocytosis and killing infected cells (Chekol Abebe et al., 2021). B cells generate antibodies that neutralise infections and tag them for eradication. Regulatory B cells, play a significant role in anti-inflammation response by stimulation of cytokine IL-10. This cytokine aids in the reduction of the activity of pro-inflammatory cells such as macrophages and Th1 cells. In addition, B cells have the ability to display antigens to regulatory T cells (Tregs), so enhancing their inhibitory role (Cano & Lopera, 2013).

T lymphocyte cells, such as cytotoxic T (Tc) lymphocytes and helper T (Th) lymphocytes, directly identify and coordinate the assault on infected cells. Tregs cells play a crucial role in reduction of inflammation. They promote the transcription factor Foxp3 that play suppressive role in immune system and promote gene expression of IL-2 receptor, which play role in the homeostasis and differentiation of effector T-cell subsets (Chekol Abebe et al., 2021; Létourneau et al., 2009). Tregs cells utilise many strategies to inhibit immune responses, such as releasing cytokines, engaging in direct cell-to-cell interactions, and disrupting metabolic processes. Th2 cells, which are mainly linked to allergic reactions, can also promote anti-inflammatory effects in some situations by releasing the anti-inflammatory cytokine IL-10 and TNF-β (Kaku et al., 2014). T helper cells, namely Th1 cells, have a complex role in inflammation. They can get fatigued and facilitate the development and growth of Tregs, which eventually leads to the suppression of inflammation (Cano & Lopera, 2013).

The probiotic in synbiotic may enhance the effectiveness of antibiotics by maintaining a balanced gut microbiota, which potentially lowers the risk of infections during cancer treatment. For instance, the use of synbiotics (inulin, *Lactobacillus rhamnosus*, and *Bifidobacterium lactis*) has been found to reduce colorectal proliferation significantly, induce necrosis in colonic cells and increase the production of interferon γ in the cancer patients (Kelesidis & Pothoulakis, 2012). Clinical studies

have also revealed positive outcomes when cancer patients took synbiotics after colorectal cancer surgery, including fewer infections, less diarrhea, quicker recovery of normal gut function, reduced use of antibiotics, lower risk of severe infections, and shorter hospital stays (Chen, Y. et al., 2022; Lei et al., 2017). Probiotics are also reported to assist in detoxifying the gut, potentially reducing the risk of cancer associated with pesticide exposure and heavy metals. *Lactobacillus* was found to sequester organophosphate pesticides, parathion, and chloropyrifos. Remarkably, this pesticide-sequestering capability was observed to be independent of the metabolic activity of the bacteria, with both live and heat-killed *Lactobacillus* strains demonstrating similar pesticide-capturing abilities (Trinder et al., 2016).

2.8 Pesticides

Pesticides are one of the leading causes to death by self-poisoning (World Health Organization, 2022). It is harmful when in contact with certain amounts, producing acute poisoning or even long-term health problems such as cancer and infertility (Goh et al., 2021; Moreira et al., 2021). The global consumption of pesticides is about 4.33 million metric tons and expected to raise up to 4.41 metric tons in 2027 (Statista Research Department, 2024). Each of the pesticide might have different method of application such as dusting, spraying, fumigation and granular application (Stejskal et al., 2021). After pesticide application, plant uptakes and degradation may cause the depletion of pesticide concentration. However, the remaining pesticide residues may spread to the environment by surface runoff, vapor drifting and leaching (Vymazal & Březinová, 2015). Persistency of pesticide residue are affected by the physical and chemical properties of pesticide such as the solubility, half-life, pH value and changing climate (Butkovskyi et al., 2021; Vryzas, 2018). According to Jallow et al. (2017), excessive and non-judicious use of pesticides are potentially causing the retention and accumulation of pesticide residue within the crops, and entering the food chain (Goh et al., 2021; Jallow et al., 2017). The growth of crop beneficial *Pseudomononas* bacteria were found to be inhibited due to the disruption on aromatic amino acid biosynthesis which was specifically targeted by glyphosate (Aristilde et al., 2017).

There are various pesticide types available in the market, including fungicide, insecticide, herbicides, rodenticides, nematicides, and other miscellaneous pesticides. The most common chemical categories of fungicides are dithiocarbonates, azoles, strobilurins, dithiolane and benzamide whereas the important chemical categories of insecticides are organochlorines, organophosphates (OPs), pyrethroids, neonicotinoids and so on (Sule et al., 2022). Pesticide mixtures that are widely applied in the rice paddy field had used in the *in-vivo* rat model study. The combination of fungicide (e.g. Monocot and Fujione) and insecticide (e.g. Broadox, Sumibassa and Takumi) had been used in the in-vivo rat model study and their toxicity profile was shown in Table 2.2.

Besides that, the gut microbiota's impact associated by pesticide exposomes on brain function and behaviours can have significant consequences for overall health and well-being. The gut brain axis (GBA) is connected the brain to the gut through a bidirectional nerve called vagus nerve (Kanwar Rajawat et al., 2022). In normal circumstances, the GBA plays a role in regulating the digestive system and maintaining balance in metabolic activities. However, environmental pollutants cause neurotoxicity can disrupt the composition of the gut microbiome, leading to an imbalance in the enteric nervous system (ENS) (Costa, 2015; Sarkar et al., 2017). As a result, the GBA's functionality is compromised, which leads to the emergence of neurodegenerative disorders that worsen over time.

Table 2.2 The detail profiles of selected pesticides used, including their active compounds, oral acute toxicity and toxicity effect

Pesticide	Active compounds	CAS No	Rat Oral LC_{50} (mg/kg)	Pictogram	Toxicity effects
Monocut	70% Benzamide (Flutolanil)	66332-96-5	3542 mg/kg (F) 4913mg/kg (M)		-highly toxic to mammal and aquatic organism
Broadox	5% Buprofezin	69327-76-0	2198 mg/kg (M) 2355 mg/kg (F)		-primarily toxic to aquatic organism
	20% Isoprocarb	2631-40-5	450 mg/kg		
Fujione	Diisopropyl 1,3-dithiolan-2-ylidenemalonate	50512-35-1	300 mg/kg (F)		low or moderate toxicity -primaruly affect aquatic organism -generally safe for human
Sumibassa	O,O-Dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate (Fenitrothion)	122-14-5	240-500 mg/kg		-highly toxic to aquatic organism -moderate toxic to mammals
	2-Sec-Butylphenyl-N-methylcarbamate (Fenobucarb / BPMC)	3766-81-2	NA		
Takumi	Flubendiamide	272451-65-7	>2000mg/kg (F)	None	-low toxicity

Notes: The information obtained from MSDS of product from Agricultural Chemical (M) Sdn. Bhd. for all pesticides.NA= not available

CHAPTER 3

CHARACTERISATION OF PROBIOTICS STRAINS AND THE EVALUATION OF STRESS TOLERANCE AND CARBON METABOLISM

3.1 Introduction

Following ingestion, the probiotics must survive in gastrointestinal tract in sufficient number in order to be effective and be compatible with the product matrix, processing, and storage conditions to maintain desired properties (Khushboo et al., 2023). It is therefore stress factor analysis is crucial to reveal the functionality, viability and efficacy of probiotic strain. Known stress tolerant probiotic is vital for maximal survival performance with enhanced product stability and maintained cell viability across various stress during the manufacture, storage and delivery process (Wendel, 2022). Furthermore, stress tolerance factors and survival rates of probiotic under the simulated GI conditions can aid in meeting regulatory requirements for probiotic claims. However, the regulatory requirement of probiotic as drug or food supplement may be vary in different product authorization category and different countries (Arora & Baldi, 2017; Venugopalan et al., 2010).

Probiotics from genus Lactobacillus, Streptococcus and Bifidobacterium are widely commercialised in the probiotics supplement industry (Vera-Santander et al., 2023). The effectiveness of probiotic consumption is often determine by the average colony forming unit (CFU) of the probiotic. The optimal of probiotic consumption are usually vary on the forms of intakes, ages and individual health condition. The consumption of liquid products are usually have lower CFU counts compared to solid

concentrated supplement due to its higher absorption potencies (Soares et al., 2023). Generally, the minimum dose of probiotic consumption is suggested on 1 billions CFU/day, especially for kids, normal adult, and specific health patients are about 1-5 billions CFU/day, 1-10 billions CFU/day and 20-50 billions CFU/day respectively (National Institute of Health, 2023).

In short, this chapter provides comprehensive evaluation on the selected eight probiotic strains including their growth patterns, genetic identification, various stress tolerance test, and carbon metabolism profiling. These results aimed to discover suitable strains of probiotic with commercialization potential.

3.2 Methodology

3.2.1 Source of probiotic strains

There are eight bacterial strains isolated from Thai fermented food provided by Assoc. Prof. Dr Vijitra Luang-In from Mahasarakham University, Thailand. Detail information about the selected bacterial strains is shown as Table 3.1.

Table 3.1. The selected probiotic strains obtained from Thai fermented food.

STRAINS LABEL	PROBIOTIC STRAIN (NCBI accession code)	ORIGINAL SOURCES	CULTURE MEDIA
P1	*Enterobacter xiangfangensis* (LC342989.1)	Fermented fish	LA/LB
P2	*Lactococcus hircilactis* (LC336444.1)	Water kefir from Nakhon Ratchasima	MRSA/ MRSB
P3	*Lactococcus lactis* (LC336446.1)	Water kefir from Nakhon Ratchasima	MRSA/ MRSB
P4	*Lactococcus lactis subsp. lactis* (AB008215.1)	Purchase from TBRC	MRSA/ MRSB
P5	*Lactiplantibacillus plantarum (NA)*	Pak Sain Dong	MRSA/ MRSB
P6	*Lactobacillus fermentum (NA)*	Pak Sain Dong	MRSA/ MRSB
P7	*Lactobacillus brevis (NA)*	Purchase from TBRC	MRSA/ MRSB
P8	*Pediococcus pentosaceus* (LC336439.1)	Water kefir from Nakhon Ratchasima	MRSA/ MRSB

Note: TBRC= Thailand Bioresource Research Center, LA= Luria agar, LB=Luria broth, MRSA= de Man, Rogosa and Sharpe agar MRSB= de Man, Rogosa and Sharpe broth, NA= not available and identified

3.2.2 Probiotic activation and storage

The eight strains are initially activated by mixing 10 mg freeze-dried powder of probiotic source with 10 mL of culture media and then incubate at 30°C in incubator shaker with rotation at 150 rpm for 48-72 h until the solvent looks turbid, indicating culture grows. About 10 µL turbid cultures are streaked on slant LA/MRSA as working stock (Luang-In et al., 2020) (Appendix 1). The single colony of probiotic was inoculated into the broth media as mentioned in Table 3.1 and incubated at 30°C with 150 rpm for 24 h. The fresh probiotic strains at 24 h incubation are constantly maintained by sub-cultured in the sterile broth media for every analysis. Probiotic working stock was preserved in two forms such as liquid suspension culture with additional of 20% (v/v) glycerol and also another form is in lyophilised form of probiotic and stored in -80°C freezer (Shekh et al., 2020).

3.2.3 Growth curve and cell count

An inoculant from agar media was selected and added into new 10 ml sterile LB or MRSB media. The probiotic strains were incubated at 30°C in incubator shaker with rotation at 150 rpm. Cell count was performed at incubation period 0, 2, 4, 6, 8, 10, 12, 24, 28,and 32 h until death phase was obtained . The cells were counted with haemocytometer. Initially, about 5 µl of cell suspension and 5 µl trypan blue stains were loaded into one of the 96 well plate and mixed evenly. The addition of trypan blue stains allows differentiation of live and dead cell with living viable cell stain white and dead cell stain purple blue when observed under light microscope (Thirabunyanon et al., 2009). About 6 µl of the mixture loaded between the haemocytometer and cover glass by using micropipette. Dilution of culture suspension were adjusted based on the abundance of 100-200 cells/square in the centre square box of haemocytometer. The number of cells were counted in 5 out 25 boxes in the centre square (Figure 3.1) using Equation 3.1.

The number of cells/ml of suspension= $A \times 5 \times 10^4 \times DF$, (Equ. 3.1)
where A= the mean number of cells per square, DF= dilution factor

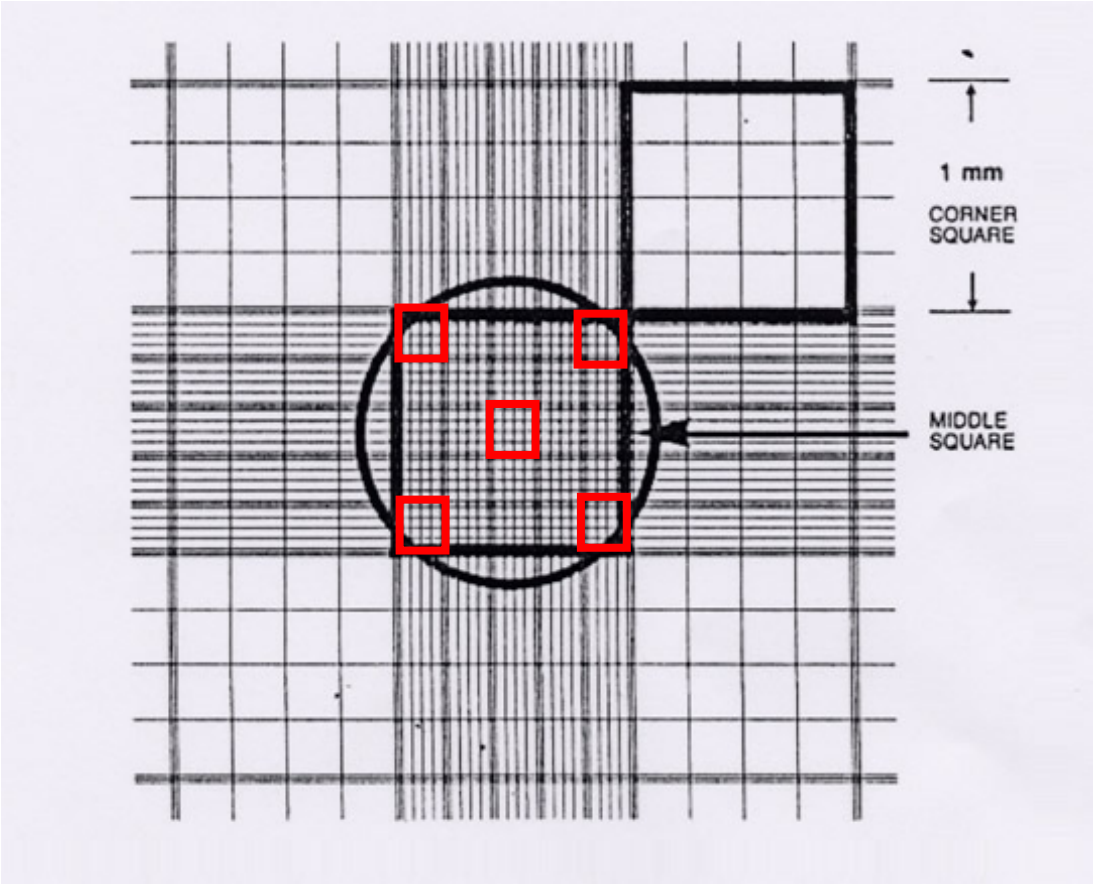


Figure 3.1 Cell counting at the centre square of the haemocytometer.

3.2.4 Morphological observation and gram staining

The morphological observation of all 8 probiotics were initially prepared from slide smear. Single loop of probiotic culture was selected with inoculation loop, and transferred to microscope slide with adding a loopful of water. The culture was spread over the slide and then slowly moved over flame, using heat to adhere the cells on the surface of the slide. Then crystal violet stain was added on the top of the slide and then rinsed off with water after 1 min carefully without losing the smear culture. The process continued by adding one drop of iodine solution for 1 min, rinsed off with water and followed by adding decolourizer ethanol for 10 s through flooding over the slide. Lastly, the slide was counterstained with safranin for 30 s and rinsed off. The slides were left to dry prior microscope observation.

3.2.5 DNA extraction and amplification

As the obtained sources might under various batch of sub-cultures in Thailand, the strains were further identified by Sanger DNA sequencing techniques. The DNA materials of each bacterial strain was extracted from liquid suspension stock using Patriot DNA Extraction Kit (Patriot Biotech Sdn. Bhd., Malaysia). About 1 mL of liquid suspension of bacterial stock was pipetted into 1.5 mL Eppendorf tube and then centrifuged at 4000 rpm for 5 mins. The supernatant was discarded, and the remaining pellet was further resuspended with 300 µL of cell lysis buffer solution (CLB). The sample was then incubated at 55°C for 30 mins. About 150 µL of salt precipitation buffer (SP) was then added and mixed well. After incubation at 4°C for 5 mins, the tube was centrifuged at 10,000 rpm for 5 mins. About 300 µL of supernatant was then transferred into another new 1.5 ml Eppendorf tube containing 300 µL of binding buffer (BB) and 15 µL of nucleic acid binding silica beads (NsB). The tube was inverting few times to promote the fixing of nucleic acid to the beads. After incubation of 5 mins, the tube was in placed in a magnetic rack for 1 min until the supernatant becomes clear. The DNA materials were expected to stick on the magnetic region, while the clear supernatant was discarded and rinsed with 75% ethanol solution for 2 times without disturbing the magnetic strip. After washing steps, the magnetic strip was removed, and air dried the tube for 5 mins. Lastly, about 100 µL of elution buffer was added into the tube that containing NsB with DNA materials and incubate at room temperature for 5 mins. The NsB was removed through placing the tube into the magnetic rack, and the final clear solution was the extracted DNA material.

The extracted DNA with approximately about 1500 basepair (bp) bacterial full-length 16S gene, was amplified using universal primers 27F (5' GAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). About 2 µl of genomic DNA (gDNA) was mixed with 10 µl of 2X mastermix stock that containing 0.3 pmol of both forward and reverse primers, 0.5 U of thermostable DNA polymerase and 400 µM of deoxynucleotides triphosphates (dNTPs). The polymerase chain reaction (PCR) was performed with 1 cycle of initial denaturation process at 94°C for 2 mins, followed by 25 cycles for denaturation, annealing and extension process (98°C for 10 s; 53°C for

30 s; 68°C for 10 s) and 1 cycle of final extension at 68°C for 1 min. The PCR products were then purified by standard PCR clean-up method. The purified PCR products were subjected to bidirectional sequencing with universal primers 785F (5' GGATTAGATACCCTGGTA 3') and 907R (5' CCGTCAATTCMTTTRAGTTT) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The PCR product were send to First Base Laboratory Sdn. Bhd. for Sanger DNA sequencing. Pre-processing of the sequences was carried out to cut off uncertain sequences including sequences contained multiple PCR products, single nucleotide polymorphism (SNP), background noise, and indel mutation using software Trace Data File Viewer (MEGA software) from https://www.megasoftware.net/web_help_11/Trace_Data_File_Viewer_Editor.htm). The pre-processed sequencing data in FASTA format was further analysed using Nucleotide Basic Local Alignment Search Tool online (BLAST, NCBI) from https://blast.ncbi.nlm.nih.gov/Blast.cgi. The top 10 blast of each sequence was compared and checked the similarity from the original sources using online alignment tools Vector Builder from https://en.vectorbuilder.com/tool/sequence-alignment.html. The primary strain annotation was identified from the highest index with max score (MS), percentage identity (PI), and query coverage (QC).

3.2.6 Stress tolerance test

3.2.6.1 Gastric acid

The simulated gastric juice, similar to intestinal environment was produced by mixing solution of pepsin (0.3% w/v), NaCl (0.5% w/v), and adjusted to pH 2.5. About 30 ml of 1×10^6 CFU/ml culture stock was centrifuged at 4000 rpm for 20 min (Vinderola & Reinheimer, 2003). The cell pellet were washed twice in 50 mM dipotassium phosphate (K_2HPO_4) (pH 6.5) and then resuspended in 3 ml of the same buffer. The supernatant was then centrifuged at 8000 rpm for 5 mins and the pellet was then resuspended in 10 ml of simulated gastric solution. The total number viable counts in colony forming unit (CFU) were counted for both before and after 3 h

incubation period at 37°C using trypan blue staining and expressed in $\log_{10}$ CFU/ml. The survival rate (%) was calculated using Equation 3.2.

Survival rate (%) = $[(\frac{Nf}{Ni}) \times 100]$, (Equ. 3.2)

where Ni=initial number of viable CFU, Nf=final number of viable CFU, DF=dilution factors and V= volume of samples

3.2.6.2 Bile salt, pH, Sodium chloride and temperature tolerance

The bile salt and pH stress analysis were performed based on the protocol in Vinderola & Reinheimer (2003) and Nascimento et al. (2019) with slight modification. About 1×10^6 CFU/ml of each culture stocks were transferred to 10 ml LB/MRSB media supply with 0, 0.3, 0.5 and 1% (w/v) of bile salt and then incubated in incubation shaker for 24 h at 37 °C with 120 rpm rotation speed. For pH tolerance test, different pH 3, 6 and 9 were adjusted by using 1N sodium hydroxide (NaOH) and 2N hydrochloric acid (HCl). About 1×10^6 CFU/ml of probiotic stock was transferred to the pH adjusted media and then incubate at 30 °C for 24 h. While for sodium tolerance, LB and MRSB were prepared in different sodium concentration by adding 0, 3, 5 and 7% (w/v) of NaCl powder. About 1×10^6 CFU/ml of probiotic stock was transferred to the pH adjusted media and then incubated at 30 °C for 24 h. For temperature tolerance analysis, about $1x10^6$ CFU/ml of probiotic stock was transferred to the media and then incubated at different incubation temperature at 25 °C, 30 °C and 37 °C for 24 h.

Measurements of bile salt were made using UV-Vis spectroscopy at 560 nm and compared to a control culture (0% bile salt). The percentage of growth in the presence of bile salts relative to the control was used to express the results by using Equation 3. The measurement of pH, NaCl and temperature tolerance were calculated by the percentage of growth detected by UV-VIS spectroscopy at 600 nm in the presence of pH, NaCl and temperature relative to the control was used to express the result using Equation 3.3

Growth (%) = [(Af-Ai)/Ai] ×100, (Equ. 3.3)

where Af =final absorbance of samples after 24h and Ai= absorbance of sample at 0 h.

3.2.7 Carbon utilisation assessment

Carbon metabolise activities were performed through Biolog Ecoplate. About 1 x 10^4 CFU concentration of each probiotic stock was counted using automated cell counter (Denovix, USA) and then centrifuged at 1000 rpm for 10 mins. The supernatant was discarded and the pellet was diluted with 10 ml of sterile phosphate buffer saline (pH 7.2-7.4), then was incubated by shaking at 150 rpm for 1 h. The Ecoplate contains 31 different metabolised carbon substrates which categorised into 5 subgroups, namely carbohydrates (G1), amino acid (G2), polymers (G3), carboxylic acid(G4), and amine (G5) as mentioned in Frąc et al. (2012). About 100 µl of diluted sample was loaded to each well of Ecoplate and incubated at 25 °C for 168 h, the absorbance was measured at 590 nm using plate reader at every 24 h interval. The amount of carbon metabolism activity of all probiotics from 0-168 h were determined in well-colour development (WCD) (Frąc et al., 2012) and carbon substrate utilisation (CSU) based on the Equation 3.4-3.6:

$$WCD = (P_a - P_c) \quad \text{(Equ. 3.4)}$$

$$pi = (P_a - P_c) / \text{sum of } (P_a - P_C) \quad \text{(Equ 3.5)}$$

$$CSU\ (\%) = \text{sum of } pi \text{ of each group} \times 100\% \quad \text{(Equ. 3.6)}$$

where pi =index value of single well, P_a = absorbance value from each well, P_c = the absorbance of control well.

3.2.8 Statistical analysis

Analytical software Graphpad Prism (Version 10, Boston) was used for the initial data analysis of most of the triplicate datasets and presented in mean± standard error. One way analysis of variance (ANOVA) is conducted for discrimination analysis of response variables between probiotic groups with Duncan Post Hoc Test with $p < 0.05$ denoted for significantly difference among the substrate treatment. Multivariate analysis for principal component analysis (PCA) was performed using SIMCA 14 software (SIMCA) and PCA biplot was plotted from online free webtool SR Plot (https://www.bioinformatics.com.cn/plot_basic_cluster_heatmap_plot_024_en). The clustering heatmap on stress tolerance response and carbon substrate utilisation had been generated using online webtool ClustVis (https://biit.cs.ut.ee/clustvis/).

3.3 Result and discussion

3.3.1 Probiotics identification by morphology and DNA confirmation

Probiotic (P1-P8) were identified by the nucleotide base pairs (bps) top score matched with species in NCBI Blast system. Table 3.2 shortlisted the NCBI accession code (AC) of identified strains with the morphological observation of each probiotic in term of gram stain behavior, cell shape and colony colour. In the NCBI Blast system, several parameters such as max score (MS), query coverage (QC), percentage identity (PI) and associate length are often used to determine the high alignment score points (HSP) between the two query and subject sequences and give the alignment quality (Stover & Cavalcanti, 2017). The top10 blast with highest MS, QC and PI were shown in Appendices 2-9. Based on the NCBI blast result, P3 and P4 as *Lactococcus Lactis WS18 gene and Lactococcus lactis subsp. lactis* with the similar AC in the original source (Table 3.1, Table 3.2). However, Sanger sequence technique for 16S RNA only reliable to identify bacteria strain up to species level under comparative criteria like MS, QC and PI, yet to resolve strain level difference. It is therefore the other 6 strains (P1-P2 & P5-P8) that displayed some variation in the genetic sequences as compared

to the original source were registered with new accession code in the NCBI GenBank database to avoid strain-specific variation and create genomic database for the future study (Table 3.2). In future, it is recommended to conduct whole genome sequencing, single nucleotide polymorphism (SNP) based analysis or multi-locus sequence typing of housekeeping genes for precise strain discrimination and robust taxonomic assignments (Goyal et al., 2022).

Table 3.2. The genetic identification of probiotics strains isolated from Thai food.

Label	Identified Species	Top Blast AC (MS, QC, PI)	Result outcome of NCBI BLAST	Gram Stain	Morphology (shape)	Colony colour
P1	*Enterobacter asburiae strain MAP1*	MT180710.1 (1810,100%,100%)	-newly register in NCBI genbank with AC: PQ620115	- (pink)	Elongate spherical shape	White to beige
P2	*Lactococcus lactis subsp.lactis strain MAP2*	MN749817.1 (1604,99%,100%)	-newly register in NCBI genbank with AC: PQ620126	+ (purple)	Spherical cocci shape	Creamy white
P3	*Lactococcus lactis WS18*	LC336446.1 (1343,100%,100%)	-*C*onfirmed same AC *LC336446.1*	+ (purple)	Spherical cocci shape	White
P4	*Lactococcus lactis subsp. lactis YIT 2008*	AB008215.1 (1315,99%,99.72%)	-*C*onfirmed same AC AB008215.1	+ (purple)	Spherical cocci shape	White
P5	*Lactiplantibacillus plantarum MAP5*	CP162015.1 (1094, 100%,100%)	-newly register in NCBI genbank with AC: PQ620119	+ (purple)	Rod shape	Creamy white
P6	*Limosilactobacillus fermentum MAP6*	CP094655.1 (1197,100%,100%)	-newly register in NCBI genbank with AC: PQ620122	+ (purple)	Rod shape	White
P7	*Levilactobacillus brevis MAP7*	CP142716.1 (1539,100%, 100%)	-newly register in NCBI genbank with AC: PQ620124	+ (purple)	Rod shape	White to slight yellow
P8	*Pediococcus pentosaceus MAP8*	CP137627.1 (793,100%,99.77%)	-newly register in NCBI genbank with AC: PQ620127	+ (purple)	Spherical cocci	Creamy white

Notes: NCBI = national center for biotechnology information, AC= NCBI accession code, MS= max score, QC = query coverage, PI= percentage identity, symbol - represent gram negative bacteria, symbol + represent gram positive bacteria.

Besides genetic identification, all eight strains were further confirmed with their morphological observation. P1 with elongate spherical shape showed pink stain during gram staining on the cell had confirmed it is a gram-negative bacterium (Figure 3.2a). While P2-P8 showed purple stain during gram staining had confirmed they are gram-positive bacteria (figure 3.2b-h). It is correctly matching that genus *Lactococcus spp., Lactobacillus spp.* and *Pediococcus spp.* were gram-positive bacteria (Taye et al. 2021; Todorov et al., 2023). Probiotic P2-P4 are identified as the relative strains of *Lactococcus lactis* (Oberg et al., 2022). Due to vast ecological diversity and genetic diversity of Lactobacillus genus, taxonomy reclassification had been done to differentiate bacteria from different source and habitat since year 2020. For instance, P5 *Lactiplantibacillus plantarum* where the genus is named as Lacti (refer to milk), Planti (refer to plant materials) and bacillus (rod shape), P6 *Limosilactobacillus fermentum* where is named Limosus (refer to viscous or slimy colony characterisation) and P7 *Levilactobacillus brevis* where the genus name has come from Levi (refer to smooth colony structure) (Huang et al., 2018). In addition, the elongated rod shape and purple stained cell of P5-P7 further confirmed their identity as gram positive bacteria with rod shape structure (Figure 3.2). Probiotic P8 is identified as *Pediococcus pentosaceus***,** gram-negative bacteria with pink stained cell and come in conformity with its scientific domain name of coccus (Todorov et al., 2023).

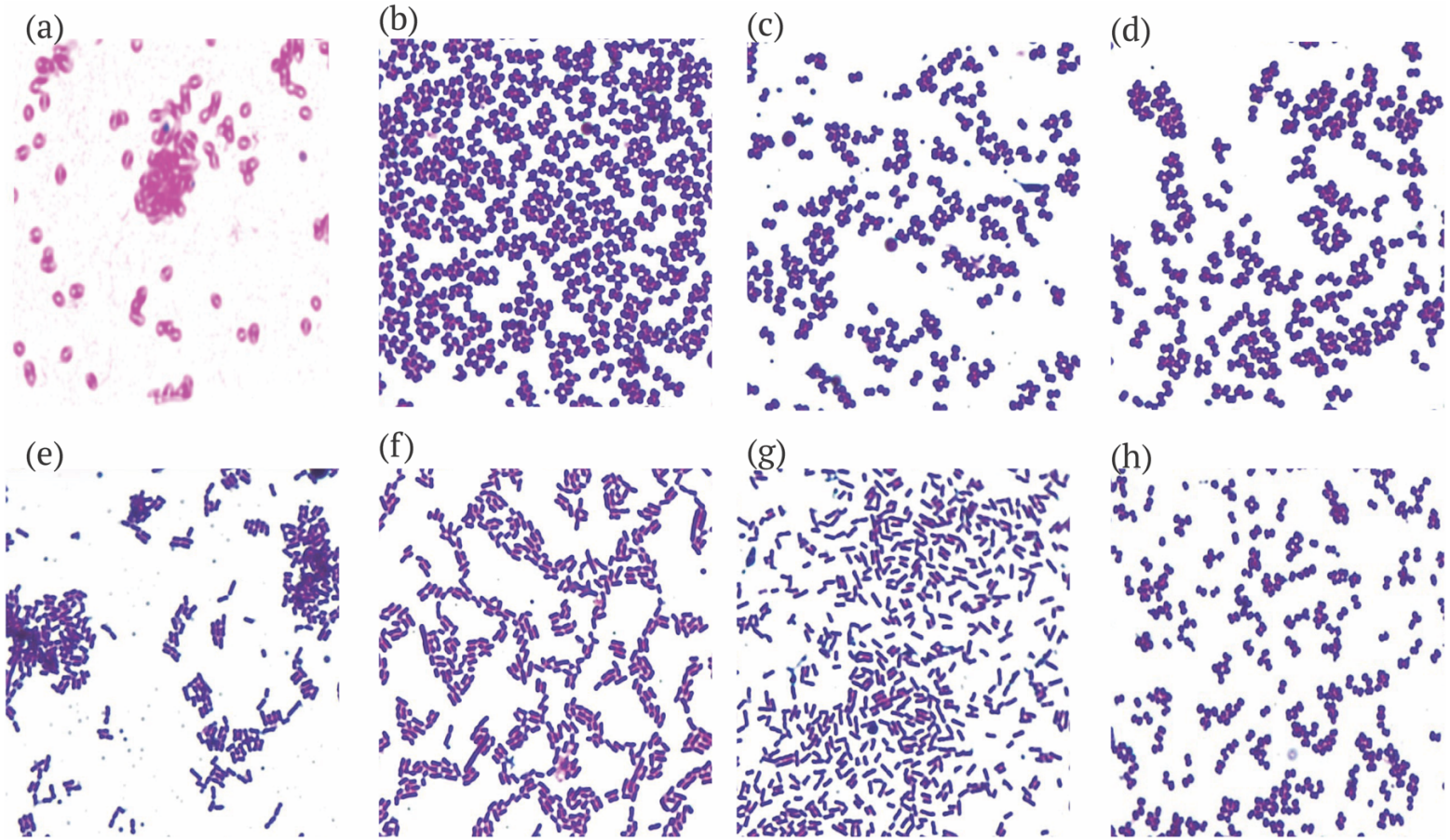
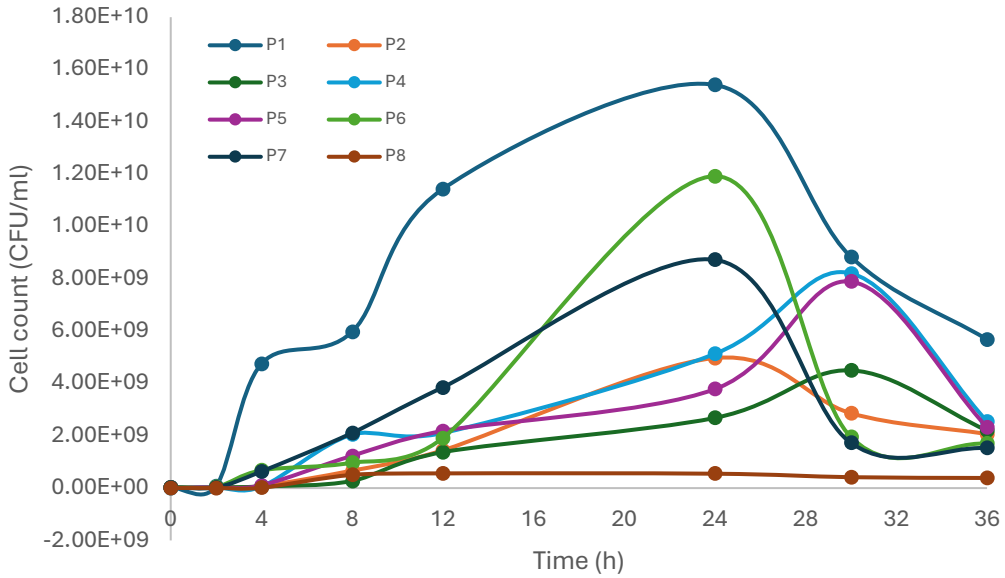


Figure 3.2 The morphological observation of probiotic strains P1-P8 under light microscope 400x magnification with displayed of its gram staining colour and cell shape. Purple colour refers to gram positive bacteria and pink color refers to gram negative bacteria. (a) P1=*Enterobacter asburiae*, (b) P2=*Lactococcus lactis subsp. lactis MAP2*, (c) P3= *Lactococcus lactis WS18*, (d) P4= *Lactococcus lactis subsp. lactis YIT 2008*, (e) P5= *Lactiplantibacillus plantarum MAP5*, (f) P6= *Limosilactobacillus fermentum MAP6*, (g) P7= *Levilactobacillus brevis MAP7* and (h) P8= *Pediococcus pentosaceus MAP8*.

3.3.2 Probiotic growth rate and growth curve

The probiotic growth curve of each probiotic in the MRS broth (except P1 in Luria broth) have been investigated and reported in Figure 3.3. The growth rate of P1 is higher than other probiotic strains, however it is not comparable to the rest of the probiotics as P1 need to culture in different agar as they could not survive in the MRS broth with a more enriched nutrient. The comparison of probiotic P2-P8 reveal the highest growth density observed in P6 *L. fermentum* (about $1.19x10^{10}$ CFU/ml) in 24 h incubation period. The factors that influence the growth pattern of probiotic is closely related to its genetic makeup, nutrient requirement, metabolic rate with different metabolite productions (Makarova et al., 2006; Peleg & Corradini, 2011). *Enterobacter asburiae* (P1) demonstrated relative shorter lag phase compared to other probiotic strains due to its high adaptation to new environment and high density of growth promoting genes (Raturi et al., 2022) (Figure 3.3b). *L. fermentum* (P6) showed rapid exponential growth from 12 h to 24 h, revealed that high resource utilisation ability that promote faster growth (Vrancken et al., 2008). The variation in growth curve may due to the difference in enzyme efficiency and metabolic pathway (Wang, S. et al., 2020). The growth curve comprises several phases like lag phase (LP)**,** exponential phase (EP**)** , and death phase (DP) (Broeckx et al., 2020). LP is a stage where cells begin to prepare for active growth but not growing significantly in number, where EP is commonly used to reflect the strain's optimal performance with high metabolic activity and proliferation rate, and lastly DP indicate for the cells lose viability and begin to lyse (Bertrand, 2019). Through understanding these phases, the growth and preservation of probiotics can be optimised in functional foods and supplements.

(a)



(b)

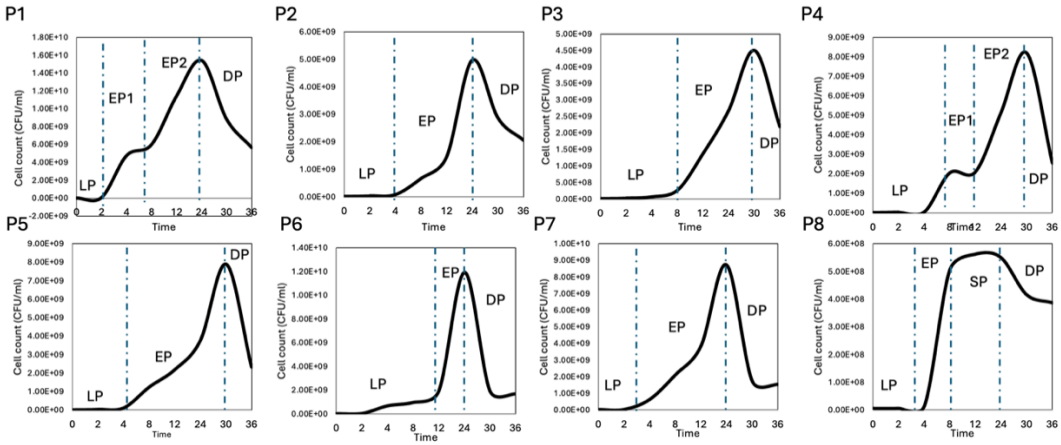


Figure 3.3 The growth curve of probiotics observed. (a) overview of cell number over 36 incubation period of all 8 probiotic strains, where (b) showed the growth pattern of probiotic P1-P8 where P1=*Enterobacter asburiae*, P2=*Lactococcus lactis subsp. lactis MAP2*, P3= *Lactococcus lactis WS18*, P4= *Lactococcus lactis subsp. lactis YIT 2008*, P5= *Lactiplantibacillus plantarum MAP5*, P6= *Limosilactobacillus fermentum MAP6*, P7= *Levilactobacillus brevis MAP7* and P8= *Pediococcus pentosaceus MAP8*. LP= lag phase, EP= exponential phase, DP=dead phase.

3.3.3 Stress characterisation of probiotics

Stress tolerance is crucial for the selection of good probiotic. A good probiotic bacteria is usually have capability to survive under acidic environments, so that they can imparting benefit to the host (Uma et al., 2019). The characterisation of all 8

probiotic strains against different stress such as under gastric acid, bile salt (BS), sodium chloride salt (NaCl), temperature (Temp) and pH demonstrated in Figure 3.4 (a-e).

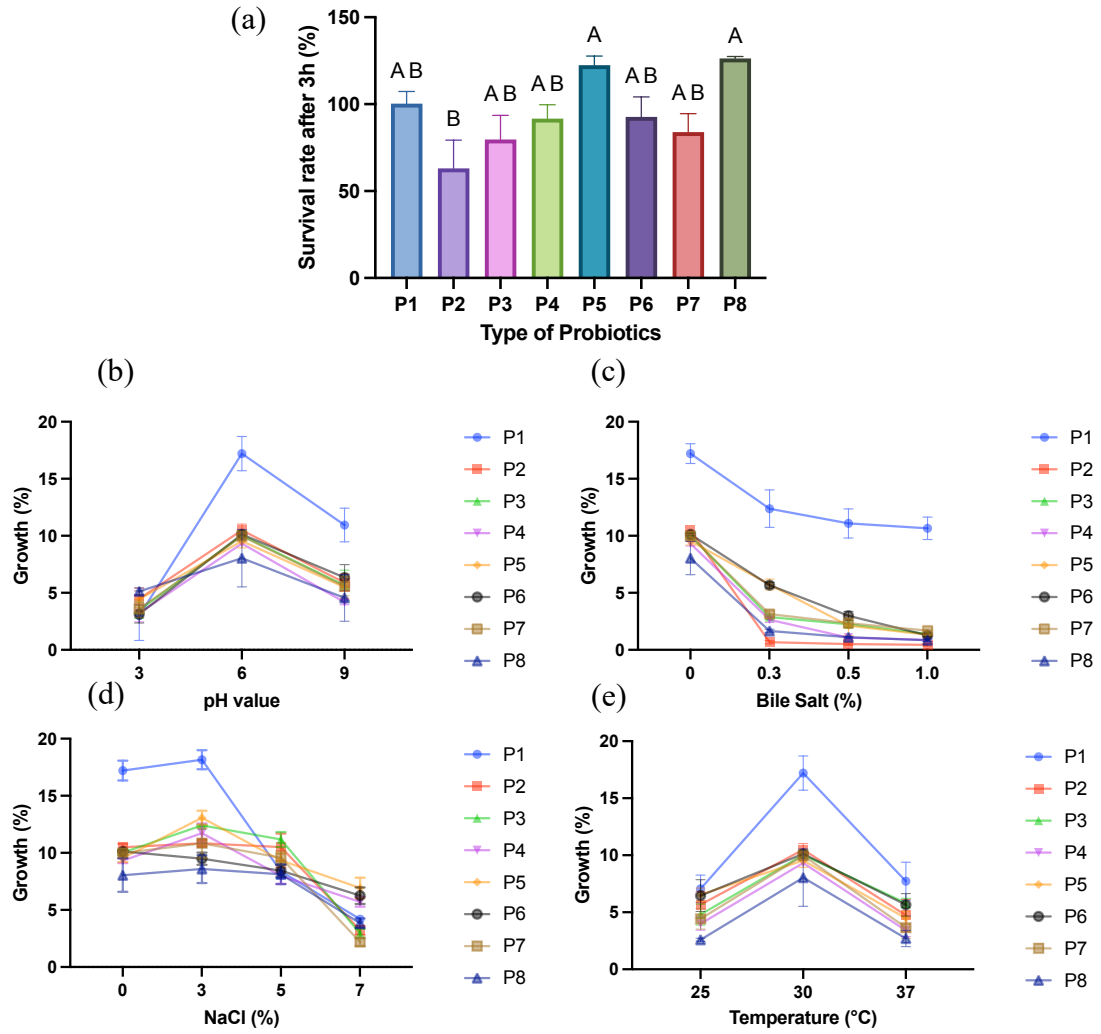


Figure 3.4 The comparison of probiotic growth rate of P1-P8 under different stress parameters including (a) gastric acid at pH 2.5, (b) pH value, (c) bile salt, (d) salt concentration (NaCl), and (e) temperature. P1=*Enterobacter asburiae*, P2=*Lactococcus lactis subsp. lactis MAP2*, P3= *Lactococcus lactis WS18*, P4= *Lactococcus lactis subsp. lactis YIT 2008*, P5= *Lactiplantibacillus plantarum MAP5*, P6= *Limosilactobacillus fermentum MAP6*, P7= *Levilactobacillus brevis MAP7* and P8= *Pediococcus pentosaceus MAP8*

Extensive studies demonstrated *L. plantarum* exhibited many beneficial effects in the gut health improvement and exhibit strong resistance towards gastric acid and bile salt (Katiku et al., 2022; Prete et al., 2020; Šeme et al., 2015; Singhal et al., 2021). It is therefore *L. plantarum* is widely recognized as a standard strain for probiotic

selection criteria. From this study, the novel strains identified in this study: P5 had identified as *L. plantarum* which have a huge potential to be widely used in functional food production was set as standard strain for comparison with other strains.

All probiotic strains except P2 showed more than 80% survival rate after 3 h incubation in the simulated gastric acid environment, which suggested that these probiotic strains possess good resistance towards gastric acid. From the findings, P8 *P. pentosaceus* showed strong resistance towards gastric acid as P5 *L. plantarum* with increase of viable cell number and displayed survival rate about 126.38% and 122.46% respectively (Figure 3.4 a). The results are in agreement with the studies reported that *P. pentosaceus* exhibited good resistance towards gastric acid up to pH 2 (Jonganurakkun et al., 2008; Mgomi et al., 2022; Qi et al., 2021). All other strains (except P2 *Lactococcus lactis subsp. lactis* is more sensitive to gastric acid with only 63% survival rate) exhibited high gastric acid resistance with more than 80% survival rate which also indicated these strains showing great potential as functional probiotic. Many literatures studies had supported the probiotic display good gastric tolerance like *L. fermentum* exhibited more than 80% and 94.4% survival rate at pH2.5 and pH3 simulated gastric juice in Bao et al., (2010) and Liu, C. et al. (2022) respectively, while more than 80% survival rate in all 4 mutant or non-mutant strains of *L. brevis* reported by Lyu et al. (2018). About 8 out of 12 strains of *L. plantarum* strains showed more than 80% survival rate at simulated gastric acid at pH3 (Wu, Q. et al., 2022).

All probiotics show pH resistance with positive growth rate with similar acidic and alkaline resistance as standard strain P5 (Figure 3.4 b). This study revealed that the growth rates of probiotics is optimal at pH 6, which could be achieved in neutral media. However, it is observed that about 3-5% of growth rate reduction in all strains in both acid and alkaline media as compared with the neutral pH6 media. These results showed that all these strains faced some degree of acidic and alkaline stress, but they are still capable to survive and maintain positive growth after 24 h of incubation. P1 strains seem to be more sensitive in low acid environment with about 10% growth reduction. According to Guan & Liu, (2020), acid tolerant probiotic can adapt to gastrointestinal acidic conditions with several mechanism such as the interaction of cell membrane and ion transport system which involved the proton pumps and ATPases to maintain pH homeostasis and also the production of protective proteins

like chaperones and enzymes to protect and refold damaged proteins caused by acid stress. The GI tract has varying pH levels, from highly acidic in the stomach to neutral or slightly alkaline in the intestines. Probiotics must adapt to these changing conditions to remain viable.

The overall growth rates of all 8 probiotic strains are gradually reduced when bile salt concentration increase to 0.3% (Figure 3.4 c). Probiotic P1 *E. asburiae* is significantly resistance to bile salt compared to other probiotic strains. This finding is in conformity with the study of Li et al. (2023) who reported that *E. asburiae* was reported with extremely high resistant up to 4% (w/v) bile salt. The probiotics strains tested in this study are all show greater survival rate below 0.5% of bile salt, which in agreement with most of lactic acid bacteria such as *L. plantarum* and *L. fermentus* showed bile salt resistance up to 0.5% and 0.3% respectively (Hao et al., 2024; Singhal et al., 2021). In very rare case like about 74.7% of *L. fermentum* can survive under 1.2% bile salt in *L .fermentum* (Chen et al., 2022) and 60-80% of *L. plantarum* survive in 1.8% of bile salt (Prete et al., 2020). Bile salts play a crucial role in the emulsification and absorption of fats in the digestive system, regulation of cholesterol metabolism and influence the gut environment (Bellesi & Pilosof, 2021). From the findings, all probiotic must resist bile salt to survive and establish themselves in the gut, and promote the modulation of bile salt metabolism and in turn contribute to a healthy gut microbiota.

All probiotic strains showed optimal growth rate in 3% NaCl (Figure 3.4 d). Bisson et al. (2023) revealed that *L. plantarum* can survive up to 7% NaCl. Another study reported that *L. fermentum* and *P. pentosaceus* showed increasing growth rate tolerate up to 6% of NaCl and extreme slower growth rate in 8% of NaCl (Zhang et al., 2020). High NaCl concentrations are usually coupled with the damage of cell surface and deformation of functional protein structure, leading to slowing of strain proliferation (Zhang et al., 2020). Salt (NaCl) tolerance ensures that probiotics can survive during food processing and storage in salty environments, as many probiotic products are incorporated into food matrices.

The temperature range selection is based on normal room temperature (25°C), probiotic laboratory incubation temperature (30°C) and gastrointestinal temperature

(37°C). From Figure 3e, all probiotic P1-P8 showed positive growth at all three temperature ranges and optimal growth at 30°C (Figure 3.4 e). The result is come in agreement with the finding of Katiku et al. (2022) that reported on good growth of *L. plantarum* at 30°C and 37°C and reduction in growth at 20°C and 45°C. In short, temperature stability is critical for probiotics to maintain viability during production, storage, and transit through the human body, where temperatures can fluctuate.

P1 showed higher growth performance across all stress parameters (Figure 3.4) and displace distinct cluster in PCA scatter plot (Figure 3.5). Due to the huge discrepancy when comparing data of P1 to P8 together, the P1 value were removed from statistical analysis in heat map and PCA biplot. Based on clustering heatmap, there are 3 close clusters (P3&P4, P6&P7 and P8, P2&P5) being observed, which mean the probiotics in the same cluster responses the same toward similar stressors. P3 and P4 demonstrated negative and low correlation across most stress parameters, but is very sensitive to NaCl concentration, whereas P6 and P7 exhibit negative correlations to GA, BS and NaCl parameters. P8, P2 and P5 showed positive correlations to GA.

