THE ROLE OF POI AS A NATURALLY FERMENTED HAWAIIAN FOOD IN THE MODULATION OF GUT HEALTH

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DEDICATION

I would like to dedicate this dissertation to my parents, Rui-ling Shen and Ji-lin Dong, for their unwavering love and support throughout my academic journey. I am forever grateful for their encouragement and sacrifices that have propelled me towards this achievement.

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ABSTRACT

Poi, a culturally significant staple food in the Hawaiian diet, is traditionally made from cooked taro and can undergo natural fermentation by lactic acid bacteria (LAB). Previous studies have suggested potential health benefits of poi, such as its prebiotic, probiotic, and anti-cancer properties. However, the specific health advantages and the underlying mechanisms related to the probiotic nature of poi remain unclear. The gut microbiota plays crucial roles in various physiological functions, and communication within this microbial community is facilitated by the production of metabolites such as short-chain fatty acids (SCFAs). Probiotics and probiotic foods can help replenish beneficial bacteria in the gut and reduce chronic inflammation in the intestines associated with gastrointestinal disorders. Poi, as a naturally fermented food, has the potential to deliver beneficial bacteria and improve gut homeostasis. This dissertation aimed to investigate the probiotic properties of poi, its anti-inflammatory effects, its regulation of lipid metabolism, and its impact on gut microbiota.

Six commercially available poi brands were subjected to simulated gastrointestinal digestion, and LAB surviving the process were isolated and identified. The analysis revealed changes in bacterial abundance and diversity in poi after digestion, identifying 13 different LAB species, including the most prevalent species: *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Enterococcus lactis*, *Leuconostoc pseudomesenteroides*, and *Lactiplantibacillus plantarum*. Some LAB isolates demonstrated antimicrobial activities against *Listeria monocytogenes* and/or *Salmonella enterica* serotype Typhimurium, as well as bile salt hydrolase activities and cholesterol assimilation. The adhesion abilities to human intestinal epithelial cells (Caco-2) varied among the LAB isolates.

Furthermore, extracts from both fresh and fermented poi were tested on Caco-2 cells to evaluate their effects on bacterial infection, inflammation, oxidative stress, and lipid metabolism. The extracts inhibited the adhesion and invasion of *L. monocytogenes* and *S.* Typhimurium, suppressed the expression of pro-inflammatory genes, promoted the expression of anti-inflammatory genes, and upregulated tight junction proteins essential for gut barrier integrity. The poi extracts also influenced gene expressions

related to lipid metabolism, favoring lipid oxidation and gastrointestinal immunoregulation, including PPARs, FIAF, and SREBP-2.

Finally, the impact of fresh and fermented poi on human gut microbiota was assessed using in vitro fecal fermentations. Fermented poi significantly increased microbial diversity. Both fresh and fermented poi altered microbial community structure and promoted the production of SCFAs, including butyrate, valerate, isobutyrate, and isovalerate. Poi affected the abundances of various bacterial phyla and genera, increasing beneficial genera such as *Bacteroides*, *Bifidobacterium*, and *Roseburia*, while decreasing potentially harmful genera such as *Fusobacterium* and *Klebsiella*. The fermented poi group exhibited more pronounced effects on gut microbiota and predicted metabolic pathways compared to the fresh poi group.

In conclusion, this study has illuminated the potential probiotic properties of poi, especially fermented poi, as demonstrated by the presence of probiotic LAB, inhibition of pathogenic bacteria, modulation of inflammatory responses, enhancement of gut barrier function, and influence on gut microbiota composition and SCFA production. Further research is needed to elucidate the contribution of specific bioactive compounds in poi to its health-promoting attributes. The findings presented in this dissertation may contribute to the development of preventive strategies against inflammatory bowel diseases and metabolic syndromes.

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16S RNA	16S ribosomal ribonucleic acid
ABC	Adenosine Triphosphate-binding cassette
ABCG5	Adenosine triphosphate-binding cassette subfamily A members 5
ABCG8	Adenosine triphosphate-binding cassette subfamily A members 8
ALDEx2	ANOVA-like differential expression analysis 2
AMPK	Adenosine 5′-monophosphate-activated protein kinase
ANCOM-BC2	Analysis of Compositions of Microbiomes with Bias Correction 2
ANOVA	Analysis of Variance
APC	Antigen-presenting cells
ASGPB	Advanced Studies in Genomics, Proteomics and Bioinformatics
ATP	Adenosine triphosphate
BIM	Body mass index
BSH	Bile salt hydrolase
CBE	Crude bacteriocin extract
CD	Crohn’s diseases
CD4	Cluster of Differentiation 4
CFS	Cell-free supernatant
CFU	Colony-forming unit
CLA	Conjugated linoleic acid
DADA2	Divisive amplicon denoising algorithm 2
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal bovine serum
FGF	fibroblast growth factor
FIAF	Fasting-induced adipose factor