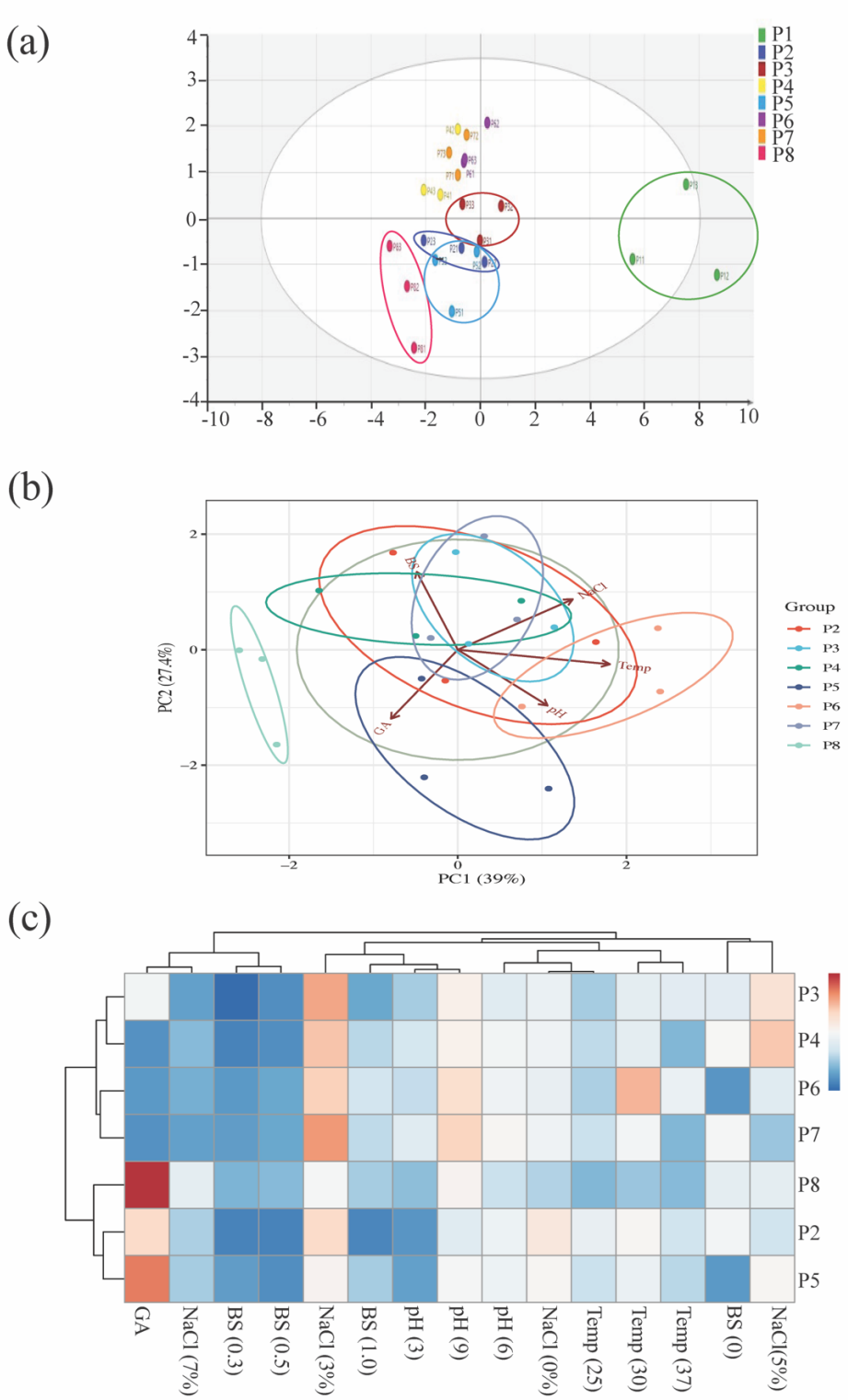


Figure 3.5 The comparison of probiotic growth rate in (a) PCA score scatter plot for P1-P8, (b) PCA biplot and (c) Clustering heatmap graph between P2-P8 with all stress parameters. GA= gastric acid, BS= bile salt and Temp =temperature. Capital letter on top of the bars represents significant different of probiotic groups at P< 0.05. P2=*Lactococcus lactis subsp. lactis MAP2*, P3= *Lactococcus lactis WS18*, P4= *Lactococcus lactis subsp. lactis YIT 2008*, P5= *Lactiplantibacillus plantarum MAP5*, P6= *Limosilactobacillus fermentum MAP6*, P7= *Levilactobacillus brevis MAP7* and P8= *Pediococcus pentosaceus MAP8*

The PCA biplot further confirmed the effect of bile salt and NaCl to P3 and P4, temperature and pH to P6 and P7, while gastric acid on P2 and P5 (Figure 3.5b). P8 demonstrated distinct cluster in both clustering heatmap and PCA biplot (Figure 3.5 b&c) compared to other probiotic group, which indicating that P8 exhibited different stress tolerance profile with other probiotic type. GA stress parameters exhibit strong influence on P5 and P8, which further supported that P5 *L. plantarum* and P8 *P. pentosaceus* exhibited unique tolerance against GA. The study of Guo et al. (2017) revealed the capability of *L. plantarum* to retain cell structure, sustain intracellular membrane integrity, and regulate pH homeostasis under acid stress, preventing the cells against damage, which account for the high tolerance of GA in *L. plantarum*.

In short, all eight probiotic showed positive growth under stress environment which proved that they have some tolerance ability against stress. They may employed different mechanisms to regulate adaptations for better survival and growth (Bustos et al., 2024). Therefore, the same cluster of probiotics could be potentially used in a combination treatment or consumption.

3.3.4 Biolog ecoplate assessment

3.3.4.1 Carbon substrate utilisation (CSU)

The carbon utilisation assessment on the selected 8 probiotic strains had been performed for cumulative of 168h. Biolog Ecoplate is used to determine the metabolic and physiological potential of microorganism based on the degradation and metabolise ability towards 31 types of carbon substrates (Perujo et al., 2020). The well colour turned purple through tetrazolium redox dyes when the probiotic metabolised the carbon substrates. The higher intensity of colour changes was corresponding to high metabolic activity. Therefore, the colour intensity of the response well were used to quantify the well colour development (WCD). The 31 carbon substrates were clustered into 5 major subgroups in the utilisation of carbon substrate (CSU) (Figure 3.6). In this study, WCD is used to provide an overview on the overall metabolic activity of probiotic against 31 carbon substrates (Frąc et al., 2012).

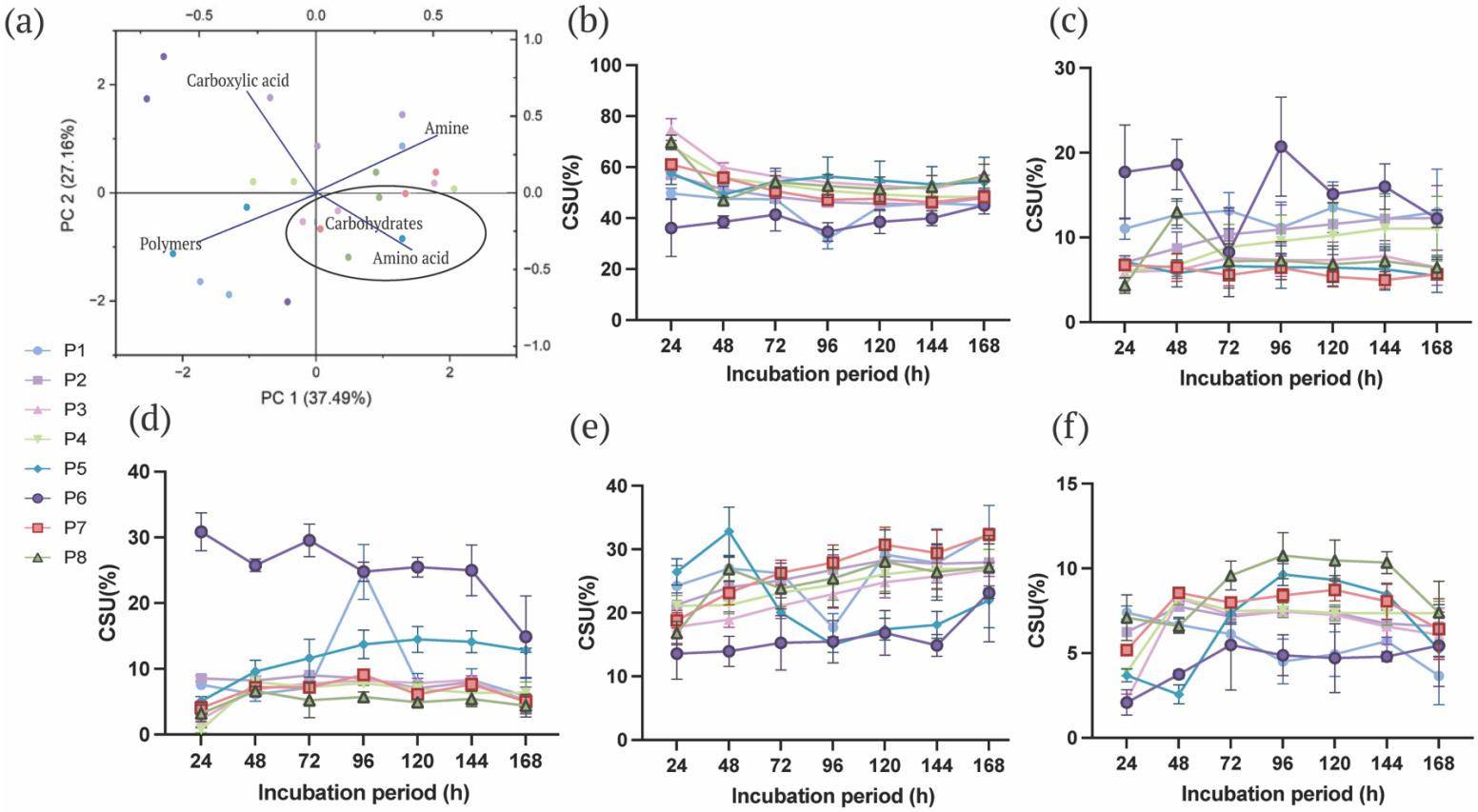


Figure 3.6 The carbon metabolism assessment based on 31 carbon substrates. Observation parameters over 168 h are plotted (a) PCA biplot and carbon substrate utilisation (CSU) (%) in respective sub-carbon groups of carbohydrate (b), polymer (c), carboxylic acid (d) amino acid (e) and amine and amide (f). P1=*Enterobacter asburiae*, P2=*Lactococcus lactis subsp. lactis MAP2*, P3= *Lactococcus lactis WS18*, P4= *Lactococcus lactis subsp. lactis YIT 2008*, P5= *Lactiplantibacillus plantarum MAP5*, P6= *Limosilactobacillus fermentum MAP6*, P7= *Levilactobacillus brevis MAP7* and P8= *Pediococcus pentosaceus MAP8.*

The PCA biplot indicates that all probiotic strains are responsible on different metabolism function (Figure 3.6 a). Biplot is the graphical presentation of score scatter and loading plot to visualise the data structure of the variables (Yan & Tinker, 2006). There is no distinct group clustering among the probiotic types, however probiotics are all cluster nearby, representing the reliable of the data. The loading plot displayed that amine subgroup fall in quadrant 2 (Q2), which reflecting that it showed strong positive association with the probiotic group fall within Q2. Carbohydrate and amino acids showed higher percentage of carbon substrate utilisation had fall within quadrant 4 (Q4). Amongst all 5 subgroups of carbon substrate, the highest percentage utilisation are carbohydrate, which achieved approximately average 40-80% amongst tested probiotics (Figure 3.6 b). The data assembled in this study showing that the growth-promoting properties of a range of carbohydrates for probiotic P3 *L. lactis*, especially in first 24 h (Figure 3.6 b). Therefore, it is not surprise that *L. lactis* have been widely used in sugar fermentation (Gunkova et al., 2021).

As compared to all probiotic strains, P6 *L. fermentum* displayed high CSU% for polymers (except 72 h incubation period) and carboxylic acid compared to other probiotic groups from 24 h to 168 h (Fig 3.6 c&d). It has higher utilisation percentage in the carboxylic acid and polymer group while lower CSU% in carbohydrate, amino acid and amine amides groups. The carbon metabolism of *L. fermentum* has been studied extensively due to its heterofermentative lactic acid bacteria nature (Capra et al., 2023; Hossain, 2022). *L. fermentum* showed its heterofermentative metabolism through the ability to metabolise different carbon source and the production of acetic acid during the exponential growth phase, and then accumulate metabolic products during the cell proliferation (Charalampopoulos et al., 2002).

3.3.4.2 Clustering heatmap

Clustering heatmap (Figure 3.7) had been generated to visualise how probiotcs bacteria utilised the carbon substrate in 168 h of culture. There are 3 distinct substrate utilisation patterns had been observed over the 168 h cultivation. The first cluster is the high utilisation group substrates, which constantly needed from 24 h till the end of the experiment at 168 h. This results may indicated that the probiotic may adapt to

metabolise specific compounds as the incubation progresses. These group of carbon substrate are N-acetyl-D-glucosamine (NAG), L-serine and L-asparagine, which suggesting that the probiotic has a strong preference for these energy sources. Amino acid L-asparagine act as energy supplier and involved in the nitrogen metabolism. Literature revealed that *Lactobacillus spp.* can metabolise L-asparagine for protein synthesis and energy in nutrient-deficient environments, and also aid in the gut colonization (Sugahara et al., 2021). By metabolising L-asparagine, these probiotics can promote their growth with more persistence in the gastrointestinal tract, and stay resilience under stress, thereby enhancing their probiotic efficacy (Broderick & Duong, 2016). While NAG is a monosaccharide derivation of glucose, which play significant components of cell well of bacteria, it is used to enhance the growth of gut microbiota, and ameliorate inflammation activity in the chronic gastrointestinal disease like ulcerative colitis (Choi et al., 2023).

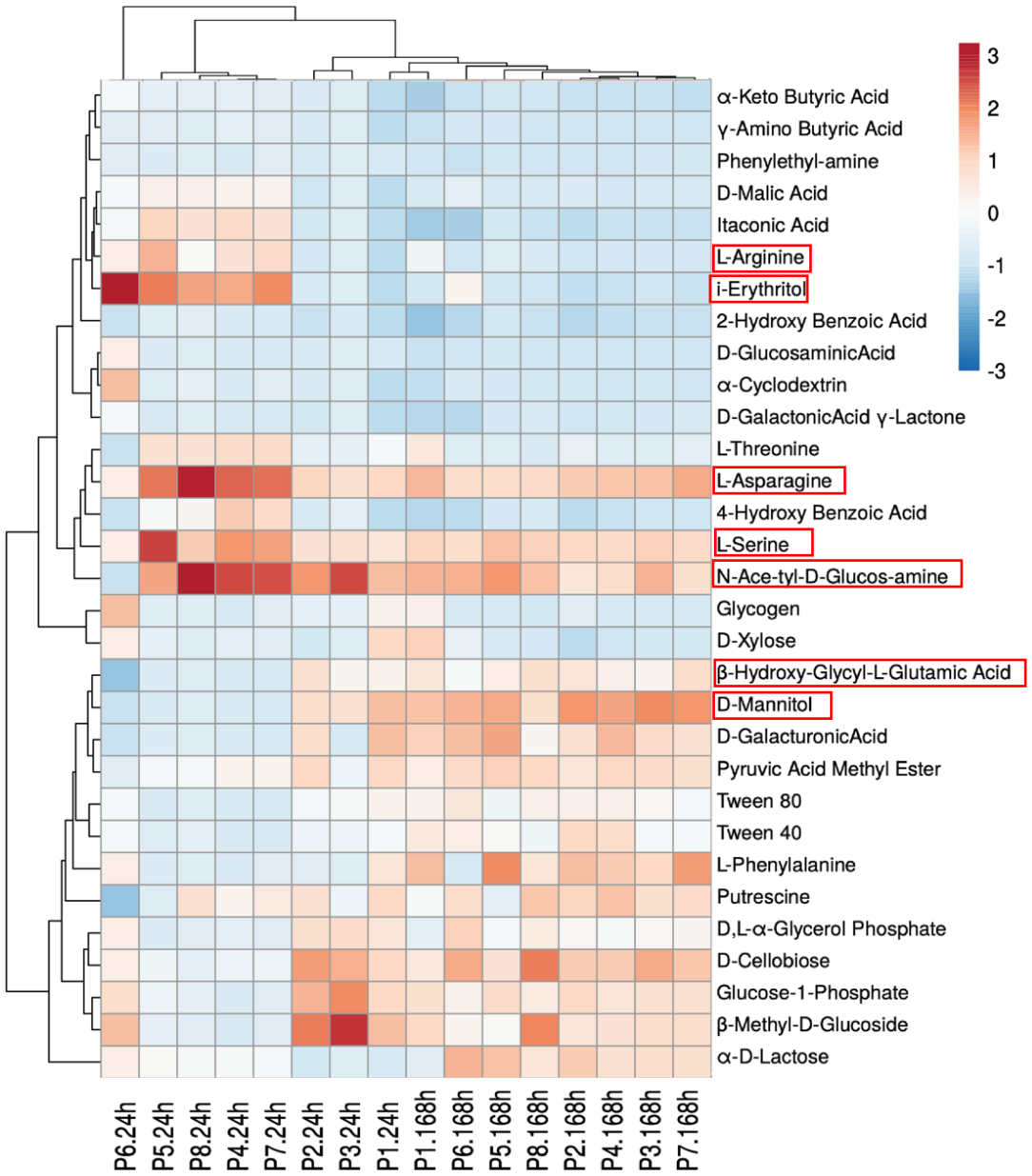


Figure 3.7 The clustering heatmap generated to compare the correlation between all 8 probiotic groups and 31 carbon substrates in two different timepoint 24 h and 168 h. P1=*Enterobacter asburiae*, P2=*Lactococcus lactis subsp. lactis MAP2*, P3= *Lactococcus lactis WS18*, P4= *Lactococcus lactis subsp. lactis YIT 2008*, P5= *Lactiplantibacillus plantarum MAP5*, P6= *Limosilactobacillus fermentum MAP6*, P7= *Levilactobacillus brevis MAP7* and P8= *Pediococcus pentosaceus MAP8*

The second cluster is carbohydrate-based substrates showed significant shift from 24 h to 168 h in all probiotic groups. There are two groups of substracts that shifted from high utilisation at the initial culture to low utilisation at 168 h culture and vise versa. This cluster represent the major cluster as two-third of metabolites carbon are in these cluster. This results may indicated that the probiotic may adapt to metabolise specific compounds as the incubation progresses. This pattern suggests that

the probiotic's metabolic activity shifts over time, possibly adapting to optimise energy extraction from preferred carbon sources as initial resources are depleted (Ma, T. et al., 2023). Carbon substrates like β-hydroxy-glycyl-L-glutamic acid, D-mannitol and D-galacturonic acid demonstrated a significant increase in utilisation over time. As energy reserves diminish at 168 h, the increased demand for energy drives a higher rate of substrate utilisation. D-mannitol and D-galacturonic both play similar role in the energy production, engaging actively in the glycolysis process (Protzko et al., 2018; Wisselink et al., 2002). There are different metabolic pathway such as M2DH-based catabolic pathway and phosphoenolpyruvate-dependent phosphotransferase system reported in the mannitol utilisation systems which had been studied in the probiotic like *Bacillus subtilis* (Heravi & Altenbuchner, 2014), and *L. plantarum (Yang, X. et al., 2019).*

Another substrate group showed an opposite utilisation trends with high utilisation on initial 24 h and then slowly depleted in utilisation over time of 168 h. These carbon substrates are I-erythritol, itaconic acid, malic acid and L-arginine are possibly been used up over time. The metabolism of all these four compounds are varied by different probiotic based on the enzymatical reaction occurs within the probiotic-carbon substration interactions. For instance, as a part of tricarboxylic acid (TCA) cycle metabolism, itaconic acid is metabolised by enzyme itaconate decarboxylase through many LAB and yeasts (Chen et al., 2016; Gopaliya et al., 2021). There are few studies reported on the malic acid can be metabolise by many LAB including *Lactobacillus* strains (particularly *L. plantarum* in most of the existing studies), *Pediococcus* strains and *Oenoccocuccs* strains with the present of malolactic enzyme (Fu et al., 2022; Gil-Sánchez et al., 2019; Krieger-Weber et al., 2020). Carbon substrate I-erythritol is a sugar alcohol, that can use as low calories sweeter and gain interest in diabetic because their consumption do not affected the insulin and glucose level (Daza-Serna et al., 2021). While, amino acid L-arginine play crucial role that act as precursor in various metabolic pathway and central intestinal metabolites in most of the microbial and mammalian organisms (Nüse et al., 2023).

The last cluster of substrate utilisation can be seen on the substrates like α-keto-butyric acid, γ-amino buttyric acid, and phenylethyl-amine, which exhibited minimal utilisation over the whole study period, suggesting they are not primary energy sources

for these probiotic under the given conditions. In short, these result suggesting that the probiotics has a strong preference for different energy sources.

In summary, the carbon substrate metabolic profiling through Biology Ecoplate assessment had underscores the unique substrate utilisation abilities among different probiotic groups (P1–P8) and across temporal variations from 24 h to 168 h. The ability of probiotic to metabolise various carbon sources, including carboxylic acids, suggests potential probiotic benefits such as modulating gut pH, producing SCFAs, and enhancing gut health by reducing pathogenic bacterial growth. This metabolic versatility positions this probiotic as potentially effective in contributing to a balanced and beneficial microbiome. The findings can provide ideas on the selection of probiotic strains based on their efficiency and metabolic versatility in CSU and contributed to the development for probiotic formulations of specific health applications.

3.3.4.3 Pearson correlation

The correlation between 31 carbon substrates were indicated from the Pearson correlation heatmap plot (Figure 3.8). The statistical analysis for Pearson correlation of CSU had been shown in Appendix 10. The individual carbon substrate showed different degree of correlations even being clustering into the same carbon substrate subgroup (G1-G5). In the carbohydrate (G1) subgroup, glucose-1-phosphate showed strong significant positive correlation with D-mannitol. Glucose-1-phosphate acts an important intermediate components in the glycolysis pathway (Salih et al., 2022), which play vital roles in the modulation of various metabolic pathways whereas D-mannitol as a stored carbohydrates that being produced from microorganism or plant. Theoretically, D-mannitol does not direct participant in the glycolysis pathway, however the production of intermediate in glycolysis pathway like fructose-6-phosphate can be from D-mannitol in the present of microorganism (Martău et al., 2020). D-cellobiose showed moderate and strong correlation with α-D-lactose and β-methyl-D-glucoside. This finding can be explained with their similar nature as disaccharides with similar break down pathway to cleave glycosidic bonds in the present of glycosidase enzyme (Deng et al., 2020; Sinha et al., 2019), which potentially

reflecting their coordination on the substrate utilisation patterns. On the other hand, NAG showed moderate negative correlations with D-cellobiose, α-D-lactose, β-methyl-D-glucoside and D-mannitol whereas D, L-α-glycerol phosphate showed a strong negative correlation with D-mannitol and glucose-1-phosphate. The negative correlation might suggest when single type of carbohydrate metabolism is high, the utilisation of another type of carbohydrate might be lower. This is possibly due to the pathway inhibition or substrate preference by the probiotic group (Bhayani et al., 2022). A potential competition for resources or inhibition effects between pathways, for instance the store carbohydrate D-mannitol might less utilised when the resources is being used to utilise other carbohydrate (e.g. NAG) (Wünsch et al., 2019). In short, the correlation among G1 provide an idea on the different interplay between different carbohydrate metabolic pathway.

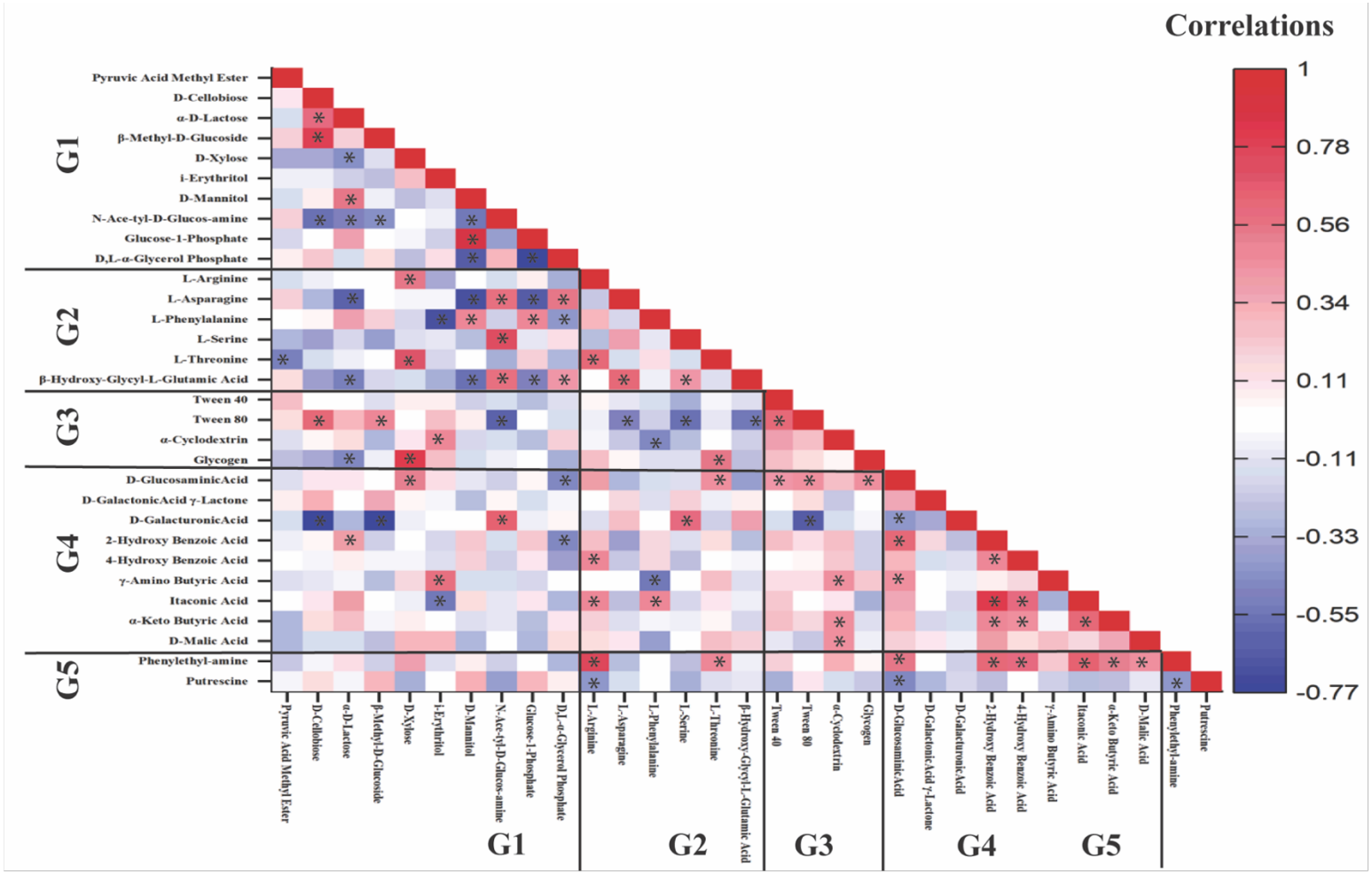


Figure 3.8. The Pearson's correlation heatmap among 31 carbon substrates, where 5 major carbon subgroup G1 = carbohydrate, G2=amino acid, G3= polymer, G4= carboxylic acid and G5=amine groups. The label of * represent the variables showed significant different at P<0.05.

In comparison among amino acid subgroup (G2), significant positive correlations are observed in the L-arginine with L-threonine and β-hydroxy-glycyl-L-glutamic acid. The positive correlation is closely associated with the similar metabolism or biosynthetic pathways as the L-threonine and L-arginine are both important key component in the nitrogen metabolism, that potentially revealed the association in the co-regulation of metabolic pathway (Ginésy et al., 2017). The same phenomenon observed in L-serine and L-asparagine with β-hydroxy-glycyl-L-glutamic acid, in which they are active components involved in the peptide and amino acid biosynthesis (De Koning, 2017; Dondoni & Marra, 2000).

There are many strong and moderate significant correlations either positive or negative observed between subgroup G1 and G2. For instance L-asparagine and β-hydroxy-glycyl-L-glutamic acid showed similar negative correlation with α-D-lactose, D-mannitol and glucose-1-phosphate whereas it showed positive correlation with NAG and D,L-α-glycerol phosphate. In this case, metabolic trade-off is potentially occurred between carbohydrates metabolism and amino acid metabolism in the negative correlation groups (Cavigliasso et al., 2023; Depraetere et al., 2015). For instance, when the demands for amino acid increase, the probiotic was selectively prioritised the amino acid metabolism and potentially convert carbohydrate metabolism into storage form. The positive correlation with NAG and D,L-α-glycerol phosphate can be explained with the involvement of similar metabolic pathway in the cell wall synthesis in the probiotic (Wintgens, 2014).

Majority of the substrates from polymer subgroup G3 showed negative correlation with G2 except the correlation between glycogen with L-threonine. Contrarily, G3 showed positive correlations with carboxylic acid subgroup (G4) except tween 80 with D-galacturonic acid showed strong positive correlation. Polymers, like glycogen are large storage molecules used in cellular metabolism. The prioritizing preference on the amino acid metabolism may reduce the resource availability for polymer metabolism (Schutter & Dick, 2001).

Majority of the individual carbon substrates from G4 carboxylic acid subgroup showed significant positive correlation among each other within the G4 except D-glucosaminic acid showed moderate negative correlations with D-galacturonic acid.

The same reasons with previous discussion that indicated shared similar intermediates and pathways can contributed to the positive correlation in the G4. For instance, D-galactonic acid γ-lactone and D-galacturonic acid are involved in the same carbohydrate metabolism. The cascade cycle of various intermediates involved in TCA and amino acid metabolism may explain the positive correlation between α-keto butyric acid (intermediate of amino acid degradation) and D-mallic acid (intermediate of TCA metabolism pathways) (Ganesan et al., 2006). Among G4, itaconic acid showed strongest positive correlation with 2-hydroxy-benzoic acid. These two compouds play vital role in the regulation of oxidative stress, maintaining balance of reactive oxygen species (ROS) and modulation of immune response (Li et al., 2017; Zhu et al., 2021). Besides that, there are 6 out 9 carbon substrates from G4 showed significant positive correlation with phenylethylamine in G5. Phenylethylamine is also acts as key stress responder with modulation of signalling pathway against oxidative stress (Kawano et al., 2000). It shares the same co-regulation signalling pathways against stress response as in the carboxylic acid compounds like 2-hydroxy-benzoic acid, 4-hydroxy-benzoic acid and itaconic acid.

In summary, these correlation heatmap provide valuable insight on the association among the individual carbon substrate. From the discussion, positive correlations may reveal the similar metabolic pathways or regulatory mechanism that upregulated in coordination whereas negative correlations often reflect competing pathways with inhibitory action and substrate preferences based on the prioritised demand of certain carbon group

3.3.5 Potential applications in food and health industry

The results of probiotic strains correlated to their key metabolic capabilities and stress tolerance properties could be potentially applied into food industry in various sectors. *L. lactis* (P3) displayed superior utilization of carbohydrates, particularly glucose-1-phosphate and D-mannitol, which are critical in sugar fermentation. Therefore, P3 could be used in fermented dairy and non-dairy products, as well as sugar fermentation for beverages like kombucha or kefir. *L. fermentum* (P6) exhibited

high efficiency in metabolising carboxylic acids (e.g., D-galacturonic acid, malic acid) and polymers (glycogen and α-cycledextrin). It can be incorporate together with P3 to enhances production of fermented foods by modulating pH during fermentation, leading to longer shelf life and improved flavour.

L. lactis (P2-P4) showed preference for amino acids like L-asparagine and L-serine, which contribute to nitrogen metabolism and gut colonization. They has a huge potential application into the development of synbiotic foods with tailored prebiotic components (e.g., N-acetyl-D-glucosamine) (NAG) to promote gut health by strengthening cell walls and competing with pathogens and resilience under stress. In many literature review, NAG could regulate and improve osteoarthritis and inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease (Choi et al., 2023; Derwich et al., 2023). Therefore, it can be used as functional probiotics for antimicrobial action and preventing infections like diarrhoea.The results also show the potential of these probiotics for various health applications. *P. pentosaceus* (P8) and *L. plantarum* (P5) exhibited exceptional resistance to gastric acid (pH 2.5) and bile salts, with survival rates exceeding 120%. They can be potentially used for treating acid reflux, irritable bowel syndrome (IBS), or gut microbiota imbalances by ensuring their viability through the GI tract (Ahuja et al., 2023). The carbon utilization of carboxylic acids, including itaconic acid and malic acid, by *L. fermentum* (P6) and *L. plantarum* (P5) suggests production and maintain of short-chain fatty acids (SCFAs) like acetate, which maintain gut pH balance is good for reducing gut inflammation and enhancing microbial diversity, with potential applications for managing inflammatory bowel diseases (IBD) (Zhang et al., 2022).

Furthermore, utilization of phenylethylamine and 2-hydroxy-benzoic acid by strains like *L. plantarum* (P5) indicates potential antioxidant effects, which is best for oxidative stress management and immune support, particularly in aging populations. The probiotics such as *E. asburiae* and *L. lactis (P2-P4)* could be manufactured in cocktail mixture as gut health supplement for post-antibiotic recovery or gut repair, as they highly utilised substrates like D-mannitol, which are known to support energy production and gut barrier function (Li et al., 2024). Strong utilization of erythritol in *L. fermentum* (P6) could ensure for effective modulate the glyceric response to control the blood glucose level (Mutlu et al., 2022). In short, strong utilization of sugar

alcohols like erythritol and D-mannitol suggests application in low-calorie probiotic foods for diabetics or weight management.

The stress tolerance analysis also provide some insight in long-term viability during storage or in food products, for instance, all the probiotic strains were preserved in both liquid suspension with glycerol and lyophilised form at -80°C, indicating a focus on maintaining viability during storage. Strains like *P. pentosaceus (P8)* and *L. plantarum (P5)*, which exhibited high stress tolerance (e.g., gastric acid survival rates exceeding 120%), are likely more resilient during storage, as stress tolerance mechanisms (e.g., production of protective proteins or enhanced membrane stability) can aid in surviving desiccation or freezing (Gao, X. et al., 2022).

Stress tolerance resistance to bile salts and NaCl suggesting strains could remain viable in high-salt environments or acidic food products (e.g., pickles, yogurts). For instance, *E. asburiae* (P1) demonstrated resilience at high NaCl concentrations, is food matrix compatible and suitable for incorporation into fermented or preserved foods. Additionally, *E. asburiae* exhibited good ability in the production of organic acid (e.g. gallic acid) and various useful enzymes (e.g. tannase, amylase, protease), which can be applied in diverse industry (Beniwal et al., 2010; Govindarajan et al., 2019).

Positive growth across a temperature range (25°C to 37°C) supports the viability of strains like *L. plantarum* and *L. fermentum* in varied storage conditions, including room temperature and refrigerated products. The combination of probiotics in ability to utilise various carbon substrates over extended incubation periods (e.g., 168 h in the Biolog Ecoplate assessment) suggests metabolic flexibility, which may contribute to maintaining activity in food matrices or during storage.

The safety regulatory bodies like the European Food Safety Authority (EFSA) and the FDA require probiotics to be non-pathogenic, non-toxic, and free of adverse side effects (Zavišić et al., 2024). The study can only confirmed the high viability of the selected strains (*L. plantarum*, *P. pentosaceus*, etc.) under simulated gastrointestinal conditions, supporting their alignment with these safety criteria. Furthermore, the ability of the strains to tolerate gastric acid, bile salts, and high NaCl concentrations demonstrates their functionality and supports claims related to gut health and survival

through the digestive tract, as required for probiotic labelling (Liang et al., 2024). The study also highlights metabolic properties such as SCFA production and carbon substrate utilization, which could substantiate health claims, provided further clinical trials are conducted. However, this study covered a limited scope of knowledges across vast challenges in the probiotic and functional food development. *In-vivo* evaluation model is needed for future research. Future studies on the pathogenicity (haemolysis tests), antibiotic resistance (to ensure it does not carry transferable resistance gene, following EPFS guideline), cell surface, hydrophobicity, and CaCO2 (to validate gut adhesion capability) are necessary to confirm their probiotic status.

3.4 Conclusion

The selected eight probiotic strains had been genetically identified and display different degree of stress tolerance across various stress factors. The probiotic demonstrates a shift in its carbon metabolic preference over time, showing an adaptive ability to utilise different carbon sources more efficiently as incubation progresses. This adaptation may indicate resilience and versatility in resource utilization, key traits for probiotics thriving in the variable environments like the gut. Based on our findings and existing supporting literature evidences, P2-P8 strains are successfully to be termed as "probiotic". However, P1 *E. asburiae* still remained question, even there are few literature studies revealed that *E. asburiae* can be a potential gut beneficial microbe. The biggest doubt is that *E. asburiae* from Enterobacter genus, belongs to phylum Proteobacteria, which had been recognised as opportunistic pathogenicity bacteria. It is therefore concerning about probiotic claims that linked to metabolic properties and safety validation still remain challenging to address. Additional research, clinical validation, and strategic claim positioning, all of which will be essential for successfully translating these findings into compliant functional food products in order to fulfil diverse regulatory requirements. In short, this research provide basic insight on the potential of local probiotics as functional food ingredients, paving the way for sustainable and innovative approaches to enhance human health.

CHAPTER 4

DEVELOPMENT OF SYNBIOTIC FUNCTIONAL FOOD WITH ENHANCEMENT OF BIOACTIVE COMPOUNDS AND CYTOTOXICITY PROPERTIES IN PROBIOTIC FERMENTED CABBAGE (PFC)

4.1 Introduction

The global functional foods market is expected to reach $275.77 billion by 2025, growing at a compound annual growth rate (CAGR) of 7.9% from 2018 to 2025 (Grand View Research, 2022). Functional foods available in various forms, including fortified beverages, probiotic yogurts, and snack bars with added vitamins and minerals. They may also contain bioactive compounds like antioxidants or phytochemicals.

Synbiotic functional food had tackled emerging interest due to its synergistic approach to amplify health advantages properties (Pandey et al., 2015; Swanson et al., 2020). Synbiotic functional food describes the food derived from the probiotic and prebiotic. Probiotics play crucial roles in the reduction of gastrointestinal cytotoxicity, para-cellular absorption, act as protective barrier in gut with modulation of host xenobiotic metabolism, in addition involve in the modulation of functional activity and enzymatic expression taking action against external stress and threat within the host. Cabbage (*Brassica oleracea*) was used as prebiotic in this study demonstrated several benefits over other vegetables, including low cost, high availability, and long shelf life (Bousquet et al., 2021). They possess minimal processing and higher versatility in its

consumption compared to other functional food that requiring lengthy processing procedure.

Fermentation is a biochemical process where microorganisms break down glucose, producing ethanol, acid, and carbon dioxide. Fermentation is the best food-processing method to preserve fresh foods and effectively extend the shelf-life of foods (Godbey, 2022). During fermentation, endogenous enzymes and autochthonous microorganisms cause reactions that alter the bioactivity profile and bioactive compounds in cabbages. For instance, fermentative microbes produce peptides, organic acids, amino acids, and antioxidant compounds (flavonoid and phenolic) and change the physiochemical aspects of the fermentation products (Zhang et al., 2021). For instance, glucosinolates (GSLs) and phenolic compounds in cabbages have been reported to be transformed into active molecules during fermentation, in which the fermented cabbage with an increased biological value that has anti-cancer properties to health (Šamec et al., 2016).

The application of natural compounds obtained from plants including *Brassica* vegetables to treat cancer had aroused much interests among researchers. This approach is expected to be more effective and economical than chemotherapy treatment with minimal side effects (Calcabrini et al., 2017). The bioactive compounds from fermented cabbage can potentially be implemented in daily diet as bio-therapeutics against cancer cells. However, limited studies were done to determine the functional properties of probiotics fermented cabbage. In this study, the use of cabbage as prebiotic coupling with different probiotics strains either single or combination strains had been investigated under fermentation process for optimal yield of total flavonoid (TFC), phenolic (TPC), glucosinolates (TGLs) and antioxidant activity. The efficacy of PFC in inhibiting growth of cancer cell line and reducing reactive oxygen species (ROS) within the cells were examined. ROS are commonly generated in oxidative metabolism events, high oxidative stress levels can cause macromolecular damage within cells (Ray et al., 2012). Therefore, oxidative stress prevention is one of the main aim in cancer prevention.

4.2 Methodology

4.2.1 Setup for cabbage fermentation

White cabbage (*Brassica oleracea*) bought from Malaysia native grower from Kai Ying Enterprise, Terengganu, Malaysia was used in this study. The experiment was designated into 2 categories to investigate the optimal yield of the bioactive compounds under the effect of individual probiotic strain and multiple probiotic strains (Table 4.1).

Table 4.1. Fermentation composition with different probiotic bacteria

(a) Fermentation with individual probiotic strain

Flask	Fermentation contents
Control (RC)	*Raw cabbage*
Positive Control (PC)	*No bacterial strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M1	1×10^6 CFU/mL P*1 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M2	1×10^6 CFU/mL P*2 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M3	1×10^6 CFU/mL P*3 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M4	1×10^6 CFU/mL P*4 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M5	1×10^6 CFU/mL P*5 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M6	1×10^6 CFU/mL P*6 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M7	1×10^6 CFU/mL P*7 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M8	1×10^6 CFU/mL P*8 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*

(b) Fermentation with multiple probiotic strains

Flask	Fermentation contents
M9	1×10^6 CFU/mL *all 8 strains + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M10	1×10^6 CFU/mL 4 *strains (P1,P3,P6,P8) + 150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M11	1×10^6 CFU/mL 3 strains of *Lactococcus* (P2,P3,P4) + *150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*
M12	1×10^6 CFU/mL 3 strains of *Lactobacillus* (P5,P6,P7) + *150g cabbage + 150 mL fermented rice water + 5% salt (w/v)*

Notes: Formulation was designed according to the protocol of Vijitra et al. (2018) with slight modifications.

4.2.2 Preparation for fermentation formulation

Approximately 7 kg of cabbage was cut into smaller pieces to maximise the surface area for fermentation. The fermented rice water was prepared at a concentration of 1 kg of rice/ 10 L of sterile water and fermented for 48 h. The final pH of fermented rice water was adjusted to pH6 prior the PFC formulation. The probiotic strains P1-P8 from liquid suspension stock were initially centrifuged at 4000 rpm for 15 mins, and the supernatant was discarded to remove residual nutrient media. The probiotic pellets (P1-P8) were collected, resuspended and mixed thoroughly with fermented rice water at 1 x 10^6 CFU/mL. The PFC formulation began by adding 150 g chopped cabbage to a 250 ml culture flask, followed by 150mL of fermented rice water containing the probiotic mixture at targeted concentration and the corresponding salt percentage as mentioned in Table 4.1.

4.2.3 Harvest of PFC and Extraction

The probiotic fermented cabbages (PFC) were harvested on 24, 48, and 72 h after fermentation. The collected PFC were sent for freeze dry prior the nutrition phytochemical and bioactive compound detection. Extraction of samples was performed by mixing 100 mg of PFC dried powder with 10 ml methanol. The mixtures were vortexed for 2 mins and centrifuged for 4000 rpm at 10 mins. The methanolic extracts were kept in 15 ml centrifuge tube prior phytochemical analysis.

4.2.4 Proximate and ICP-OES analysis

Proximate analysis was performed to analyse the contents of moisture, ash, crude carbohydrate, crude protein, crude fat and fibre of PFC (Tayyeb et al., 2017) (Appendix 11). The mineral contents such as potassium (K), calcium (Ca), Iron (Fe), Copper (Cu), Manganese (Mn), Zinc (Zn), Aluminium (Al), and baron (B) had been analysed with inductively coupled plasma-optimal emission spectrometry (ICP-OES)

measurement (7300 DV, Perkin Elmer, USA) (Khoo et al., 2022). The analysis procedure begin with the addition of 1 g freeze dried powder of PFC into crucible containing 2 ml concentrated hydrochloric acid (HCl) solution and then allowed to evaporate on hot plate at 200 °C. The dried samples were further dissolved in 10 mL of 20% nitric acid (HNO_3) and soaked in water bath for one hour, and then diluted with 100 mL distilled water before loading into the ICP-OES analysis. The multi-element standard solution 1 for ICP (Sigma Aldrich Merck, Malaysia) was used as standard to calibrate standard curve for each element.

4.2.5 Detection of bioactive compounds

4.2.5.1 Total flavonoids contents (TFC)

The TFC of PFC was determined by employing the aluminium chloride ($AlCl_2$) colorimetric method (Lee et al., 2022). Approximately 100 µL methanolic sample extract were combined with 100 µL 2% $AlCl_2$ solution and loaded into the 96 well plates. The mixtures were left in room temperature for 60 mins incubation prior analysed under microplate reader at a wavelength of 415 nm. Standard concentrations of quercetin (QE) of 20, 40, 60, 80, and 100 µg/ml (ppm) were used as standard for quantifying the activity of flavonoids in the samples. The TFC was obtained from the standard calibration curve and expressed as QE mg / 100 g.

4.2.5.2 Total phenolics contents (TPC)

The measurement of TPC was conducted using the Folin−Ciocalteau's method, with slight modification of protocol as mentioned in Chandra et al. (2014). Five serial standard solution of gallic acid (GA) were prepared using a serial dilution method. The analysis began with the mixture of 10 µL of sample extract or GA with 50 µL distilled water and 10 µL Folin−Ciocalteu's phenol reagent, and then kept at room temperature for 10 mins. Subsequently, addition of 50 µL distilled water and 100 µL 7% (w/v) sodium carbonate into the mixture. The resulting solution was then placed in a dark environment and incubated at room temperature for 30 mins. The absorbance of all the

samples/standards was measured at 765 nm using microplate reader, relative to a reagent blank. TPC value were expressed in mg GA/ 100 g of samples using the standard curve of serial dilution of GA.

4.2.5.3 Total glucosinolate contents (TGLs)

The spectrophotometric analysis was conducted following the methodology with minor adjustments as specified in Mawlong et al. (2017) and Ishida et al. (2012). A volume of 5 µL sample extract was combined with 150 µL of 2 mM sodium tetrachloropalladate. The sample was left to incubate at room temperature for 1 hour. After incubation, the absorbance was determined at a wavelength of 425 nm using a UV-Vis spectrophotometer (Labomed UV-VIS Double beam UVD-3500). A control was established using the same methodology, with replaced sample extract with 100% pure methanol. The standard powder of glucosinolates produced from rapeseed colza (European Reference Material, BC367) (RS) was utilised as the reference standard, and the resulting value was represented as mg RS/100 g.

4.2.6 Antioxidant properties of PFC

The antioxidant properties of PFC were examined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Rahman et al., 2015). About 4 mg of DPPH were dissolved in 1000 µL of methanol for making 0.1mM DPPH working solution. Serial dilution of sample extract / standard L-ascorbic acid (AA) (Merck, Germany) was dissolved with methanol at different concentration of 2,4,6,8,10 mg/mL. The reaction started with the pipette of 150 µL of 0.1 mM DPPH solution into each well of the 96 wells plate that containing 100 µL of extracts or standard. The 96 well plate was immediately incubated in the dark for 30 mins at room temperature. The absorbance of the mixture was examined in a spectrophotometer at 517 nm prior 5 mins of shaking in the microplate reader. The antioxidant present within the samples were expressed in mg AA/ 100 g of samples using the standard curve of serial dilution of AA. The working stock of 0.01 mM DPPH in triplicates was act as negative control

blank (D_0) and the standard ascorbic acid was act as positive control. Percentage of inhibition of DPPH radical scavenging activity (IDRS) and half maximal inhibitory concentration (IC_{50}) were calculated by the following equation:

$$\text{IDRS (\%)} = \frac{(D0-D1)}{D0} \times 100, \quad \text{(Equ.4.1)}$$

$$IC_{50}\,(\text{mg/mL}) = \frac{50-m}{c} \quad \text{(Equ.4.2)}$$

where D_0 is the absorbance of the DPPH blank, and D_1 is the absorbance of the extractive / standard, m and c get from the linear trendline equation y=mx+c obtained from the graph of IDRS against sample concentration.

4.2.7 Correlation analysis

Pearson correlation analysis of TFC, TPC, TGLs and IC_{50} were performed to visualise the relationship between each other. The normalised dataset of each parameters were analysed and visualised in the heatmap plot using Origin Pro software (Origin Lab,version 2024, United State). The clustering heatmap was plotted using online software ClustVis from https://biit.cs.ut.ee/clustvis/.

4.2.8 Optimisation of fermentation condition

The optimisation of PFC were further performed using the combination of 8 probiotic strains (M9) with the aim to yield optimal bioactive compounds. Response surface modelling methodology (RSM) was used to visualise the optimisation response between 3 manipulating variables and 3 responding variables. RSM coupled with randomised box-behnken design was performed using Design Expert Software (Version 13). A step by step guideline for Design Expert software had been shown in Appendix 11. The manipulating variables include the X_1: fermentation period of 24, 48 and 72 h., X_2: salt percentage at 3, 5 and 7% and X_3: fermentation temperature at 25, 31 and 37 °C. The responding variables include Y_1: TFC, Y_2: TPC and Y_3: TGLs.