FOS	Fructooligosaccharide
FXR	Farnesoid X receptor
GAPDH	Human glyceraldehyde 3-phosphate dehydrogenase
GDCA	Sodium glycodeoxycholic acid
GI	Gastrointestinal tract
GLM	Generalized linear model
GLP1	Glucagon-like peptide 1
GPCR	G-protein-coupled receptors
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylases
HDL	High-Density lipoprotein
HFD	Hight-fat diet
HSD	Honestly significant difference
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
IE	Inhibition efficiency
IFN	Interferon
IL	Interleukin
IRB	Institutional review board
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
LGG	*Lacticaseibacillus rhamnosus* GG
LPS	Lipopolysaccharides
LXR	Liver X receptor
MAPK	Mitogen-activated-protein kinase
MEGA	Molecular evolutionary genetics analysis
MOI	Multiplicity of infection
MRS	De Man, Rogosa, and Sharpe
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide

NCBI	National Center for Biotechnology Information
NEAA	Non-essential amino acids
NIFA	National Institute of Food and Agriculture
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NPC1L1	Niemann-Pick C1-like 1
NF-κB	Nuclear factor-kappa B
OD	Optical density
PBS	Phosphate-buffered saline
PCA	Plate count agar
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptors
PYY	Peptide YY
RAPD-PCR	Random amplification of polymorphic DNA-polymerase chain reaction
RIG-I	Retinoic acid-inducible Gene 1
RLR	RIG-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SCFA	Short chain fatty acid
SD	Standard deviation
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SREBP	Sterol regulatory element binding protein-1
SSF	Simulated salivary fluid
SV	Sequence variants

T2DM	Type 2 diabetes mellitus
TAE	Tris-Acetate-EDTA buffer
TAME	Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride
TDCA	Sodium taurodeoxycholic acid
TGF-β	Transforming growth factor-β
TGR5	G protein-coupled bile acid receptor 1
TJ	Tight junction
TLR	Toll-like receptor
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TNF-α	Tumor necrosis factor α
TPS	Taro polysaccharides
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UC	Ulcerative colitis
US	United States
USA	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization
ZO	Zonula occluden

CHAPTER 1 DISSERTATION OVERVIEW

1.1 Introduction

Cardiometabolic disorders, including obesity and obesity-associated conditions such as type 2 diabetes mellitus, non-alcoholic fatty liver disease, cardiovascular diseases, and chronic kidney disease, have reached epidemic proportions worldwide (Aron-Wisnewsky & Clément, 2016). These diseases result in over 36 million deaths annually, accounting for 63% of the total annual deaths worldwide. By 2030, the estimated global cost for these diseases is projected to reach $13 trillion (Arena et al., 2015). In just two decades, the prevalence of obesity and severe obesity in the United States has increased from 30.5% and 4.7% in 2000 to 42.4% and 9.2%, respectively (Hales, 2020). Meanwhile, inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), have been rapidly emerging not only in developed countries like the US and Europe but also in newly industrialized countries within the Asia-Pacific, Middle East, South America, and Africa. As of 2017, the global number of people suffering from IBD reached 6.8 million (Jairath & Feagan, 2020; Molodecky et al., 2012). Estimates indicate that more than 1 million individuals in the US and 2.5 million in Europe are affected by IBD (G. G. Kaplan, 2015). The prevalence of IBD has also rapidly increased in China since the first case was reported in 1956, reaching more than 260,000 cases now (Jairath & Feagan, 2020). IBD imposes a significant burden on individuals, causing morbidity and loss of societal productivity due to its chronic, relapsing inflammation. The direct annual healthcare cost for IBD patients in the US is three times higher than that of non-IBD patients ($22,987 vs $6956 per-member per-year paid claims), and the out-of-pocket costs for IBD patients are twice more than the non-IBD control ($2213 vs $979 per-year reported costs; K. T. Park et al., 2020).

The human gut microbiota, which comprises more than 10^{14} microorganisms primarily residing in the colon, plays a crucial role in various aspects of human health (Fouhy et al., 2012). With around 3.3 million genes, the gut microbiota possesses a gene count approximately 150 times that of human genes (Qin et al., 2010). It exerts a significant influence on energy balance, immune function, cell proliferation, and brain

function. Disruption of the gut microbiota is associated with the development of cardiometabolic diseases, IBD, mental disorders, and other conditions (Lazar et al., 2019). Short-chain fatty acids (SCFAs), produced during the microbial fermentation of non-digestible fibers, serve as a critical means of communication between the gut microbiota and the host (Canfora et al., 2019). SCFAs, such as acetate, butyrate, and propionate impact the host's energy harvesting, satiety, and energy expenditure through the activation of G-protein-coupled receptors and the stimulation of hormone secretion involved in lipid and glucose metabolism (L. Yu et al., 2021). Butyrate, in particular, serves as a primary energy source for colonocytes, promoting cell proliferation and maintaining the integrity of the gut barrier (Louis & Flint, 2009). The presence of SCFAs-producing bacteria in the gut, such as *Bifidobacterium*, *Roseburia*, *Blautia*, *Akkermansia*, *Faecalibacterium*, and *Bacteroides,* is associated with positive health outcomes (Parada Venegas et al., 2019). Conversely, an abundant of potentially pro-inflammatory and pathogenic genus like *Eubacterium., Ruminococcus*, *Streptococcus*, *and Megasphaera* is often found in individuals with metabolic disorders or IBD (Barathikannan et al., 2019; Palmas et al., 2021; Parada Venegas et al., 2019).