4.2.9 Gas chromatography mass spectroscopy (GCMS) analysis

GCMS was performed to qualify the present of isothiocyanates, flavonoid and phenolic compounds. The RC and PFC fermented under optimal fermentation condition were selected for this analysis. RC and PFC samples were extracted with dichloromethane, followed by centrifugation at 4000 rpm for 5 min. After centrifugation, the supernatants were transferred to new vials and the lower layer of the extractive was dried over 0.5 g magnesium sulphate ($MgSO_4$). The extractive in vials were used for the GC-MS analysis with GCMS QP2010 Ultra (Shimadzu, Japan). A capillary column, ZB-5MS (5% Phenyl-arylene, 30 m × 0.25 mm x 0.25 m) was used with hydrogen as the carrier gas. The GCMS setting are design as follow: column oven temperature: 50°C, splitless injection mode at injection temperature 300°C, linear velocity of 35.5 cm/s, purge flow at 3 mL/mins, and pressure at 50.2 kPa. The temperature was kept at 50°C for 1 min and ramped up to 300°C at the rate of 5°C /min for 5 min and then to 320°C at the rate of 5°C /min for 5 min with the flow at 1 mL/min. The mass spectra were obtained by electron ionization over a range of 50−600 m/z with the ion source temperature at 200°C with interface temperature of 300°C, and the electron multiplier voltage 1.18kV. The detected mass spectrum with its corresponding retention indices of the samples were further loaded in the free online GCMS-ID platform from https://gcms-id.ca for peak identification and compound identification. The identification of components was further compared to the known reference material mass spectra in the National Institute Standard and Technology (NIST) library database. The automated mass spectral deconvolution and identification system by NIST was adopted for initial peak identification. This process included removal of background noise, spectral deconvolution and compound identification.

4.2.10 Cytotoxicity properties

4.2.10.1 Cell line sources

Three cell lines including two cancerous cell lines (breast cancer cell line (MCF-7) & colon cancer cell line (HCT-116)) and one non-cancerous cell line 3T3-L1 were obtained from research members of Faculty of Science and Marine Environment, Universiti Malaysia Terengganu (UMT).

4.2.10.2 Culture of cell lines

All cell line cultures were conducted in animal cell laboratory, Institute Marine Biotechnology of Universiti Malaysia Terengganu. The non-cancerous cell lines (3T3-L1) and cancer cell lines (MCF-7 and HCT116) were cultured in Dulbecco's Modified Eagle Media (DMEM) and Roswell Park Memorial Institute (RPMI) complete media respectively. There are 3 stages involved in the cell culture process which are revive, subculture and cryopreserve of cell line are conducted.

4.2.10.2.1 Preparation of cell culture stock solutions

The DMEM and RPMI media were purchased from Beijing Solarbio Science & Technology Co., Ltd., China. The DMEM contained 4500 ml/L D-glucose, 4 mM L-glutamine, 3700 mg/L sodium bicarbonate ($NaHCO_3$), 1 mM sodium pyruvate and 15 mg/L phenol red, while the RPMI-1640 media contained 2000 mg/L D-glucose, 2000 mg/L $NaHCO_3$, 300 mg/L L-glutamine and 5 mg/L phenol red. Both DMEM and RPMI-1640 complete media were prepared with supplement of 10% fetal bovine serum, and 1% penicillin-streptomycin (Appendix 12). Phosphate saline buffer (PBS) solution was made up of 8g of sodium chloride (NaCl), 0.04 g potassium chloride (KCl), 0.2 g of monopotassium phosphate (KH_2PO_4), and 2.16 g disodium phosphate (Na_2HPO_4), and top up to a total volume of 1L with deionised water, and adjusted pH to 7. About 100 mL trypsin-ethylenediaminetetraacetic acid (EDTA) was prepared from 0.8 g NaCl, 0.04g KCl, 0.035 g $NaHCO_3$, 0.1 g D-glucose , 0.02g EDTA salt, and 0.05g of trypsin. The freezing media stock (10 mL) was prepared by adding 9.5

mL sterile fetal bovine serum and 0.5 mL dimethyl sulfoxide (DMSO) (Rahaman et al., 2017).

4.2.10.2.2 Cell revives and thawing

The cell line sources were obtained in cryopreserved vial in the liquid nitrogen tank. The vial was initially removed from liquid nitrogen tank and then transferred to -80°C refrigerator for 30 mins, followed by temporarily stored at -20°C refrigerator for 1 hours and lastly thawed at 37°C water bath. At the same time, the ready complete media of DMEM or RPMI were warmed at water bath at 37°C. The revive process began with adding 3-4 mL of media into new 15 mL centrifuge tube, then gently resuspended the cells in the cryovial and pipetted all the cell sources into the centrifuge tube. The centrifuge tube was centrifuged at 1000 rpm for 5 mins, then discarded the supernatant. About 1 mL of media was added to the centrifuge tube that containing only pellet of cell source, then resuspended gently and transferred into a new 25 cm^2 cell culture flask with filled of 7 mL of culture media. Lastly, the culture flask was incubated at incubator with supplied of 5% carbon dioxide (CO_2) for 24 h.

4.2.10.2.3 Sub-culture and Cryopreservation

After 24 h of incubation, the cells were observed under inverted phase contrast microscope connected to the computer (Olympus IX51, Tokyo) to check the status of cell adherence on the bottom of flask. The cell culture was also examined if there is a sign of contamination. Cells that reached 85-90 % confluence were sub-cultured. The sub-culture process began with the removal of old media, followed by washing with (PBS) solution for 2-3 times. After washing, PBS was discarded and about 1.5 mL of trypsin-EDTA was added into the culture flask. The culture flask was incubated in the incubator at 37°C with 5% CO_2 for 5 mins. The cells were observed again under microscope to ensure the cell are detached from the bottom of culture flask. About 4 mL of media was added into the detached cell source, and then completely pipetted into new 15 mL centrifuge tube and then centrifuged at 1000 rpm for 5 mins. After centrifugation, the supernatant was discarded, and then 1 mL of media was added into the tube. The cells were resuspended gently and transferred into 3-4 new culture flasks

that containing 7 mL of new culture media. To note, the number of flask for new batch of sub-culture can be determined by personal usage with pipetted lower volume of cell source. The remaining cell source can preserved through cryopreservation technique. The remaining cell source in pellet form after centrifugation was mixed gently with 1ml of freeze media and transferred into a new sterile cryovial tube. The tube was storage on icebox, then transferred to -20°C refrigerator overnight, then transferred to -80°C for short term storage and liquid nitrogen tank for long term storage (Appendix 13).

4.2.10.3 PFC treated cell line

The optimal condition of PFC extract (PFC) and three types of control including raw cabbage (RC), glucosinolate standard reference rapeseed (RS) and drug doxorubicin hydrochloride (PC) were selected for the *in-vitro* cell culture analysis. Rapeseed standard reference (ERM-BF434a) and doxorubicin hydroxide powder (HPLC standard, CAS No 25316-40-9) were purchased from European Commission, Europe and Merck Sigma Aldrich, USA. Initially, about $2x10^4$ cells/well were loaded into each well of 96 wells plate. The cells were seeded in the CO_2 incubator at 37°C for 24 h. The seeded cells were stimulated with 10 serial dilutions of all 4 samples ranged from 3.125 µg/ml to 300 µg/ml (4 replicate for each concentration) and incubated in CO_2 incubator at 37°C for 48 h and 72 h.

4.2.10.4 Morphological observation

The morphological observation on the cell changes had been conducted using inverted phase contrast microscope connected to the computer (Olympus IX51, Tokyo). The images of cell structure were taken with Leica microscope imaging software. The morphological changes of PFC-treated cell line in cytotoxicity analysis were observed and compared with the untreated cell lines.

4.2.10.5 *In-vitro* cytotoxicity (MTT assay)

The cytotoxicity activity of sample extracts were examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT assay). Detail protocol of MTT assay includes cell counting, cell seeding, dilution of PFC samples, MTT reagent preparation and MTT treatments (Appendix 14). About 8-12 passages of cell lines was used for this analysis. The cell lines with about 90% confluency were washed with PBS and trypsinised following the same procedure as mentioned in the sub-culture section. About 10 µL of cell suspension was pipetted and loaded in the automated cell counter (DenoVix, United State). The cell suspension was then diluted at 2×10^5 cells/ mL (equivalent to 2×10^4 cells/ well for 100 µL in the cell seeding process). About 88 out of 96 wells in the plate that containing 2×10^4 cells/ well and the remaining 8 wells was set as blank. The 96 well plate was then incubated at 37 °C overnight in 5% CO_2 incubator. After seeding overnight, the PFC treatment was loaded at 10 serial concentrations (6.25, 12.5, 25,50,75,100,150,200,250,300 µg/ mL). The negative control was on the treat cells with culture medium only while the positive control was 10% dimethyl sulfoxide (DMSO) in the treated cells (Figure 4.1).

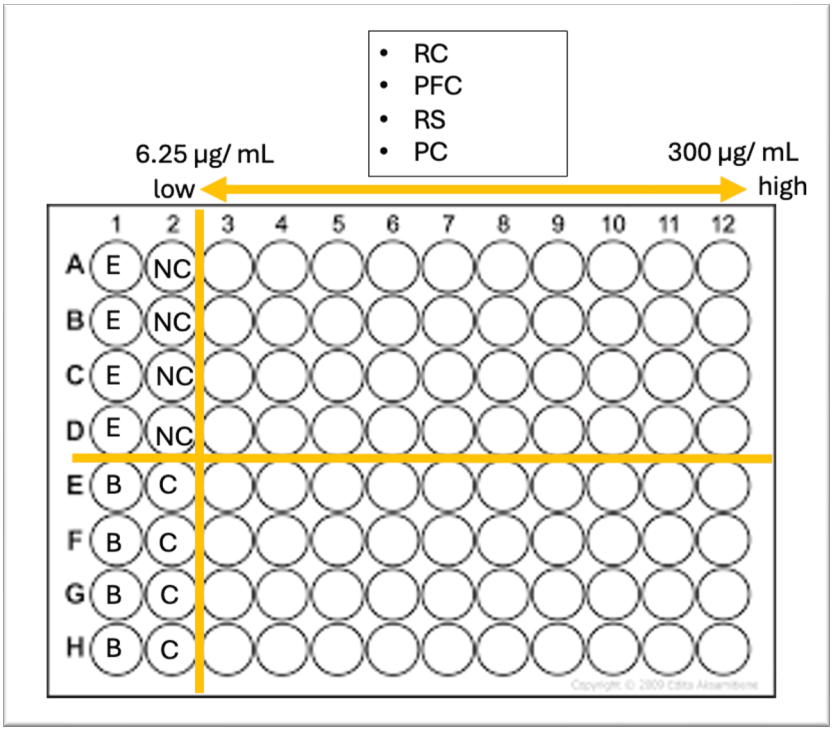


Figure 4.1 The distribution of samples (4 replicates each) loaded in the 96 well plate for MTT cytotoxicity analysis. E denotes for empty well without media and cells, B denotes for blank well with media only, NC denotes for wells that contain media with cells without treatment, C denotes for cells treated with 10% DMSO solution. There are 4 types of samples (PFC= probiotic fermented cabbage, RC= raw cabbage, RS= glucosinolate standard reference rapeseed (RS) and drug doxorubicin (PC) were loaded at 10 serial dilution concentration from low to high are 6.25, 12.5, 25, 50, 100, 150, 200, 250 and 300 µg/ mL.

After 48 h and 72 h of incubation, 100 µl of 0.5 mg/mL MTT reagent was added into each well, and incubated for 4 h in a CO_2 incubator. The MTT solution were then discarded without touching the purple stain residue on the well. Then, about 100 µl DMSO was loaded into the well to solubilise the MTT dyes and dissolve the colour substance. For complete solubilization, the plates were agitated for 5 min at room temperature, and then the colour changes were measured on a microplate reader at wavelength 570 nm. MTT with DMSO was used as the blank, while doxorubicin was used as the positive controls. Each assay was performed with 4 replications. The full protocol had been conducted as mentioned in Wangsawat et al. (2021). The 50% growth inhibition concentrations (IC_{50}) of the extracts were calculated from fitted response curves of growth inhibition percentage against serial dilution of samples. The selectively index (SI) was used to compare the cytotoxicity effect between cancerous and non-cancerous cell line (Lica et al., 2021). The formula used for cytotoxicity analysis were shown as follow:

Growth inhibition percentage (%) = $[1-(AB_{smp}-Ab_b)/(ABnc- AB_b)] \times 100$ (Equ.4.3)

IC_{50} = (50-b)/a, from growth inhibition curve Y=aX +b (Equ.4.4)

Selectively Index (SI) = IC_{50} NC / IC_{50} C (Equ. 4.5)

where AB_{smp} = absorbance of loaded samples, Ab_b= absorbance for blank (label B) and AB_{nc} = absorbance for negative control where the well that contain media with cells without treatment, a= gradient and b= y-intercept value of the growth inhibition curve, IC_{50} NC = IC_{50} of non-cancerous cell line, IC_{50} C = IC_{50} of cancerous cell line.

4.2.10.6 Intracellular ROS density

The cell line treated with probiotic fermented cabbage and raw cabbage with IC_{50} concentration (result obtained from section 4.2.9.5) for 48 h and 72 h were examined for their oxidative stress level using dihydroethidium (DHE) assay kit (Abcam, ab236206, United Kingdom) (Appendix 17). The full protocol was conducted as mentioned in the protocol guideline of Abcam (Abcam, 2018). The fluorescence of each well was measured using microplate fluorometer at excitation wavelength of 480-520 nm and emission 570/600 nm. The data was presented as DHE in relative fluorescence unit (RFU).

4.2.11 Statistical analysis

All the data obtained in triplicate were process with Statistical Package for Social Science (SPSS) software (SPSS Version 23, IMB Worldwide, USA) for the initial statistical analysis. One-way analysis of variance (ANOVA) was used for discrimination analysis. In the ANOVA analysis, the homogeneity variance test and Post Hoc Test using Duncan equal variances, where n=3 for each parameter were conducted. The treatments that fall in the different homogenous subset group and Duncan Post Hoc Test with $p< 0.05$ denoted for significantly difference among the substrate treatment.

4.3 Results and Discussion

4.3.1 Nutritional profiling

Proximate analysis is commonly used to assess food quality control and utilise information for nutritional labeling (Ganogpichayagrai & Suksaard, 2020). The nutritional profile of PFC from different probiotic fermentations were analysed and recorded in Table 4.2 a. Ash content represent the total mineral residue leftover after the combustion of the samples, associate with the mineral profile analysed with ICP-OES. The raw cabbage without fermentation and addition of probiotic (RC) and cabbage with fermentation without addition of probiotic (PC) showed significantly lower ash contents (Table 4.2 a) and mineral contents (Table 4.2 b) compared to all probiotic fermented cabbages. All PFC groups showed an average 89-91% moisture contents. Moisture content is a great indicator for predicting the shelf life of a food product as high moisture content always associate with high proliferation of microorganism (Md Noh et al., 2020). In food fermentation, maintaining an adequate water content is crucial to ensure that vegetables remain fully submerged, which helps prevent the growth of harmful microbes. The percentage of crude fat revealed the overall fat accumulated within the samples, including steroid, fat soluble vitamin, and oil soluble dyes beside the commonly known fatty acid compounds. The crude fibre and crude protein represent the total amount of the dietary fibre and the protein present in the PFC sample. In proximate analysis, the protein is indirectly measured from the total nitrogen contents within the samples.

Table 4.2 Nutrition profile from (a) proximate analysis and (b) ICP-OES analysis.

(a)

PFC Formulation	Ash (%)	Moisture (%)	Crude Fat (%)	Crude Protein (%)	Crude Fibre (%)	Carbohydrates (%)
RC	1.24±0.02 ^{a}	90.73±0.4 ^{g,h}	1.41±0.06 ^{f,g}	1.15±0.11 ^{d,e}	2.66±0.28 ^{b}	2.80±0.30 ^{f,g}
PC	1.46±0.01 ^{b}	90.90±0.07 ^{h}	1.47±0.07 ^{g}	1.03±0.01 ^{b,c}	2.39±0.07 ^{a}	2.75±0.14 ^{f}
M1	**2.16±0.05** ^{h}	89.43±0.08 ^{a,b,c}	**2.96±0.05** ^{l}	**1.41±0.06** ^{f}	3.45±0.18^{d}	0.58±0.23 ^{a}
M2	2.05±0.08 ^{f,g,h}	89.8±0.20 ^{c,d}	0.99±0.04 ^{d}	1.12±0.03 ^{c,d,e}	4.43±0.16 ^{h,i}	1.60±0.30 ^{c,d}
M3	2.05±0.03 ^{f,g,h}	90.39±0.06 ^{e,f,g}	0.37±0.06 ^{a}	0.99±0.02 ^{b}	4.43±0.10 ^{h,i}	1.78±0.06 ^{d}
M4	**2.16±0.01** ^{h}	90.24±0.08 ^{e,f}	1.32±0.06 ^{e,f}	1.04±0.01 ^{b,c}	2.97±0.06 ^{c}	2.26±0.06 ^{e}
M5	2.11±0.05 ^{g,h}	89.14±0.18 ^{a,b}	1.25±0.01 ^{e}	**1.33±0.07** ^{f}	2.90±0.13 ^{c}	**3.27±0.28** ^{g}
M6	1.94±0.04 ^{e,f,g}	89.07±0.16 ^{a}	2.55±0.07 ^{k}	1.05±0.01 ^{b,c}	3.85±0.10 ^{e}	1.54±0.32 ^{c,d}
M7	1.67±0.04 ^{c,d}	89.58±0.03 ^{b,c}	0.80±0.10 ^{c}	1.03±0.02 ^{b,c}	3.66±0.02 ^{d,e}	3.26±0.04 ^{g}
M8	1.77±0.27 ^{c,d,e}	90.02±0.45 ^{d,e}	0.69±0.09 ^{b}	0.87±0.03 ^{a}	2.89±0.06 ^{c}	**3.77±0.23** ^{h}
M9	1.72±0.27 c,d	89.33±0.05 ^{a,b}	0.65±0.03 ^{b}	**1.42±0.02** ^{f}	**4.61±0.12** ^{j}	2.47±0.45 ^{e,f}
M10	1.84±0.02 ^{c,d,e}	89.53±0.47 ^{b,c}	2.32±0.11 ^{j}	1.21±0.10 ^{e}	4.25±0.05 ^{f,g}	0.84±0.30 ^{a,b}
M11	1.64±0.05 ^{c}	90.35±0.27 ^{e,f,g}	1.63±0.06 ^{h}	1.10±0.10 ^{c,d}	4.14±0.04 ^{f}	1.14±0.37 ^{b,c}
M12	1.86±0.06 ^{d,e,f}	90.57±0.20 ^{f,g,h}	2.00±0.03 ^{i}	1.10±0.05 ^{c,d}	3.66±0.18 ^{d,e}	0.82±0.41 ^{a,b}
Average (n=39)	1.83±0.28	89.93±0.62	1.46±0.75	1.13±.16	3.59±0.72	2.06±1.03

(b)

	Ca (mg/L)	Fe (mg/L)	Cu (mg/L)	Mn (mg/L)	Zn (mg/L)	Al (mg/L)	K (mg/L)	B (mg/L)
RC	0.02±0.01 ^{a}	0.04±0.02 ^{a}	0.03±0.02 ^{a,b}	0.01±0 ^{a}	0.09±0.01 ^{a}	1.03±0 ^{a}	42.790.47 ^{a}	0.02±0 ^{a}
PC	0.03±0.02 ^{a,b}	0.04±0.01 ^{a}	0.02±0 ^{a,b}	0.01±0 ^{a}	0.06±0.01 ^{a,b}	0.06±0.02 ^{a}	43.26±0.9 ^{a}	0.03±0.01 ^{a}
M1	0.04±0 ^{a,b}	0.09±0.01 ^{a,b}	0.05±0.01 ^{b}	0.01±0.01 ^{a,b}	0.08±0.01 ^{a,b}	0.06±0 ^{a}	45.86±2.61 ^{a,b}	0.03±0.01 ^{a}
M2	0.03±0.01 ^{a,b}	0.08±0.02 ^{a,b}	0.01±0.01 ^{a,b}	0.01±0 ^{a}	0.07±0.01 ^{a,b}	0.06±0.01 ^{a}	47.39±0.3 ^{b,c}	0.03±0 ^{a,b}

M3	0.03±0 ^{a,b}	0.41±0.05 ^{c}	0.19±0.02 ^{c}	0.02±0.01 ^{c,d}	0.18±0.05 ^{c,d}	0.11±0.02 ^{c}	52.84±0.63 ^{e,f}	0.04±0.01 ^{a}
M4	0.02±0.01 ^{a,b}	**1.35±0.05** ^{d}	**0.25±0.05** ^{d}	**0.04±0.01** ^{d}	**0.27±0.05** ^{e}	**0.2±0.03** ^{d}	**53.16±1.21** ^{f}	0.02±0.01 ^{a}
M5	0.02±0 ^{a}	0.47±0.06 ^{c}	**0.27±0.05** ^{d}	0.02±0 ^{a,b,c}	0.17±0.02 ^{c}	**0.24±0.02** ^{d}	52.07±2.04 ^{d,e,f}	0.02±0 ^{a}
M6	**0.04±0.01** ^{b}	0.53±0.02 ^{c}	**0.24±0.02** ^{d}	0.03±0.01 ^{c}	0.19±0.02 ^{c,d}	**0.23±0.01** ^{d}	53.73±1.22 ^{f}	0.02±0.01 ^{a}
M7	0.03±0.01 ^{a,b}	0.08±0.02 ^{a,b}	0.01±0.01 ^{a}	0.01±0 ^{a,b}	0.11±0.02 ^{b}	0.17±0 ^{b}	49.59±0.46 ^{c,d,e}	0.03±0.01 ^{a}
M8	0.04±0.01 ^{a,b}	0.21±0.03 ^{b}	0.03±0.01 ^{a,b}	0.01±0 ^{a,b}	0.16±0.02 ^{c}	0.12±0 ^{b}	**54.59±1.44** ^{f}	0.03±0.01 ^{a}
M9	0.03±0.01 ^{a,b}	0.09±0.02 ^{a,b}	0.02±0.01 ^{a,b}	0.02±0 ^{b,c,d}	0.07±0.01 ^{a,b}	0.12±0 ^{b}	**54.12±0.87** ^{f}	0.03±0.01 ^{a}
M10	0.03±0.01 ^{a,b}	0.08±0.01 ^{a,b}	0.03±0 ^{a,b}	0.02±0 ^{a,b,c}	0.08±0.01 ^{a,b}	0.09±0.01 ^{a}	51.62±0.49 ^{d,e,f}	0.03±0.01 ^{a}
M11	0.03±0.01 ^{a,b}	0.09±0.05 ^{a,b}	0.02±0 ^{a,b}	0.02±0.01 ^{c,d}	0.21±0.03 ^{d}	0.1±0.03 ^{b,c}	43.23±0.63 ^{a}	0.10±0.02 ^{c}
M12	0.03±0.01 ^{a,b}	0.04±0.03 ^{a}	0.02±0 ^{a,b}	0.02±±0 ^{b,c,d}	0.09±0.01 ^{a,b}	0.14±0.02 ^{b,c}	48.89±0.2 ^{b,c,d}	0.05±0.01 ^{a}

Notes: The significant different at P value < 0.05 (N=3) are labelled with different alphameric number to represent the homogenous subset group.

Among all sample group, M9 showed highest crude protein content of 1.42±0.02 % and crude fibre contents of 4.61±0.12 % while having the lowest crude fat content about 0.65±0.03 %. This result suggests the probiotic combination in M9 warrants further research to explore the efficacy and effectiveness in different disciplinary health prospective. Assuming the the probiotic couldn't alter the protein and fibre contents, as their primary function isn't to directly synthesise protein or fibre within the food itself, the higher protein and fibre is expected due to the either the high protein content in the probiotic bacteria itself and indirectly break down of complex plant cellulose molecule into simpler forms, thus making protein or fibre components more bioavailable (Heller, 2001; Jäger et al., 2020). While comparing the mineral contents, surprisingly M4 with *L. lactis subsp. lactis* showed highest mineral contents of iron (Fe) with 1.35±0.05 mg/L copper (Cu) with 0.25±0.05 mg/L, manganese (Mn) with 0.04±0.01 mg/L, zinc (Zn) with 0.27±0.05 mg/L and aluminum (Al) with 0.2±0.03 mg/L. However, to the best of our knowledge, currently there is no report reported about the probiotic can directly increase the mineral contents. Instead, there are emerging study focus on the influence of probiotic stimulate mineral absorption in the gut (Varvara & Vodnar, 2024). In sum, table 3 portraited an overview of the total nutritional profile, it plays crucial role in new food product development, especially establish baseline composition for standardization of different product batch, in addition offer declaration of specific nutrients for regulatory compliance.

4.3.2 Total flavonoids contents (TFC)

Flavonoids are increasingly recognised as an essential element in a wide range of nutraceutical, pharmacological, therapeutic, and cosmetic uses. They are diverse class of plant pigments that are widely found in vegetable, stems, fruits and flowers (Panche et al., 2016). In this study, the effect of probiotic strains either single or combination of multiple strains on the flavonoid contents had been examined (Figure 4.2).

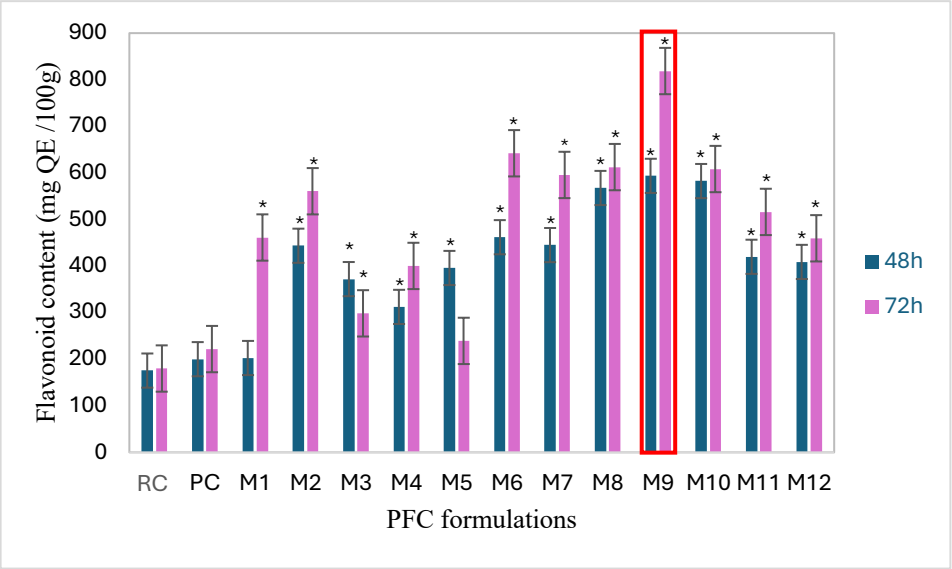


Figure 4.2. Comparison of total flavonoids contents of the PFC produced from different probiotic strains. RC represent for raw cabbage without fermentation, PC is fermented raw cabbage without the addition of probiotics, M1-M8 represent PFC with the addition of single probiotic strain and M9-M12 represent PFC with the addition of combination probiotic strains. The significant difference of PFC formulation compared to RC at $p<0.05$ (n=3) are labelled with *.

When comparing all probiotic groups, the inclusion of probiotics (M1-M12) significantly increased TFC compared to both PC and RC during the fermentation phase. This findings provide a strong evidence that the addition of probiotic can improve the overall flavonoid contents of PFC, which are come in agreement with the findings of Chandra et al. (2014) and Khan et al. (2020). PFC from M9 which contained all eight probiotic strains, resulted in the significantly highest TFC up to 818.6 mg QE/ 100 g after 72 hours of fermentation. In comparison the effect of fermentation period on the TFC, fermentation period 72 h showed an overall higher TFC compared to PFC with 48 h fermentation.

In short, the coupling effect probiotic and fermentation process demonstrated great improvement on the total flavonoid contents. It is predicted that the capability of probiotics to produce specific enzymes such as enzyme β-glucosidase and β-galactosidase for breaking down complex compounds. This process can result in the release of flavonoids or the conversion of precursor compounds into different flavonoid derivatives (Maina et al., 2021) and finally resulting in higher bioavailability of flavonoids within the fermentation products (Adebo & Gabriela Medina-Meza, 2020; Beganović et al., 2011). In addition, a study demonstrated that probiotics can

break down the membrane of cell walls, make it easier for the body to access the by-product flavonoids compounds (Liang et al., 2019). The variation in the flavonoid contents is contingent upon various factors, such as fermentation duration, specific conditions, and live viable probiotic concentration. Distinct probiotic strains can exhibit diverse enzymatic activity, resulting in various impacts on the flavonoid composition.

4.3.3 Total phenolic contents (TPC)

The effect of probiotic strains on the TPC was shown in Figure 4.3. Similar findings found in M9 showed the highest TPC about 494.62 mg GA/ 100g on 72 h fermentation period. The finding provides strong evidence that the probiotic consortium from M9 enhance the bioavailability of TPC after probiotic fermentation process. Probiotic could induce fermentation rate with high production of bioavailability phenolic compounds through conversion of large phenolic acid into more potent antioxidant form (Sharma et al., 2022). Phenolics compounds can be known as polyphenol compound, they represent a large group of small molecules that characterised by their specific phenol structure unit, typically aromatic ring with one or more hydroxyl groups (Saranraj et al., 2019). In screening the effect of single probiotic, PFC from M6, M7 and M8 with value 396.67, 383.33, 396.67 mg GA/ 100g respectively showed significantly higher TPC contents than RC and PC in both 48 h and 72 h fermentation period. The findings might suggest *Lactobacillus fermentum* , *Lactobacillus brevis* and *Pedicoccus pentosaceus* could be potent probiotic strains for the improvement of phenolic contents of food samples. *Lactobacillus fermentum* had been reported with the improvement of bioactive compounds in whole grain sorghum (Adebo et al., 2018), and brown rice (Khan et al., 2020).

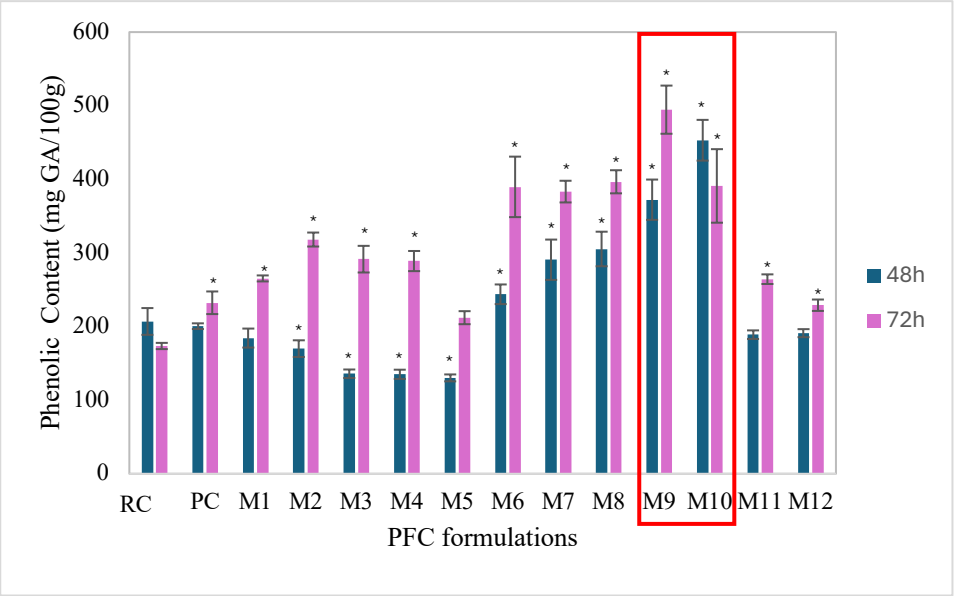


Figure 4.3. Comparison of total phenolic contents of the PFC produced from different probiotic strains. RC represent for raw cabbage without fermentation, PC is fermented raw cabbage without probiotics, M1-M8 represent PFC with the addition of single probiotic strain and M9-M12 represent PFC with the addition of combination probiotic strains. The significant difference of PFC formulation compared to RC at $p<0.05$ (n=3) are labelled with *.

4.3.4 Total glucosinolate contents (TGLs)

The glucosinolate contents of PFC formulated from all probiotic strains had been examined (Figure 4.4). PFC from M9 and M10 showed significant higher contents of TGLs with about 25.097 mg RS/ 100g and 24.486 mg RS/100g respectively compared to others PFC formulations. The finding further confirmed the synergistic effect of the combination of 8 probiotic strains. With the synergistic interactions of all probiotic strains, the diversity in the microbial community creates a robust fermentation process, leading to the productions of specific metabolites that potentially enhance the bioavailability of glucosinolates, and finally resulting in the raise of TGLs contents (Rosés et al., 2023; Zhang et al., 2022). In comparison of the effect of single probiotic on TGLs, M6 with probiotic fermentation of *Lactobacillus fermentum* displayed highest TGLs about 20.306 mg RS/ 100g. The result revealed that *Lactobacillus fermentum* may poses specific enzymes that selectively metabolise big complex glucosinolates components, contributing to higher TGLs in free form. Rapeseed colza was selected as standard of TGLs in this study due to its wide varieties

of glucosinolate and isothiocyanates compounds, including progoitrin, sinigrin, sinalbin, gluconapoliferin, glucoalyssin, gluconapin, glucobrassicanapin, gluconasturtiin, neoglucobrassicin, methyl-pentyl glucosinolate, butyl glucosinolate and other more glucosinolate deriviatives just to name a few (Miklavčič Višnjevec et al., 2021). Through probiotic fermentation process, the breakdown of vegetables' cell wall release different variety of glucosinolates though enzymatic hydrolysis process, contributed to high more bioavailable glucosinolate and isothiocyanates compounds in the final product (Mocniak et al., 2023).

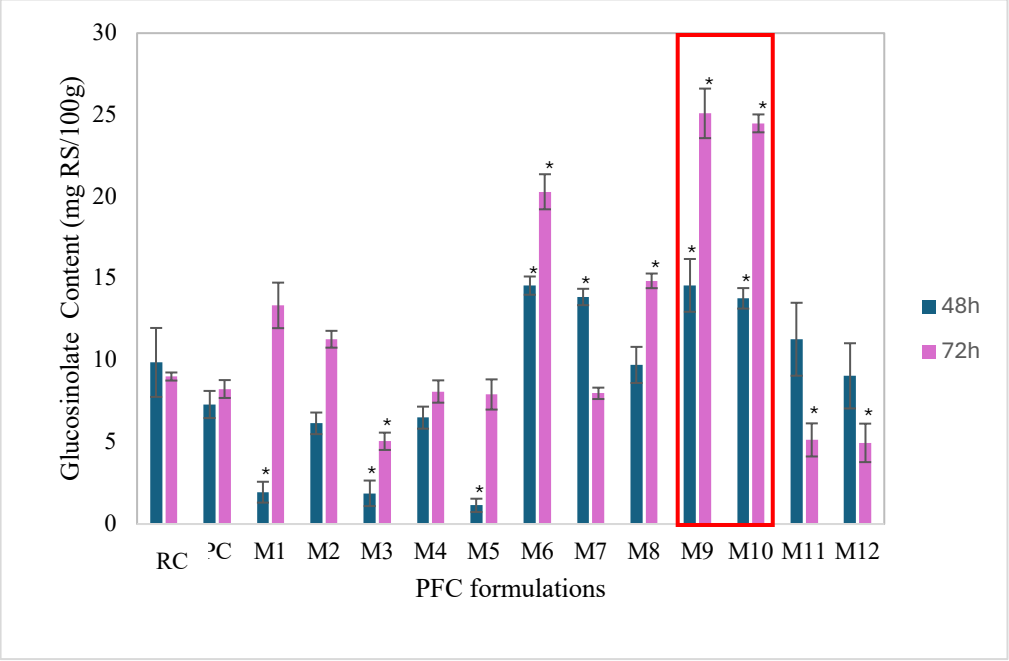


Figure 4.4. Comparison of TGLs of the PFC produced from different probiotic strains. RC represent for raw cabbage without fermentation, PC is fermented raw cabbage without probiotics, M1-M8 represent PFC with the addition of single probiotic strain and M9-M12 represent PFC with the addition of combination probiotic strains. The significant difference of PFC formulation compared to RC at $p<0.05$ (n=3) are labelled with *.

4.3.5 Antioxidant analysis

Antioxidant activity (%) can be examined from the DPPH radical scavenging activity (IDRS) and IC_{50} of PFC. The present of antioxidant equivalent to ascorbic acid (AA) was obtained from the AA standard curve (Figure 4.5 a&b). High IDRS activity can reflect higher present of antioxidant agent to bind with the DPPH (Figure 4.5 c).

Besides that, IC_{50} of PFC were obtained to provide information of maximal concentration of PFC needed to inhibit 50% DPPH radicals (Figure 4.5 d). Therefore, the lower IC_{50} value of PFC corresponds to higher antioxidant properties. In comparison of single strain PFC formulation, M6-M8 demonstrated higher present of antioxidant, higher IDRS activity with lower IC_{50} value (Figure 4.5 b-d). Many literature evidences reported that *Lactobacillus fermentum* as potent probiotic that can enhanced antioxidant activity in crops and plants (Grishina et al., 2023; Li et al., 2024; Paulino Do Nascimento et al., 2022). Similar finding on probiotic *Lactobacillus brevis* and *Pedicoccus pentosaceus* demonstrated that they improved antioxidant activities of crops, plants and dairy product through fermentation (Meng et al., 2025a; Meng et a., 2025b; Song et al., 2021; Song et al., 2023; Wang, Z. et al., 2023).

Among all 14 PFC formulations, M9 and M10 comprising combination of all 8 probiotics and 4 probiotics strains respectively displayed highest antioxidant properties for both 48 h and 72 h fermentation period with highest antioxidant content (Figure 4.5 b) and highest DPPH scavenging activity (Figure 4.5 c). This result is aligned with the results obtained in the TFC and TPC (Figure 4.2 and 4.3). Phenolic and flavonoid compounds are known to be the key contributors of antioxidant activities in Brassica vegetables (Chandra et al., 2014; Rahman et al., 2022). Many literature studies showed a positive correlation of TPC and TFC with antioxidant activity in Brassica vegetable (Bhandari & Kwak, 2015; Dos Reis et al., 2015; Wang et al., 2018). Phenolic and flavonoid compounds chemically function as good antioxidant agent, demonstrating significant capacity as hydrogen and electron donors to neutralise free radical of reactive oxygen species (ROS) such as hydroxyl radical, superoxide radical and hydrogen peroxide (Kumar et al., 2020; Speisky et al., 2022). In short, this study demonstrated that the probiotic fermentation can significantly enhance the antioxidant properties. However, PFC can't claim as high antioxidant compounds due to their extremely high IC_{50} value ranged from 3-6 mg/ mL as compared to IC_{50} in standard AA pure compounds about 114.38 µg/mL (Figure 4.5a).

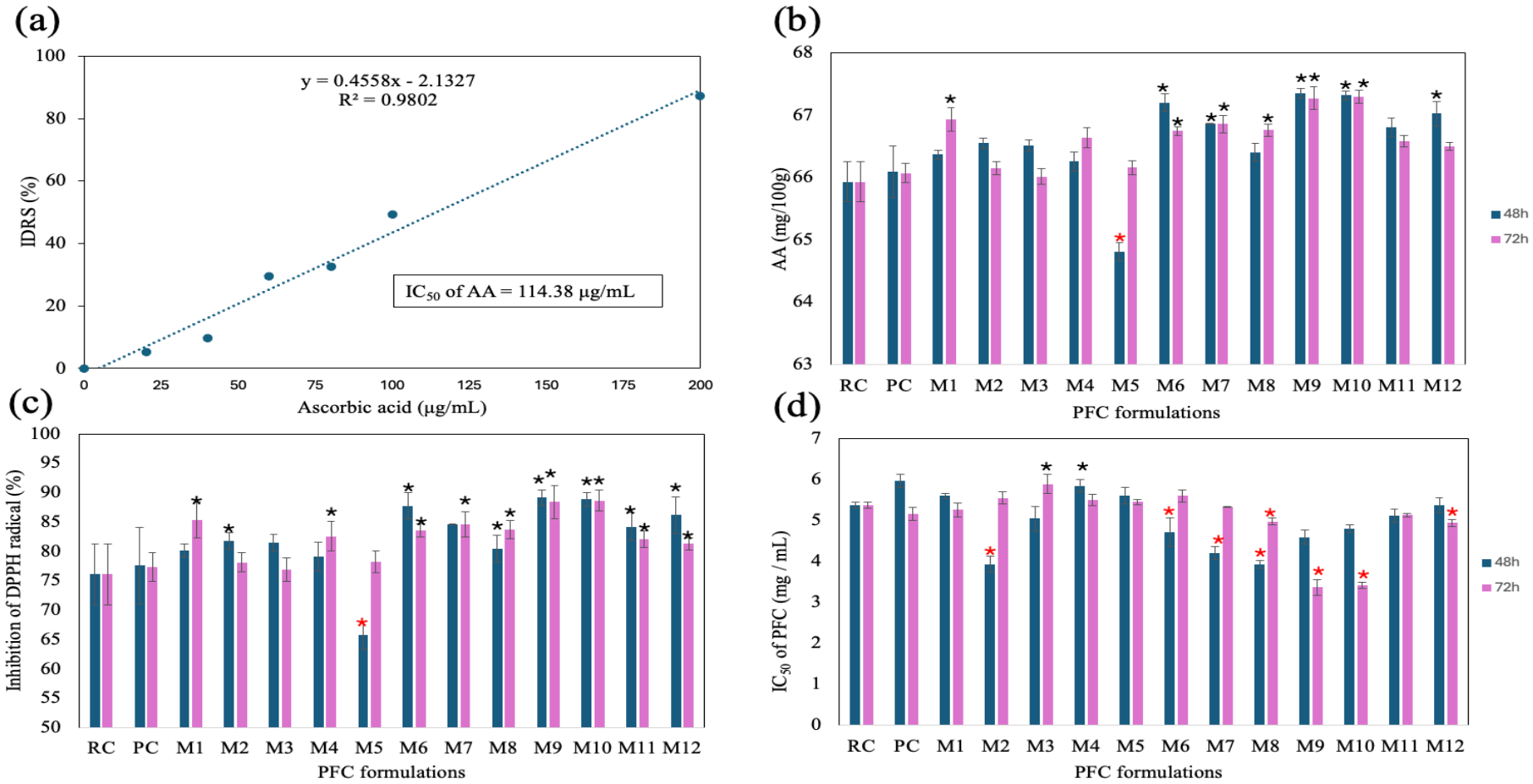


Figure 4.5 Antioxidant properties. (a) Standard curve for ascorbic acid (AA). (b) Antioxidant present within the PFC samples. (c) Inhibition of DPPH radical scavenging activity (IDRS) of PFC formulation. (d) The concentration (IC_{50}) of PFC to inhibit 50% of DPPH scavenging radical. The significant increase and decrease of PFC samples compared to RC at $p<0.05$ (n=3) are labelled with* and * respectively.

4.3.6 Correlation analysis

Correlation analysis were adopted to further illustrate the relationships between antioxidant capability in TFC, TPC, TGLs and IC_{50} in DPPH scavenging activity of all datasets using heatmap (Figure 4.6). Based on Figure 4.6, TFC,TPC and TGLs are positively correlated with each other while they negatively correlated with IC_{50} of DPPH in both 48 h and 72 h fermentation period. The results are in agreement with the lower the IC_{50} value of PFC, the higher the antioxidant properties (Adebo & Gabriela Medina-Meza, 2020). Phenolic compounds are natural antioxidants that can neutralise free radicals, reducing cellular damage while flavonoids are a type of phenolic compound with strong antioxidant properties. There are plenty of studies also show positive correlation between TPC, TFC and antioxidant activity such as reported in red cabbage and purple carrot (Mizgier et al., 2016), plant extract of *Barleria prionitis* (Singh et al., 2023), and white tea (Wang et al., 2023). It is evidence that an improvement in antioxidant capacity often associated with enhanced growth performance of the beneficial gut microbiome such as *Bifidobacteria* spp. and *Lactobacillus* sp. in the gut (Zhang et al., 2023). However, not all phenolics or flavonoids contribute equally to antioxidant capacity, it is highly influenced by its specific types and structures, and resulting in different degree of effectiveness. In sum, in depth study is needed to explore the intricacies of the overall interaction of bioactive compounds with antioxidant capacity. A stronger correlation between TFC, TPC and TGLs in 72 h fermentation period was observed (higher correlation value with darker red highlight) compared to 48 h fermentation period, but not observed in the correlation with IC_{50} of antioxidant activities (Figure 4.6 a&b). The similar finding had been found in the solid state fermentation of soybean flour with *Lactobacillus casei* showed a significant increase of phenolic compounds at 72 h fermentation period compared to 24 h and 48 h fermentation period (Li, S. et al., 2020).

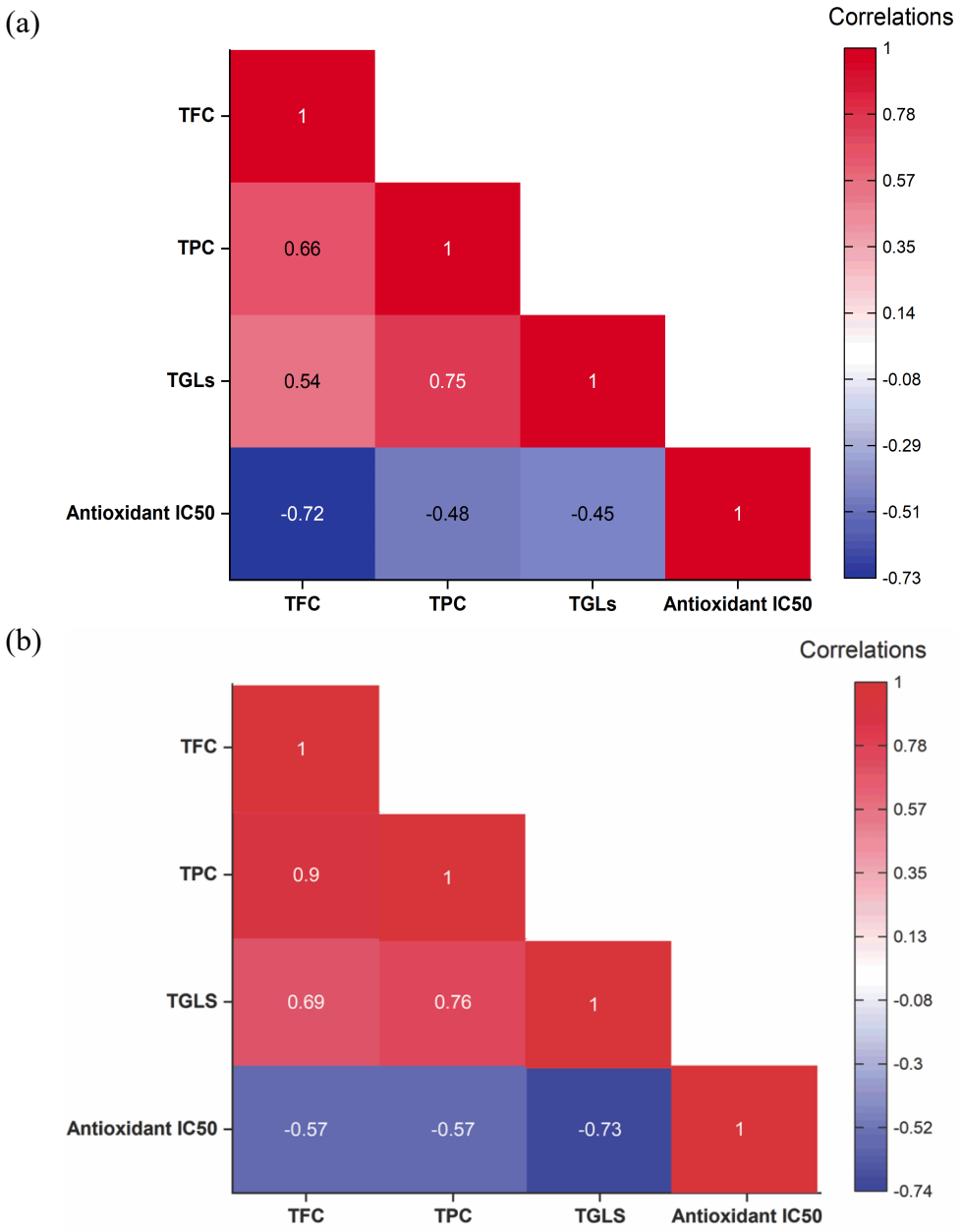


Figure 4.6 Clustering analysis using heatmap to visualise the spearman correlation between TFC, TPC, TGL and DPPH in (a) 48 h fermentation and (b) 72 h fermentation.

4.3.7 GCMS analysis

The GCMS chromatogram between raw cabbage (RC) and probiotic fermented cabbage (PFC_{M9}) had been reported in the Figure 4.7. The detected compounds were compared in Table 4.3. It is observed that there are more high intensity peaks with small molecular mass compounds detected in PFC extract compared with RC extract.

This finding showed that the probiotic fermentation process had significantly break down complex molecule into smaller bioactive molecules in PFC. Glucosinolate degradation compounds such as 1-isothiocyanato-4-methyl sulfonyl butane, 4-Isothiocyanato-1-butene and its thiols derivative compounds like 2-Methyl-3-furanthiol and 2-amino-4,5-dihydro-5-methyl-thiazole were found in PFC extract only instead RC. Compound 1-isothiocyanato-4-methyl sulfonyl butane is known as sulforaphane, which had been widely reported with good anticancer properties (Asif Ali et al., 2023; Devi & Thangam, 2012), antioxidant properties (De Figueiredo et al., 2015), anti-inflammation (Treasure et al., 2023) and so on. Sulforaphane showed critical protective role against oxidative stress, regulating NF-κB signaling pathway that stimulate inflammatory and immunomodulatory responses towards cancer and external stress (Qin et al., 2018). GLs derivative 4-Isothiocyanato-1-butene has been identified as a potent antibacterial agent and displayed good plant defense mechanisms (Cao et al., 2016; Du et al., 2022). In addition, there are more bioactive phenolic and flavonoid compounds detected in PFC compared to RC (Table 4.3), which further supported PFC with higher TFC and TPC in Figure 1 and 2. Bigger molecule of phenolic compounds with higher retention time such as 2,5-dihydroxyacetophenone and 2,4-bis(1,1-dimethylethyl) phenol are detected in raw cabbage, whereas smaller phenolic compounds like butyrolactone, 2-hydroxy-gamma-butyrolactone, 5-Hydroxymethyl-2[5H]-furanone, 5-Hydroxymethylfurfural (HMF) just to name a few had detected in the PFC extract.

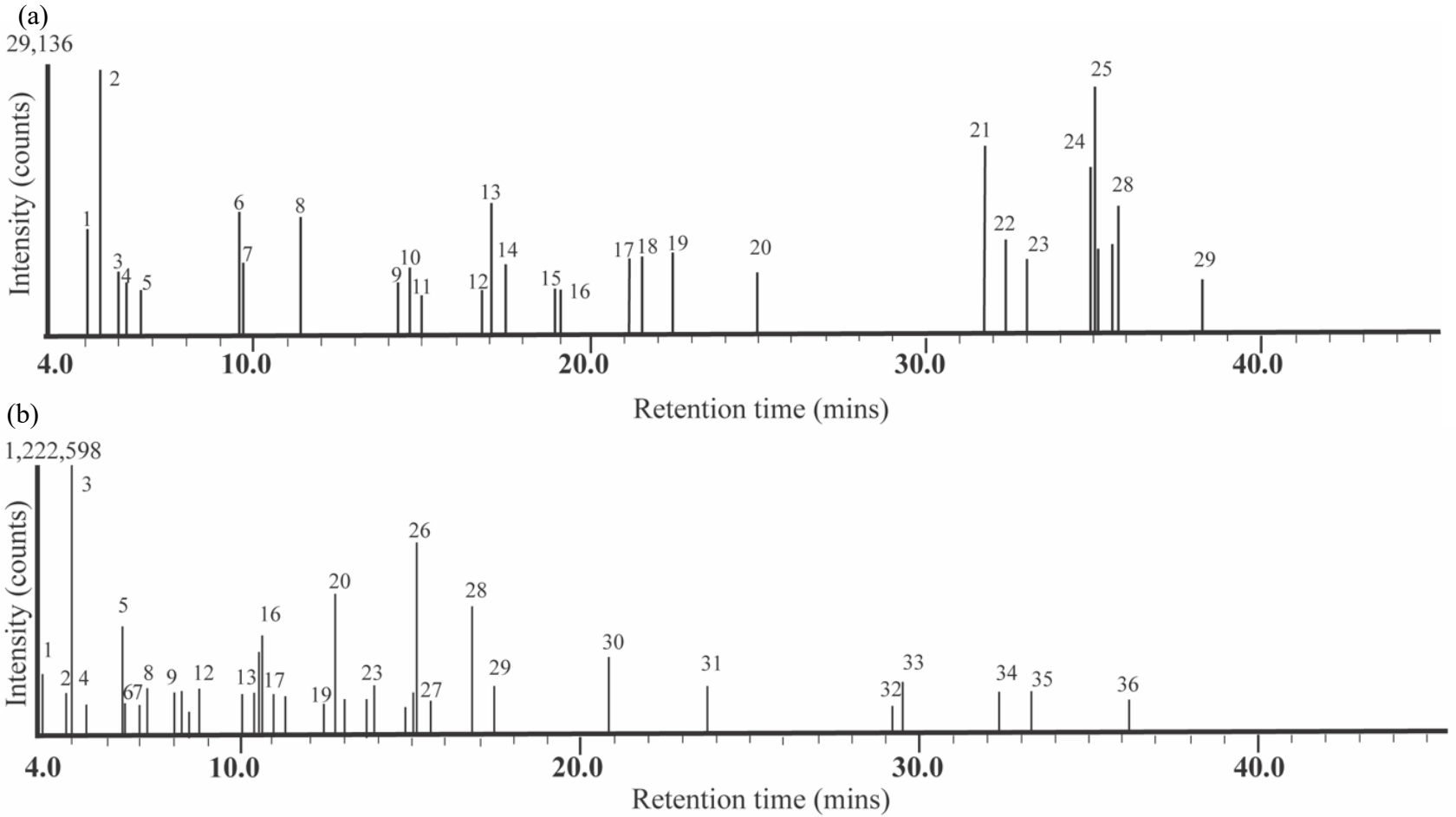


Figure 4.7 The chromatogram obtained for (a) raw cabbage (RC) and (b) probiotic fermented cabbagePFC_{M9}.

Table 4.3 The chemical compounds detected from GCMS for RC and PFC

Chemical functional group	Chemical compounds detected	Retention time (mins)	Label in chromatogram	
			RC (Fig.6a.)	PFC (Fig.6b.)
GLs and ITC derivative	1-isothiocyanato-4-methyl sulfonyl butane	4.156	-	1
	4-Isothiocyanato-1-butene	8.090	-	10
	2-Methyl-3-furanthiol	10.010	-	14
	Thiazole, 2-amino-4,5-dihydro-5-methyl-	11.254, 12.494	-	18,19
Phenolics	2,5-Dihydroxyacetophenone, bis(trimet)	19.132	16	-
	Phenol, 2,4-bis(1,1-dimethylethyl)	22.449	19	-
	Butyrolactone	6.984	-	7
	2-hydroxy-gamma-butyrolactone	8.760	-	12
	(S)-5-Hydroxymethyl-2[5H]-furanone	13.727	-	23
	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	10.386	-	15
	5-Hydroxymethylfurfural (HMF)	15.119	-	26
	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-	12.817	-	20
	Benzeneacetaldehyde	9.892	-	13
	Benzaldehyde, 2,5-bis[(trimethylsilyl)o	10.899	-	17
Flavonoids	3',5'-Dimethoxyacetophenone	23.765	-	31
Alcohol	3-Methylbenzyl alcohol	5.092	1	-
	1-(1-Methoxyethyl)-4-methoxymethylb	5.455	2	-
Aromatic acid and derivatives	2-Amino-6-methylbenzoic acid	5.937	3	-
	Arsenous acid, tris(trimethylsilyl) ester	9.552	6	-
Siloxanes and silicon-based compounds	Cyclotetrasiloxane, octamethyl-	14.710,16.802, 17.414	9,12,29	24,28,29
	Cyclopentasiloxane, decamethyl-	20.828	15	30

	Silanediol, dimethyl(2-methylpent-3-yloxy)	24.958	20	
	Silane, ethoxytriethyl-	5.428	-	4
	Cyclotrisiloxane, hexamethyl-	12.998	-	21
	Oxime-,methoxy-phenyl-	6.455	-	5
Ketones	1,2-Cyclopentanedione	7.200	-	8
Fatty acid and their esters	Hexadecanoic acid, methyl ester (Methyl Palmitate)	32.370	21	34
	9,12Octadecadienoic acid (Z,Z)-, methyl ester (Methyl Linoleate)	34.940	24	-
	Octadecanoic acid	36.160	-	36
	Methyl stearate	35.568	27	-
	11,14,17-Eicosatrienoic acid, methyl ester	35.717	28	-
	1-Propanol, 2,2-dimethyl-, acetate	4.826,4.939	-	2,3
	3-Acetoxy-3-hydroxypropionic acid, methyl ester	15.590	-	27
	Dimethyl trisulfide	7.840	-	9
Indole derivative	Pyrido[2,3-g]indole, 5-methoxy-2,3,7,9	9.621	7	-
	1H-Indole-3-acetonitrile	29.287	-	32
	2-Pyrrolidinone, 1-methyl-5-(1-methylp)	29.493	-	33
Amides	4-Acetonylquinazoline	33.285	-	35
Hydrocarbon	Undecane	11.397	8	-
	Naphthalene, 1-methyl-	17.082, 17.502	13,14	-
Metal-containing compounds	Nickel, nitrosyl[(1,2,3,4,5-.eta.)-1,2,3,4-	6.213	4	-

Notes: RC: raw cabbage, PFC: probiotic fermented cabbage

4.3.8 Response surface modelling (RSM)

Optimisation data on the optimal yield of PFC had been presented in RSM (Figure 4.8, Table 4.4 & Appendix 15). The RSM modelling for TFC, TPC and TGLs showed significant with P value of 0.0465, 0.0074, and 0.0067 with R^2 value of 0.8297, 0.9054, and 0.9083 respectively. From the RSM plot, highest TFC, TPC and TGLs of PFC was observed optimal under 3% salt concentration at 25°C and 48 h fermentation period (Figure 4.8). The bioactive compounds produced in PFC fermented with 3% salt showed higher yield in TFC, TPC and TGLs contents compared to 5% and 7% salt concentration in 25 °C fermentation (Figure 4.8). Elevated salt concentration imposes osmotic stress on plant tissues and microorganisms, with the potential to reduce the cell wall permeability and substantially influence the liberation of flavonoid, phenolics and glucosinolates (Ćosić et al., 2023; Dabravolski & Isayenkov, 2023). In addition, excessive amount of salt might cause enzyme denaturation, for instance enzymatic activities was slower down when salt concentration increased (Rysová & Šmídová, 2021).

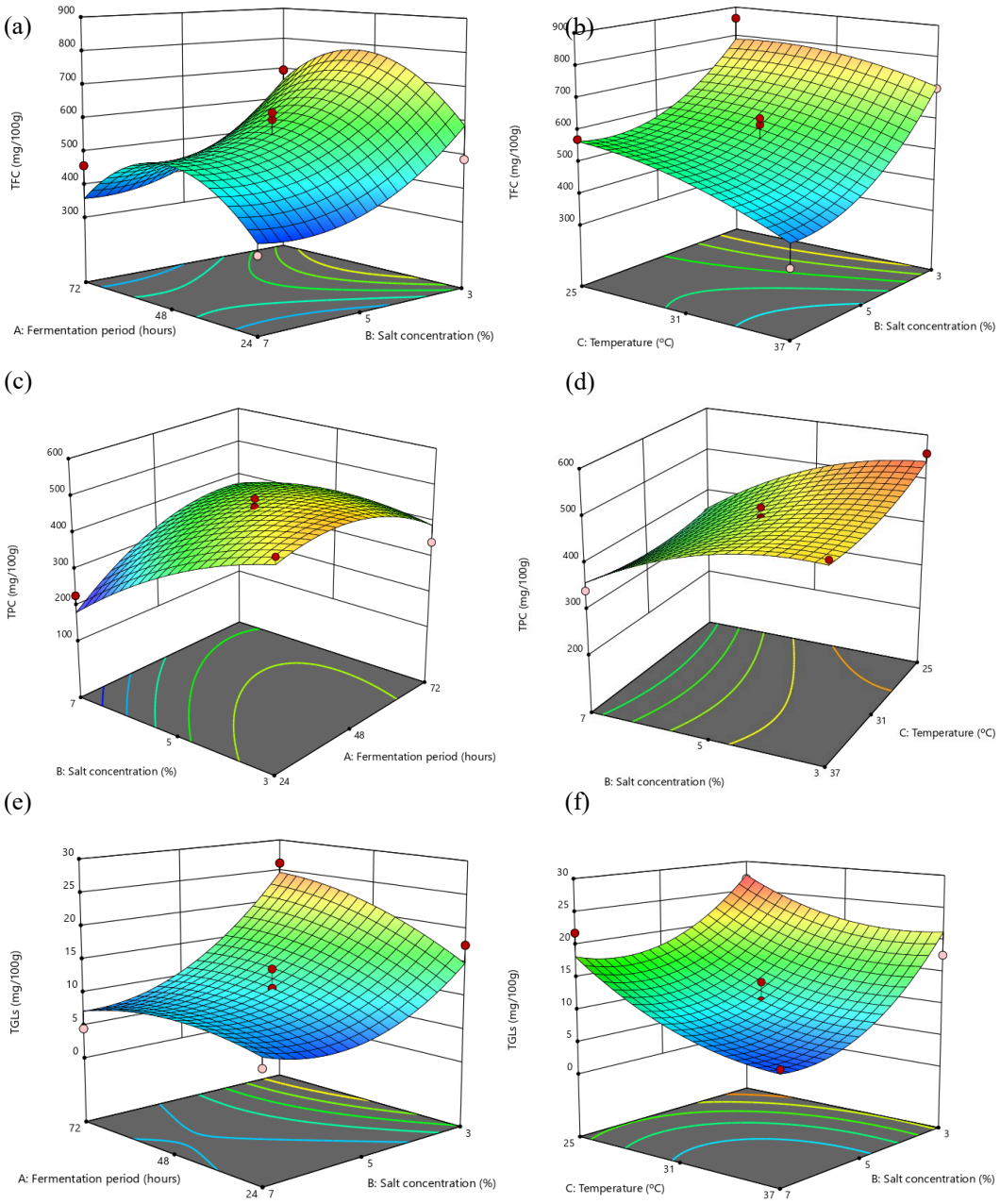


Figure 4.8. Response surface modelling of three manipulating variables such as fermentation period (X_1), salt concentration (X_2) and fermentation temperature (X_3) with responding variable (a) TFC with variables X_1 and X_2, (b) TFC with variables X_2 and X_3, (c) TPC with variables X_1 and X_2, (d) TPC with variables X_2 and X_3,, (e) TGLs with variables X_1 and X_2 (f) TGLs with variables X_2 and X_3,. (i) comparison between fermentation period and salt concentration and (ii) comparison on the salt concentration and fermentation temperature.

The 3% of salt concentration is optimal to kill harmful microbial organism and promote growth of salt-tolerant beneficial probiotic strains. PFC fermented under 25°C displayed highest TFC, TPC and TGLs values (Figure 4.8 b,d&f). As the temperature increase from 25°C to 37°C, the contents of all responding variables decrease in all three salt concentration. This findings suggest that 25°C is suitable for the optimal enzymatic reaction to take place and optimal probiotic growth to further break down the complex compounds within the cabbages. According to He et al. (2020), *Lactococcus spp.* and *Lactobacillus spp.* were predominant at 20-25°C during vegetable salt fermentation, the result aligned with our study, given that our probiotics strains used also included some Lactobacillus sp..

The bioactive compounds such as TFC, TPC and TGLs showed significant variation over times. TFC and TPC increased from 24 h to 48 h and then declined were observed at 72 h under all three salt concentrations for TFC and 3% salt for TPC (Figure 4.8 a&c). While TGL exhibited a graduate increase from 24-72 h at all three salt concentrations. During fermentation process, the production of lactic acid leading to lower pH, thus it might contributed to lower viability of probiotics under acidic environment (Calinoiu et al., 2016).

In short, all these fermentation parameters can work either synergistically or antagonistically, to give combined effects on the degradation of complex compounds to release more useful bioactive compounds and enhancement of antioxidant factors. Modification of these fermentation parameters can lead to the optimisation of the functional characteristics of fermented food products, therefore augmenting their health advantages by meticulous regulation of fermentation conditions. In conclusion, the production of PFC is optimal under fermentation condition at 3% salt concentration at 25°C with 48 h fermentation periods. This formulation of PFC was selected for upcoming analysis for *in-vitro* and *in-vivo* study.

Table 4.4. The result of responding variables obtained to generate RSM modelling with randomised Box- Behnken design.

Runs	Manipulating Variables			Responding Variables		
	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	24	3	31	487.091	493.343	17.614
2	24	5	37	357.842	393.884	6.56355
3	24	5	25	467.474	312.828	10.794
4	24	7	31	316.285	228.049*	6.17897
5	48	3	25	861.288**	558.595**	27.0656**
6	48	3	37	699.684	496.115	17.6395
7	48	5	31	515.988	456.077	10.9222
8	48	5	31	599.905	454.004	13.845
9	48	5	31	620.688	456.077	10.9222
10	48	5	31	500.006	435.346	9.07617
11	48	5	31	543.352	478.881	9.84533
12	48	7	37	311.433*	341.673	6.74303
13	48	7	25	571.487	348.869	21.793
14	72	3	31	695.452	342.75	26.1773
15	72	5	25	322.214	535.542	18.4857
16	72	5	37	323.837	414.616	10.3581
17	72	7	31	456.701	356.273	4.4868*

Notes: X_1 = fermentation period, X_2 =salt concentration, X_3 = fermentation period, Y_1 =total flavonoid contents (TFC), Y_2 =total phenolic contents (TPC), Y_3 = total glucosinolate contents (TGLs). Label * showed the lowest value and ** showed the highest value.

4.3.9 Cytotoxicity properties

4.3.9.1 Cytotoxicity analysis

In this study, the efficient concentration to inhibit 50% cell growth (IC_{50}) had been observed on the PFC extract comparing to 3 types of control: raw cabbage (RC), glucosinolate standard reference rapeseed (RS) and drug doxorubicin (PC). MTT assay is commonly used to act an indicator for cell viability, and IC_{50} is used to represent the concentration of samples or drugs required to inhibit 50% of cell viability (He et al., 2016). The lower IC_{50} value in cytotoxicity analysis often indicated that a sample shows higher potent in killing cell or inhibit cell growth with lower concentration. This indirectly demonstrated that it is more effective in inducing cytotoxic effect and possess higher efficacy in cytotoxicity properties. High cytotoxicity properties displayed on PC> RS>PFC>RC (Figure 4.9). In this study, PFC extract being our target compound showed higher cytotoxicity properties than raw cabbage with IC_{50} value 134.85 ug/ml, 163.935 ug/ml and 195.22 ug/ml after 48 h treatment and 108.35 ug/ml, 102.47 ug/ml, and 184.91 ug/ml after 72 h treatment in MCF-7, HCT-116 and 3T3-L1 cell line respectively. This result demonstrated that the addition of probiotics improved the cytotoxicity properties of cabbage. The literature reviews Tasdemir & Sanlier (2020) and Pyo et al. (2024) had detail report on the fermented food and probiotic supplemented food with high cytotoxicity properties.

The standard reference compound (RS) had been examined to reflect the cytotoxicity effect of pure glucosinolate (GLs) and isothiocyanate (ITC) compounds that are commonly found in cabbage. Glucosinolate and ITCs like alkyl-ITC exhibited DNA damage and alteration of protein repair in MCF-7 (Liao et al., 2021). The RS treated cell and PC treated cell showed higher cytotoxicity properties than PFC is reasonable as these two compounds are the standard pure compounds. Drug Doxorubicin used as positive control that is widely used as anticancer drug belonging to the anthracyclic class (Sritharan and Sivalingam, 2021). It showed lowest IC_{50} value about 4.23 ug/ml, 6.37 ug/ml and 4.82 ug/ml inMCF-7, HCT-116 and 3T3-L1 cell line after 48 h and 2.67 ug/ml, 1.48 ug/ml and 1.98 ug/ml after 72 h treatment. In comparison of cell line type, non-cancerous cell line 3T3-L1 showed higher IC_{50}

compared to cancerous cell line. The result demonstrated that there is less cytotoxicity effect in the non-cancerous cell than cancerous cell line. There are literature evidences that drug or sample exhibit more cytotoxic effect on cancerous cell line compared to non-cancerous cell lines (Frenkel, 2009; Itharat et al., 2004; Uğur et al., 2017; Zuco et al., 2002).

Selective index (SI) was used to compare cancerous cell line MCF-7 and HCT-116 with non-cancerous cell line was shown in Table 4.5. SI revealed different cytotoxicity effect, where SI value <1 represent the drug given is more toxic than normal cells, SI value =1 represent non-selectively toxicity, and SI value >2 represent a benchmark for selective cytotoxicity in favor to cancer cell line. The selective cytotoxicity usually suggested for better potential as targeted cancer therapy candidate with reduced effect on the non-cancerous cell lines or tissues (Selvendiran et al., 2010). The PFC with SI>1 showed slightly selectively cytotoxicity towards cancerous cell line MCF-7 and HCT-116 as compared to 3T3-L1. Based on Lica et al. (2021), SI>1 is considered desirable cytotoxicity, however, in most of the published paper revealed that SI>2 is considered as the ideal drug selection in the development of new anticancer agents (Demirgan et al., 2016; Oliveira et al., 2015; Peña-Morán et al., 2016). The possible explanation is that PFC is a crude compound, may generate membrane disruption compounds such as lipopeptides, enzymes and organic acid like SCFAs during probiotic fermentation process (Cheng et al., 2021). These by products from fermentation can disrupt the cell membranes non-specifically on the cancerous or non-cancerous cell line. For instance, surfactins as a negatively charge lipoprotein displayed membrane lysis mechanism over cells (Buchoux et al., 2008). It is recognised as potent biosurfactant that commonly produced by microbes like *Bacillus subtilis* (Dan et al., 2021). However, PC doxorubicin in this study displayed cytotoxicity effect towards both cancerous and non-cancerous cell line, which come in agreement in the findings of Wang et al. (2004).

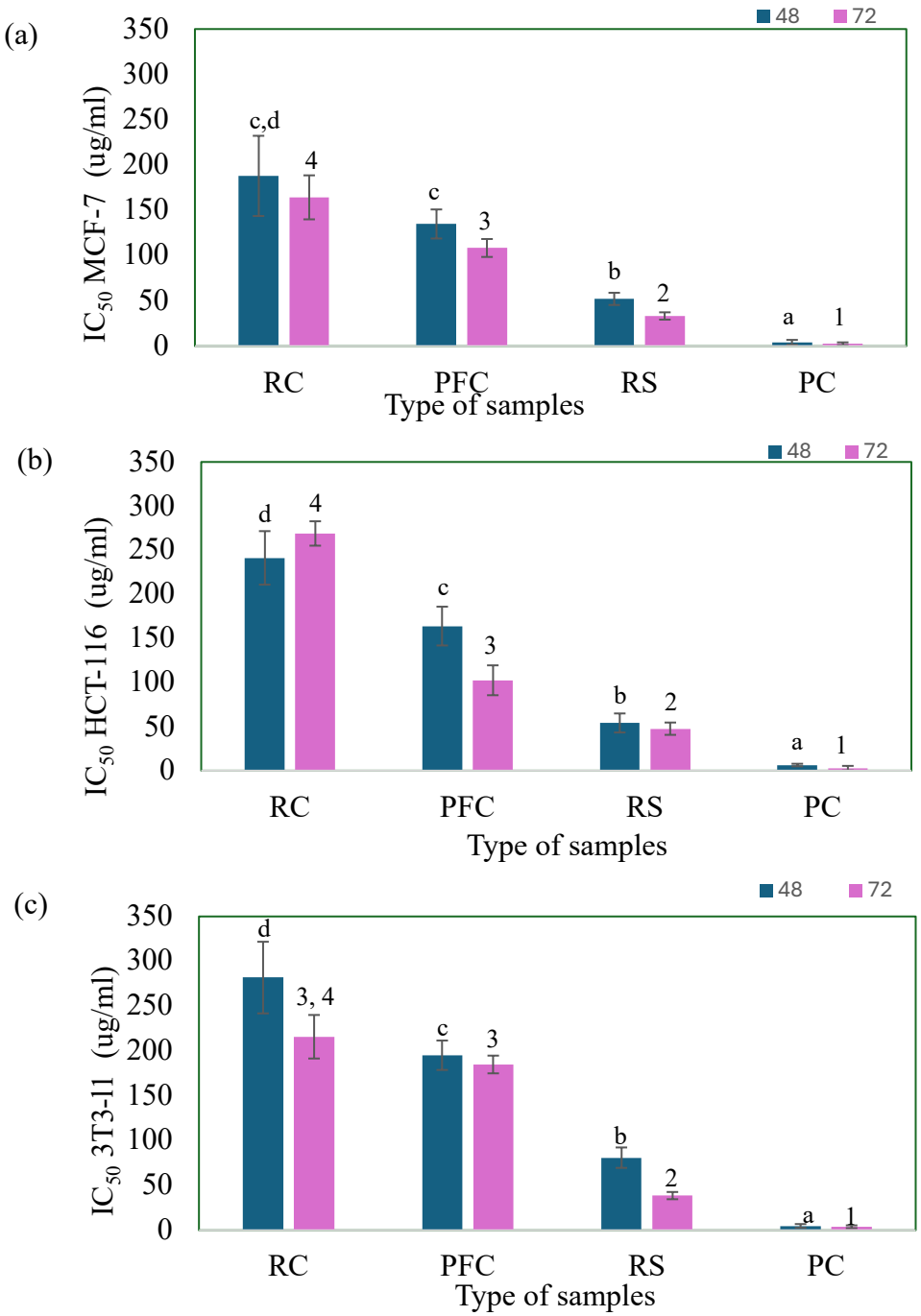


Figure 4.9. The efficient concentration to inhibit 50% cell growth (IC_{50}) for 48 h and 72 h treatment (a) cancer cell line MCF-7, (b) cancer cell line HCT-116, and (c) non-cancerous cell line 3T3-L1. RC = raw cabbage, PFC = Probiotic fermented cabbage from M9, RS = rapeseed glucosinolate reference standard and PC = positive control doxorubicin. The different alphameric symbol and numbers represent the homogenous subset group with significant different $P<0.05$ at 48h and 72h respectively.

Table 4.5 Selective index for cytotoxicity analysis

Fermentation period (h)	Samples	SI for MCF-7/ 3T3-L1	Cytotoxicity effects	SI for HCT-116/ 3T3-L1	Cytotoxicity effects
48	RC	1.50	SC	1.17	NST
	PFC	1.45	SC	1.19	NST
	RS	1.55	SC	1.50	SC
	PC	1.14	NST	1.16	NST
72	RC	1.31	SC	0.80	T
	PFC	1.71	SC	1.80	SC
	RS	1.16	NST	0.81	T
	PC	1.51	SC	1.36	SC

Four cytotoxicity status: T (SI value <1) = drug is more toxic than normal cells, NST (SI=1) = non-selectively toxicity, SC (1<SI<2) = the drug is selectively cytotoxicity to cancerous cell line, BSC (SI>2) = benchmark for selective cytotoxicity to cancer cell line. RC = raw cabbage, PFC = Probiotic fermented cabbage from M9, RS = rapeseed glucosinolate reference standard and PC = positive control drug doxorubicin.

4.3.9.2 Morphological changes

The morphological assessment of cell lines treated with PFC at IC_{50} concentration for 72 hours revealed significant differences between cancerous and non-cancerous cells (Figure 4.10). Different degree of morphological changes with cell shrinkage and shape changes had been observed in these 3 cell lines. Untreated MCF-7 cells appear in polygonal shape and uniformly attached to the surface, contrarily there is a marked morphological disruption, the cells become shrunken and rounded (Figure 4.10 a). The presence of membrane blebbing, cell clustering, numerous floating cells and debris on the surface of treated MCF-7 cell line might reflect the occurrence of apoptosis and modulation of cellular metabolism leading to cell death (Figure 4.10 aii) (Purnama et al., 2023). Similarly, PC at IC_{50} demonstrated high cytotoxicity effect role towards all cells with significant damage and cause cell rupture in structure (Figure 4.10 aiii).

For cancerous cell line HCT-116 cell line, the untreated cells appear in spherically shape, grow in dense with well adherence and clear boundaries. However, the cell structure change obviously after PFC and PC treatment with distinct cell shrinkage, disrupted cell clusters, cell burst and reduced in cell density (Figure 4.10

bi-iii). The alteration of metabolic environment triggered by PFC, leading stress and causing cell to shrink and slowly to cell death (Vanden Berghe et al., 2013). While doxorubicin as potent anticancer agent, plays its cytotoxicity role through inhibition of DNA replication, causing genomic instability, ultimately trigger cell apoptosis (Sliwinska et al., 2009).

While look on the non-cancerous cell line 3T3-L1, a mild cell shrinkage following PFC treatment was observed as compared to other two cancerous cell lines, which compliment with the findings in MTT cytotoxicity analysis with higher IC_{50} values (Figure 4.10). Most cells retained the spindle-like fibroblast morphology, and it is comparatively lower rounded and detached cell debris observed in treated 3T3-L1 cell line. Conversely, totally cell damage was observed in PC treatment where all the spindle fibroblast cell turned rounded and ruptured (Figure 4.10 ciii). In sum, these observations provide solid evidence on the PFC extracts with enhanced the cytotoxicity effect on the cancerous cell line as compared to non-cancerous cell line, indicating their partial selectivity of cytotoxicity. However, the limited difference in morphological response between cancerous and non-cancerous lines is insufficient to make any solid claim. This result is well aligned with the previously observed selectivity index (Table 4.5), highlighting the need for further in-depth research to enhance anticancer specificity and clarify the mechanisms of action.

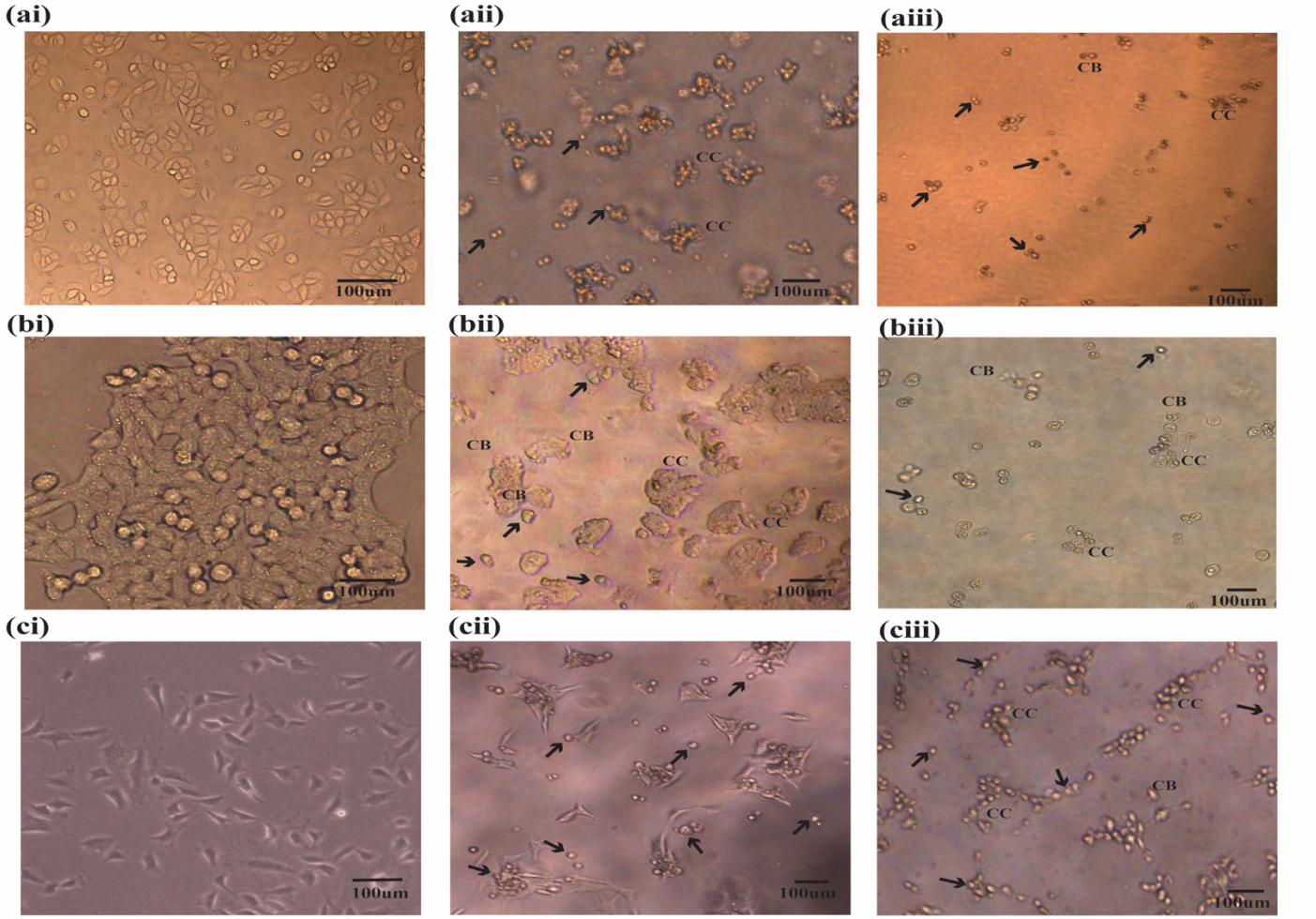


Figure 4.10. The morphological changes of cell structure. (i) untreated cell line, (ii)treated cell line at IC_{50} of PFC, (iii) treated cell line at IC50 of PC after 72 h at 10x magnification of inverted microscopy. a) MCF-7 cell line, b) HCT-116 cell line and c) 3T3-L1 cell line. Black arrow =rounded rupture cells, CC= cell clusters, CB= cell burst.

4.3.9.3 Reactive oxygen species (ROS)

DHE assay is used to measure the total ROS level especially superoxide and hydrogen peroxide directly in the live cells. DHE is highly sensitive to ROS radicals especially superoxide anions. It can oxidise to 2-hydroxyethidium, giving fluorescence signals that use to quantify the ROS level across different treatment or experimental conditions. Generally, the intracellular ROS is used to reflect the non-cancerous cell metabolism performance (Di Meo et al., 2016). The total ROS fluorescence intensity of raw cabbage (RC) and probiotic fermented cabbage (PFC) in all different cell lines had been analysed and compared with positive control (PC) and negative control (NC) (Figure 4.11). Antimycin A (AA) as a good inducer of ROS production had been used as a positive control (PC) in this analysis to establish the maximum ROS level and validates the sensitivity of the assay (Batjuka & Skute, 2017). It is the reason why PC is highest in fluorescence intensity compared to RC and PFC samples. N-acetyl cysteine (NAC) had been used as negative control (NC) due to its well establish roles in reducing ROS level, which commonly used as the baseline (Momeni et al., 2011). NAC is a powerful intracellular antioxidant and serves as a precursor to glutathione, and can neutralise intracellular ROS, particularly superoxide anions, through restoring intracellular glutathione levels and directly depleting free radicals within the cells (Mokhtari et al., 2017).

In this study, all the fluorescence intensity of raw cabbage (RC) and probiotic fermented cabbage (PFC) fall within the fluorescence intensity range of PC and NC. This result might suggest that the treatment of RC and PFC might potentially trigger oxidative stress response upon intact with the cell lines. However, the ROS stress level of RC and PFC is mild or moderate since their RFU values are lower than PC that might resulting in the oxidative damage (Jalili-Nik et al., 2020). In addition, it is spotted that the RFU values of RC and PFC are higher in IC_{50} of 48 h treatment than IC_{50} 72 h. The changes of ROS level between 48 h and 72 h might be vary depend on several factor such as continuous stress that induce ROS production and delayed response due to adaptive antioxidant defence (Huchzermeyer et al., 2022; Zandi & Schnug, 2022). The result might explained with active stimulation of ROS production in 48 h and the stress level gradually reduced at 72 h. PFC showed significance lower oxidative stress with 3743 RFU than RC with 4642 RFU in the MCF-7 cell line.

Sample with higher antioxidant properties is expected to neutralise and reduce the ROS radicals, and subsequently reduce the amount of free radicals to react with DHE (Huchzermeyer et al., 2022).

The findings demonstrated that the fluorescence intensity in cancerous cell line MCF-7 and HCT-116 are higher than non-cancerous cell line 3T3-L1. The results also further support the findings in the MTT assays where cancerous cell line (MCF-7 and HCT-116) displayed lower IC_{50} value, implied that they faced higher oxidative stress with increased oxidative damages within the cell lines. The proliferation of cancerous cell lines are faster than non-cancerous cell line, which resulting in the increase of metabolic activity and elevate of oxygen consumption (Sahoo et al., 2022). Interestingly, HCT-116 exhibited lower ROS radicals with 3743 RFU and 2730 RFU for 48 h and as 72h respectively compared to MCF-7 cell line following PFC treatment. Similar findings had been reported by Ahmad et al. (2019) and El-Hawary et al. (2021) revealed that higher antioxidant defense in HCT-116 than MCF-7. This phenomenon may be attributed to different counteract mechanisms to oxidative stress and cellular redox systems. HCT-116 exhibited more robust mechanisms like detoxification system against high glutathione activities, intercalation of the SOD radicals and catalase against stress, promote more ROS neutralization, leading lower oxidative stress (Ko et al., 2013; Su et al., 2019). While MCF-7 only relied on the mitochondrial oxidative phosphorylation for ROS production in response to stress-induced agents like organic acid, peptides, GLs and ITC detected in PFC, leading weaker antioxidant defense as compared to HCT-116 (Cao et al., 2010; Kamada et al., 2022). This finding reflects differential in antioxidant defense response can provoke intrinsic variations in metabolic vulnerability and redox regulation between the two cancerous cell types.

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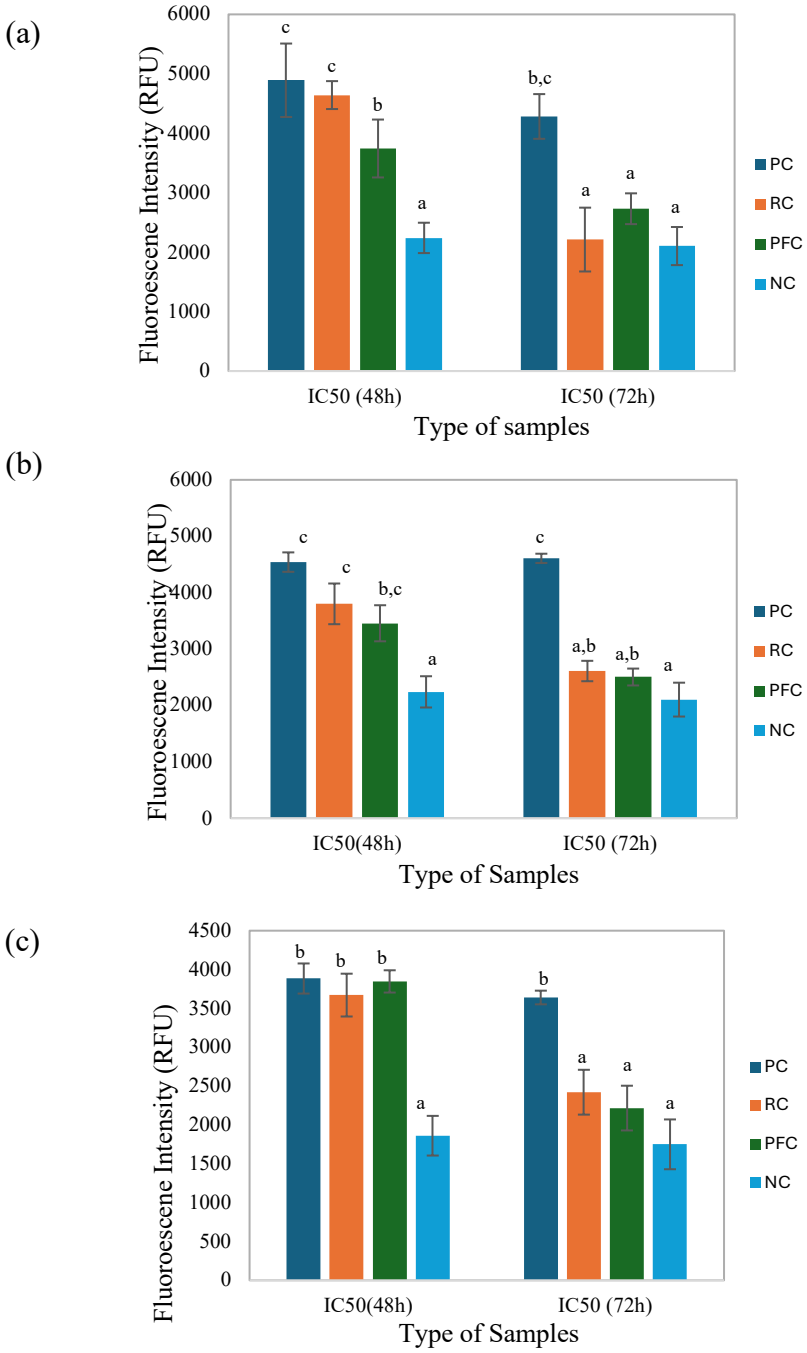

Figure 4.11. The ROS of PFC and RC for 48 h treatment and 72 h treatment in cancer cell line (a) MCF-7, (b) HCT116 cell line and (c) fibroblast cell line 3T3-L1. RC = raw cabbage, PFC = Probiotic fermented cabbage from M9, RS = rapeseed glucosinolate reference standard and PC = positive control doxorubicin. The different alphameric symbol represent the homogenous subset group with significant different $P<0.05$.

4.4 Conclusion

The addition of 8 probiotic strains significantly improved the overall bioactive compounds and antioxidant properties of PFC compared to the raw cabbage and fermented cabbage without probiotic. PFC that produced at optimal fermentation conditions of 3% salt concentration under 25 °C for 48 hours showed higher cytotoxicity property and reduced oxidative stress in MCF-7, HCT116, and 3T3-L1 cell lines compared to raw cabbage. Higher intensity of glucosinolates and its derivatives such as sulforaphane and 4-isothiocyanato-1-butene detected in PFC can support the findings with higher cytotoxicity activity. These findings highlight the potential of symbiotic PFC to provide significant nutritional value and bioactive substances that promote health.

CHAPTER 5

GUT MICROBIOTA MODULATION AND OXIDATIVE STRESS RESPONSE IN SPRAGUE DAWLEY RAT WITH FEEDING OF PESTICIDES AND PFC

5.1 Introduction

The gut is important for nutrient absorption, health regulation, and immune defence mechanisms (Li et al., 2018). Gut microbiota is a diverse array of microorganisms inhabiting the digestive tract forming a mutualistic relationship with their host and contributing to a wide range of physiological functions (Xiong et al., 2017a; Xiong et al., 2017b). Recent studies highlight the close association of gut microbiota with factors such as rearing conditions, health status, diet, and developmental stages, particularly in mice models (Xiong et al., 2017b). This underscores the importance of investigating gut microbiota as a critical determinant of host health. Exploring the gut microbiota in rats provides valuable insights into the impact of functional foods, such as PFC, on nutrient metabolism, gut health, and overall physiological well-being. Such studies offer a foundation for understanding the potential of PFC and similar functional foods in promoting health and mitigating adverse effects in animal models and, potentially, in humans.

Considering the runoff of pesticide residues into agricultural fields, this study investigates the potential of probiotic-fermented foods (PFC) in mitigating the effects of pesticide exposure on gut microbiota in rats. The research also examines the influence of PFC on gut microbiota profiles following pesticide exposure and subsequent feeding. Prolonged pesticide exposure has been reported to disrupt sex hormones, leading to endocrine disruption and mimicking the actions

of endogenous hormones (Ji *et al.*, 2020; Mazaheri *et al.*, 2020). For example, Akoto *et al.* (2015) detected organophosphate contamination in eggplant, tomatoes, and okra in Ghana, with risk indices of 87.78%, 95.84%, and 97.94%, respectively. The increasing use of pesticide in agriculture is linked to a rise of cancer cases, while epidemiological studies have highlighted the potential role of different pesticide classes—such as organophosphates, organochlorines, pyrethroids, and carbamates—in the development of cancers, including breast cancer (Ellsworth et al., 2018; Rebouillat et al., 2021; Ventura et al., 2019; Yang et al., 2020), thyroid cancer (Lerro et al., 2021), and brain cancer (Bhat et al., 2010). In addition, it is evident that pesticide exposure induce changes of gut micro flora diversity (Liu, J. et al., 2021) and cancer (Matich et al., 2021). Previous experiment proven that the gut microbiota could be artificially manipulated to improve gut health and cancer prevention (Legesse Bedada et al., 2020; Panebianco et al., 2020). The interplay between pesticides, probiotics, and the gut microbiome in cancer regulation remains unclear. Therefore, this study aims to investigate the role of probiotics in regulating the physiological responses of Sprague Dawley rats exposed to pesticide residues through dietary consumption. Therefore, this study aim to examine the function of probiotic regulation of physiological responses of SD rats under pesticide residue effects via consumption.

5.2 Methodology

5.2.1 Animal ethnics approval

This current experimental study was conducted at the Animal Laboratory of the Institute of Climate Change and Marine Biotechnology (ICAMB), Universiti Malaysia Terengganu (UMT), Malaysia. A total of 24 Sprague Dawley rats had been used throughout this research. Prior to the start of the study, an animal ethical proposal was approved from Institutional Animal Care and Use Committee (IACUC) of UMT with approval reference no. UMT/JKEPHMK/2022/78 (Appendix 16). All experimental procedures performed were aligned with the standards set forth in the IACUC guidelines. All members of this research team

declared that all ethical and legal aspect of this study was fulfilled throughout the whole study.

5.2.2 Freeze dried probiotic fermented cabbage preparation

Probiotic fermented cabbage (PFC) were produced by fermenting cabbage with 8 selected probiotic strains at 1×10^6 CFU/mL in 3% salt concentration at 25 °C. The PFC were harvested after 48 hours of fermentation period and sent for freeze dried to remove all the water contents to mimic the degradation and metabolic changes. The PFC was blended into powder form and stored at refrigerated condition at 2-8 °C to maintain its biological activity. The extraction of PFC was performed by dissolving 1g of PFC in 150 mL of water. The mixture was mechanically vertexed for 5 mins and centrifuged at 4000 rpm for 20 mins. The supernatant was harvested and sent for freeze dry to get the extractive. The final extractive yield was measured and used to prepare the PFC oral gavage concentration at 50, 125 and 500 mg/ kg body weight of rats.

5.2.3 Pesticide preparation

The stock of pesticide mixture had been prepared from 5 different pesticides that are commonly used for rice cultivation in Malaysia. The actual dosage (AD) given to each rat daily was given based on the sum of maximum residue limits (MRL) of each active ingredient (Table 5.1). As there is maximal volume of administration volume in rat, so the administrated volume for rat is fixed at 1 mL / kg of rat body weight. Therefore, the pesticide mixture stock (PMS) are prepared with concentration of 2 mL/L Fujione, 4 mL/L Monocut, 1.5 mL/L Sumibassa, 10 g/L Broadox and 50 mg/L Takumi for 6-week oral feeding treatment period.

Table 5.1. The dosage information of pesticides used.

Pesticide	Types	Active Ingredients	MRL	Actual dosage/ BW
Fujione	Fungicide	Isoprothiolane 40%	1mg/kg	1 mg/kg
Monocut	Fungicide	Benzamide 70%	2mg/kg	2 mg/kg
Sumibassa	Insecticide	Fenitrothion 20% + Fenobucard 20%	2.5mg/kg	2.5 mg/kg
Broadox	Insecticide	Buprofezin 5% + Isoprocarb 20%	10mg/kg	10 mg/kg
Takumi	Insecticide	Flubendiamide	0.5mg/kg	0.5 mg/kg

Notes: MRL= maximal residue limit of pesticides in food, BW= body weight of rat.

5.2.4 Animal source and housing condition

A total of 24 pathogen free male rats *Rattus norgegicus* of 6 weeks old (weight 100-120 g) were purchased from Animal Research and Service Center, Universiti Sains Malaysia. All rats were individually caged in a plastic cage (dimension 500 mm x 360 mm x 200 mm) and located at room temperature approximately 25±3°C with constant humidity (relatively 50-70%). They were allowed to acclimatise to hygienic laboratory conditions for two week before starting the experiment in the Animal Holding Facility of ICAMB, UMT. Water was provided *ad libitum* and standard rat diet (Altronmin 1324) (Appendix 17) was supplied daily at 10% (w/w) of rat's body weight throughout the experimental periods.

5.2.5 Design of animal study

Oral gavage feeding method were conducted with slight modification of UBC Animal Care Committee (2021). The rats were orally fed by steel gavage at size of 16G &18G (based on the growth of rats) following to the experimental design (Table 5.2). The oral gavage feeding method and the monitoring health condition of rats had been conducted based on the guidelines in Appendix 18 &19. The oral gavage feeding was conducted once daily for continuous 6 weeks to track the physiological and gut microbiota in rats. The consideration for pesticide, PFC and probiotic were set based on the health monitoring results from preliminary

study in rats. The final concentrations of all treatments were set as in Table 5.2 with slightly modified after referring to some existing relevant literature study. The pesticide concentration was designed according to the Organisation of Economic Co-operation and Development (OECD) guideline for testing of chemical (Test no.420) (OECD, 2001). The maximal intake volume in rat was about 2 mL for 200 g rat, therefore the dosage intake daily for rat in this study was set on 1 mL / 100 g.

Table 5.2. Rats used and treatments administrated

Treatment Group	Treatment	Description	Number of rats used
A	Control	No treatment	N=3
B	Pesticide only	1 ml/ kg BW of PMS	N=3
C	MD PFC only	125 mg of PFC /kg BW	N=3
D	Probiotic group	Mixture of 8 probiotic strains at 1 x 10^6 CFU/ml = 8x 10^6 CFU/ kg	N=3
E	Pesticide + LD PFC	1 ml/ kg BW of PMS + 50 mg PFC /kg body weight	N=4
F	Pesticide + MD PFC	1 ml/ kg BW of PMS + 125 mg PFC /kg body weight	N=4
G	Pesticide + HD PFC	1 ml/ kg BW of PMS + 500 mg PFC /kg body weight	N=4

Notes: LD = low dose, MD = middle dose, HD = high dose, BW= body weight, PMS = pesticide mixture stock, PFC = probiotic fermented cabbage, CFU = colony forming unit. They are extra one rat in Group E-G to prevent any mortality happened during the analysis. All collected data were presented in triplicate (N=3), one outlier from group E-G were eliminated from the study.

5.2.6 Physiological response analysis

The physiological assessment of rat such as biometry growth parameters including weekly and cumulative weight gain had been examined after each treatment. All rats were weighed weekly to visualise the growth performance. The percentage of weekly weight gain and accumulative weight gain were calculated using the formula below:

$$\text{Weekly Weight Gain (\%)} = \frac{W(n+1)-Wn}{Wn} \times 100 \qquad \text{(Equ 5.1)}$$

Cumulative Weight Gain (CWG) (%) = $\frac{WF-WI}{WI} \times 100$ (Equ 5.2)

where Wn= number of weeks (n=1,2,3,4,5,6) , WF = final weight, WI = initial weight

5.2.7 Handling of rat samples

Anaesthesia procedure was conducted prior blood collection section. Ketamine and xylazine were given at the dosage of 0.1ml /100g of rat weight through intraperitoneal (IP) injection. Clinical signs including temporary loss of consciousness shown by unresponsiveness to painful stimuli, lack of awareness and lack of movement were observed to ensure anaesthesia are adequate. Monitoring during anaesthetic and anaesthesia recovery were conducted as mentioned in IACUC IOWA University (2020). Rats were sacrificed after 42 days of treatment (6 weeks). During scarification, the rats were given euthanasia with sodium pentobarbital (Barbiturates) at 200 mg/kg though intraperitoneal (IP) injection to kill the rat in rapid unconsciousness and pain free condition. The administration protocol was practised according the protocol as mentioned in Laferriere and Pang (2020) (Appendix 20).

5.2.8 Blood collection and analysis

About 7 ml of fresh blood was collected from each rat prior sacrifice and distributed into three types of blood collection tubes: 2 ml into a plain blood tube, 2 ml into an EDTA blood tube, 1 ml into a fluoride blood collection tube, 1 mL of EDTA tube and 1mL of plain tube. The first 5 mL blood collection tubes were promptly sent to BP Clinical Lab Sdn. Bhd., Kuala Terengganu Branch, Malaysia, for biochemical analyses. The remaining 1 mL each tube was used to collect plasma with EDTA tube and to collect serum with plain tube for oxidative stress analysis. The plain blood tube is used to collect serum samples for the analysis of liver , protein, renal and lipid profiles, including alanine aminotransferase (ALT),

aspartate aminotransferase (AST), alkaline phosphate (ALP), total protein, albumin, globulin, sodium, potassium, chloride, urea, creatinine, uric acid, calcium, phosphate, total cholesterol and triglyceride. The EDTA tube used to prevent blood clotting for conducting full blood analysis such as total red blood cell (RBC) count, haemoglobin, red cell distribution width (RDW), packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), total white blood cell (WBC) count, lymphocyte, neutrophil, monocyte, eosinophil and basophil. The fluoride tube was used to determine the glucose concentration. The remaining 2 mL blood in plain tube and EDTA tube were centrifuged at 4000 rpm for 15 minutes, two hours after collection. The supernatants obtained were serum (from the plain tube) and plasma (from the EDTA tube), which were subsequently stored at -20 °C for oxidative stress biomarker analysis.

5.2.9 Biological indicator of oxidative stress

The blood plasma of each treatment group was used to determine the oxidative stress level of the rat. Total protein contents were conducted prior the start of the catalase (CAT), superoxide dismutase (SOD), total glutathione (GSH) and lipid peroxidation activity (LPO).

5.2.9.1 Catalase activity (CAT)

CAT activity was assessed indirectly by tracking the consumption of hydrogen peroxide (H_2O_2) according to the protocol described in Rasheed et al. (2007). In brief, 200 uL of the sample was diluted with 8.3 mL of 62.5 mM phosphate buffer at pH 7.2 in the spectrophotometric cuvette. About 1.5 ml of H_2O_2 (30 mM) was then added in the sample mixtures. The absorbance of control and sample group were measured with UV-Vis spectrometer in microplate reader at 240 nm. The serial dilution of protein albumin was used as standard to generate

standard curve of the total protein. CAT activity (U/mL) was calculated according to the following formula:

$$\text{TP} = \frac{[(Ac-As)-C]}{M} \quad \text{(Equ 5. 3)}$$

$$\text{CAT viability (U/mL)} = \text{TP} \times \frac{\text{Vst}}{\text{Vsp}} \times \text{DF} \times \frac{1}{Tr} \quad \text{(Equ 5..4)}$$

where TP= total proteins in CAT activities, Ac= absorbance of control (reaction mixture without sample) , As = absorbance of samples, C and M obtained from the standard curve of standard protein albumin Y=Mx+C, Vst= volume of substrate, Vsp=volume of sample, DF= dilution factor and Tr= reaction time

5.2.9.2 Superoxide dismutase activity (SOD)

SOD activity was analysed through spectroscopy measurements taken at 420 nm. Specifically, cuvettes were prepared by forming 1 L stock mixtures containing 50 µl of plasmatic supernatant (20x dilution factor of the blood plasma), 930 µl of 1mM TRIS 50 mM/EDTA buffer (pH 8.2) and 4 µl of 30 µM catalase. To initial the reaction, 16 µl of 24 mM pyrogallol prepared in 10 mM HCl was the final component added to the stock mixture solution. The sample absorbance was measured at 420 nm using UV-Vis spectrometer in the microplate reader with 1min time intervals. The SOD activity was presented in U/mL based on the calculation formula below:

$$\text{SIR (\%)} = [(A_c\text{-}Ac_b) - (\frac{AS-ASb}{Ac-Acb})] \times 100 \quad \text{(Equ. 5.5)}$$

$$\text{SOD activity (U/mL)} = \text{SIR} \times \frac{Vr}{Vsp} \times \text{DF} \quad \text{(Equ. 5.6)}$$

where SIR= SOD inhibition rate, Ac = Absorbance of reaction mixture without samples with enzyme, As= Absorbance of reaction mixture of samples with enzyme, Acb and Asb = absorbance of C and S without addition of enzyme, Vr= volume of reaction, Vsp= volume of sample, DF= dilution factors

5.2.9.3 Total reduced glutathione activity (GSH)

The assay of GSH was measured with Ellman reagent method. About 200 µl of plasma was mixed with 2.3 ml of 0.2 M potassium phosphate at pH 7.6. About 0.5 ml of 0.001 M 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was added to the mixture. After allowing the reaction to proceed for 5 minutes, the absorbance of the reaction product in the cuvette was detected at 412 nm using a UV/Visible double-beam spectrophotometer. Standard curve was plotted from the reduced GSH with concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mM GSH, as outlined by Khan et al., (2012). The GSH activity was calculated from the standard curve of GSH.

5.2.9.4 Lipid peroxidation analysis (LPO)

Lipid peroxidation analysis was measured using thio-barbituric acid reactive substance (TBARS) method according to the protocol of Stefani et al. (2014). About 62.5 µl of blood plasma (20X DF) was mixed with 187.5 µl of 10% trichloro-acetic acid (TCA) for 5 mins and then centrifuged for 10 mins. About 125 µl of supernatant was further mixed with 125 µl of 0.67% of 2-thiobarbituric acid (TBA), agitate, and followed by continuous heating for 15 mins at 100 °C in a water bath. Lastly, about 375 µl of n-butanol was added to the mixture after the cooling. The mixture was agitated and centrifuged at 3000 rpm for 5 mins. The supernatant was measured under UV-Vis Spectrometer in microplate reader at 535 nm. The 1,1,3,3-tetrametoxypropane (TMTP) was used as standard and a standard curve was plotted.

5.2.10 Gut microbiota DNA extraction and polymerase chain reaction (PCR)

The faeces DNA was extracted using Patriot DNA Extraction Kit (Patriot Biotech Sdn Bhd, Malaysia). The faeces were firstly homogenised via bead beating of zirconia beads (combination size of 0.1and 0.5mm) at 3000 rpm for 30 minutes. The DNA extraction procedure are based on instruction of

manufacturer's instruction with magnetic rack (Appendix 3C). The PCR amplification process has conducted on the bacterial 16s rRNA of V3 hypervariable region by adding 2 ul extracted DNA to 10 ul primers mix encoded 341F: CCTACGGGNGGCWGCAG and 518R: ATTACCGCGGCTGCTGG and 10 ul 2x of Master Mix REDiant II (Axil Scientific Pte Ltd, Singapore).

PCR was carried out using SolarBio PCR mastermix (SolarBio, China) using the following setting: initial denaturation at 95 °C for 3 minutes followed by 30 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s. The barcode amplicons were viewed on agarose gel through Bio-Rad imaging system. SPRI bead (0.8X) was used to purify the amplicons, and the purified amplicons served as the template for an index PCR (8 cycles) were added to the Illumina-compatible dual-index barcodes and Illumina adapter. The resulting libraries were size-selected using 0.8 X SPRI bead, pooled into a single tube and quantified using Denovix high sensitivity assay. The sequencing service were done by Patriot Biotech Sdn Bhd, Malaysia on sequencer machine Novaseq 600 (Illumina, SanDiego).

5.2.11 Gut microbiota data analysis

The process of separating and removing unnecessary parts of the raw sequence DNA databased paired-end readings was performed using cutadapt v1.18 (M. Martin, 2011). The processed readings were merged using fastp v0.21 (S. Chen et al., 2018) and subsequently imported into QIIME2 v.2022.8 (Bolyen et al., 2019). The reads were denoised with dada2 (Callahan et al., 2016) to generate Amplicon Sequence Variants (ASVs). Taxonomic assignment of the ASV was performed using the q2-feature-classifier (Bokulich et al., 2018), trained with the latest GreenGenes2 database (2022.10) (McDonald et al., 2024). Only ASVs that were assigned to the phylum and family level were chosen for further investigation. The ASV table and taxonomic classification table were exported using QIIME2 tools. Several plots for relative abundance analysis and alpha diversity were analysed using online software Microbiome Analyst from

https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/ModuleView.xhtml. (Appendix 21). The function of each ASV was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) analysis . The ASV is mapped with Green gene reference phylogeny to predict KEGG orthologs (KOs). The KOs was further mapped to its corresponding enzyme commission number (EC) and assigned metabolic pathway (PWK) based on the KEGG database. The results were finally statistically examined in the Statistical Analysis of Metagenomic Profiles (STAMP) software (Douglas et al., 2020; Parks et al., 2014).

5.2.12 Statistical analysis

All the data obtained in triplicate were process with Statistical Package for Social Science (SPSS) software (SPSS Version 23, IMB Worldwide, USA) and GraphPad prism software for the initial statistical analysis. One-way analysis of variance (ANOVA) was used for discrimination analysis with Duncan equal variances assumed for the homogeneity variance test and Post Hoc Test, where n=3 for each parameter, to test for difference among treatment groups. The treatments that fall in the different homogenous subset group and Post Hoc Test with $p< 0.05$ denoted for significantly difference among the substrate treatment. Principle component analysis (PCA), and Pearson correlation heatmap had been generated from Origin Pro data analysis and graphing software (Origin Lab Corporation, Version 2024). Clustering heatmap had been generated from online web tool ClustVis (https://biit.cs.ut.ee/clustvis/). Partial least squares discriminant analysis (PLS-DA) was further performed using SIMCA 14.1 software.

5.3 Result and Discussion

5.3.1 Physical assessment

Tracking the growth rate of rat groups is crucial as the data obtained can be used as health indicator and growth monitoring measure to evaluate the effectiveness of the diets being tests. Rat group C receiving individual dose of 125mg PFC /kg rat body weight (BW) showed the highest growth (97.92%) after 6 weeks of treatment (Figure 5.1 a&b). This result may suggest that PFC derived from useful probiotic strains and prebiotic capable of enhance growth fitness over 6 weeks treatment periods. PFC contains useful probiotic strains improving gut microbiota's health, contributing to the improvement of nutrient absorption and digestions (Dahiya & Nigam, 2022b; Erdoğan & Ertekin Filiz, 2023). In addition, fermented products like PFC increase the nutrient bioavailability through fermentation leading to large complex break-down into more easily absorbable compounds (Beganović et al., 2011; Khushboo et al., 2023). In addition, probiotic treatment D show significant growth in the 1st treatment week. The probiotic fermentation process in PFC improve production of short chain fatty acid (SCFAs), which can increase energy metabolism and promote gut health (Qayyum et al., 2023). However, all treatment groups with pesticide treatment (B, E, F, G) showed a lower growth rate after 6 weeks treatment compared to the treatment group C. This result might imply that the pesticide exposure had impacted on the appetite, and the overall weight gain. Treatment group G demonstrated the lowest growth rate in the first three week of treatment, which indicated that the high dose of PFC coupled with pesticides caused burden in their suggesting cell response and adjustment in that condition.

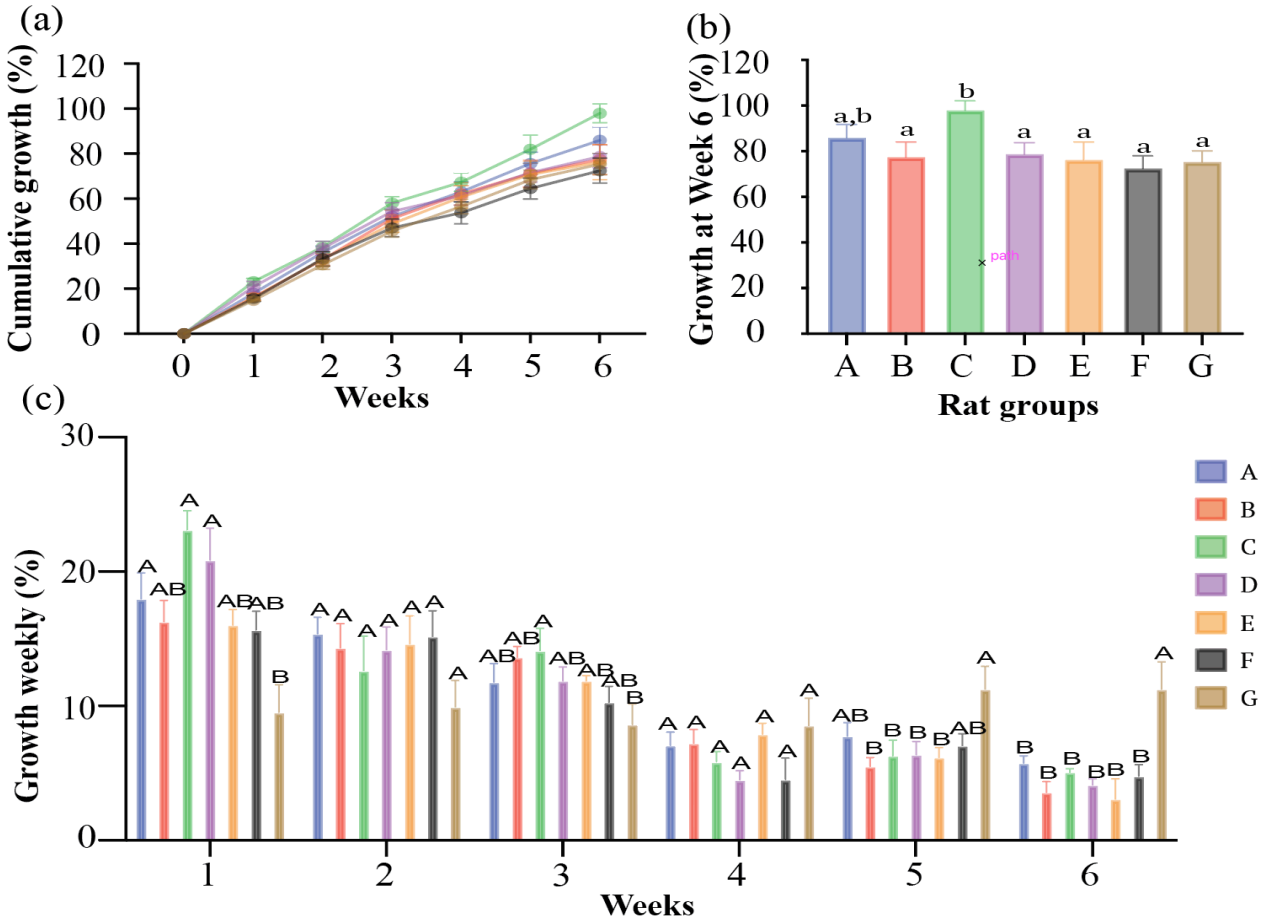

Figure 5.1. The physiological parameters analysis of rat group (n=3), where a (a) cumulative growth, (b) growth rate at week 6, and (c) growth weekly. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture

5.3.2 Blood biochemical profile

5.3.2.1 Hematological analysis

Full blood analysis is critical to provide insight on fitness condition throughout the experimental period. Statistically, there is no significant difference between control (group A) and treatment groups in the comparison of full blood analysis components in term of red blood cell (RBC) and white blood cell (WBC) (Table 5.3). This result suggests that all the treatment groups are considered a healthy state like control group A. Therefore, PCA and PLS-DA were used to visualise data variation and pattern distribution of RBC and WBC dataset (Figure 5.2). Different components of RBCs such as total RBC count, red cell distribution width (RDW), total hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and packed cell volume (PCV) was compared (Figure 5.2 a-c). There is no distinct cluster of treatment group being observed in Figure 5.2 a&b, however, distinct cluster of treatment groups from control A group can be observed in 3D score scatter plot (Figure 5.2 c).

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Table 5.3. Full blood analysis includes red blood cell profile and whie blood cell profile for each treatment group A-G (A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture).

(a) Full Blood	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**Std range**
Total RBC ($x10^{12}$/L)	7.00±0.00[a]	6.33±0.56 [a]	6.63±0.57 [a]	6.63±0.43 [a]	6.68±0.25 [a]	6.73±0.17 [a]	6.78±0.27 [a]	6.60-7.20
Haemoglobin (gm/L)	154.67±1.45 [a]	136.00±12.74 [a]	142.00±12.01 [a]	143.67±10.4 [a]	144.75±6.41 [a]	142.75±3.33 [a]	143.75±5.36 [a]	130.00-170.00
PCV (L)	0.41±0.01 [a,b]	0.40±0.02 [a,b]	0.37±0.04 [a,b]	0.42±0.01 [a]	0.40±0.03 [a,b]	0.40±0.00 [a,b]	0.37±0.01 [b]	0.40-0.50
MCV (fl)	58.33±0.88 [a]	58.33±0.88 [a]	58.33±0.33 [a]	59.33±1.20 [a]	58±0.58 [a]	57.50±0.65 [a]	57.25±0.75 [a]	49.00-63.00
MCH (pg)	22.33±0.33 [a]	21.33±0.33 [a]	21.6±0.33 [a]	21.67±0.67 [a]	21.5±0.29 [a]	21.25±0.25 [a]	21.25±0.25 [a]	19.60-22.20
MCHC (g/L)	375±4.08 [a,b]	370±0.00 [b]	370±0.00 [b]	370±0.00 [b]	370±0.00 [b]	370±0.00 [b]	380±8.16 [a]	329.00-375.00
RDW (%)	14.23±0.26 [a]	14.10±0.21 [a]	14.30±0.17 [a]	14.53±0.62 [a]	14.40±0.10 [a]	14.85±0.06 [a]	14.68±0.18 [a]	11.10-15.20
Total WBC ($x10^9$/L)	9.05±0.86[a,b]	6.50±3.18 [b]	9.45±3.55 [a,b]	8.55±1.51 [a,b]	10.55±0.45 [a,b]	12.70±0.90 [a]	6.20±0.33 [b]	6.00-18.00
Lymphocytes (%)	78.00±7.35 [a-d]	79.50±2.04 [a,b,c]	85.50±4.49 [a]	81.00±1.63 [a,b]	76.00±2.45 [b,c,d]	70.50±0.41 [d]	72.00±0.00 [c,d]	66.60-90.30
Neutrophils (%)	18.00±8.16 [a,b]	16.50±1.22 [a,b]	9.50±4.49 [b]	14.00±1.63 [b]	16.50±4.49 [a,b]	24.50±0.41 [a]	24.00±0.82 [a]	6.20-26.70
Monocytes (%)	1.33±0.88 [a]	3.67±0.88 [a]	4.67±0.88 [a]	3.33±0.33 [a]	5.00±1.29 [a]	4.00±0.58 [a]	3.50±0.87 [a]	0.80-3.80
Eosinophils (%)	0.00±0.00 [a]	1.00±0.58 [a]	0.67±0.33 [a]	1.67±0.33 [a]	1.00±0.41 [a]	1.00±0.41 [a]	0.75±0.25 [a]	0.20-3.50
Basophils (%)	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00-0.80

Notes: Std range = standard range for rat study, RBC= red blood cell, RDW=red cell distribution width, PCV=Packed cell volume, MCHC= mean corpuscular haemoglobin concentration, MCH= mean corpuscular haemoglobin, MCV=mean corpuscular volume, WBC=white blood cell

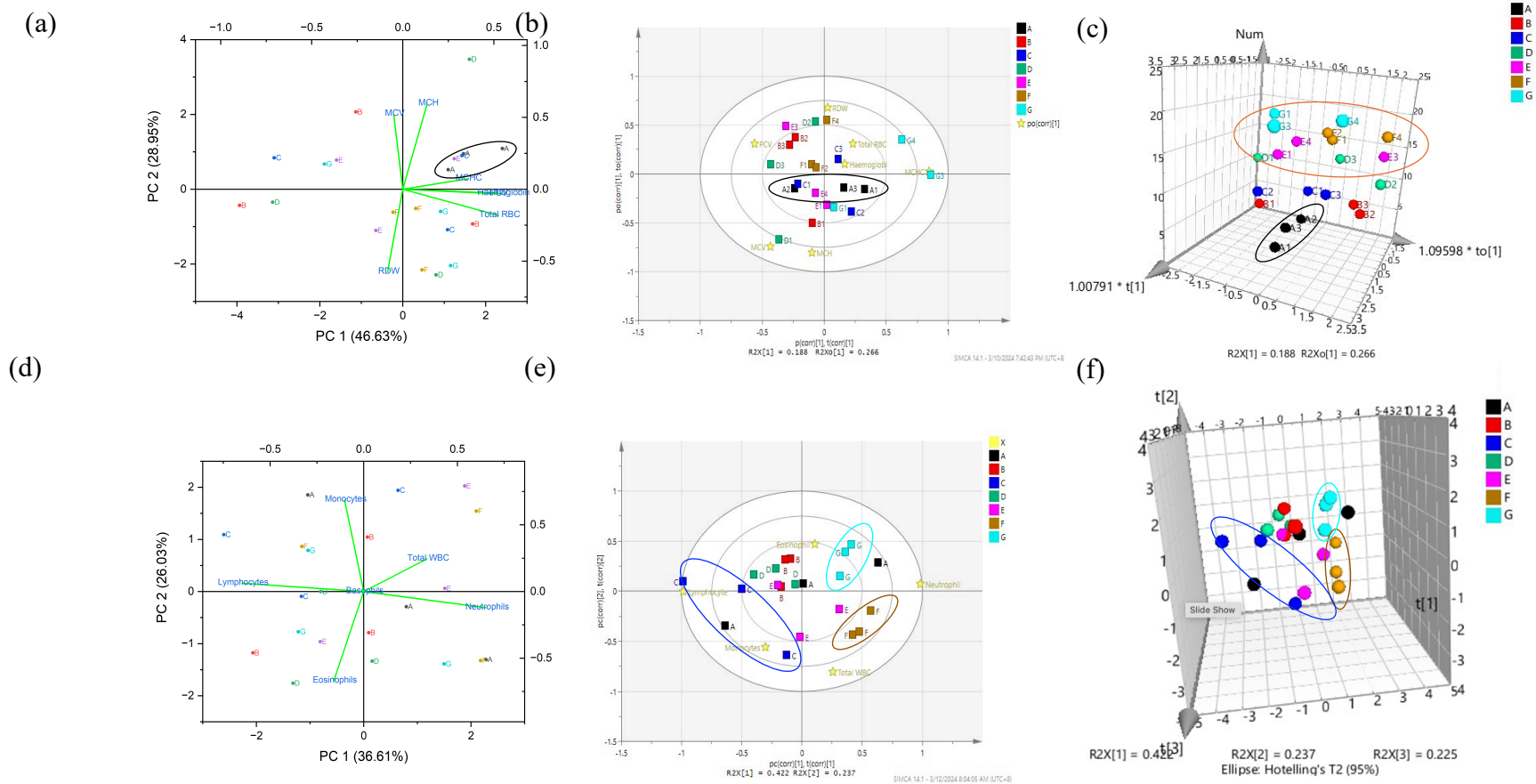

Figure 5.2. Comparison of the RBC and WBC parameters of rat groups. a-c) PCA biplot, PLS-DA 2D biplot and PLS-DA 3D score scatter plot of RBC parameters, d-f) PCA biplot, PLS-DA biplot and PLS-DA 3D score scatter plot of WBC parameters. The colour code for rat group A=black, B=red, C=blue, D=green, E=pinkish purple, F=brown, G=tiffany blue. The yellow star shape in b and e are the RBC parameters and WBC parameters respectively.

Although the WBCs of all treatment groups (A-G) in this study falls within the normal range of healthy rats (River, 2008) (Table 5.3). However, it is interesting to observe the WBC of treatment groups of C, F and G forming three distinct clusters (Figure 5.2 e&f). WBCs are critical components of immune system, they play significant role in defense mechanism against abnormal cell, infection, and foreign invaders (Sattler, 2017). Treatment group G showed positive correlation towards eosinophilia suggesting that treatment group G may encounter with allergic and inflammation problem with the presence of eosinophil (Puzzovio et al., 2023). The high association of neutrophils in both treatment group F and G could also indicate that they are in stress as neutrophils serve as first defense responders activated to response towards the external antigens and chemokines being part of the complement system (Tang et al., 2022). Treatment group C are highly associated with lymphocytes and may suggest that the ingestion of PFC can enhance the activation and proliferation of lymphocytes like B cells being responsible for antibody production. In addition, T cells (either T helper or T cytotoxic cells) that stimulate immune signaling molecules, contributed to an overall improved adaptive immunity (Maldonado Galdeano et al., 2019).

5.3.2.2 Serological analysis for renal, liver, lipid, protein and glucose profile

The blood profiles of the control group (A) were similar to those of the middle-dose PFC-fed (C) and probiotic-fed (D) groups, with the exception of total cholesterol (TC) levels (Table 5.4). This indicates that the consumption of fermented cabbage and probiotics is safe and promotes healthy regulation. The total cholesterol level was reduced in treatment group C rats, suggesting a synergistic effect of probiotics and prebiotics in cholesterol regulation. The role of prebiotics and probiotics in lowering total cholesterol has been well-documented through several mechanisms, including bile salt metabolism, production of short-chain fatty acids (SCFAs), direct cholesterol assimilation, and the synergistic interaction of synbiotics during fermentation (Bock et al. , 2021; Larkin et al., 2009; Ooi & Liong, 2010). Furthermore, PFC consumption led to lower total cholesterol levels in treatment groups E, F, and G all exposed to pesticides (Table 5.4 d). These findings further demonstrate the potential of PFC in reducing total cholesterol levels in rats.

Table 5.4 Serological analysis comprising (a) renal profile, (b) liver profile, (c) protein profile, (d) lipid profile and (e) glucose for each treatment group A-G (A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture).

(a) Renal Profile	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**Std range**
Sodium (mmol/L)	138.67±0.88 [a]	141±1.73 [a]	140.33±0.88 [a]	140.67±0.88 [a]	138.50±0.96 [a]	139.50±0.29 [a]	139.00±1.15 [a]	135.00-155.00
Potassium (mmol/L)	5.43±0.44 [a]	5.15±0.20 [a]	4.97±0.44 [a]	5.97±0.59 [a]	6.63±0.38 [a]	6.03±0.93 [a]	5.80±0.57 [a]	3.60-6.00*
Chloride (mmol/L)	102.00±0.00 [a]	103±1.15 [a]	98.50.29 [b]	102.00±1.15 [a]	101.50.87 [a]	102.00±0.58 [a]	99.00±0.00[a]	95.00-110.00
Calcium (mmol/L)	2.47±0.07 [a]	1.93±0.54 [a]	2.50±0.10 [a]	2.59±0.01 [a]	2.34±0.23 [a]	2.46±0.09 [a]	2.54±0.03 [a]	0.60-0.7*
Phosphorus (mmol/L)	2.57±0.45 [a]	3.30±0.54 [a]	2.79±0.27 [a]	3.45±0.32 [a]	3.23±0.24 [a]	2.92±0.63 [a]	2.84±0.19 [a]	0.31-0.58*
Urea (mmol/L)	5.36±0.05[b,c]	6.38±0.36 [a,b]	5.23±0.44 [b,c]	5.17±0.19 [c]	**6.53±0.27 [a]**	5.43±0.28 [b,c]	**6.50±0.30 [a]**	5.00-20.00
Creatinine (umol/L)	55.00±0.58 [b,c]	**62.67±3.18 [a]**	50.33±2.85 [c]	57.00±1.86 [a,b,c]	**63.33±4.41 [a]**	58.33±3.51 [a,b]	59.00±1.86 [a,b]	17.68-70.72
Uric acid (umol/L)	173.67±35.67 [a]	213.67±85.53 [a]	143.67±60.07 [a]	283.00±74.73 [a]	219.75±62.04 [a]	200.50±61.19 [a]	282.75±63.62 [a]	132.60-442.00
(b) Liver Profile	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**Std range**
AST (U/L)	212.67±7.36 [a,b]	141.00±32.67 [a,b]	111.33±28.26 [b]	145.67±10.69 [a,b]	239.67±9.81 [a]	241.33±14.99 [a]	245.00±10.37 [a]	60.00-230.00
ALT (U/L)	55.00±4.62 [b]	55.00±3.79 [b]	44.50±2.89 [b]	42.00±2.89 [b]	**87.50±3.18 [a]**	66.00±4.36 [a,b]	**87.50±2.60 [a]**	30.00-80.00
ALP (U/L)	133.67±15.19 [a]	152.33±16.01 [a]	128.33±7.02 [a]	125.67±24.36 [a]	124.67±24.66 [a]	117.00±5.24 [a]	127.33±7.88 [a]	45.00-170.00
(c) Protein Profile	A	B	C	D	E	F	G	**Std range**
Total Protein (g/L)	56.33±1.33 [a]	56.33±1.33 [a]	53.33±1.86 [a]	57.00±0.58 [a]	56.33±1.11 [a]	56.25±0.48 [a]	57.50±1.85 [a]	55.00-75.00
Albumin (A)(g/L)	26.50±0.29 [a]	26.50±0.29 [a]	26.50±0.87 [a]	26.50±0.29 [a]	**24.50±0.29 [b]**	**24.50±0.29 [b]**	28.00±0.58 [a]	25.00-40.00
Globulin (G) (g/L)	30.00±0.00 [a]	30.00±0.00 [a]	30.00±0.00 [a]	30.00±0.00 [a]	30.00±0.00 [a]	30.00±0.00 [a]	30.00±0.00 [a]	20.00-35.00
A/G Ratio	0.90±0.00 [a,b]	0.90±0.00 [a,b]	0.85±0.03 [b]	0.90±0.00 [a,b]	0.80±0.00 [c]	0.80±0.00 [c]	0.95±0.03 [a]	1.00-2.00
(d) Lipid Profile	A	B	C	D	E	F	G	**Std range**
TC (mmol/L)	1.80±0.08 [a,b]	1.85±0.04 [a]	**1.45±0.04 [d]**	1.65±0.12 [b,c]	**1.60±0.00 [c,d]**	1.65±0.12 [b,c]	**1.60±0.08[c,d]**	2.22-15.61*

Triglyceride (mmol/L)	1.43±0.34 [a]	1.13±0.15 [a]	1.10±0.10 [a]	1.20±0.31 [a]	1.43±0.24 [a]	1.00±0.15 [a]	1.30±0.00 [a]	1.67-22.72*
HDL (mmol/L)	0.47±0.03 [a]	0.50±0.00 [a]	0.40±0.00 [a]	0.47±0.03 [a]	0.47±0.03 [a]	0.43±0.03 [a]	0.43±0.03 [a]	2.22-3.89*
LDL (mmol/L)	0.60±0.12 [a]	0.83±0.03 [a]	0.57±0.03 [a]	0.67±0.20 [a]	0.60±0.15 [a]	0.53±0.13 [a]	0.53±0.09 [a]	2.00-6.00*
TC/HDL	3.57±0.03 [a]	3.67±0.03 [a]	3.70±0.06 [a]	3.47±0.09 [a]	3.67±0.12 [a]	3.533±0.12 [a]	3.63±0.03 [a]	>3.00
(e) Glucose	A	B	C	D	E	F	G	**Std range**
Glucose (mmol/L)	12.90±2.86 [c]	**20.30±1.47 [b]**	16.25±1.92 [b,c]	13.35±1.10 [c]	**22.45±5.51 [b]**	**21.45±5.92 [b]**	**31.35±1.43 [a]**	3.89-11.56*

Notes: Std range = standard range for rat study, ALT=alanine aminotransferase, AST=aspartate aminotransferase, ALP=alkaline phosphatase, TC=total cholesterol, HDL=high density lipoproteins, LDL=low density lipoproteins. The statistical analysis was performed with n=3, where the alphameric numbers (a,b,c,d) present as the significant homogenous subset group ($p<0.05$) between the 7 treatment groups. Symbol * represent the treatment groups do not fall within the standard range reported by (David Geffen, 2013; River, 2008; Taconic Biosciences, 2004). The bold highlight showed the treatment group is significant than control.

Urea content in blood is generally higher in rat treated by pesticide with or without PFC (treatment groups B, E, F & G), which is not significant between these groups. While treatment group control (A), middle dose of PFC (C) and Probiotic (D) show lower urea content (Table 5.4 a). As kidney function in urea removal through urine, the high urea content in all pesticide treatment group might imply the impact of pesticide in kidney function. However, the kidney function could also affected by external factors such as dehydration, liver function, and dietary protein intake (Lauverjat et al., 2006; Martin et al., 2005). Treatment group G with higher dose of PFC treatment might have higher protein and salt intake, which indirectly causing water retention within body and leading the accumulation of urea within the blood.

The creatine content in treatment group control (A) and pesticide treatment group (B) is significantly different, the increment of creatine could be modulated by consumption of middle or high dose of PFC (treatment group F&G) which shows no differences to control rat (Table 5.4 a). Therefore, similar with the urea profile, the high creatinine profile in pesticide treatment group indicate kidney problems, such as kidney damage, infection, or reduced blood flow (Fuentes-Delgado et al., 2018; Hassanin et al., 2018). Both creatinine and urea are commonly used as key indicators of renal function. However, creatinine is often regarded as a more reliable marker for assessing kidney function because its levels are less influenced by dietary protein intake, dehydration, or liver function compared to urea (Salazar, 2014). As a waste product of muscle breakdown, creatinine is a good measure of kidney function because it is produced continuously, is less affected by nutrition and hydration, and has a direct correlation with the glomerular filtration rate (GFR). Although these are less common, high creatinine levels can be a sign of impaired kidney function, acute kidney injury (AKI) or chronic kidney disease (CKD) and muscle damage (Bragadottir et al., 2013; Levey et al., 2015).

Liver profile shows that treatment groups E, F, G that received pesticide and PFC together showed significant higher ALT and AST levels compared to other treatment groups (Table 5.4 b). Liver enzymes AST and ALT are commonly used to indicate liver cell damage, with elevated levels indicating hepatocellular injury (Lala et al., 2023). Surprisingly, pesticide treatment alone (B) has lower AST and ALT compared to pesticide and probiotics combination. In comparison of protein profile,

treatment groups E and F showed significant lower albumin with about 24.50±0.29 g/L compared to the control with albumin level of 26.50±0.29 g/L (Table 5.4 c). From the findings, the combination dosage of pesticide with PFC (treatment group E-G) seem to elevate the biomarker enzyme of liver indicators, and cause reduction in albumin level (treatment groups E&F).

All pesticides treatments groups (B, E, F and G) showed significant increase in glucose level compared to the control A group, while treatment group C and D did not (Table 5.4 e). This may indicate that pesticide exposure trigger gluconeogenesis and cause glucose intolerance against the toxic effect of the pesticide mixtures (Velmurugan et al., 2017, Nath 2002). Several literature studies reported an elevation of glucose level during pesticide exposure which showed that pesticide induced disruption on the glucose metabolism causing alteration in glucose homeostasis (He et al., 2020; Rahimi & Abdollahi, 2007)

Treatment groups A, C, and D were positioned close to each other at the centre of the biplot (Figure 5.3a), while all pesticides treatment group (B, E, F and G) showed distinct cluster at bottom region when visualised under 3D scatter plot (Figure 5.3 b). To test if the glucogenesis pathway, bile salt responses and cholesterol level are correlated, all blood regulation and treatment responses are tabulated into a hierarchical clustering heatmap (Figure 5.4). A positive correlation means that when one variable increases, the other variable also increases and vice versa (Fernandez et al., 2017). There are 2 major clusters observed from Figure 5.4, where urea, lymphocytes, ALP, sodium, albumin, MCV and MCH fall within one cluster, and the remaining parameters in another cluster. Treatment group C showed stronger positive correlation with urea, lymphocytes and ALP, and treatment group E showed strong positive correlation with AST, ALT, calcium, creatinine, potassium, and phosphorus.

In short, the overall blood profile revealed the negative impact of pesticide exposure on elevation of the liver damage and glucose level. Most importantly, the consumption of PFC are potentially contributed in the total cholesterol reduction and potential modulation against kidney function damage brought by pesticide exposure.

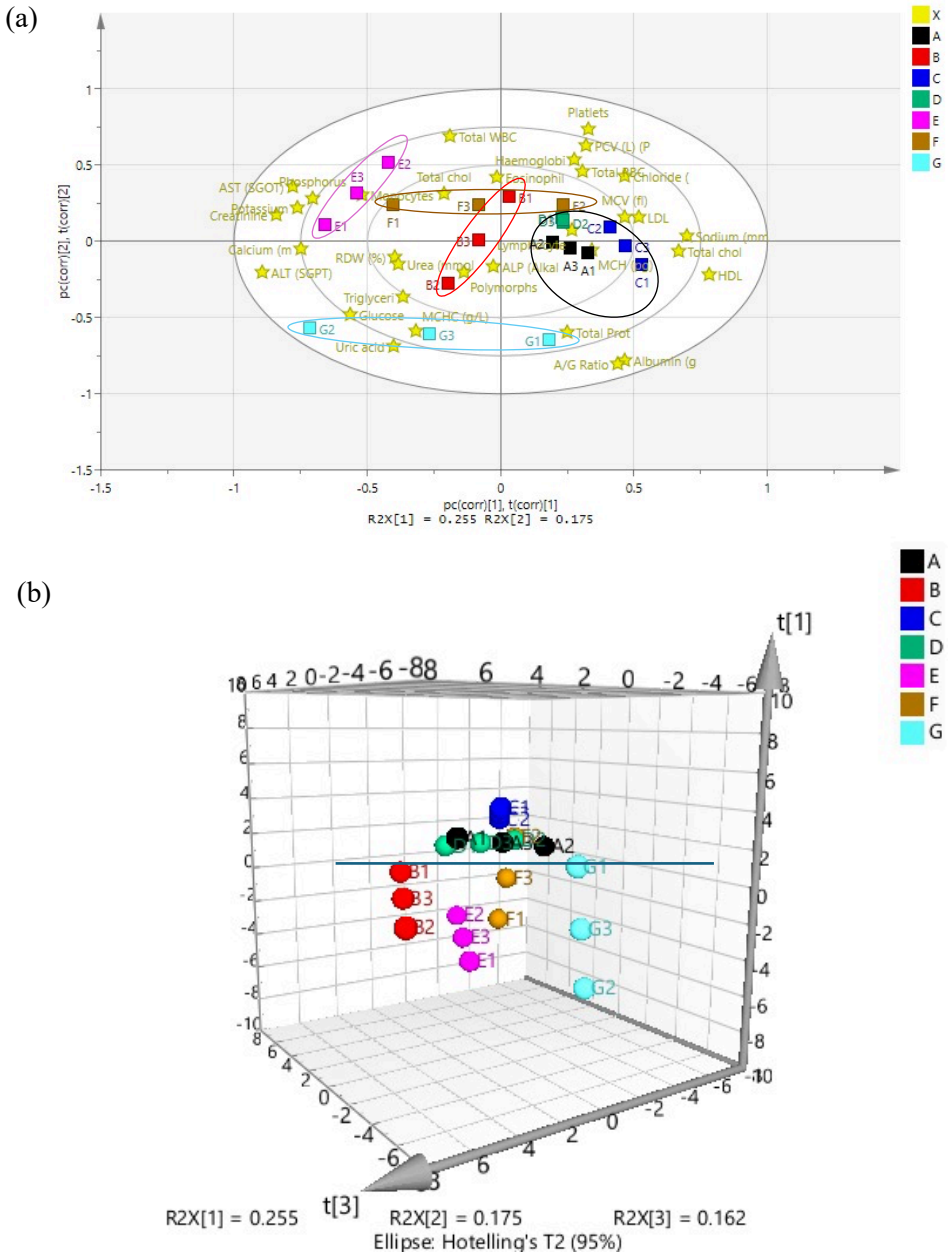

Figure 5.3. Overview on the correlation and associate neighboring cluster of all blood parameters with mean dataset of the rat groups. (a) PLS-DA biplot of all components, (b) PLS-DA 3D score scatter plot. Rat groups A = control, B = pesticide mixture, C = middle dose of PFC, D = probiotic and E-G = combination dose of low, middle, and high dose of PFC with pesticide mixture

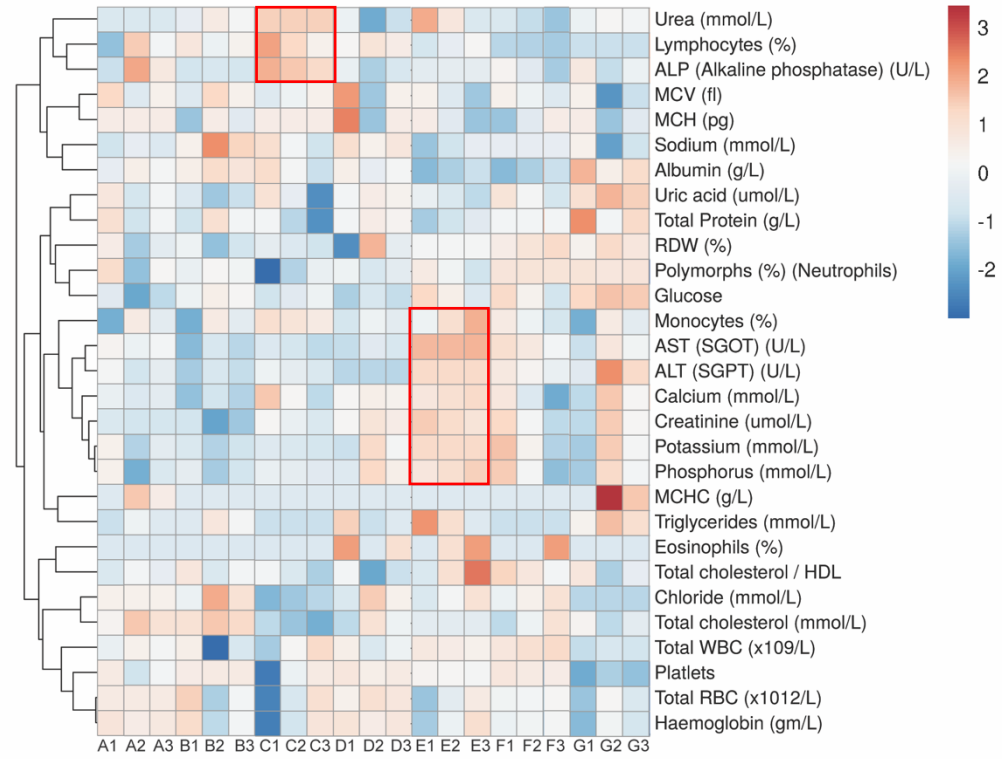

Figure 5.4 The clustering heatmap between control and all treatment groups. The mean dataset values are ln(x) transformed with unit variance scaling. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture

5.3.3 Oxidative stress analysis

Oxidative stress (OS) analysis is important to give information about the potential effect of PFC and pesticides on the overall health of animals. Generally, OS occurs when there is imbalance production between reactive oxygen species (ROS) and their elimination by antioxidant system (Aranda-Rivera et al., 2022). ROS such a as peroxides, hydroxyl and superoxide radicals are highly reactive molecules that damage DNA, protein and lipids and subsequently leading to several cascade immunology response and inflammation (Juan et al., 2021). In this study, four oxidative stress analysis had been performed (Figure 5.5).

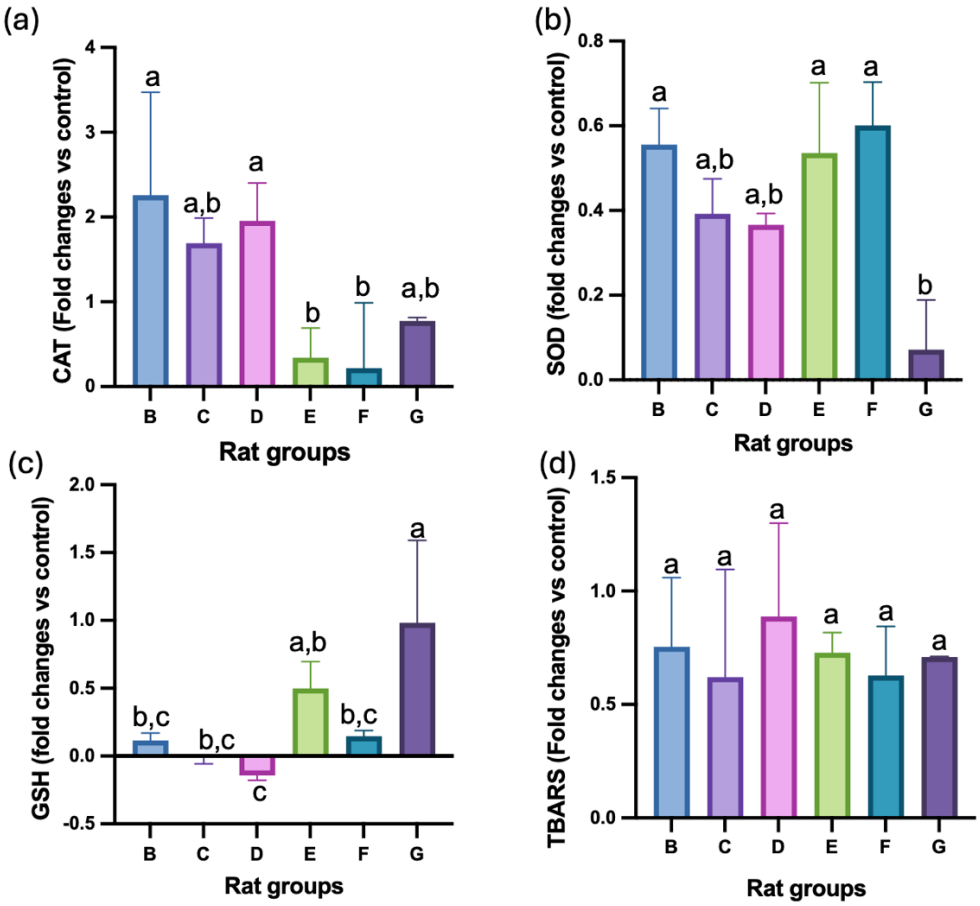

Figure 5.5 The oxidative stress analysis a) CAT, b) SOD, c) GSH and d) TBARs were presented in the fold changes towards control rat group A. Rat groups B = pesticide mixture, C = middle dose of PFC, D = probiotic and E-G = combination dose of low, middle, and high dose of PFC with pesticide mixture. The statistical analysis was performed with n=3, where the alphameric numbers (a,b,c) present as the significant homogenous subset group ($p<0.05$) between 7 rat groups.

Apparently, all treatment groups show an increment in oxidative responses compared to control, except GSH reduction in probiotic fed rats (rat group D). From the result, rat B feed with pesticide showed almost double fold in CAT activity but not in the treatment groups with combination dosage of pesticide and probiotic (E, F &G), implying that PFC promote protection of rat from pesticide (Figure 5.5 a). About 0.4 to 0.6-fold higher SOD were detected in all treatment rats compared to control, except treatment group G (Figure 5.5 b). Various pesticide types display different degree of oxidative stress across diverse cell type and animal models by disrupting redox homeostasis, leading to alterations in biochemical, physiological, and morphology systems (Alipanah et al., 2022; Chatterjee et al., 2021; Katić et al., 2021; Shah & Parveen, 2022). In this study, the oral feeding of maximum residue limit dosage of

pesticide was given to simulate the similar amount of pesticide residues content in the agricultural crops. The significant increase in CAT and SOD revealed that more antioxidants were elicited as oxidative stress responses toward low pesticide residue. This is closely associated with their roles (CAT and SOD) as first line defense antioxidant enzymes among all the antioxidant enzymes (Rajput et al., 2021).

The low GSH levels in all treated rats imply intestinal oxidative stress being in contradict to other experimental model which show the function of probiotics induce the rise of GSH and reduce intestinal oxidative stress (Peran et al., 2007; Yadav et al., 2007). The GSH function as primary intracellular antioxidant to neutralise ROS and prevent lipid peroxidation through direct interaction with lipid radicals to stop the chain reaction of lipid peroxidation (Yan et al., 2023). Lipid peroxidation is a harmful and destruction process, where it compromises for the membrane damage, protein and DNA damage and even up to cell apoptosis (Juan et al., 2021). This finding demonstrates that all treatment groups might experience mild or moderate degree of oxidative stress, yet it didn't achieve lipid peroxidation stage.

5.3.4 Fecal gut microbiome profiling

In this study, fecal microbiome is used as a proxy for gut microbiome of the animal host. In fact, fecal microbiome is a subset to the gut microbiome, used to reflect the microbial community within the distal gut and colon, not comprising upper GI tracts such as small intestine and stomach (Ahn et al., 2023). The fecal microbiome profiling is widely used in analyses of gastrointestinal dysfunction, gut microbiota modulation and alteration in various diseases and disorders (Soldán et al., 2024). The findings provide significant insights towards microbial composition, microbial taxonomy and metabolic shift, and its associated gut health risk with dysbiosis (Dinsmoor et al., 2021). Additionally, fecal studies helped in understanding the full complexity of the gut microbial functions such as defense against infection and SCFAs productions (Flint et al., 2015; Peterson et al., 2022).

5.3.4.1 Alpha diversity

The alpha diversity of the treatment groups can be visualised under 4 indexes, namely Chao1, ACE, Simpson and Shannon index (Figure 5.6). These 4 indexes are widely used to describe the richness and diversity of microbial communities. Chao1 emphasis on the overall richness, where rare species is being focus, while ACE emphasis on the abundance-based richness with moderate rare species impact. Shannon index was used to indicate the overall distributed species in both richness and evenness of the communities and Simpson index focus more on the evenness and emphasised on the abundance of the most common species (Thukral, 2017). Neither of the treatment groups showed significant different at $P<0.05$ (two tailed) in term of species richness diversity index Chao 1 and ACE and species diversity index Shannon and Simpson (Figure 5.6). This study failed to assess statistically significant differences in the alpha diversity of the gut microbiota up to 2515 identified taxa despite previous studies related to probiotic and pesticide might cause significant different in alpha diversities (Kanwar Rajawat et al., 2022; Wu & Su, 2022). However, other similar studies on gut microbiota showed similar results with no significant different in alpha diversity of gut microbiota but significant different in the relative abundance (Worsley et al., 2021; Zhang, J. et al., 2022).

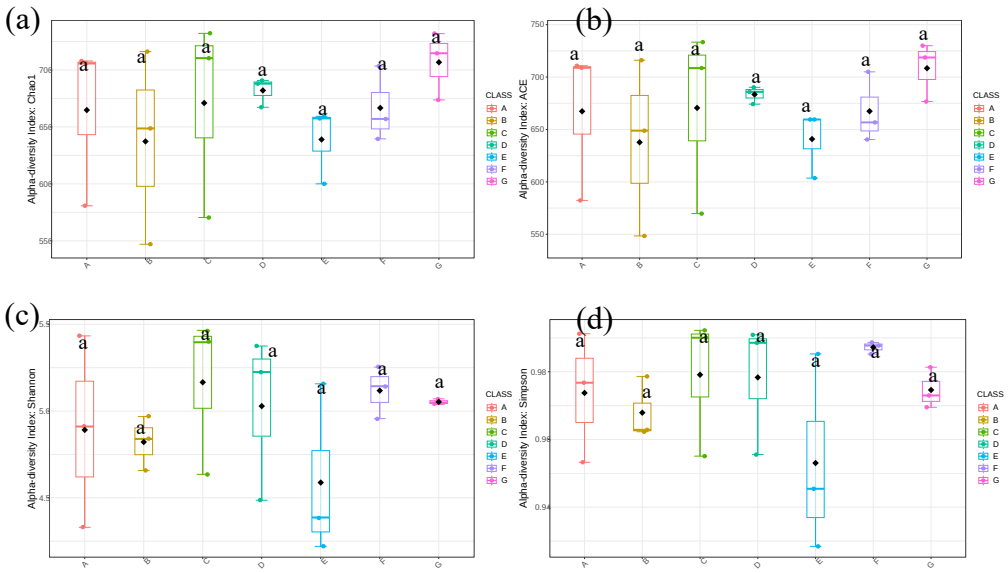

Figure 5.6 Alpha diversity index. (a) Chao1, (b) ACE, (c) Shannon, (d) Simpson diversity index for all rat groups. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture

5.3.4.2 Changes in taxa and functions

The relative abundance of gut microbiota detected in the fecal samples of all treatment groups had been plotted in Figure 5.7. Following preprocessing, about 2264 and 2123 amplicon sequencing variances (ASVs) of gut microbiota were detected at phylum and family level respectively. It is observed that the gut microbiota of rats were dominated by three phyla: Bacteroidota, Firmicutes, and Proteobacteria, collectively accounting for over 95% of the total abundance. This is a common observation in rodent gut microbiota studies, as these phyla are typically the most prevalent in mammalian gut microbiomes (Figure 5.7 a & Figure 5.8 a).

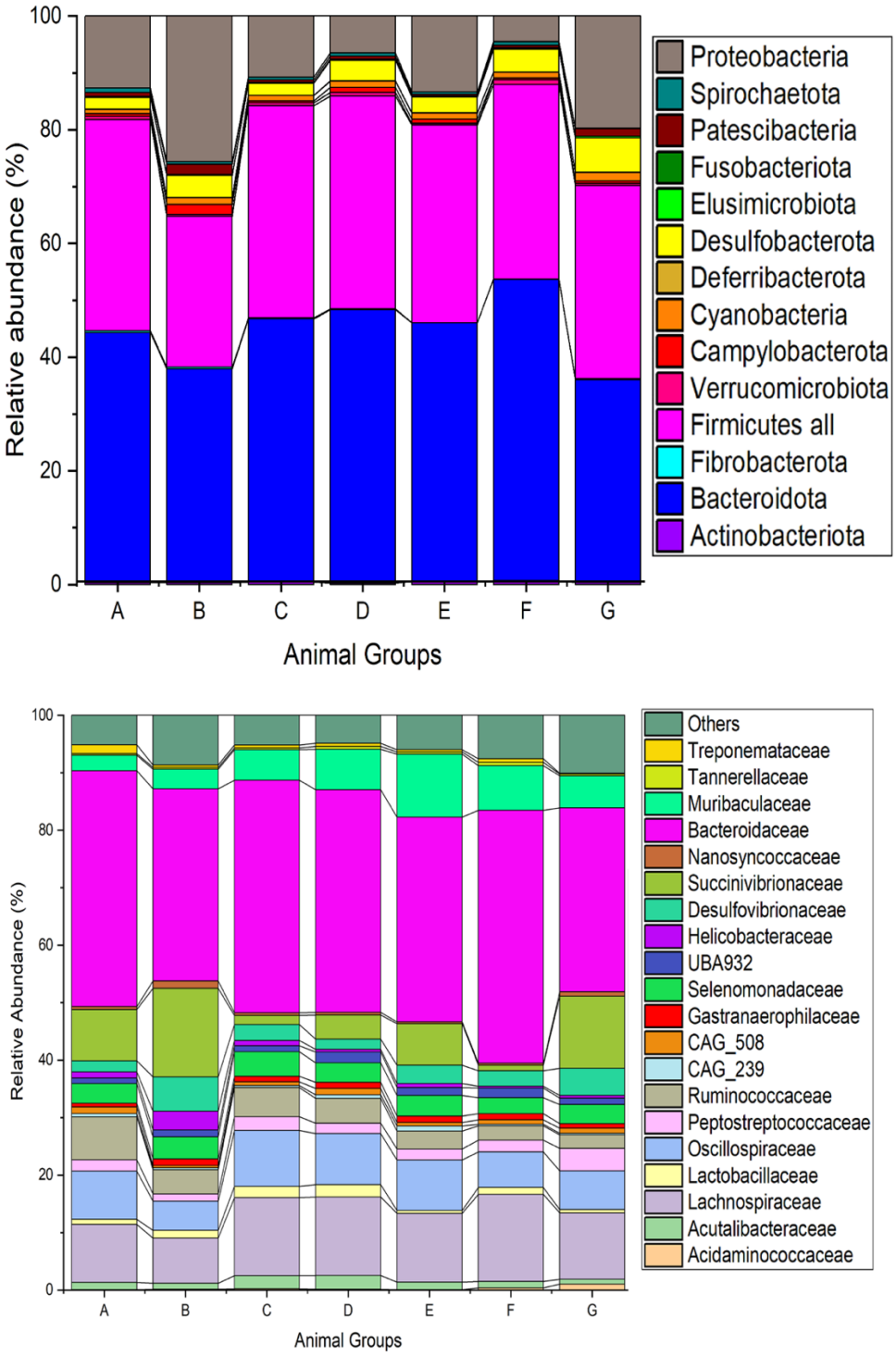

Figure 5.7 The stack bar graph on the relative abundance of gut microbiome in (a) phylum level and (b) family level. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture.

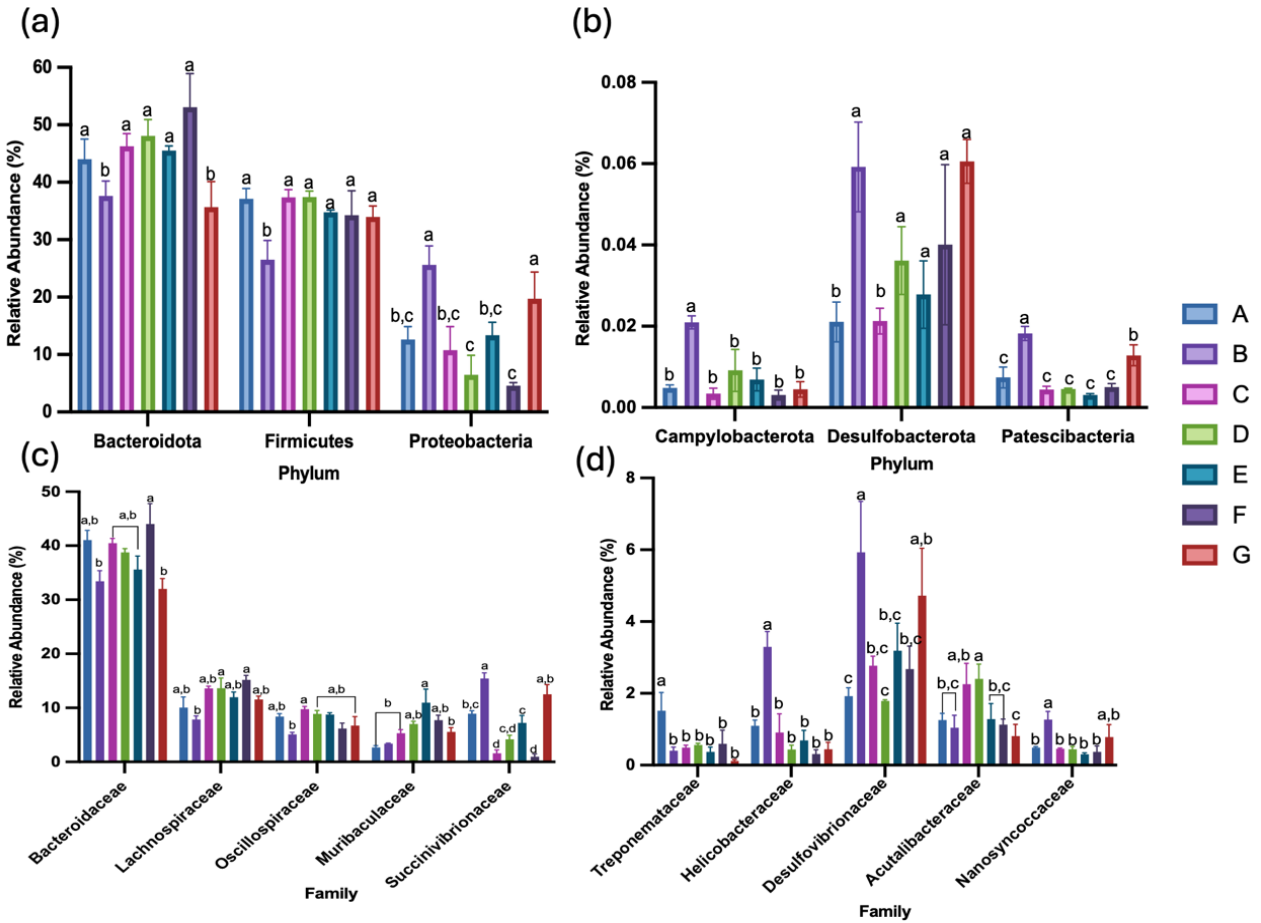

Figure 5.8 The relative abundance of gut microbiome in all rat groups. (a) major 3 phylum gut microbiome groups, (b) phylum microbiome groups that showed significantly different at P<0.05, (c) 5 major family gut microbiome groups, and (d) family microbiome groups that showed significantly different at P<0.05. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture.

Pesticide treatment rat group B and G demonstrated a significant elevation of harmful microbiota such as Proteobacteria, Desulfobacterota and Patescibacteria and showed significant decrease in the healthy Bacteroidota as compared to other rat groups (Figure 5.8 a&b). Proteobacteria, Desulfobacterota and Patescibacteria are all recognised as harmful microbiome causing negative impacts on the host health, correlated with the development of various diseases such as cardiovascular disorder, obesity, autoimmune disorder, inflammatory related disease such as inflammatory bowel disease (IBD) and metabolic disorders (Fakharian et al., 2023; Zhu et al., 2023). Besides that, there is a significant increase of Campylobacteria in pesticide treated group B which indirectly indicated that this rat group faced gut dysbiosis problem. This is because Campylobacteria is pathogenic bacteria that is greatly involved in the gut dysbiosis and inflammation response through the disruption of the gut mucosal barrier function (Huang et al., 2021).

A significant increment of Proteobacteria in pesticide feeding rat, might indicate that the rats undergoes gut dybiosis and inflammation activities (Rizzatti et al., 2017). Proteobacteria can trigger the production of lipopolysaccharide, which indirectly activated toll-like receptor 4 (TLR4), causing inflammation response (Shi et al., 2021; Vasques-Monteiro et al., 2021). Desulfobacteria are a group of sulfate-reducing bacteria being crucial in the sulfur metabolism in the presence of low abundance. High abundance of desulfobacteria can result in the raise of hydrogen sulfide level and production of harmful metabolites, which subsequently increase oxidative stress and induced inflammatory action that causing several disease such as IBD, ulcerative colitis and obesity (Li et al., 2022; Liu et al., 2021). Patescibacteria is little studied in detail, but the association of this phylum and other pathogenic bacteria had been reported with promoting role in inflammatory response and exacerbation of metabolic disorders (Kang et al., 2023). Campylobacteria is pathogenic bacteria that show great involvement in gut dysbiosis and inflammation response through the disruption of the gut mucosal barrier function (Huang et al., 2021).

It is interesting to observed that rat group E and F with low and middle dose PFC treatment showed no significant gut microbiota changes in other 5 phyla (except Desulfobacterota) as compared to rat group A and C (Figure 5.8 a&b). Meanwhile, PFC consumption at low and middle dose can also promote the growth of healthy gut

microbiome such as Bacteroidota and Firmicutes. These findings may suggest that low and middle dose of PFC may support gut microbiome recovery, helping to restore the growth of healthy microbiome in pesticide treated group to normal level. Contrarily, rat group fed with high dose PFC (G) suggested that there might create a negative shift of healthy gut microbiome may happen when the PFC was overdose.

Phyla Bacteroidota and Firmicute had been reported as healthy microbiome due to their functionality in the SCFAs production, immunomodulatory properties and gut barrier function (Al-Madboly et al., 2023). These two phylum contribute to a balanced microbiome that support inflammation regulation, energy balance and the overall metabolic health (Stojanov et al., 2020). The increment of Bacteroidota population modulate the digestive ability of dietary fibre into SCFAs (Hu et al., 2024). SCFAs are crucial in maintaining gut health and greatly influence the immune and metabolic function of the host (Parada Venegas et al., 2019).

In family level, the distribution in relative abundance of the top 10 abundance family had been plotted in Figure 5.7b. In the family level, Bacteroidaceae is the most predominantly family encounter about 32-44% of total abundance, followed by Lachnospiraceae with 7-15%, Murribaculaceae with 3-14% and Oscillospiraceae with 3-11%. Bacteroidaceae and Murribaculaceae belonged to phylum Bacteriodota whereas Lachnospiracea and Oscillospiraceae belongs to phylum Firmicute. The gut microbiota of control A and pesticide B groups showed significant different at the family of Succinivibrionaceae, Desulfovibrionaceae, Helicobacteraceae, Nanosyncoccaceae and Treponemataceae from the phylum Proteobacteria (Figure 5.8 c&d). Succinivibrionaceae, for example, are known to be involved in the production of short-chain fatty acids and fermentation of carbohydrates, which contributed to good gut health. However, their increased abundance in the context of pesticide exposure may suggest a shift towards a more pathogenic profile, potentially linked to inflammation and metabolic disturbances (Li, Y. et al., 2020). Similarly, Desulfovibrionaceae, which are associated with the production of endotoxins, have been implicated in inflammatory responses and obesity (Van Hecke et al., 2019). This up-regulation in pesticide-treated rats may exacerbate gut inflammation and contribute to systemic effects, including metabolic syndrome (Zhang, X. et al., 2021).

In family level, rat group E and F were further proven to show gut health benefit with the increasing in the healthy microbiota population from Bacteroidaceae, Lachnospiraceae and Murribaculaceae, while reduction in harmful microbiota from Succinivibrionaceae, Desulfovibrionaceae, Helicobacteraceae and Nanosyncoccaceae compared to B rats. Families like Bacteroidaceae, Lachnospiraceae and Murribaculaceae are known for their role in producing SCFAs, such as acetate, propionate and butyrate, which can promote gut health and improve the modulation of immune function (Zeng et al., 2017).

5.3.4.3 Discrimination analysis

Discrimination analysis of the fecal microbiome profile is conducted using PCA analysis and PLS-DA analysis. PCA and PLS-DA are statistical methodologies employed in gut microbiota analysis to streamline and elucidate intricate datasets, uncovering patterns and classifications associated with alterations in microbial communities (Figure 5.9). From PLS-DA, treatment group B and G formed distinct cluster at Q1&Q3, which showed close association among each other and having similar microbial composition (Figure 5.9 b&c). These two groups also showed negative correlation with other rat groups from Q2 and Q4 in the 2D-score scatter plot. treatment group C, D and F also demonstrated close association, which means they might share similar microbiota profiles. From Figure 5.9 c, it is observed that control A group distributed on the top cluster, which possess some difference of gut microbiome profile compared to all the treatment groups.

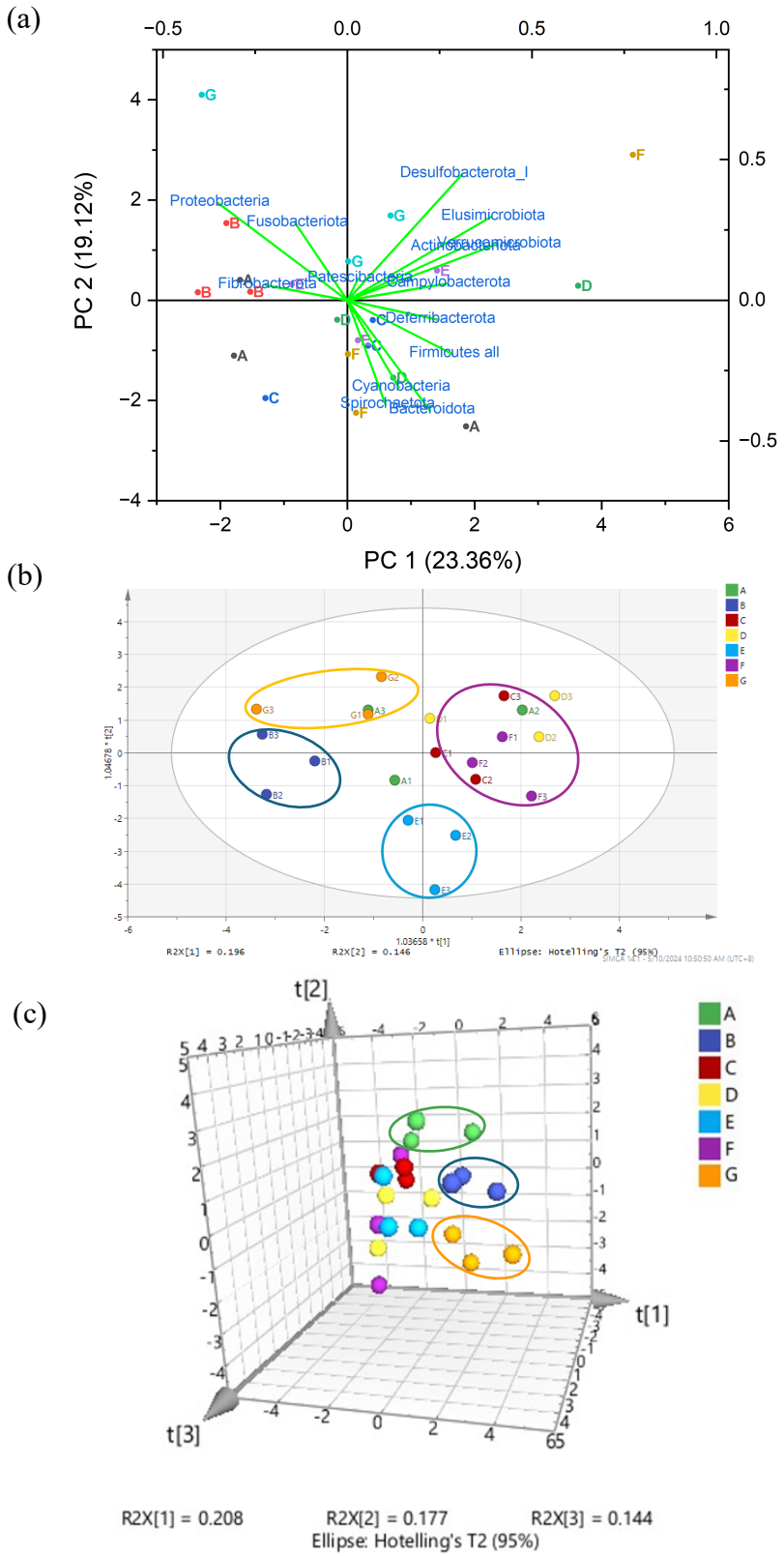

Figure 5.9 PCA and PLS-DA analysis. (a) PCA biplot for gut microbiota at phylum level, b) PLSDA 2D and C) PLSDA- 3D score plot. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture.

5.3.4.4 Correlation analysis

Clustering heatmap revealed the correlation distance and average linkage between the gut microbiomes. Treatment group B and G formed one relative cluster in both phylum and family clustering of gut microbiome compared to other treatment groups (Figure 5.10). These two treatment groups showed stronger correlations in the phylum such as Fibrobacteria, Fusobacteria, Cyanobacteria, Patescibacteria, and Proteobacteria and weaker correlation in the healthy microbiome like family Bacteriodetes, Lachnospiraceae and Oscillospiraceae and Lactobacillaceae belonging to the phylum Bacteroita and Firmicute (Figure 5.10a & 5.11b). Proteobacteria typically persisted at low concentration at normal healthy gut microbiome. However, an increase abundance of proteobacteria is often associated to gut dysbiosis and had been recognised as signature of disease (Rizzatti et al., 2017; Shin et al., 2015). A high level of Proteobacteria particularly from family Succinivibrionaceae, Desulfovibrionaceae, Helicobacteraceae and Nanosyncoccaceae can implicated for negative gut health. Contrarily, treatment groups A, C, D E and F formed another cluster, displaying positive correlation in the healthy microbiota from the phylum of Bacteroidota including family of Bacteroidaceae and Muribaculaceae and phylum Firmicute (Figure 5.10). Both Bacteriodota and Firmicute are considered as healthy gut microbiome, however they show different mechanism in the metabolic activity. Bacteroidetes produce various type of enzymes to metabolise soluble polysaccharides whereas Firmicute do also consists of many carbohydrate-digesting enzymes that is beneficial in gut function but is greatly limited by the structure of plant cell wall (Y. J. Zhang et al., 2018).

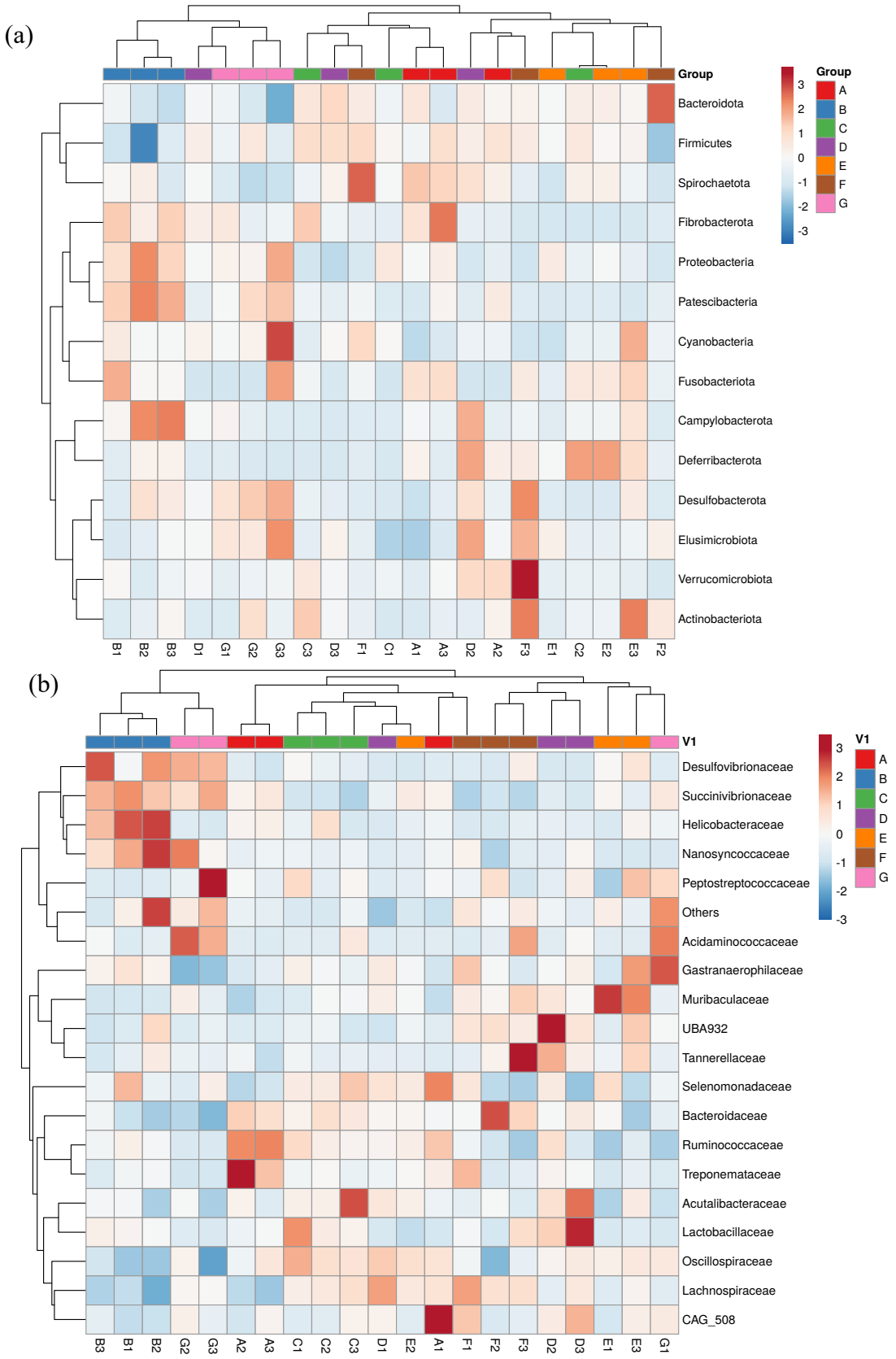

Figure 5.10. The clustering heatmap between rat groups with gut microbiome (a) at phylum level and (b) at family level. Both x-axis and y-axis of clustering heatmap are clustered using correlation distance and average linkage. Rat groups A=control, B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture.

The Pearson correlation analysis was used to examine symbiotic microbes at the phylum and family levels (Figure 5.11). Interestingly, certain phyla exhibited significant strong positive correlations, such as Verrucomicrobiota with Bacteroidota, Desulfobacterota with Cyanobacteria and Elusimicrobiota, and Proteobacteria with Patescibacteria. Conversely, significant negative correlations were observed between several phyla, including Proteobacteria with Bacteroidota, Firmicutes, and Verrucomicrobiota; Patescibacteria with Bacteroidota and Firmicutes; Spirocheatota with Cyanobacteria; and Fibrobacterota with Actinobacteriota.

In short, this study provided an overview on the association of gut microbiome with the treatment group. The correlation observed can reveal the specific microbial alterations upon receiving the pesticide or PFC treatment, in addition displaying co-occurrence patterns in complex microbial communities.

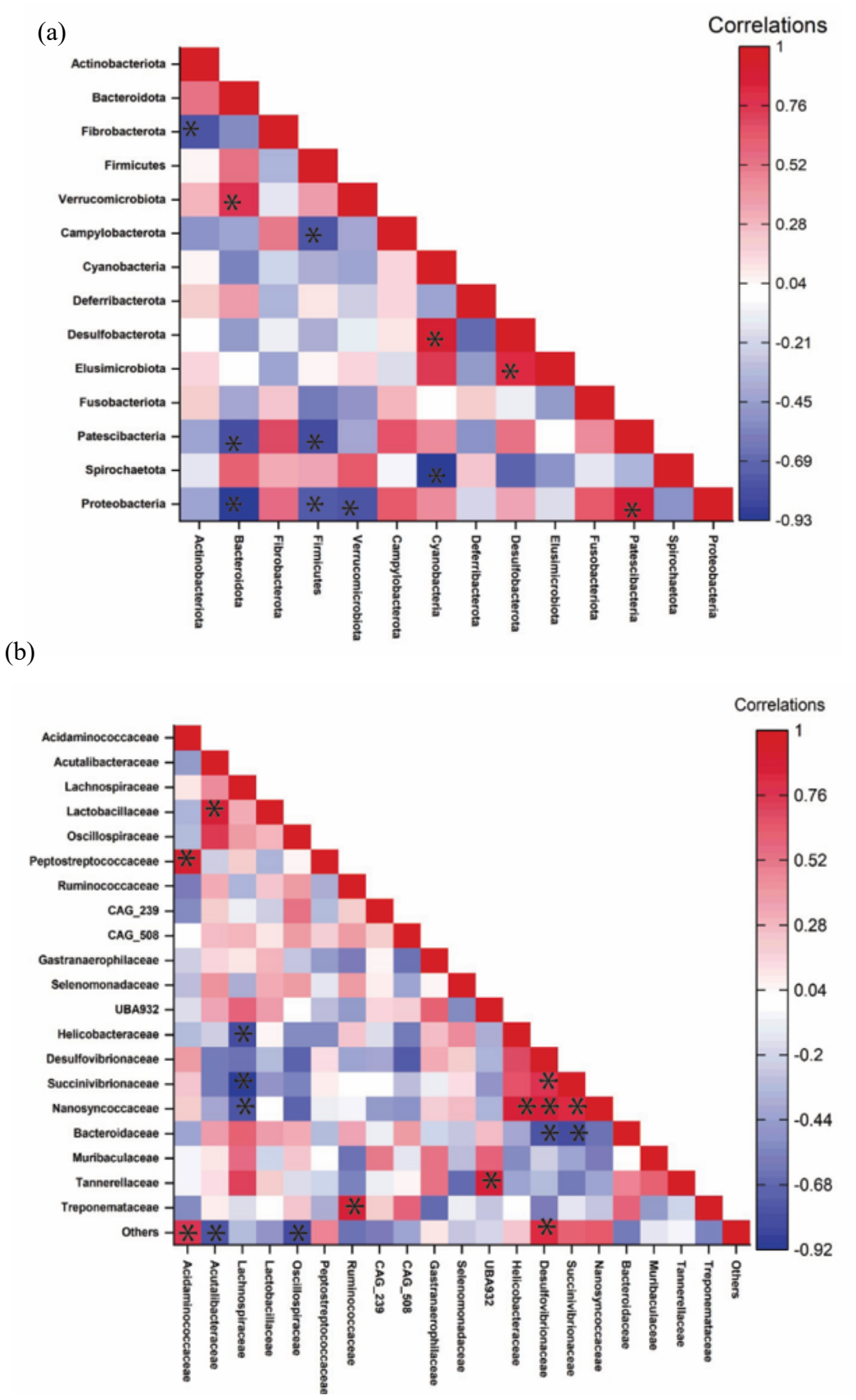

Figure 5.11 Correlation analysis presented in Pearson correlation at (a) phylum level and (b) family level. The symbol of * represent significant different at P<0.05.

5.3.5 Metabolic functional gene prediction

PICRUSt2 is an useful bioinformatics tool in the metagenomics analysis to predict the functional profiles of microbial communities based on marker gene data, typically from 16S rRNA gene sequences (Douglas et al., 2020). There are a total 2210 out of 7000 EC and 387 out of 530 pathways from KEGG database being predicted from the 16S rRNA sequencing dataset of all treatment groups. Through screening from the metabolic pathway and enzymes databases, there are several metabolic pathways and enzymes of SCFAs metabolism and amino acid metabolism showing significant differences among treatment groups under Duncan Post Hoc variance analysis had showed in the Figure 5.12.

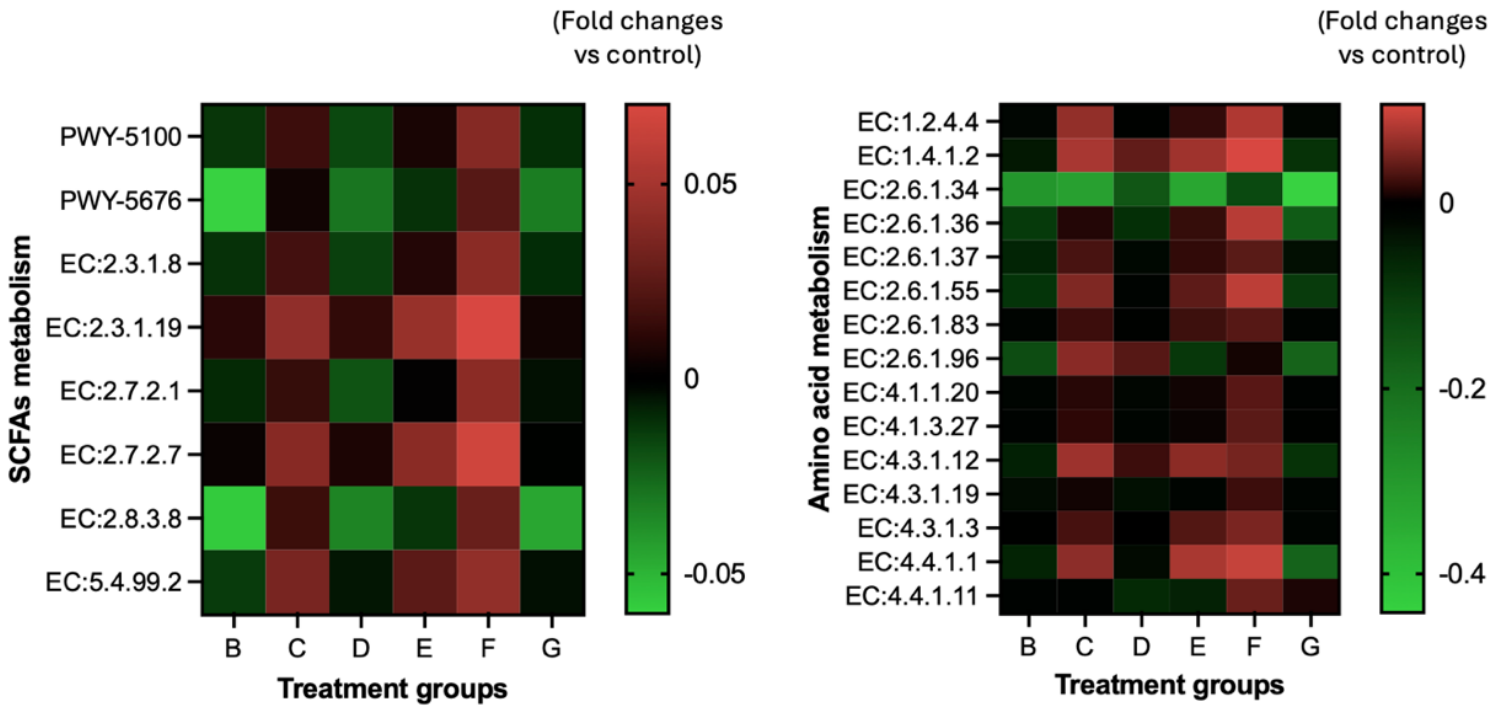

Figure 5.12 The heatmap comparison on the EC and pathways related to the SCFAs metabolism (a) and amino acid metabolsim (b). The significant difference under PosHoc analysis under Duncan equal variance test. Rat groups B=pesticide mixture, C=middle dose of PFC, D= probiotic and E-G= combination dose of low, middle, and high dose of PFC with pesticide mixture.

Notes: PWY5100 = pyruvate fermentation to acetate and lactate II, PWY-5676 = acetylene CoA fermentation to butanoate II, EC2.3.1.8 = phosphate acetyltransferase, EC2.3.1.19 = phosphate butyryltransferase, EC2.7.2.1 = acetate kinase, EC2.7.2.7 = Butyrate kinase, EC2.8.3.8 = acetate CoA-transferase, EC5.4.99.2 = methylmalonyl-CoA mutase. Amino acid metabolism like EC1.2.4.4 = 3-methyl-2-oxobutanoate dehydrogenase, EC1.4.1.2 = glutamate dehydrogenase, EC2.6.1.34 = UDP-N-acetylbacillosamine transaminase, EC2.6.1.36 = L-lysine 6-transaminase, EC2.6.1.37 = 2-aminoethylphosphonate-pyruvate transaminase, EC2.6.1.55 = taurine-2-oxoglutarate transaminase, EC2.6.1.83 = LL-diaminopimelate aminotransferase, EC2.6.1.96 = 4-aminobutyrate-pyruvate transaminase, EC4.1.1.20 = diaminopimelate decarboxylase,EC4.1.3.27 = anthranilate synthase, EC4.3.1.12 = ornithine cyclodeaminase, EC4.3.1.19 = threonine ammonia-lyase, EC4.3.1.2 = histidine ammonia-lyase, EC4.4.1.1 = cystathionine gamma-lyase and EC4.4.1.11 = methionine gamma-lyase)

As mentioned in the previous section, SCFAs play various roles in the gut health and disease through modulation of various metabolic pathways including gene transcription, immune system and specific receptors and enzymatic activities (Wu, Y et al., 2021). From Figure 5.12 a, treatment group C and F showed positive reads (red colour) of PWY-5100, PWY-5676, EC2.3.1.8, EC2.8.3.8, and EC2.7.2.1 whereas treatment group B and G showed negative reads (green colour) as compared to the control group across all SCFAs metabolism pathway and enzymes. An increase abundance of PWY-5100 showed an increase conversion of pyruvate into acetate and lactate level, which promote higher energy production (Caspi, 2007; Zhu, C. et al., 2023). The production of lactate support the growth beneficial bacteria within the gut, indirectly support the microbial diversity and thus enhance the gut health function (Luo, F. et al., 2024). Elevation of EC2.3.1.8 and EC2.7.2.1 worked together in the activation of the acetate production. EC2.3.1.8 increase conversion of acetyl-CoA to acetyl phosphate, which later act as precursor for acetate production while EC2.7.2.1 convert acetyl phosphate into acetate with ATP energy production (Zhang, B. et al., 2021). Acetate is one of the beneficial SCFAs that are crucial for maintaining gut health function with balanced gut microbiota (Nogal et al., 2021). Metabolic pathway PWY-5676 plays in roles to convert acetylene-CoA intermediates into butyrate, where EC 2.8.3.8 which facilitate the transformation of acetyl-CoA to butyrate precursors for butyrate production (Duncan et al., 2002). The findings of treatment group C and F further supported by the high positive reads (bright red) of EC2.3.1. 19 and EC 2.7.2.7 that acts as key enzyme in the butyrate production. Higher abundance of key enzymes for butyrate production implied for better good health as butyrate had been proven to stimulate anti-inflammatory activity, improve gut barrier integrity, and regulation of various metabolic activities such as energy metabolism, lipid metabolism, glucose homeostasis and appetite regulations (Donohoe et al., 2011; Hodgkinson et al., 2023; Zhang, D. et al., 2023).

Besides SCFAs metabolism, amino acid metabolism plays essential roles in maintaining good health. Various roles within amino acid metabolism, including protein synthesis, energy production, synthesis of bioactive compounds, synthesis of non-essential amino acid, antioxidant defence, and immune function (Dai et al., 2022; Demirci-Çekiç et al., 2022; Endicott et al., 2021; Kelly & Pearce, 2020). In addition, amino acid metabolism is crucial for the regulation of signalling pathway and gene

expression, which indirectly support gut health with production of SCFAs and regulation of gut microbial metabolism (Dai et al., 2022; Kelly & Pearce, 2020). From Figure 5.12b, the comparison of colour scheme between treatment groups had been observed. Taking treatment group C and F as target observed group, they showed positive correlation in almost all 15 ECs except EC2.6.1.34 (Figure 5.12 b). Notably, enzyme commission such as EC1.4.1.2, EC2.6.1.36, EC2.6.1.55, and EC4.4.1.1 of treatment group C and F showed strong positive correlation (bright red) towards control. EC4.4.1.1 represent Cystathionine Gamma-Lyase, which facilitates the breakdown of cystathionine into cysteine, ammonia, and α-ketobutyrate and contributed to the production of antioxidant agent glutathione (Cano-Galiano et al., 2021). EC1.4.1.2 is glutamate dehydrogenase plays which catalyst the conversion of glutamate to α-ketoglutarate and ammonia, while EC2.6.1.36 (L-Lysine 6-Transaminase) and EC2.6.1.55 (Taurine-2-Oxoglutarate Transaminase) involved in the lysine catabolism and taurine metabolism respectively (Fujii et al., 2000; Schomburg et al., 2007).

Other enzymes like EC2.6.1.83, EC4.1.1.20, EC4.1.3.27, EC4.3.1.19, and EC4.3.1.3 also play vital roles in the production of important amino acids. For instance, EC2.6.1.83 and EC4.1.1.20 promote lysine biosynthesis which is crucial for gut epithelial repair and immune function (Hasebe et al., 2023), whereas EC 4.1.2.27 synthesise anthranilate being important for tryptophan biosynthesis pathway that contribute to improved indole and serotonin production and involved in the anti-inflammatory activities (Gao et al., 2020; Naz et al., 2024). EC4.3.1.19 and EC4.3.1.3 are involved in the isoleucine production and histidine metabolism respectively, which benefit for microbial growth and gut function (Holeček, 2020; F. Zhang et al., 2022).

In summary, the predicted improvement in SCFA and amino acid metabolism provides fundamental insights, suggesting that treatment groups C and F exhibited enhanced metabolic activity, contributing to improved gut health and overall metabolic balance. SCFA metabolism serves as a key indicator of active microbial growth, energy production, and enhanced gut barrier integrity (Ma, J. et al., 2022). Meanwhile, amino acid metabolism supports metabolic function by modulating amino acid synthesis and utilization in various cellular processes. However, these predicted metabolic functions are rough estimations (Tomé, 2021). Validation through actual

protein expression studies and genome annotation is essential to confirm the functional metabolic pathways.

5.3.6 Correlation between the blood, ROS and gut microbiota

The correlation of all phylum group of gut microbiota with the 10 selected parameters from blood and ROS factors is shown in Figure 5.13. The microbiota displayed different degree of correlation across the 10 parameters. Cyanobacteria showed significant positive correlation with total creatinine level and negative correlation with total GSH level. Cyanobacteria can produce hepatotoxins called microcystins which induces oxidative stress. Intense oxidative stress can subsequently reduce the antioxidant defenses including the gluthathione molecules that serves as primary intracellular antioxidant agent, which responsible for the cellular protection (Cai et al., 2013; Okamura & Pennathur, 2015). Altyar et al. (2023) report similar findings from microcystins exposure had causing elevation in oxidative stress markers, followed by depletion of GSH levels. In addition, high production of microcystins from cyanobacteria can also give rise to kidney impairment, which exacerbates to the creatinine accumulation (Romeu et al., 2010). In short, microcystins toxins produced from cyanobacteria showed great impact on the elevation of oxidative stress and creatinine level, which in turn causing the reduction of antioxidant agent like GSH. This interaction highlighted the harmful effects of cyanobacterial toxins on renal function and emphasise the role of oxidative stress in the development and progression of kidney-related disorders.

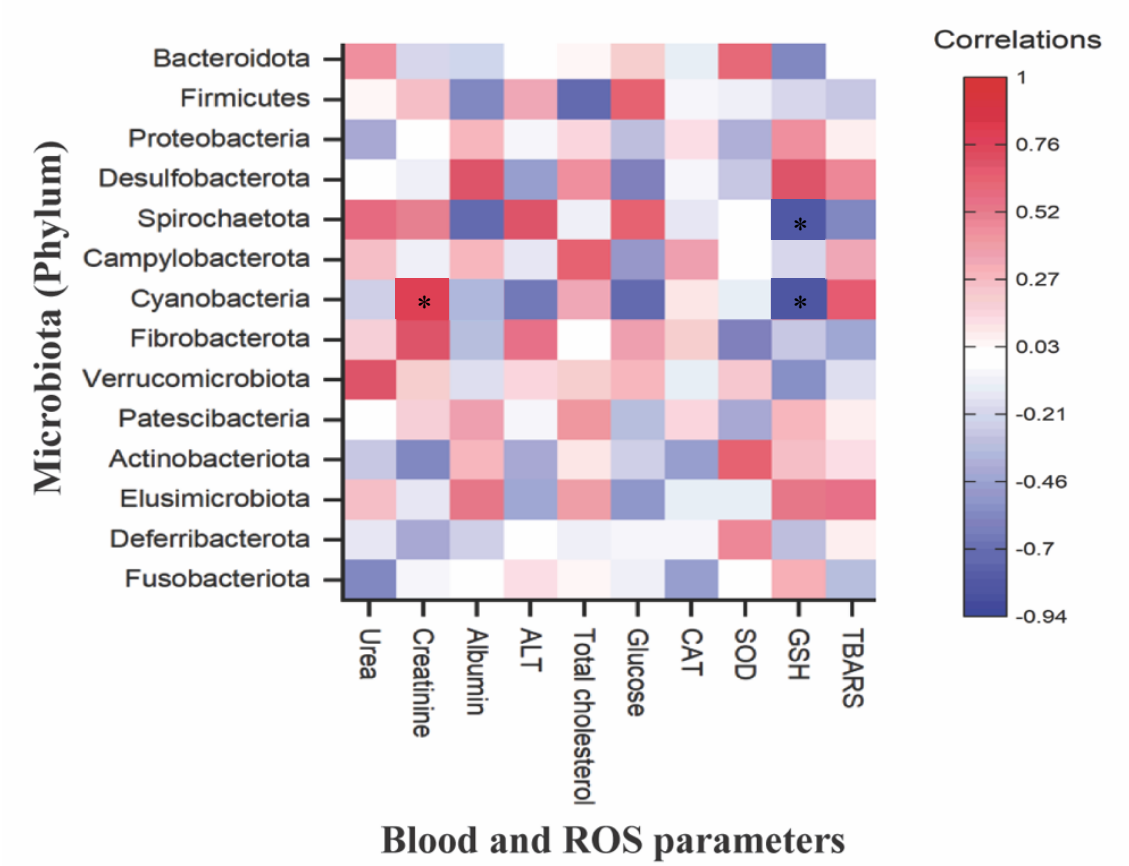

Figure 5.13. The correlation heatmap between gut microbiota in phylum level with the selected blood parameters and ROS factors. The * symbol represent the significant different at P<0.05

Besides Cyanobacteria, microbes from phylum Spirocheatota also demonstrate significant negative correlation with GSH level (Figure 5.13). The depletion of GSH might highly impact from the metabolic activities of Spirocheate bacteria. These bacteria groups often considered as harmful bacteria and implied for gut dysbiosis and infections (Thingholm et al., 2021). Immune and inflammatory response against infection can be triggered and serial cascade of inflammatory immune responses often resulted in increased ROS production, which later oxidise the available reduced form of GSH (Lauridsen, 2019; Liu et al., 2022). Furthermore, the oxidative stress caused by the elevated ROS can impair the activity of key enzymes involved in the synthesis and recycling of GSH, such as glutathione reductase and glutathione peroxidase, exacerbating the depletion of GSH (Kettle et al., 2014).

In summary, gut microbiome study offers key information about the regulation of physiological processes within the host, including metabolism activity, cell immune

response, induction of inflammatory-modulation factors. The imbalance of gut microbiome (dybiosis) is often used to influence the blood parameters and indirectly reflecting the progression or onset of certain diseases. The key motivation to examine the composition of the gut microbiome in this study provide a clear image of the impact from PFC and pesticide exposure on gut microbiome diversity. The gut microbiota profiling in this study provides basic insights on the associated microbial groups and associated cluster groups, but does not yet achieve an in-depth understanding of the origins and effects of physical and mental health disorders or the optimisation of treatment strategies. The functional prediction from 16s rRNA showed limitations and may require solid validation using whole-metagenome sequencing (WMS) or experimental studies for more precise insights.

5.4 Conclusion

This study highlights the potential of probiotic-fermented cabbage (PFC) as a dietary intervention to mitigate the adverse effects of pesticide exposure on gut microbiota, oxidative stress, and physiological health in Sprague Dawley rats. The PFC consumption, particularly at moderate doses, improves metabolic balance, reduces oxidative stress, and promotes the restoration of gut microbial composition disrupted by pesticide exposure. Enhanced production of short-chain fatty acids (SCFAs) and improved amino acid metabolism were observed, suggesting a positive impact on gut health and overall metabolic activity. Moreover, PFC reduce harmful gut microbiota populations, such as Proteobacteria and Desulfobacterota, while increasing beneficial microbes like Bacteroidota and Firmicutes. These changes were associated with improved physiological markers, including reduced total cholesterol levels and a partial restoration of renal and liver function under pesticide stress. However, the study also underscores the potential risks of high PFC doses, which may exacerbate microbial imbalances and physiological disturbances. Despite these promising results, the predicted functional pathways and enzymatic activities require further validation through protein expression studies and genome annotation. Additionally, the study's limited sample size and focus on fecal microbiota warrant cautious interpretation and encourage future research to explore these findings in

broader contexts, including human trials. In conclusion, this research provides valuable insights into the protective and restorative potential of PFC against pesticide-induced toxicity. It underscores the importance of incorporating functional foods in dietary strategies to promote gut health, reduce oxidative stress, and mitigate environmental health risks.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

In conclusion, this study has successfully addressed all three research objectives and validated the corresponding hypotheses. Firstly, the selected 8 probiotic strains were successfully characterized based on their growth behaviours, stress tolerance, and carbon metabolic adaptability. Notably, *Lactobacillus plantarum* and *Pediococcus pentosaceus* exhibited high resistance to gastric acid and bile salts, confirming their viability through gastrointestinal transit. High metabolic adaptability enhanced the overall efficacy of functional food. These findings support hypothesis 1**,** demonstrating that the selected strains possessed strong stress tolerance and metabolic capabilities suitable for functional food applications. Secondly, the fermentation process for probiotic-fermented cabbage (PFC) was optimized at 3% salt concentration, 25 °C, and 48 hours, yielding high levels of bioactive compounds. The resulting PFC extract showed enhanced antioxidant capacity and cytotoxicity effects against cancer cell models, although the IC_{50} of cytotoxicity MTT values remained above the threshold for potent anticancer claims. Therefore, the hypotheses are considered partially validated. Nevertheless, the improved bioactivity in PFC supports hypothesis 2**,** confirming that probiotic fermentation significantly enhanced cabbage's functional properties. Thirdly, *in-vivo* assessment demonstrated that PFC supplementation mitigated pesticide-induced physiological damage in rats, including reductions in blood cholesterol, glucose, and creatinine levels, as well as improvements in gut microbiota composition and oxidative stress resilience. These outcomes support hypothesis 3**,** verifying the protective and restorative effects of PFC against pesticide-induced disturbances.

To narrow the current research knowledge gap, future studies are recommended to focus on several key areas building upon the findings of this work.

Additional probiotic characterisation should be conducted, including pathogenicity, antibiotic resistance profiling, cell surface hydrophobicity as these analyses necessitate clinical safety evaluations and regulatory scrutiny before considering commercial application. Additionally, whole-genome sequencing and comparative genomics should be performed to better understand virulence factors and strain-specific probiotic properties. The demonstrated enhancement of bioactive compounds, particularly glucosinolate derivatives like sulforaphane—during optimized fermentation of probiotic-fermented cabbage (PFC) highlights its nutraceutical potential, yet further studies using advanced metabolomics and proteomics are required to elucidate the full spectrum of bioactive compounds and their stability in different formulations. A more comprehensive evaluation on the cytotoxicity properties of PFC against cancer cells is required to elucidate the mode and mechanism of cell death through advanced assays such as apoptotic gene regulation and activation as well as cell migration and invasion studies. *In-vivo* PFC consumption showed promising therapeutic effects in pesticide-exposed rat models, including microbiota modulation, SCFA enhancement, and metabolic improvements; however, the paradoxical effects observed at higher doses stress the need for dosage standardization. High variability within treatment groups due to limited sample sizes led to large standard errors, reducing statistical significance. Therefore, increasing biological replicates under ethical approval is recommended in the future analysis. Future work should incorporate long-term feeding trials, larger and more balanced animal groups, and more precise microbiota analysis techniques such as shotgun metagenomics. Additionally, mechanistic validation through transcriptomic and proteomic profiling will be essential to clarify the pathways by which PFC mediates detoxification and host protection. Expanding to human clinical trials and investigating delivery systems will further support the translation of PFC into safe and effective functional food products aimed at mitigating environmental toxin exposure and improving gut health.

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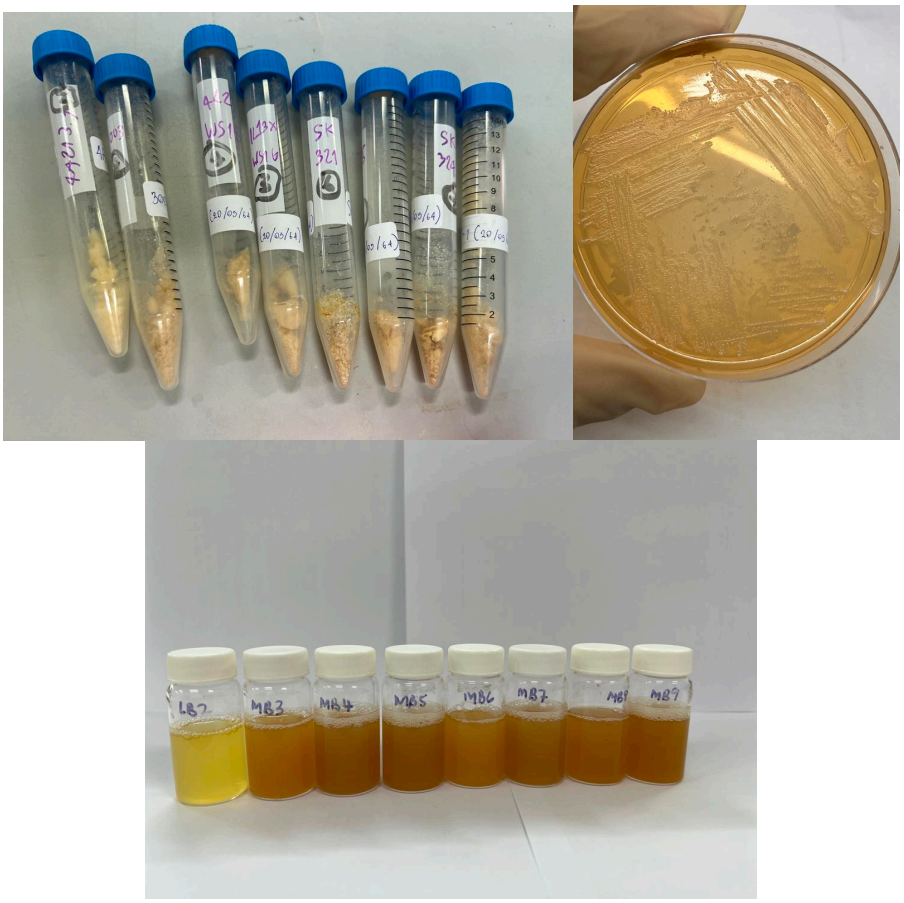
APPENDICES

Appendix 1

Bacterial Culture Stock, agar plate and liquid suspension



Appendix 1. Bacteria culture in (a) freeze dried source of probiotic strains, (b) streaking plate in MRS media agar and (c) liquid suspension culture of 8 selected probiotic strains.

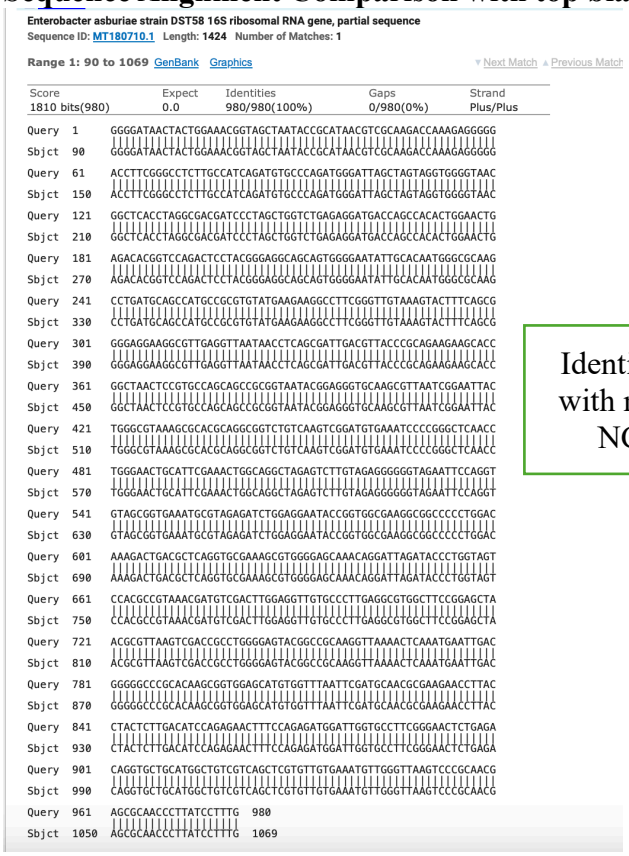
Appendix 2

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P1

(a) Top 10 Blast

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Enterobacter asburiae strain DST58 16S ribosomal RNA gene, partial sequence	Enterobacter asburiae	1810	1810	100%	0	100	1424	MT180710.1
Enterobacter cloacae strain AH1 16S ribosomal RNA gene, partial sequence	Enterobacter cloacae	1810	1810	100%	0	100	1436	MT613361.1
Enterobacter ludwigii strain AA1 16S ribosomal RNA gene, partial sequence	Enterobacter ludwigii	1810	1810	100%	0	100	1497	MT613360.1*
Enterobacter mori strain AS5 16S ribosomal RNA gene, partial sequence	Enterobacter mori	1810	1810	100%	0	100	1427	MT613379.1
Enterobacter roggenkampii CCI9 gene for 16S ribosomal RNA, partial sequence	Enterobacter roggenkampii	1810	1810	100%	0	100	1409	LC529204.1
Enterobacter sp. strain HK117 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1810	1810	100%	0	100	1250	MT581480.1
Enterobacter sp. strain HK110 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1810	1810	100%	0	100	1250	MT576966.1
Enterobacter sp. strain LSB16 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1810	1810	100%	0	100	1382	MK600532.1
Pantoea sp. strain HK111 16S ribosomal RNA gene, partial sequence	Pantoea sp.	1810	1810	100%	0	100	1250	MT576967.1
Pantoea sp. strain OsEp_A&N_15A15 16S ribosomal RNA gene, partial sequence	Pantoea sp.	1810	1810	100%	0	100	1400	MT367845.1

(b) Sequence Alignment Comparison with top blast MT180710.1



Identified species with max score in NCBI Blast

Appendix 3

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P2

(a) Top 10 Blast

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Lactococcus lactis strain CJNU 3001 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1604	1604	99%	0	100	1546	MN749817.1*
Lactococcus lactis strain MRKAK8 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1604	1604	99%	0	100	1490	OP218356.1
Lactococcus lactis subsp. lactis strain 37MoSuero 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1604	1604	99%	0	100	1466	JQ973599.1
Lactococcus lactis subsp. lactis strain M179 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1602	1602	99%	0	99.89	1386	MH669384.1
Lactococcus lactis subsp. lactis bv. diacetylactis strain SD96 chromosome, complete genome	Lactococcus lactis subsp. lactis bv. diacetylactis	1602	9612	99%	0	100	2416272	CP043523.1
Lactococcus lactis strain IL1403 chromosome	Lactococcus lactis	1602	9607	99%	0	100	2365672	CP033607.1
Lactococcus lactis strain IL6288 chromosome, complete genome	Lactococcus lactis	1602	9607	99%	0	100	2208218	CP033606.1
Lactococcus lactis subsp. lactis strain G423 chromosome, complete genome	Lactococcus lactis subsp. lactis	1602	8010	99%	0	100	2414663	CP024958.1
Lactococcus lactis subsp. lactis strain F44 chromosome, complete genome	Lactococcus lactis subsp. lactis	1602	8010	99%	0	100	2432058	CP024954.1
Lactococcus lactis subsp. lactis strain CBA3648 chromosome, complete genome	Lactococcus lactis subsp. lactis	1602	9612	99%	0	100	2484036	CP159484.1

(b) Sequence Alignment Comparison with the top BLAST MN7498.17

Lactococcus lactis strain CJNU 3001 16S ribosomal RNA gene, partial sequence

Sequence ID: MN749817.1 Length: 1546 Number of Matches: 1

Range 1: 26 to 893 GenBank Graphics ▼ Next Match ▲ Prev

Score	Expect	Identities	Gaps	Strand
1604 bits(868)	0.0	868/868(100%)	0/868(0%)	Plus/Minus

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Query  1    GCGGAGTGCTTATTGCGTTAGCTGCGATACAGAGAACTTATAGCTCCCTACATCTAGCAC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  893  GCGGAGTGCTTATTGCGTTAGCTGCGATACAGAGAACTTATAGCTCCCTACATCTAGCAC

Query  61   TCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  833  TCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGA

Query  121  GCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  773  GCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATC

Query  181  TACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTCTACCAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713  TACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTCTACCAG

Query  241  TTTCCAATGCATACAATGGTTGAGCCACTGCCTTTTACACCAGACTTAATAAACCACCTG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  653  TTTCCAATGCATACAATGGTTGAGCCACTGCCTTTTACACCAGACTTAATAAACCACCTG

Query  301  CGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  593  CGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTG

Query  361  CTGGCACGTAGTTAGCCGTCCCTTTCTGGGTAGTTACCGTCACTTGATGAGCTTTCCACT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  533  CTGGCACGTAGTTAGCCGTCCCTTTCTGGGTAGTTACCGTCACTTGATGAGCTTTCCACT

Query  421  CTCACCAACGTTCTTCTCTACCAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473  CTCACCAACGTTCTTCTCTACCAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACG

Query  481  CGGCGTTGCTCGGTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  413  CGGCGTTGCTCGGTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTA

Query  541  GGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCACCCTCTCAGGTCGGCTATGTA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  353  GGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCACCCTCTCAGGTCGGCTATGTA

Query  601  TCATCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATACAACGCGGGATCATCTTTG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  293  TCATCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATACAACGCGGGATCATCTTTG

Query  661  AGTGATGCAATTGCATCTTTCAAACTTAAAACTTGTGTTTAAAGTTTTTATGCGGTATTA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  233  AGTGATGCAATTGCATCTTTCAAACTTAAAACTTGTGTTTAAAGTTTTTATGCGGTATTA

Query  721  GCATTCGTTTCCAAATGTTGTCCCCCGCTCAAAGGCAGATTCCCCACGCGTTACTCACCC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  173  GCATTCGTTTCCAAATGTTGTCCCCCGCTCAAAGGCAGATTCCCCACGCGTTACTCACCC

Query  781  GTTCGCTGCTCATCCAGTCGGTACAAGTACCAACCTTCAGCGCTCAACTTGCATGTATTA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  113  GTTCGCTGCTCATCCAGTCGGTACAAGTACCAACCTTCAGCGCTCAACTTGCATGTATTA

Query  841  GGCACGCCGCCAGCGTTCGTCCTGAGCA  868
            ||||||||||||||||||||||||||||
Sbjct  53   GGCACGCCGCCAGCGTTCGTCCTGAGCA  26
```

Identified species with max score in NCBI Blast

Appendix 4

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P3

(a) Top 10 Blast Result

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Lactococcus lactis WS18 gene for 16S ribosomal RNA, partial sequence	Lactococcus lactis	1343	1343	100%	0	100	1073	LC336446.1*
Lactococcus lactis strain FPR1 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1084	PQ056995.1
Lactococcus sp. strain QZ-E29 16S ribosomal RNA gene, partial sequence	Lactococcus sp.	1332	1332	100%	0	99.72	1453	PQ047727.1
Lactococcus lactis subsp. lactis strain CP 6/3d 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1332	1332	100%	0	99.72	1349	PQ041287.1
Lactococcus lactis strain AN49 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1478	PP657545.1
Lactococcus lactis strain AN42 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1488	PP657544.1
Lactococcus lactis strain AN40 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1482	PP657542.1
Lactococcus lactis strain AN22 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1452	PP657537.1
Lactococcus lactis strain AN59 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1491	PP657536.1
Lactococcus lactis strain AN20 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1332	1332	100%	0	99.72	1480	PP657527.1

(b) Sequence Alignment Comparison with the top BLAST LC336446.1

Lactococcus lactis WS18 gene for 16S ribosomal RNA, partial sequence

Sequence ID: LC336446.1 Length: 1073 Number of Matches: 1

Range 1: 105 to 831 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1343 bits(727)	0.0	727/727(100%)	0/727(0%)	Plus/Plus

```
Query  1    ACCAAGGCGATGATACATAGCCGATCTGAGAGGGTGATCGGCCACGTTGGGACTGAGACA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  105  ACCAAGGCGATGATACATAGCCGATCTGAGAGGGTGATCGGCCACGTTGGGACTGAGACA  164

Query  61   CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165  CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGA  224

Query  121  CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225  CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAA  284

Query  181  GAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285  GAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCT  344

Query  241  AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345  AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGG  404

Query  301  CGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405  CGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTG  464

Query  361  TATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465  TATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGC  524

Query  421  GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  525  GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAA  584

Query  481  CTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  585  CTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG  644

Query  541  CCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  645  CCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCA  704

Query  601  ATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  705  ATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGG  764

Query  661  CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  765  CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT  824

Query  721  CTTGACA  727
            |||||||
Sbjct  825  CTTGACA  831
```

Sequence alignment in NCBI

Appendix 5

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P4

(a) Top 10 Blast Result

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Lactococcus lactis subsp. lactis gene for 16S rRNA, partial sequence, strain: YIT 2008 (= ATCC 19435)	Lactococcus lactis subsp. lactis	13 15	13 15	98 %	0	99. 72	15 40	AB008 215.1
Lactococcus lactis subsp. lactis strain L30 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	13 12	13 12	98 %	0	99. 58	14 44	OQ405 759.1
Uncultured bacterium clone nck203g11c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	13 12	13 12	98 %	0	99. 58	13 68	KF095 145.1
Lactococcus lactis subsp. lactis strain CNM439_12 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	13 12	13 12	98 %	0	99. 58	15 19	KC699 176.1
Lactococcus lactis subsp. lactis strain CNM381_12 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	13 12	13 12	98 %	0	99. 58	14 26	KC699 133.1
Lactococcus lactis strain FPR1 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	13 10	13 10	98 %	0	99. 58	10 84	PQ056 995.1
Lactococcus sp. strain QZ-E29 16S ribosomal RNA gene, partial sequence	Lactococcus sp.	13 10	13 10	98 %	0	99. 58	14 53	PQ047 727.1
Lactococcus lactis subsp. lactis strain CP 6/3d 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	13 10	13 10	98 %	0	99. 58	13 49	PQ041 287.1
Lactococcus lactis strain AN49 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	13 10	13 10	98 %	0	99. 58	14 78	PP6575 45.1
Lactococcus lactis strain AN42 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	13 10	13 10	98 %	0	99. 58	14 88	PP6575 44.1

(b)Sequence Alignment Comparison with the top BLAST AB008215.1

Lactococcus lactis subsp. lactis gene for 16S rRNA, partial sequence, strain: YIT 2008 (= ATCC 19435)

Sequence ID: AB008215.1 Length: 1540 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 274 to 992 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1315 bits(712)	0.0	717/719(99%)	1/719(0%)	Plus/Plus

```
Query  6    GGCGATGATACATAGCCG-CATGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCC  64
            ||||||||||||||||| | ||||||||||||||||||||||||||||||||||||||
Sbjct  274  GGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCC  333

Query  65   CAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAG  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  334  CAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAG  393

Query  125  CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACG  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACG  453

Query  185  TTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTA  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454  TTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTA  513

Query  245  CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAA  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  514  CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAA  573

Query  305  AGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGC  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  574  AGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGC  633

Query  365  ATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGA  424
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  634  ATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGA  693

Query  425  AATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGAC  484
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  694  AATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGAC  753

Query  485  ACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA  544
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  754  ACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA  813

Query  545  AACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAG  604
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  814  AACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAG  873

Query  605  CACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGC  664
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  874  CACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGC  933

Query  665  ACAAGCGGTGGAGCATGTGGTTTAATTCGAAACAACGCGAAGAACCTTACCAGGTCTTG  723
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  934  ACAAGCGGTGGAGCATGTGGTTTAATTCGAAACAACGCGAAGAACCTTACCAGGTCTTG  992
```

Sequence alignment in NCBI

Appendix 6

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P5

(a) Top 10 Blast

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Lactiplantibacillus plantarum strain BR2-12 chromosome, complete genome	Lactiplantibacillus plantarum	1094	5466	100%	0	100	3218310	CP162015.1
Lactiplantibacillus plantarum strain AUSA004 chromosome, complete genome	Lactiplantibacillus plantarum	1094	5471	100%	0	100	3197748	CP160637.1
Lactiplantibacillus plantarum strain AUSA002 chromosome, complete genome	Lactiplantibacillus plantarum	1094	5460	100%	0	100	3085076	CP160858.1
Lactiplantibacillus plantarum strain V2546 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	1094	1094	100%	0	100	1424	PQ013786.1
Lactiplantibacillus pentosus strain PCZ4 chromosome, complete genome	Lactiplantibacillus pentosus	1094	5471	100%	0	100	3578347	CP162863.1
Lactiplantibacillus sp. strain FRL11862 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus sp.	1094	1094	100%	0	100	1458	PQ034770.1
Lactiplantibacillus sp. strain FRL11861 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus sp.	1094	1094	100%	0	100	1469	PQ034769.1
Lactiplantibacillus sp. strain FRL11852 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus sp.	1094	1094	100%	0	100	1460	PQ034765.1
Lactiplantibacillus sp. strain FRL11846 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus sp.	1094	1094	100%	0	100	1464	PQ034763.1
Lactiplantibacillus sp. strain FRL11844 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus sp.	1094	1094	100%	0	100	1415	PQ034761.1

(b) Sequence Alignment Comparison with the top BLAST CP162015.1

Lactiplantibacillus plantarum strain BR2-12 chromosome, complete genome

Sequence ID: CP162015.1 Length: 3218310 Number of Matches: 5

Range 1: 427624 to 428215 GenBank Graphics ▼Next Match

Score	Expect	Identities	Gaps	Strand
1094 bits(592)	0.0	592/592(100%)	0/592(0%)	Plus/Plus

```
Query  1       TTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGC
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427624  TTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGC

Query  61      AGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427684  AGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA

Query  121     TCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427744  TCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTA

Query  181     ACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427804  ACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG

Query  241     GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427864  GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGT

Query  301     GGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427924  GGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC

Query  361     TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427984  TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCG

Query  421     ACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAAC
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428044  ACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAAC

Query  481     CGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428104  CGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT

Query  541     CCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAA  592
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428164  CCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAA  42821
```

Sequence alignment in NCBI

Appendix 7

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P6

(a) Top 10 blast

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Limosilactobacillus fermentum strain SCB0035 chromosome, complete genome	Limosilactobacillus fermentum	1197	5988	100%	0	100	2016236	CP094655.1
Limosilactobacillus fermentum strain M4 chromosome, complete genome	Limosilactobacillus fermentum	1197	5988	100%	0	100	2032186	CP089305.1
Limosilactobacillus fermentum strain 858 chromosome	Limosilactobacillus fermentum	1197	5988	100%	0	100	2181798	CP082946.1
Limosilactobacillus fermentum strain A51 chromosome, complete genome	Limosilactobacillus fermentum	1197	5988	100%	0	100	2188538	CP132541.1
Limosilactobacillus fermentum strain JL-1 chromosome, complete genome	Limosilactobacillus fermentum	1197	5988	100%	0	100	1982264	CP139052.1
Limosilactobacillus fermentum strain JF9 chromosome, complete genome	Limosilactobacillus fermentum	1197	5988	100%	0	100	2154733	CP157980.1
Limosilactobacillus fermentum strain FOSU-YHD19 chromosome, complete genome	Limosilactobacillus fermentum	1197	5988	100%	0	100	2165664	CP160833.1
Limosilactobacillus fermentum strain KUFM408 chromosome, complete genome	Limosilactobacillus fermentum	1197	5983	100%	0	100	2077616	CP119425.1
Limosilactobacillus fermentum strain KUFM423 chromosome, complete genome	Limosilactobacillus fermentum	1197	5983	100%	0	100	2077616	CP119406.1
Limosilactobacillus fermentum strain EFEL6800 chromosome, complete genome	Limosilactobacillus fermentum	1197	5983	100%	0	100	2068538	CP124737.1

(b) Sequence Alignment Comparison with the top BLAST CP094655.1

Limosilactobacillus fermentum strain SCB0035 chromosome, complete genome

Sequence ID: CP094655.1 Length: 2016236 Number of Matches: 5

Range 1: 161505 to 162152 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1197 bits(648)	0.0	648/648(100%)	0/648(0%)	Plus/Plus

```
Query  1       TCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAA  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161505  TCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAA  161564

Query  61      GGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161565  GGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT  161624

Query  121     CGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGCCAACCCTAGAGATAGGG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161625  CGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGCCAACCCTAGAGATAGGG  16

Query  181     CGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAG  24
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161685  CGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAG  16

Query  241     ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTG  30
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161745  ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTG  16

Query  301     GGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCAT  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161805  GGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCAT  161864

Query  361     CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAA  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161865  CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAA  161924

Query  421     CTCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCGGACTGCAGGCTGCAACTC  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161925  CTCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCGGACTGCAGGCTGCAACTC  161984

Query  481     GCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC  540
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161985  GCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC  162044

Query  541     CCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGG  600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162045  CCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGG  162104

Query  601     GGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGACAGATGATTAGGG  648
               ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162105  GGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGACAGATGATTAGGG  162152
```

Identified species with max score in NCBI Blast

(c)

Appendix 8

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P7

(a) Top 10 Blast

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Levilactobacillus brevis strain A156 chromosome, complete genome	Levilactobacillus brevis	1539	96	100%	0	100	2305485	CP142716.1
Levilactobacillus brevis strain KG Sourdough 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1402	PP152323.1
Levilactobacillus brevis strain NGRI03 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1599	PP037932.1
Levilactobacillus brevis strain TF13 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1462	OR857375.1
Levilactobacillus brevis strain TF03 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1443	OR856558.1
Levilactobacillus brevis strain PC-2 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1463	OR793163.1
Levilactobacillus brevis strain V2138 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1407	OR755016.1
Levilactobacillus brevis strain NWAFU 1652 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1434	OR554152.1
Levilactobacillus brevis strain HBUR51322 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1469	OR502342.1
Levilactobacillus brevis strain HBUR51314 16S ribosomal RNA gene, partial sequence	Levilactobacillus brevis	1539	1539	100%	0	100	1496	OR502338.1

(b) Sequence Alignment Comparison with the top BLAST CP142716.1

Levilactobacillus brevis strain A156 chromosome, complete genome

Sequence ID: CP142716.1 Length: **2305485** Number of Matches: **5**

Range 1: 87717 to 88549 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1539 bits(833)	0.0	833/833(100%)	0/833(0%)	Plus/Plus

```
Query  1      AAGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAATCCGCATGG  60
Sbjct  87717  AAGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAATCCGCATGG  87776

Query  61     ATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATCCCGCGGCGTATTAGTTA  120
Sbjct  87777  ATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATCCCGCGGCGTATTAGTTA  87836

Query  121    GTTGGTGAGGTAAAGGCCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATCGG  180
Sbjct  87837  GTTGGTGAGGTAAAGGCCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATCGG  87896

Query  181    CCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTT
Sbjct  87897  CCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTT

Query  241    ACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCG
Sbjct  87957  ACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCG

Query  301    AAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTAT
Sbjct  88017  AAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTAT

Query  361    AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG  420
Sbjct  88077  AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG  88136

Query  421    TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAG  480
Sbjct  88137  TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAG  88196

Query  481    CCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACA  540
Sbjct  88197  CCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACA  88256

Query  541    GTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAG  600
Sbjct  88257  GTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAG  88316

Query  601    GCGGCTGTCTAGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTA  660
Sbjct  88317  GCGGCTGTCTAGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTA  88376

Query  661    GATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCT  720
Sbjct  88377  GATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCT  88436

Query  721    TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAA  780
Sbjct  88437  TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAA  88496

Query  781    CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC  833
Sbjct  88497  CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC  88549
```

Identified species with max score in NCBI Blast

Appendix 9

DNA identification in NCBI BLAST with Top 10 Blast result for probiotic P8

(a) Top 10 Blast

Description	Scientific Name	MS	TS	QC	EV	PI	AL	AC
Pediococcus pentosaceus strain LA0061 chromosome, complete genome	Pediococcus pentosaceus	793	3966	100%	0	99.77	1847864	CP137627.1
Pediococcus pentosaceus strain RNP2058 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	677	OR811222.1
Pediococcus pentosaceus strain JN18 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1488	KT719234.1
Pediococcus pentosaceus strain JN3 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1488	KT719233.1
Pediococcus pentosaceus strain JN1 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1489	KT719232.1
Pediococcus pentosaceus strain JT3 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1487	KT719224.1
Pediococcus pentosaceus strain WiKim20 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1499	KX890131.1
Pediococcus pentosaceus strain IOPBL-4 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1437	KU844108.1
Pediococcus pentosaceus strain DSM 20336 T 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	793	793	100%	0	99.77	1521	KX886792.1
Pediococcus sp. UN32 partial 16S rRNA gene, isolate UN32	Pediococcus sp. UN32	793	793	100%	0	99.77	1466	LT604461.1

(b) Sequence Alignment Comparison with the top BLAST CP137627.1

Pediococcus pentosaceus strain LA0061 chromosome, complete genome

Sequence ID: CP137627.1 Length: **1847864** Number of Matches: **5**

Range 1: 117458 to 117889 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
793 bits(429)	0.0	431/432(99%)	0/432(0%)	Plus/Plus

```
Query  1       AAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGG  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117458  AAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGG  117517

Query  61      CGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117518  CGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAG  117577

Query  121     AGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117578  AGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA  117637

Query  181     GGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGT  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117638  GGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGT  117697

Query  241     TTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCCG  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117698  TTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAG  117757

Query  301     TGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117758  TGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA  117817

Query  361     GGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTC  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117818  GGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTC  117877

Query  421     TAATGTGAAAGC  432
               ||||||||||||
Sbjct  117878  TAATGTGAAAGC  117889
```

Identified species with max score in NCBI Blast

Appendix 10

Statistical analysis for Pearson correlation of Carbon Substrate Utilisation

Correlations

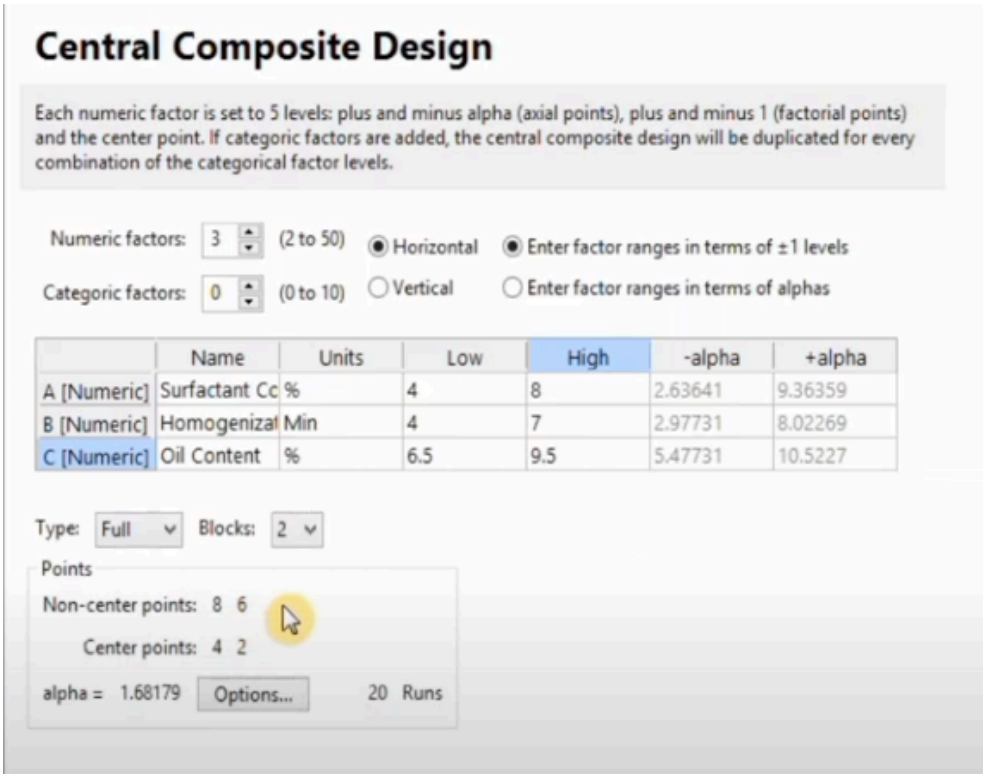
		P1	P2	P3	P4	P5	P6	P7	P8
P1	Pearson Correlation	1	.675**	.672**	.702**	.497**	.476**	.672**	.531**
	Sig. (2-tailed)		<.001	<.001	<.001	<.001	<.001	<.001	<.001
	N	93	93	93	93	93	93	93	93
P2	Pearson Correlation	.675**	1	.916**	.949**	.533**	.631**	.917**	.696**
	Sig. (2-tailed)	<.001		<.001	<.001	<.001	<.001	<.001	<.001
	N	93	93	93	93	93	93	93	93
P3	Pearson Correlation	.672**	.916**	1	.932**	.580**	.713**	.939**	.761**
	Sig. (2-tailed)	<.001	<.001		<.001	<.001	<.001	<.001	<.001
	N	93	93	93	93	93	93	93	93
P4	Pearson Correlation	.702**	.949**	.932**	1	.544**	.686**	.897**	.724**
	Sig. (2-tailed)	<.001	<.001	<.001		<.001	<.001	<.001	<.001
	N	93	93	93	93	93	93	93	93
P5	Pearson Correlation	.497**	.533**	.580**	.544**	1	.446**	.569**	.520**
	Sig. (2-tailed)	<.001	<.001	<.001	<.001		<.001	<.001	<.001
	N	93	93	93	93	93	93	93	93
P6	Pearson Correlation	.476**	.631**	.713**	.686**	.446**	1	.618**	.612**
	Sig. (2-tailed)	<.001	<.001	<.001	<.001	<.001		<.001	<.001
	N	93	93	93	93	93	93	93	93
P7	Pearson Correlation	.672**	.917**	.939**	.897**	.569**	.618**	1	.756**
	Sig. (2-tailed)	<.001	<.001	<.001	<.001	<.001	<.001		<.001
	N	93	93	93	93	93	93	93	93
P8	Pearson Correlation	.531**	.696**	.761**	.724**	.520**	.612**	.756**	1
	Sig. (2-tailed)	<.001	<.001	<.001	<.001	<.001	<.001	<.001	
	N	93	93	93	93	93	93	93	93

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

Appendix 11

Step by step guideline for Design Expert software

1. Select 'NEW DESIGN'
2. Select Response Surface Methodology
3. Select NUMERIC FACTORS – 2 mean 2 parameter such as pH and temp
4. Select Categoric Factors – usually 0
5. Fill the content for numeric factors

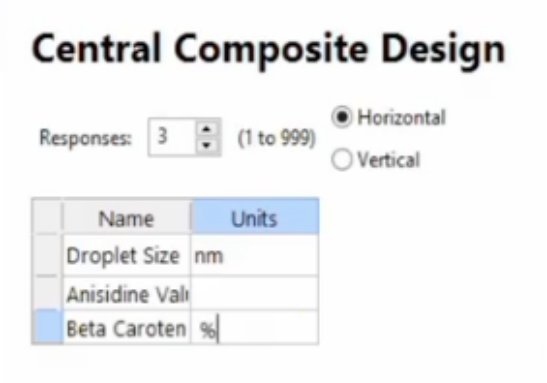


Example writing format

Table 1
Independent variables and their corresponding levels for β- Carotene nanoemulsion.

Independent variable	Symbol	Coded levels				
		−α	−1	0	+1	+α
Surfactant Concentration (%)	X_1	2.64	4	6	8	9.36
Homogenization Time (min)	X_2	2.98	4	5.5	7	8.02
Oil Content (%)	X_3	5.48	6.5	8	9.5	10.52

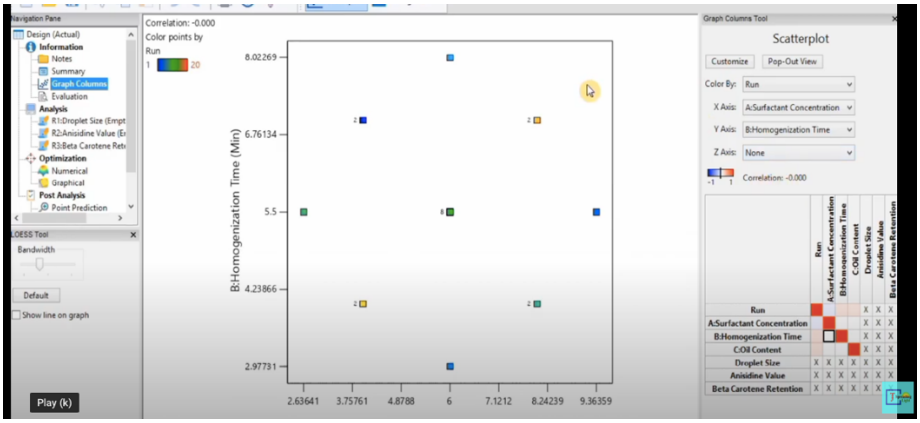
6. Select Type as FULL
7. Select BLOCKs as 1 (most of the time) , then press NEXT
8. Fill up the infor of response with respective unit , then press NEXT



9. Fill out the result of the response

Std	Run	Space Type	Factor 1 A:Surfactant Con... %	Factor 2 B:Homogenizati... Min	Factor 3 C:Oil Content %	Response 1 Droplet Size nm	Response 2 Anisidine Value	Response 3 Beta Carotene R... %
1	6	Factorial	4	4	6.5			
2	2	Factorial	8	4	6.5			
3	13	Factorial	4	7	6.5			
4	12	Factorial	8	7	6.5			
5	16	Factorial	4	4	9.5			
6	8	Factorial	8	4	9.5			
7	1	Factorial	4	7	9.5			
8	17	Factorial	8	7	9.5			
9	9	Axial	2.63641	5.5	8			
10	3	Axial	9.36359	5.5	8			
11	4	Axial	6	2.97731	8			
12	5	Axial	6	8.02269	8			
13	18	Axial	6	5.5	5.47731			
14	20	Axial	6	5.5	10.5227			
15	11	Center	6	5.5	8			
16	14	Center	6	5.5	8			
17	19	Center	6	5.5	8			
18	7	Center	6	5.5	8			
19	15	Center	6	5.5	8			
20	10	Center	6	5.5	8			

10. Save files as DESIGN FILE (.dxpx)
11. Can open file and continue keep in results
12. Then click graph colomn to view the graph, then check on the right box on the correlation, can change x-axis and y-axis on different parameter to see the correlation



13. Analysis of each response – Pls learn from this link: https://www.youtube.com/watch?v=CjnNzv8HlQs&list=PLd8ec3CmFk7394Xo3c4L7v9QcrG9Je6P4&index=3

Appendix 12

Cell culture media and stock solvent preparation

1. **Preparation of Phosphate Buffer Saline** (pH 7)(0.1M)

Refer https://www.aatbio.com/resources/buffer-preparations-and-recipes/phosphate-buffer-ph-5-8-to-7-4

Table 1. Required components

Component	Amount	Concentration
$Na_2HPO_4 \cdot 7H_2O$ (mw: 268.07 g/mol)	15.487 g	0.0578 M
$NaH_2PO_4 \cdot H_2O$ (mw: 137.99 g/mol)	5.827 g	0.0422 M

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 15.487 g of $Na_2HPO_4 \cdot 7H_2O$ to the solution.
3. Add 5.827 g of $NaH_2PO_4 \cdot H_2O$ to the solution.
4. Adjust solution to final desired pH using HCl or NaOH
5. Add distilled water until volume is 1 L.

Method 2: FSSM protocols in animal cell culture lab)

1. **Preparation of PBS (pH7,0.1M)**

Chemical	Weight(g)
NaCl	8
KCl	0.04
KH_2PO_4	0.2
Na_2HPO_4	2.16
Deionised water	1L

Chemical	Weight(g)
NaCl	9.5
KH_2PO_4	1.1
K_2HPO_4	3.7
Deionised water	1L

2. **Preparation of Trypsin-EDTA (100ml)**

Chemical	Weight(g)
NaCl	0.8
KCl	0.04

NaHCO3	0.035
D-glucose	0.1
EDTA	0.02
Trypsin	0.05

- Sterile filtered instead autoclave
- Can't be autoclaved

3. Freezing media (10mL)

-About 9.5 ml serum (Fetal Bovine Serum) + 0.5ml DMSO for 10ml stock

-Add only 1ml freeze media into cryovial.

Appendix 13

Animal cell culture technique: thawing, sub-culture and freeze cell for cryopreservation

List of items for Autoclave

1. Deionised water
2. Aspiration pump filter set and adapter x2
3. Cellulose acetate filter
4. Waste bottle x2
5. PBS (pH7.2-7.4)

Sterile Filter ITEMS

1. Trypsin EDTA
2. Freezing media
3. Pen-strep
4. FBS

Preparation of RPMI media

1. RPMI powder +NaHCO3 + deionised water for 1L stock
2. The stock is filtered with aspiration pump filter + cellulose acetate filter
3. Need to syringe filter (0.2um size) for both Pen- strep and FBS solution prior adding it to the media stock
   (Mixing ratio is 1L media +100ml FBS and 10ml Pen-strep)
4. Gentle shake until homogenize
5. The filtered stock is separately kept in 500ml blue cap bottle + 10 (50ml) centrifuge tube to prevent contamination.
6. Then parafilm all the stock bottles.

Revive Cell (Thawing)

1. Remove the vial from liquid N_2 tank and hold the cells at -80˚C refrigerator for 30 minutes, then transfer to -20˚C refrigerator for 30 minutes and lastly thaw at 37˚C water bath.
2. Prepare the media for the designated cell, and warm at 37˚C water bath.
3. Add 3-4ml media into new 15ml centrifuge tube, then resuspend the cell gently in the cryovial tube and then pipette all the cell solution into the 15ml centrifuge tube
4. Centrifuge the tube at 1000rpm for 5min
5. Transfer the fresh media to cell culture flask.
    a. $75cm^2$ cell culture flask = 15ml media
    b. $25cm^2$ cell culture flask = 8ml media
6. Discard the supernatant and Gently transfer the cell into the flask. Do not shake the vial.
7. Incubate the flask at 37˚C + 5% CO_2 for 1 day.
8. When the cells were attached to the bottom of the flask, replace the old media with the new ones.
9. Once the cell reaches confluent, subculture the cell into a new flask, and then when the cell reaches confluent again, subculture the cell into a new flask. This is to ensure the removing of the DMSO.

Sub Culture

1. Discard old media.
2. Wash the cells with Phosphate Buffer Saline (PBS) (2-3 times)
    a. $75cm^2$ cell culture flask = 5ml PBS
    b. $25cm^2$ cell culture flask = 3ml PBS
3. Discard the PBS
4. Add Trypsin-EDTA to the flask.
    a. $75cm^2$ cell culture flask = 3ml Trypsin
    b. $25cm^2$ cell culture flask = 1.5ml Trypsin
5. Incubate the flask in incubator at 37˚C + 5% CO_2 for 5 - 10 minute.
6. Observe under inverted light microscope to ensure all cells are detached.
7. Add media into the culture flask, mix it well with trypsin, and transfer to 15ml centrifuge tube.
8. Centrifuge the tube at 1000rpm for 5 minutes.
9. Remove the supernatant.
10. Add 1 ml of media into the tube, resuspend well with the sediment.
11. Prepare a cell culture flask with the media.
    a. $75cm^2$ cell culture flask = 15ml media
    b. $25cm^2$ cell culture flask = 8ml media
12. Transfer designated amount of 1ml of resuspend cell into the new flask.
13. Incubate at 37˚C + 5% CO_2.

Freeze Cell (Cryopreservation)

1. Make sure the cell were free from any contamination (yeast, fungi, bacteria, virus & mycoplasma).
2. Repeat step 1 to step 9 from Sub Culture.
3. Add 1 ml of Freeze media into the centrifuge tube, resuspend until no visible sediment.
4. Transfer the cell into the cryovial tube, then transfer into icebox prior transfer into refrigerator
5. Stored on -20˚C refrigerator for one day then transfer cryovial to 80 ˚C refrigerator (can store for 6months).

Appendix 14

MTT assay protocol

Make an outline of a 96-well plate to determine the number of treated/wells needed. Calculate the concentration and volume to be used.

Day 1

Cell Counting

1. Remove culture media of cells (in a small flask) that reach >90% confluency.
2. Wash cells with 3ml of PBS & then remove
3. Add 1 mL of Trypsin LE and gently shake the flask.
4. Incubate the cells for 30 s at 37 °C incubator with 5% CO_2.
5. View the cells under microscope to make sure the cells are detached.
6. Add 3 ml of fresh DMEM medium to stop the trypsinization.
7. Resuspend the cells and transfer them into a 15 mL conical tube.
8. Centrifuge 2 x 100g for 5 mins
9. Remove the supernatants.
10. Pipette 5 mL DMEM and gently resuspend cell pellet
11. Aliquot 20 µL into Eppendorf tube.
12. Add 20 µL of trypan blue into the tube with the cells.
13. Pipette 10 µL of the cell suspension onto haemocytometer.
14. View the clear cells under light microscope.
15. Count the cells on the grids
16. Calculate the concentration of the cells using formula
    Concentration of cells = A x DF x 10^4
    where A = average of viable cells per square, DF = dilution factors

Cell Seeding

1. Dilute the cells with DMEM medium to make up 1 x 10^5 cells/ well (prepare to the volume you need).
2. Mix well and pipette 100 µL of the cell suspension into each well of a flat bottom 96-well microtiter plate.
3. Incubate the cell at 37 °C overnight in 5% CO_2.

Day 2

Dilution of Disinfectant (usually refer to treatment used if used in anticancer)

1. Label all tubes.
2. Prepare different concentration of disinfectants by diluting the active ingredients in DMEM medium.
3. For negative control, treat cells with DMEM medium only.
4. For positive control, use 10% DMSO (50 µL DMSO in 450 µL DMEM).

Treatment of cells with disinfectant

1. Verify the confluency (80-90%) and morphology of the cells before conducting the experiment.
2. Label plate according to the layout of your 96 well treatment.
3. Remove culture medium (do this for every 3 wells to avoid the cells from drying)
4. Pipette 100 µL of the disinfectant dilution into each well.
5. Perform triplicate wells of each concentration.
6. Incubate the plate for 4 hour and 24 hours at 37 °C in 5% CO_2 incubator.
7. Remove the test solution and wash the wells twice with 100 µL fresh DMEM for 2-3 months
8. Replace with 100 µL fresh DMEM and proceed to MTT assay.

Day 3

MTT reagent preparation (5mg/ml)

1. Weight 5mg of the MTT powder into 1.5ml microcentrifuge tube covered in the aluminum foil.
2. Add 1 mL sterile PBS (concentration??), vortex until the powder completely dissolved.
3. Using 10 µL of the stock solution each in each treatment.

MTT Treatments

1. Add 10 µL of MTT solution into each well and mix well.
2. Incubate for 4 hours at 37°C in the dark (covered with aluminium foil)
3. After 4 hours, remove all media and add 100 µL of DMSO into each well. Use the common lab DMSO instead analytical DMSO.
4. Incubate the plate for 10 mins at RT (cover with aluminium foil)
5. Put on plate shaker for 5mins at 300 rpm.
6. Read the absorbance at 570 nm.

Day 4

1. Do MTT assay for samples incubated at 24 hours.

Appendix 15

Optimization result with Response Surface Modelling

X.1 Overall Statistical Setting

Build Information

File Version	13.0.5.0		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	17.00
Design Model	Quadratic	**Blocks**	No Blocks
Build Time (ms)	2.00		

Factors

Factor	Name	Units	Type	SubType	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Fermentation period	hours	Numeric	Continuous	24.00	72.00	-1 ↔ 24.00	+1 ↔ 72.00	48.00	16.97
B	Salt concentration	%	Numeric	Continuous	3.00	7.00	-1 ↔ 3.00	+1 ↔ 7.00	5.00	1.41
C	Temperature	°C	Numeric	Continuous	25.00	37.00	-1 ↔ 25.00	+1 ↔ 37.00	31.00	4.24

X.2 TFC

Fit Summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0348	0.0306	0.3514	-0.0490	
2FI	0.9520	0.0167	0.1839	-1.3958	
Quadratic	0.0431	0.0475	0.6106	-1.3225	Suggested
Cubic	0.0475		0.8883		Aliased

ANOVA for Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.271E+05	9	36349.73	3.79	0.0465	significant
A-Fermentation period	3591.84	1	3591.84	0.3743	0.5600	
B-Salt concentration	1.479E+05	1	1.479E+05	15.41	0.0057	
C-Temperature	35068.44	1	35068.44	3.65	0.0975	
AB	1154.13	1	1154.13	0.1203	0.7389	
AC	3094.41	1	3094.41	0.3225	0.5879	
BC	2423.10	1	2423.10	0.2525	0.6307	
A^2	1.013E+05	1	1.013E+05	10.56	0.0141	
B^2	32615.86	1	32615.86	3.40	0.1078	
C^2	4592.92	1	4592.92	0.4786	0.5113	
Residual	67170.23	7	9595.75			
Lack of Fit	56161.81	3	18720.60	6.80	0.0475	significant
Pure Error	11008.42	4	2752.10			
Cor Total	3.943E+05	16				

Factor coding is **Coded**.
Sum of squares is **Type III – Partial**

X.3 TPC

Fit Summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0434	0.0025	0.3273	-0.0785	
2FI	0.0934	0.0041	0.5261	-0.2484	
Quadratic	0.0371	0.0126	0.7839	-0.3993	Suggested
Cubic	0.0126		0.9686		Aliased

ANOVA for Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.099E+05	9	12211.26	7.45	0.0074	significant
A-Fermentation period	6109.36	1	6109.36	3.73	0.0949	
B-Salt concentration	47422.64	1	47422.64	28.93	0.0010	
C-Temperature	1500.02	1	1500.02	0.9149	0.3707	
AB	19434.73	1	19434.73	11.85	0.0108	
AC	10199.31	1	10199.31	6.22	0.0413	
BC	764.09	1	764.09	0.4660	0.5168	
A^2	15943.07	1	15943.07	9.72	0.0169	
B^2	6549.14	1	6549.14	3.99	0.0858	
C^2	1629.89	1	1629.89	0.9941	0.3519	
Residual	11476.47	7	1639.50			
Lack of Fit	10522.37	3	3507.46	14.70	0.0126	significant
Pure Error	954.10	4	238.52			
Cor Total	1.214E+05	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

X.4 TGLs

Fit Summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0018	0.0303	0.5991	0.3747	Suggested
2FI	0.6294	0.0213	0.5583	-0.1831	
Quadratic	0.0422	0.0614	0.7905	-0.2188	Suggested
Cubic	0.0614		0.9314		Aliased

ANOVA for Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value

Model	694.44	9	77.16	7.71	0.0067	significant
A-Fermentation period	42.12	1	42.12	4.21	0.0794	
B-Salt concentration	303.74	1	303.74	30.34	0.0009	
C-Temperature	169.59	1	169.59	16.94	0.0045	
AB	26.29	1	26.29	2.63	0.1491	
AC	3.80	1	3.80	0.3793	0.5575	
BC	7.91	1	7.91	0.7899	0.4036	
A^2	17.42	1	17.42	1.74	0.2286	
B^2	94.04	1	94.04	9.40	0.0182	
C^2	29.84	1	29.84	2.98	0.1279	
Residual	70.07	7	10.01			
Lack of Fit	56.96	3	18.99	5.79	0.0614	not significant
Pure Error	13.11	4	3.28			
Cor Total	764.51	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

Appendix 16

Animal ethnic approval letter

Jawatankuasa Etika Penyelidikan UMT
(UMT Research Ethics Committee)

Pejabat Pengurusan Penyelidikan (PPP)
Universiti Malaysia Terengganu,
21030 Kuala Terengganu

Tel : 09-6684415/4951/5078
Faks : 09- 6684944

8th November 2023

Assoc. Prof. Ts. Dr. Ma Nyuk Ling
Faculty of Science and Marine Environment
Universiti Malaysia Terengganu
21030 Kuala Terengganu
Terengganu

Dear Dr. ,

Research Ethics Approval

The UMT Research Ethics Committee (UMT REC) has approved the above research project:

No. of Animal Ethics Approval	**: UMT/JKEPHMK/2022/78**
Title	**: The Feeding Effects of Probiotic Fermented Cabbage on the Gut Microbiota and Physiochemical Responses in Rat with Exposure of Pesticide**
Name of Principal Investigator	**: Assoc. Prof. Ts. Dr. Ma Nyuk Ling**
Name of Co- Investigator	**: 1) Prof. Ts. Dr. Mohd. Effendy Abd. Wahid** **2) Assoc. Prof. Dr. Suvik A/L Assaw** **3) Dr. Shahidee Zainal Abidin**
Name of Student Investigator	**: 1) Ms. Khoo Shing Ching** **2) Ms. Tai Zhi Ting**

Thank you.

Yours sincerely,

PROFESOR CHM. DR. MARINAH BINTI MOHD ARIFFIN
Deputy Vice-Chancellor (Research and Innovation)
Universiti Malaysia Terenganu

Cc : File

Jawatankuasa Etika Penyelidikan UMT **JKEP UMT**
UMT Research Ethics Committee (UMT REC)

Appendix 17

Standard diet nutrition profile of Altromin rat feed 1324

Nutrition Profile	Contents (mg/kg)
Polysaccharides	358,852.33
Disaccharides	49,463.05
Energy (kcal/kg)	3188,49
Crude Protein	191, 970.40
Crude Fat	40,803.01
Crude Fibre	60,518.48
Ash	69,364.89
Moisture	112, 946.89
Vitamin A (I.E./kg)	25,000.00
Vitamin C	36.00
Vitamin D3 (I.E./kg)	600.00
Vitamin E	110.35
Vitamin K3	3.00
Vitamin B1	18.00
Vitamin B2	12.00
Vitamin B6	9.00
Vitamin B12	0.024
Calcium	7114.94
Phosphorus	5090.56
Magnesium	2436.93
Sulfur	1198.20
Chlorine	3541.00
Iron	198.04
Manganese	97.69
Sodium	2156.57
Potassium	9214.90
Zink	94.88
Copper	13.58
Iodine	1.62
Molybdenum	1.13
Fluorine	2.19
Selenium	0.27
Cobalt	0.35

Appendix 18

Oral gavage feeding method

PROCEDURE

1. Weigh the animal and calculate the volume to be administered (refer to Table 1 for maximum recommended volume and Page 6 for how to calculate volume).
2. Measure the distance from the oral cavity or tip of nose to the end of the xiphoid process (caudal point of the sternum) or last rib with the gavage tube placed on the outside of the restrained animal (see Figures 1A, 1B and 1C). This will be the distance the tube will be inserted into the esophagus.
    a. Tip: Mark this distance on the gavage tube using a permanent marker or a small piece of tape so this distance can be used on all rats of the same strain and weight.
    b. **NOTE:** *If using a gavage tube that is too long, there is a greater chance of injuring the animal if the tube is inserted fully. Using a gavage tube that is too short may allow the substance to enter the oropharynx and cause the animal to aspirate into the lungs.*

Figure 1

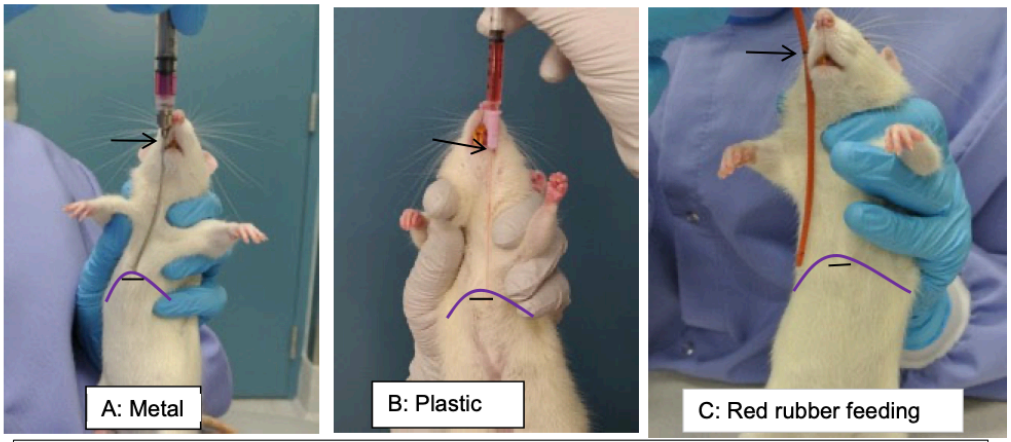


Figure 1 – Gavage tubes showing the distance to be inserted (Black arrows pointing to marks or "landmarks" on the tubes).
Purple line: bottom of rib cage
Black line: xyphoid process

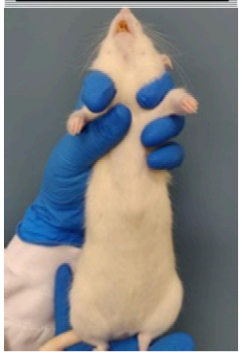
3. Pre-fill the syringe and gavage tube with the correct volume of the compound to be administered.
4. Wipe the outside of the gavage tube with gauze to remove any of the compound coating the outside of the needle/tube (to ensure accurate dosing and prevent animal from tasting potentially bitter compounds).

**Tip: If permitted on the study protocol, dipping the end of the gavage tube in a sweet substance, such as sugar water, can stimulate licking and swallowing to improve the gavage process so rodents better tolerate the procedure, especially with repeat gavaging.

5. Restrain the rat in an appropriate manner. Ensure at least the hind feet are on a stable surface.
    a. If using a metal or plastic gavage tube, restrain the rat in an upright position with your non-dominant hand and facing the handler. Use a "V-hold" to immobilize the head and neck. See Figure 2A.
        i. Ensure an adequate restraint is achieved so that the legs are controlled (otherwise the rats will grasp the gavage needle/tube and may cause injury to itself or remove the tube from its mouth) and the **head and neck are immobilized** (cannot move up or down or side to side).
        ii. Ensure the animal can breathe freely (observe if the chest is moving and the nose and feet remain pink).
    b. If using a flexible red rubber feeding tube, the rat can be gently restrained by hand, or by wrapping in a towel, while they are sitting flat on a table or counter. The wrapping should prevent the rat from using its front feet to grasp the rubber tube. Red rubber feeding tubes are softer and cannot damage the esophagus as easily, so the head/neck restraint is not as important and the restraint required is minimized. See Figure 2B as well as Appendix 2.

Figure 2

A: V-hold for Metal or Plastic tubes

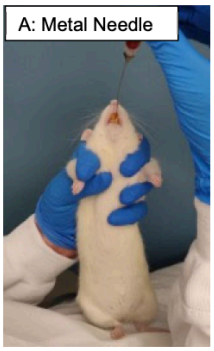


B: Towel wrapping for Red Rubber Feeding tube

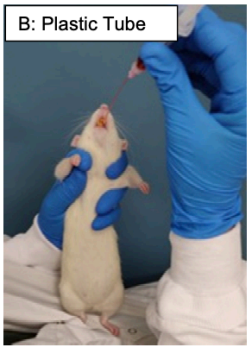
6. If restraint has taken more than a few minutes, immediately before performing gavage, ensure the plunger in the syringe is moving freely. Pull back on the plunger then eject any air out of the gavage tube.
7. Insert the end of the gavage tube into the left side of the animal's mouth in the gap behind the upper incisors and in front of the first molar (the "diastema"). For curved metal tubes, ensure the concave curve of the needle is facing the mandible (lower jaw) of the rat so that it will follow the curve of the oropharynx. See Figures 3A, 3B and 3C.

Figure 3

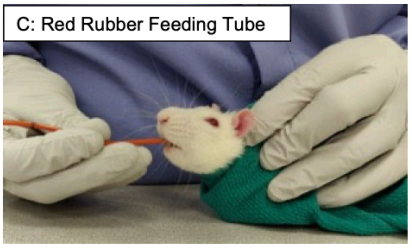
A: Metal Needle



B: Plastic Tube



C: Red Rubber Feeding Tube



8. Advance the gavage tube along the roof of the animal's mouth slightly towards the animal's left side all the way to the back of the mouth and tongue.
    a. You may feel the ridges of the hard palate as you slide the gavage tube back.
    b. The animal usually "gags" at this stage – opens its mouth wide and extends tongue.
9. Once the gavage tube is at the back of the mouth/behind the tongue:
    a. Metal gavage tubes: tilt the head back by gently tipping the syringe up and back towards the animal's spine. This aligns the esophagus in a straight line to the stomach. See Figure 4 A.
    b. Plastic gavage tube or red rubber feeding tubes: tilting the head back is not necessary because the tubing is flexible and will follow the curve of the oropharynx. See Figures 4B and 4C.

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12. If the animal is breathing normally, inject a very small "test" dose (~0.05 ml). If there is no change in breathing effort, then slowly inject the solution (over 2-3 seconds) to minimize the fluid coming back up the esophagus. If injecting an oily or viscous substance, use the smallest volume possible and inject more slowly (over 5 – 10 seconds).
    NOTE: *If the animal is **not breathing normally**, **do not administer substance** and immediately remove the gavage tube from the esophagus and release the restraint. Monitor animal's breathing closely. Do not attempt the procedure again until the animal is behaving and breathing normally.*
13. Once the entire substance has been administered, remove the gavage tube slowly (over 1-2 seconds), in a smooth arcing motion, and return the animal to its cage. Removing the gavage tube slowly helps ensure none of the substance is brought back up the esophagus into the back of the throat. Inhaling any substance into the lungs will likely result in the death of the animal.
14. Return the animal to its cage and observe for any complications (see below). Monitor immediately after gavage for 10 minutes, at the end of the day and the next day (or as described in the approved Animal Care Protocol). Do not leave animal for the day unless you are satisfied the animal is behaving normally.
15. Note procedure (drug, dose, route, volume and any complications) on cage card/monitoring records.

→CALCULATING VOLUME (IN ML) TO BE ADMINISTERED:

- Convert animal's weight from grams to kilograms
    - Divide the weight in grams by 1000
    - E.g. 250g rat ÷ 1000 = 0.25kg
- Calculate the volume to give in ml
    - Volume (mL) = dose (ml/kg) x weight of animal (kg)
    - E.g. For a 250g rat getting 10 ml/kg
      Volume (ml) = (10 ml/kg x 0.25 kg) = **2.5 ml**

Appendix 19

Monitoring criteria for rats

Score	0	2	5	10	15	20
Body Weight	0-4% weight loss	5-9% weight loss	1-14% weight loss		15-19% weight loss	20% above weight loss (IE)
Physical appearance	Normal		Generate lack of grooming or alopecia	Dermatitis or minor porphyrin or ocular/ nasal discharge	Piloerection, hunched up or severe porphyrin (IT)	Hunched and eyes half closed (IE)
Behavioural/ activity	Normal mobility	Less mobile, but social and alert	Less mobile and isolated, but alert	Less mobile and very still, not alert (IT)	still, not alert (IT)	No response when stimulated (IE)
Clinical signs	None		Soft stools or blood in the urine	Shallow or increased respiratory effort	Severe diarrhea or skin tenting (1 sec) (IT)	Irregular gasping respirations or pale skin; seizures (IE)

Appendix 20

Guidelines preparation and delivery methods for anaesthesia agent ketamine/xylazine and euthanasia sodium pentobarbital

GUIDELINES - PREPARATION OF KETAMINE/XYLAZINE COCKTAIL FOR RATS

- Use of a sterile injection vial is required (e.g. redtop blood collection tube; commercial injection vial)
- Mixing instructions:
  - Verify the concentration of your drugs prior to mixing
  - For a 10mL vial using ketamine 100 mg/mL and xylazine 100 mg/mL add:
    - 10 mL ketamine (100 mg/mL)
    - 1 mL xylazine (100 mg/mL)
- Use of the following template for a label is recommended:
  - *Rat Anesthetic Mix:* Ketamine/Xylazine
  - *Dosage: 0.1 ml/ 100gm IP*
  - Delivers: 91 mg/kg Ketamine, 9.1 mg/kg Xylazine
  - Concentration: 91 mg/mL Ketamine, 9.1 mg/mL Xylazine
  - Expires: _____
  - The expiration date for the cocktail is determined by either six months from the mixing date, or whichever of the components expires first (if less than 6 months)
    - E.g.: Diluted on 8/13/11, ketamine expires 12/10/2012, xylazine expires 10/10/11 and sterile water for interjection expires 1/12/2013, the expiration date for the cocktail is 10/10/11

HANDLING GUIDELINES FOR EUTHANASIA

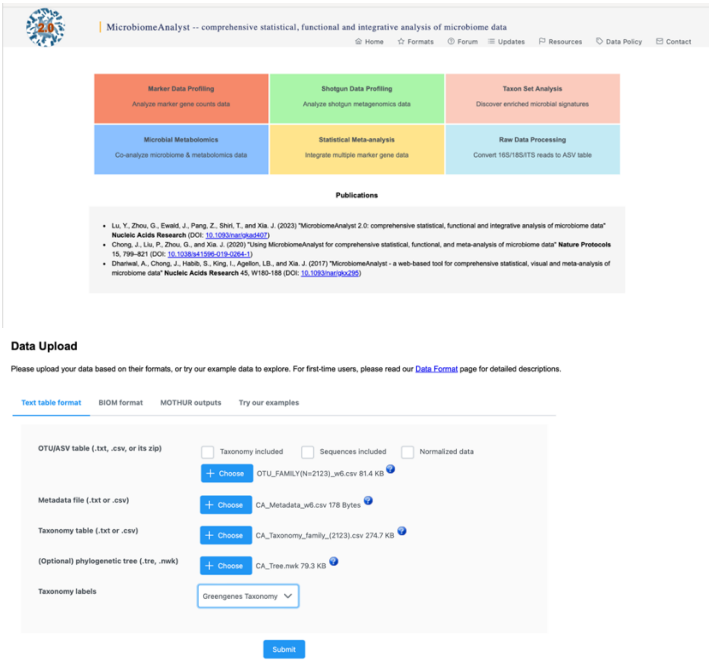
Two person technique with one holder and one injector will be practised during euthanasia to avoid mis-injection. The holder will hold the limb and head of the animals and maintain the animal in a horizontal position. It is suggested to hold the animal's head lower than its abdomen. The injector using one hand to hold the right hindlimb of the animal, visualizes the abdomen as though divided into 4 quadrants and injects into the caudal left quadrant (the animal's right side). Within the caudal left quadrant, the injection should be made at the level of the coxo-femoral joint, approximately halfway between midline and the lateral abdominal wall. The goal of the injection is to deposit injectate into the peritoneal cavity without piercing any of the abdominal organs. To avoid doing so, the injection angle should be approximately 10 to 20 degrees relative to the body wall in mice and 20 to 45 degrees in rats, with the needle directed cranially. Before injection, it is often suggested to aspirate the needle to assure its correct placement in the peritoneal cavity, although there is no evidence supporting the usefulness of this practice.

Appendix 21

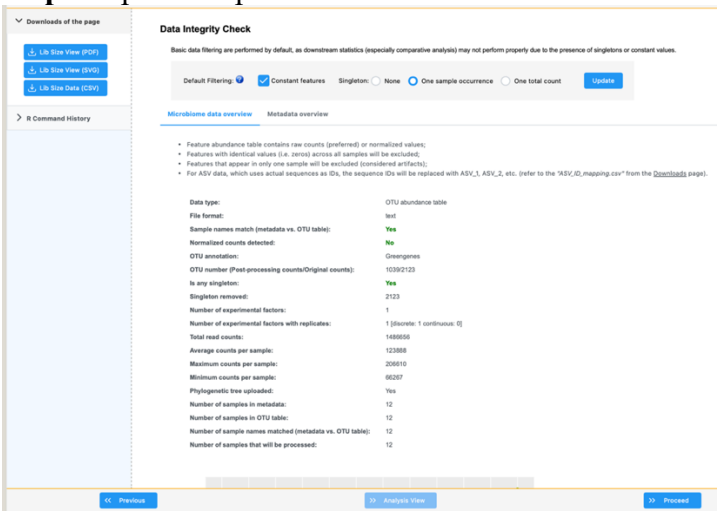
Microbiome Analyst Online

Website:
https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/

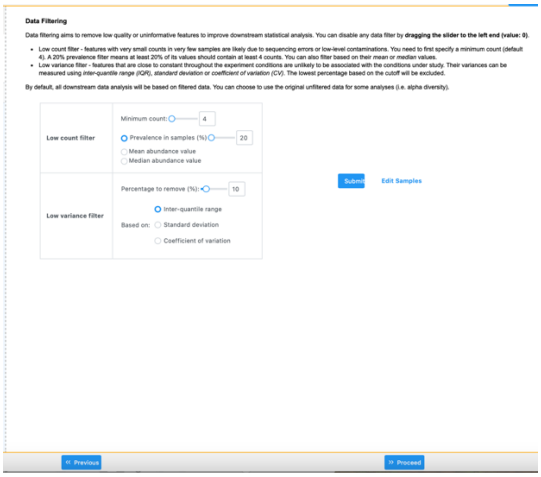
Step 1:
Home page -> Marker profiling -> data upload



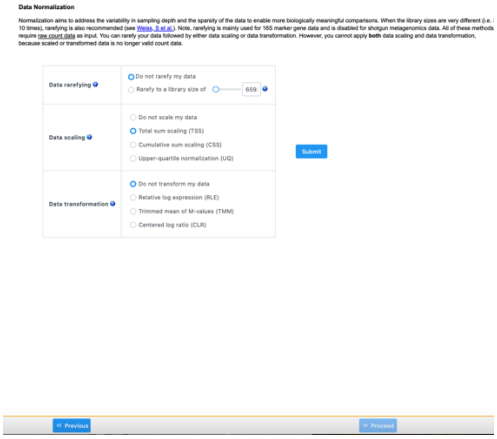
Step 2: update -> proceed



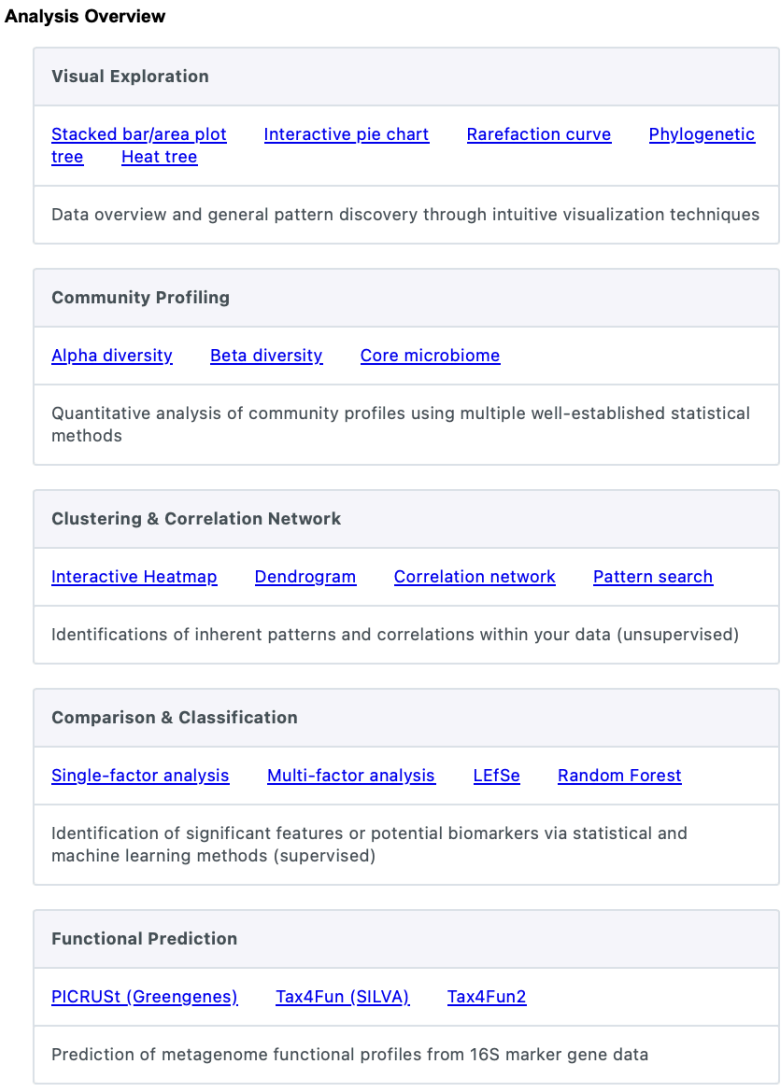
Step 3: submit -> proceed



Step 4: submit -> proceed



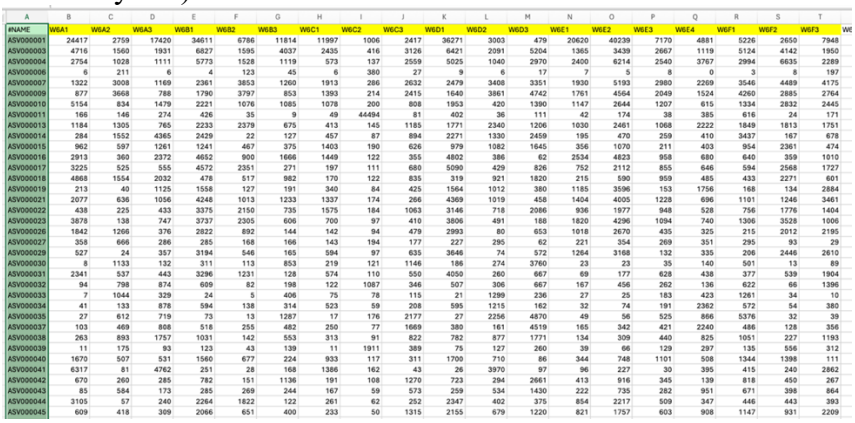
Step 5: can play around the analysis overview



Notes for editing for files

1) OTU files

– need to edit the sample name based on the target group to analyse
-need to edit the ASV that being remove from taxonomy file (must tally with taxonomy file)



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ASV000004	2754	1028	1111	5773	1528	1119	573	137	2559	5025	1040	2970	2400	6214	2540	3767	2994	6635	2289
ASV000006	6	211	6	4	123	45	6	380	27	9	6	17	7	5	8	0	3	8	197
ASV000007	1322	3008	1169	2361	3653	1260	1913	286	2632	2479	3408	3351	1930	5193	2980	2269	3546	4489	4175
ASV000009	877	3668	788	1790	3797	853	1393	214	2415	1640	3861	4742	1761	4564	2049	1524	4260	2885	2764
ASV000010	5154	834	1479	2221	1076	1085	1078	200	808	1953	420	1390	1147	2644	1207	615	1334	2832	2445
ASV000011	166	146	274	426	35	9	49	44494	81	402	36	111	42	174	38	385	616	24	171
ASV000013	1184	1305	765	2233	2379	675	413	145	1185	1771	2340	1206	1030	2461	1068	2222	1849	1813	1751
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ASV000015	962	597	1261	1241	467	375	1403	190	626	979	1082	1645	356	1070	211	403	954	2361	474
ASV000016	2913	360	2372	4652	900	1666	1449	122	355	4802	386	62	2534	4823	958	680	640	359	1010
ASV000017	3225	525	555	4572	2351	271	197	111	680	5090	429	826	752	2112	855	646	594	2568	1727
ASV000018	4868	1554	2032	478	517	982	170	122	835	319	921	1820	215	590	959	485	433	2271	601
ASV000019	213	40	1125	1558	127	191	340	84	425	1564	1012	380	1185	3596	153	1756	168	134	2884
ASV000021	2077	636	1056	4248	1013	1233	1337	174	266	4369	1019	458	1404	4005	1228	696	1101	1246	3461
ASV000022	438	225	433	3375	2150	735	1575	184	1063	3146	718	2086	936	1977	948	528	756	1776	1404
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ASV000027	358	666	286	285	168	166	143	194	177	227	295	62	221	354	269	351	295	93	29
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ASV000030	8	1133	132	311	113	853	219	121	1146	186	274	3760	23	23	35	140	501	13	69
ASV000031	2341	537	443	3296	1231	128	574	110	550	4050	260	667	69	177	628	438	377	539	1904
ASV000032	94	798	874	609	82	198	122	1087	346	507	306	667	167	456	262	136	622	66	1396
ASV000033	7	1044	329	24	5	406	75	78	115	21	1299	236	27	25	183	423	1261	34	10
ASV000034	41	133	878	594	138	314	523	59	208	595	1215	162	32	74	191	2362	572	54	380
ASV000035	27	612	719	73	13	1287	17	176	2177	27	2256	4870	49	56	525	866	5376	32	39
ASV000037	103	469	808	518	255	482	250	77	1669	380	161	4519	165	342	421	2240	486	128	356
ASV000038	263	893	1757	1031	142	553	313	91	822	782	877	1771	134	309	440	825	1051	227	1193
ASV000039	11	175	93	123	43	139	11	1911	389	75	127	260	39	66	129	287	135	556	312
ASV000040	1670	507	531	1560	677	224	933	117	311	1700	710	86	344	748	1101	508	1344	1398	111
ASV000041	6317	81	4762	251	28	168	1386	162	43	26	3970	97	96	227	30	395	415	240	2862
ASV000042	670	260	285	782	151	1136	191	108	1270	723	294	2661	413	916	345	139	818	450	267
ASV000043	85	584	173	285	269	244	167	59	573	259	534	1430	222	735	282	951	671	398	864
ASV000044	3105	57	240	2264	1822	122	261	62	252	2347	402	375	854	2217	509	347	446	443	393
ASV000045	609	418	309	2066	651	400	233	50	1315	2155	679	1220	821	1757	603	908	1147	931	2209

2) Metadata file
-edit grouping for the group clustering

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W6B1	B	WEEK6
W6B2	B	WEEK6
W6B3	B	WEEK6
W6C1	C	WEEK6
W6C2	C	WEEK6
W6C3	C	WEEK6
W6D1	D	WEEK6
W6D2	D	WEEK6
W6D3	D	WEEK6
W6E1	E	WEEK6
W6E2	E	WEEK6
W6E3	E	WEEK6
W6E4	E	WEEK6
W6F1	F	WEEK6

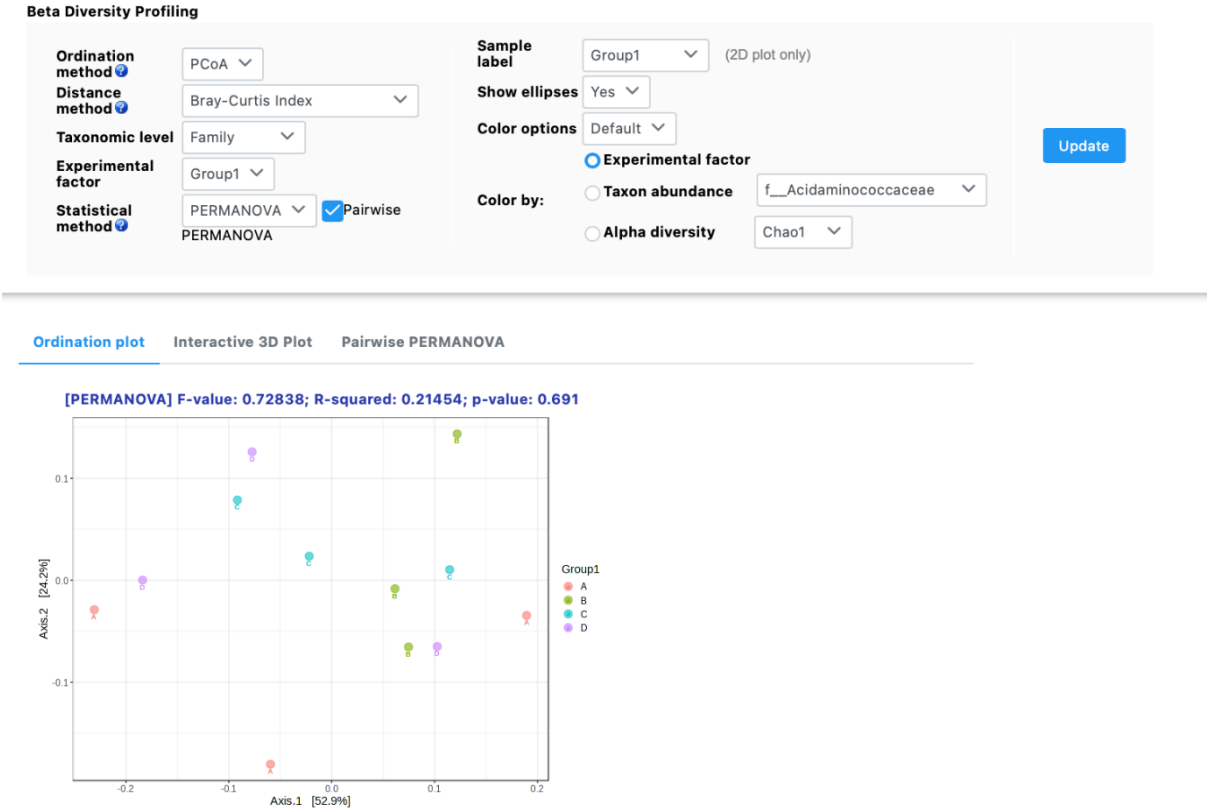
3) taxonomy file

-crosscheck on genus and family for the same level detection
-remove the undetectable genus or sp

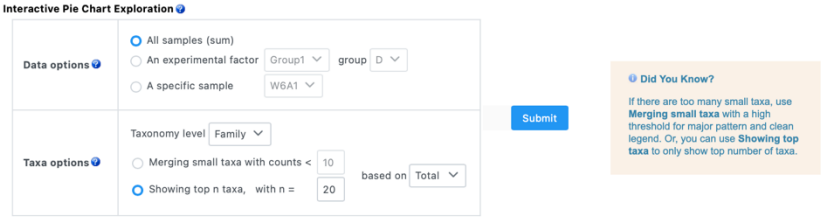
	A	B	C	D	E	F	G	H	I	J
1	#TAXONOMY	kingdom	phylum	class	order	family	genus	species		
2	ASV000001	d__Bacteria	p__Proteoba	c__Gammapr	o__Enteroba	f__Succinivib	g__Anaerobiosp	s__Anaerobiospirillum_A_succiniciproducens		
3	ASV000002	d__Bacteria	p__Firmicute	c__Clostridia	o__Peptostre	f__Peptostrep	f(U)_Peptostrep	f(U)_Peptostreptococcaceae		
4	ASV000003	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	g(U)_Prevotella		
5	ASV000004	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Alloprevotell	s__Alloprevotella_timonensis		
6	ASV000005	d__Bacteria	p__Firmicute	c__Negativic	o__Selenomc	f__Selenomo	f(U)_Selenomon	f(U)_Selenomonadaceae		
7	ASV000006	d__Bacteria	p__Proteoba	c__Gammapr	o__Enteroba	f__Enterobac	g__Escherichia	g(U)_Escherichia		
8	ASV000007	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Alloprevotell	g(U)_Alloprevotella		
9	ASV000008	d__Bacteria	p__Firmicute	c__Clostridia	o__Lachnosp	f__Lachnospi	f(U)_Lachnospir	f(U)_Lachnospiraceae		
10	ASV000009	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Alloprevotell	g(U)_Alloprevotella		
11	ASV000010	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	g(U)_Prevotella		
12	ASV000011	d__Bacteria	p__Firmicute	c__Bacilli	o__Lactobac	f__Lactobaci	g__Lactobacillu	s__Lactobacillus_intestinalis		
13	ASV000012	d__Bacteria	p__Firmicute	c__Clostridia	o__Lachnosp	f__Lachnospi	f(U)_Lachnospir	f(U)_Lachnospiraceae		
14	ASV000013	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	g(U)_Prevotella		
15	ASV000014	d__Bacteria	p__Firmicute	c__Clostridia	o__Lachnosp	f__Lachnospi	g__UBA3282	s__UBA3282_sp003611805		
16	ASV000015	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__UBA4334	s__UBA4334_sp900316505		
17	ASV000016	d__Bacteria	p__Proteoba	c__Gammapr	o__Enteroba	f__Succinivib	g__Anaerobiosp	s__Anaerobiospirillum_A_succiniciproducens		
18	ASV000017	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	s__Prevotella_sp004792655		
19	ASV000018	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	g(U)_Prevotella		
20	ASV000019	d__Bacteria	p__Desulfoba	c__Desulfovi	o__Desulfovi	f__Desulfovit	g__Mailhella	s__Mailhella_massiliensis		
21	ASV000021	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	s__Prevotella_scopos		
22	ASV000022	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	g(U)_Prevotella		
23	ASV000023	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	s__Prevotella_copri		
24	ASV000024	d__Bacteria	p__Firmicute	c__Clostridia	o__Lachnosp	f__Lachnospi	f(U)_Lachnospir	f(U)_Lachnospiraceae		
25	ASV000025	d__Bacteria	p__Firmicute	c__Negativic	o__Selenomc	f__Selenomo	f(U)_Selenomon	f(U)_Selenomonadaceae		
26	ASV000026	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	s__Prevotella_sp004792655		
27	ASV000027	d__Bacteria	p__Firmicute	c__Clostridia	o__Oscillosp	f__Oscillospi	g__Faecousia	s__Faecousia_sp000434635		
28	ASV000028	d__Bacteria	p__Firmicute	c__Clostridia	o__Lachnosp	f__Lachnospi	f(U)_Lachnospir	f(U)_Lachnospiraceae		
29	ASV000029	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	s__Prevotella_sp004792655		
30	ASV000030	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Phocaeicola	s__Phocaeicola_A_sp900291465		
31	ASV000031	d__Bacteria	p__Bacteroic	c__Bacteroic	o__Bacteroic	f__Bacteroid	g__Prevotella	s__Prevotella_sp004792655		
32	ASV000032	d__Bacteria	p__Patescib	c__Saccharir	o__Saccharir	f__Nanosync	g__Nanosyncoc	g(U)_Nanosyncoccus		
33	ASV000033	d__Bacteria	p__Firmicute	c__Clostridia	o__Lachnosp	f__Lachnospi	g__UBA3282	s__UBA3282_sp003611805		
34	ASV000034	d__Bacteria	p__Desulfoba	c__Desulfovi	o__Desulfovi	f__Desulfovit	g__Mailhella	s__Mailhella_massiliensis		

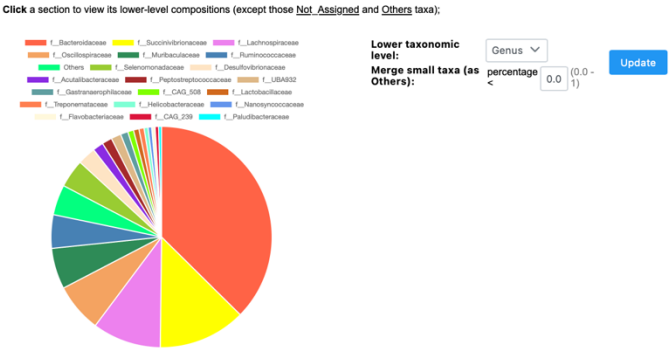
4) Setting for ALPHA DIVERSITY

5) Setting for BETA DIVERSITY

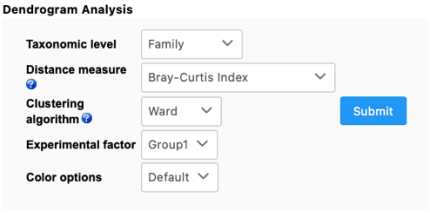


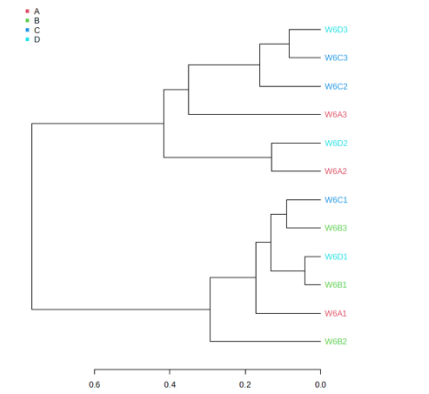
6) Setting for PIECHART



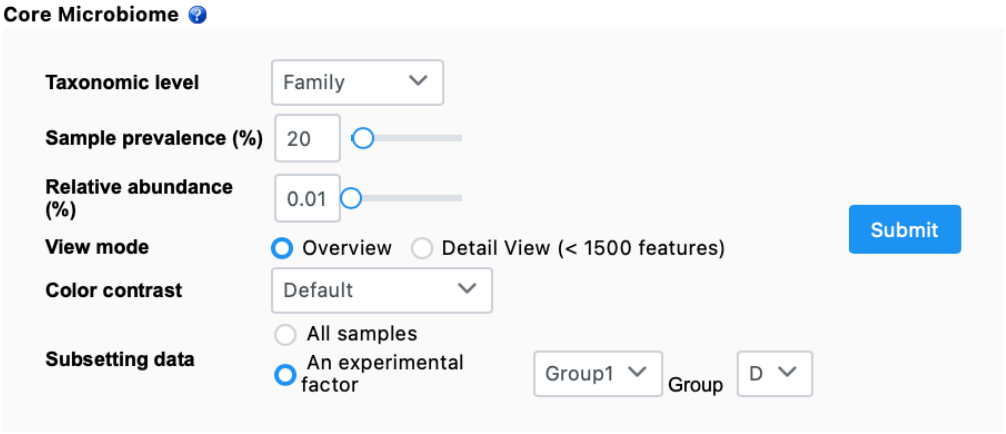


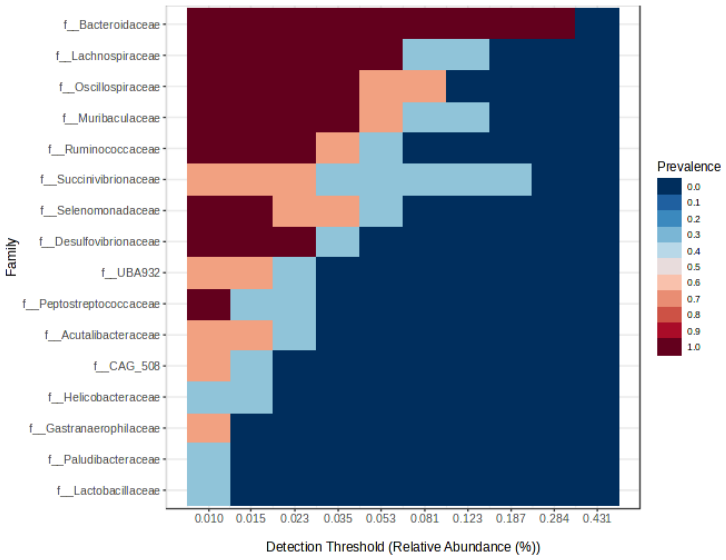
7) Setting for DENDROGRAM





8) Setting for CORE MICROBIOME



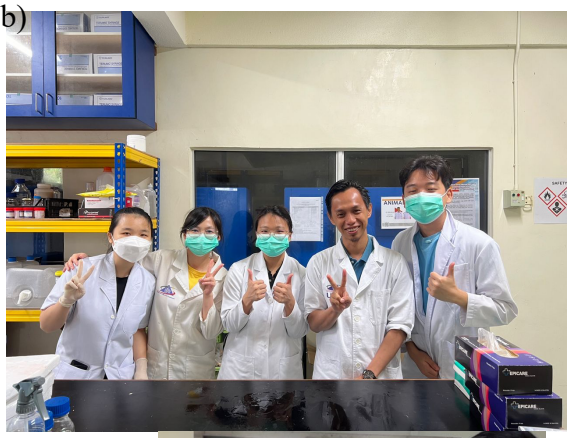


Appendix 22

Photograph on the in-vivo animal study: rat handling, treatment, oral gavage and dissection

(a)

(b)



(c)

(d)

Figure A1: (a) Photograph for rat dissection, (b) group photo of animal dissection team (c) rat culture room and (d) rat oral gavage.

Publications

Scopus Author ID: 57205419800
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Total published papers: 19
Published paper during PhD: 13
h-index: 10 (google scholar), 8 (Web of Science & Scopus)
Total Citation: 520 (google scholar), 354 (scopus)

No	Journal /Article	Index	Impact factor, Quartile ranking
1.	**Khoo, S. C.,** Chin, K. W., Ting, T. Z., Luang-In, V., Lan, J. C. W., & Ma, N. L. (2025). Stress Tolerance and Metabolism Profiling of Selected Functional Probiotic Strains. *Food Bioscience*, 105919. https://doi.org/10.1016/j.fbio.2025.105919	SCI	IF4.8, Q1
2.	Gao, L., **Khoo, S. C.**, Zhang, Z., & Wu, X. (2025). Trends in sustainable single-cell protein from non-grain feedstocks. *Trends in Biotechnology*. https://doi.org/10.1016/j.tibtech.2025.04.018	SCI	IF14.3, Q1
3.	Chin, K. W., **Khoo, S. C**., Luang-In, V., Tan, S. H., & Ma, N. L. (2025). Probiotic fermented Carica papaya water kefir with chemopreventive potential. *Food Bioscience*, *68*, 106343. https://doi.org/10.1016/j.fbio.2025.106343	SCI	IF4.8, Q1
4.	See, M. S., Ching, X. L., **Khoo, S. C.,** Abidin, S. Z., Sonne, C., & Ma, N. L. (2025). Aquatic Microbiomes Under Stress: The Role of Gut Microbiota in Detoxification and Adaptation to Environmental Exposures. *Journal of Hazardous Materials Advances*, 100612. https://doi.org/10.1016/j.hazadv.2025.100612	SCI	IF5.5, Q1
5.	**Khoo, S.C.,** Zhang, N. Luang-in, V., Goh, M.S., Sonne, C. Ma, N.Y. (2024). Exploring environmental exposomes and the gut-brain nexus: Unveiling the impact of pesticide exposure. Environmental Research, 118441. https://doi.org/10.1016/j.envres.2024.118441	SCI	IF8.431, Q1
6.	Chin, K. W., **Khoo, S. C**., Paul, R. P. M., Luang-In, V., Lam, S. D., & Ma, N. L. (2024). Potential of Synbiotics and Probiotics as Chemopreventive Agent. *Probiotics and Antimicrobial Proteins*, 1-17. https://doi.org/10.1007/s12602-024-10299-z	SCI	IF4.4, Q1
7.	Lee, C. H., Hamdan, N., Ling, L. I., Wong, S. L., Nyakuma, B. B., **Khoo, S. C.**, ... & Lee, T. H. (2024). Antioxidant and antidiabetic properties of bioactive peptides from soursop (Annona muricata) leaf biomass. *Biomass Conversion and Biorefinery*, *14*(16), 19849-19862. https://doi.org/10.1007/s13399-023-03993-5	SCI	IF3.5, Q2

8.	**Khoo, S. C.,** Shoji, Y., Teh, C. H., & Ma, N. L. (2023). Discovery of Biomarkers for Myogenous Temporomandibular Disorders Through Salivary Metabolomic Profiling: A Pilot Study. *Journal of Oral & Facial Pain & Headache*, *37*(3). **https://doi.org/10.11607/ofph.3353**	SCI	IF2.5, Q3
9.	Lam, S. S., Show, P. L., Peter, A. P., Chew, K. W., Tham, P. E., Ma, N. L., **Khoo. S.C.** ... & Sonne, C. (2023). Detection methods of Covid-19 to build resilience for environmental changes in the community. *Urban Governance*. https://doi.org/10.1016/j.ugj.2023.10.001	DOAJ	NA
10.	Lee, C. H., Lee, T. H., Wong, S. L., Nyakuma, B. B., Hamdan, N., **Khoo, S. C**., ... & Jamaluddin, H. (2023). Characteristics and trends in global Edible Bird's Nest (EBN) research (2002–2021): a review and bibliometric study. *Journal of Food Measurement and Characterization*, 1-22. https://doi.org/10.1007/s11694-023-02006-3	SCI	IF3.4, Q2
11.	Lee, C. H., Hamdan, N., Ling, L. I., Wong, S. L., Nyakuma, B. B., **Khoo, S. C.,** ... & Lee, T. H. (2023). Antioxidant and antidiabetic properties of bioactive peptides from soursop (Annona muricata) leaf biomass. *Biomass Conversion and Biorefinery*, 1-14. https://doi.org/10.1007/s13399-023-03993-5	SCI	IF4.0, Q2
12.	**Khoo, S. C.,** Ma, N. L., Peng, W. X., Ng, K. K., Goh, M. S., Chen, H. L., ... & Sonne, C. (2022). Valorisation of biomass and diaper waste into a sustainable production of the medical mushroom Lingzhi Ganoderma lucidum. *Chemosphere*, *286*, 131477. https://doi.org/10.1016/j.chemosphere.2021.131477	SCI	IF8.943, Q1
13.	**Khoo, S. C.,** Goh, M. S., Alias, A., Luang-In, V., Chin, K. W., Michelle, T. H. L., ... & Ma, N. L. (2022). Application of antimicrobial, potential hazard and mitigation plans. *Environmental Research*, 114218. https://doi.org/10.1016/j.envres.2022.114218	SCI	IF8.431, Q1
14.	Azmi, W. A., **Khoo, S. C.** (Co-first author), Ng, L. C., Baharuddin, N., Abd Aziz, A., & Ma, N. L. (2022). The current trend in biological control approaches in the mitigation of golden apple snail Pomacea spp. *Biological Control*, 175, 105060. https://doi.org/10.1016/j.biocontrol.2022.105060	SCI	IF4.2, Q1
15.	Lee, T. H., Lee, C. H., Ong, P. Y., Wong, S. L., Hamdan, N., Ya'akob, H., ... & Cheng, K. K. (2022). Comparison of extraction methods of phytochemical compounds from white flower variety of Melastoma malabathricum. *South African Journal of Botany*, *148*, 170-179. https://doi.org/10.1016/j.sajb.2022.04.026	SCI	IF3.1, Q2
16.	**Khoo, S. C.,** Peng, W. X., Yang, Y., Ge, S. B., Soon, C. F., Sonne, C., & Ma, N. L. (2020). Development of formaldehyde-free bio-board produced from mushroom mycelium and substrate waste. *Journal of Hazardous Materials*, 123296. https://doi.org/10.1016/j.jhazmat.2020.123296	SCI	IF 14.224, Q1
17.	Ma, N. L**., Khoo, S. C.** (Co-first author)**,** Peng, W. X., Ng, C. M., Teh, C. H., Park, Y. K., & Lam, S. S. (2020). Green application and toxic risk of used diaper and food waste as growth substitute	SCI	IF 11.072, Q1

	for sustainable cultivation of oyster mushroom (Pleurotus ostreatus). *Journal of Cleaner Production*, 122272. https://doi.org/https://doi.org/10.1016/j.jclepro.2020.122272		
18.	Peng, W.X., Ma, N.L., Zhang, D.Q., Zhou, Q., Yue, X.C., **Khoo,S.C.**, Yang, H., G. R.R., Wang, Y.C., Suo, C.F., Peng, Y.H., Yang, Y.F., Lam, S.S., Sonne, C. A review of historical and recent locust outbreaks: Linked to global warming, food security and mitigation strategies. *Environmental Research*, 191, 110046. https://doi.org/10.1016/j.envres.2020.110046	SCI	IF 8.431, Q1
19.	**Khoo, S.C.,** Phang, X.Y., Ng, C.M., Lim, K.L., Lam, S.S., Ma, N.L. 2019. Recent Technologies for Treatment and Recycling of Used Disposable Baby Diapers. *Process Safety and Environmental Protection*. https://doi.org/10.1016/j.psep.2018.12.016	SCI	IF 7.926, Q1
20.	Ma, N.L., **Khoo, S.C.** (Co-first author), Lee, J.X., Soon, C.F., Nor Aini, A.S. 2020. Efficent micropropagation of Dendrobium aurantiacum from shoot explant. Plant Science Today. https://doi.org/10.14719/pst.2020.7.3.724	SCI	IF0.15,Q4
21.	Ma, N. L., **Khoo, S. C.** (Co-first author)**,** Loke, L., Lam, S. S., Tan, S. H. (2019). Growth media derived from solid waste for orchid *Dendrobium kingianum* culture. *Malaysian Applied Biology Journal*, *48*(1), 73-78.	Scopus	IF 0.194, Q3

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