The integrity of the gut barrier is essential for maintaining selective gut permeability and preventing the translocation of pathogenic bacteria (Zuo et al., 2020). This selective permeability is mediated by tight junctions that seal the paracellular spaces between the epithelial cells (Paradis et al., 2021). In metabolic disorders and IBD, the gut permeability increases, the mucosa layer becomes thinner, and the tight junctions are damaged (Rohm et al., 2022). These alterations allow bacterial antigens, lipopolysaccharides, and bacterial metabolites, to freely enter the gut epithelial cells and sub-epithelial tissues, triggering innate and adaptive inflammatory responses. This, in turn, leads to the activation of immune cells and the secretion of inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8 (Yeshi et al., 2020). The chronic inflammatory response observed in IBD and metabolic disorders contribute to the detrimental damage to the gut epithelium and may even stimulate neoplastic processes (Genua et al., 2021).

Thus, mitigating inflammation in the gut epithelium, restoring the integrity of the gut barrier, and favorably modulating the gut microbiota hold promise for the treatment and prevention of chronic cardiometabolic diseases and IBD.

Research has shown the potential benefits of fermented foods in reducing inflammation, combating obesity, and modulating the gut microbiota (Beena Divya et al., 2012). These beneficial properties are attributed to the presence of indigenous bioactive compounds such as dietary fibers and polyphenols, a diverse microbial community (predominantly lactic acid bacteria–LAB), and bioactive metabolites generated during the fermentation process (postbiotics; Shahbazi et al., 2021).

Probiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host have been extensively studied for their health-promoting properties (Hill et al., 2014). These include the production of antimicrobial compounds, anti-inflammation effects, preservation of the gut barrier, and cholesterol assimilation (Sanders et al., 2019) LAB strains exhibiting probiotic properties have been isolated from various sources, including fermented foods (Ghatani et al., 2022; Luo et al., 2011; Todorov et al., 2007).

Poi, a native fermented food in Hawaii, is prepared by cooking, peeling, and mashing taro (*Colocasia esculenta*) corms, which are then mixed with water (Allen & Allen, 1933). Poi is a nutritious starchy food that not only provides starch but also supplies dietary fiber, vitamins, minerals, and bioactive compounds such as anthocyanins, flavonols, flavanols, and carotenoids (Champagne et al., 2011; A. S. Huang et al., 2000). Previous studies using conventional culturing methods have identified LAB species, including *Weissella confusa*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Leuconostoc citreum*, and *Leuconostoc lactis*, and *Enterococcus faecium,* in fresh poi (L. He, 2003; Pirazzini, 2008). However, the probiotic potential of LAB in poi remains underexplored. Furthermore, resistant starch, a type of starch that resists digestion and serves as a substrate for beneficial gut bacteria, has been found in cooked taro. *In vitro* fermentation of taro with human feces has shown alterations in the microbial community structure and increased production of SCFAs (Saxby, 2020). The presence of resistant starch may facilitate bacterial fermentation in poi as well as selectively promote LAB proliferation in the gut. Additionally, considering that fermented poi likely contains not only prebiotics, but also probiotic LAB and microbial metabolites produced during fermentation, we speculated that fermented poi may exhibit more pronounced effects on the gut microbiome, as well as properties similar to probiotics

and postbiotics, such as anti-inflammation and anti-obesity properties, compared with fresh poi. This dissertation aimed to investigate and address these hypotheses.

1.2 Problem statement and objectives

Probiotics have been exploited for their benefits in alleviating and preventing obesity and obesity-related risk factors, inflammation, and overall gut health through the modulation of gut microbiota. However, achieving persistent engraftment of probiotics in the human intestinal tract is challenging due to the inherent resistance of the gut microbiome to colonization by exogenous microorganisms (Schmidt et al., 2018). To overcome this limitation, it has been suggested that probiotic supplementation should be tailored to the individual's specific gut microbiota, considering strains that complement the recipient's baseline microbiota (León Aguilera et al., 2022). Furthermore, combining prebiotics and probiotics as synbiotics has the potential to synergistically enhance their effects on the gut microbiota. This highlights the potential of fermented foods as a promising approach for modulating the gut microbiota. Fermented foods are naturally abundant in LAB, delivering a substantial quantity of commensal microbes to the gut. The food matrix plays a crucial role in preserving the viability of these microbes, ensuring their thriving in the gut to contribute to its health (Marco et al., 2017). Additionally, the co-existing microbial substrates present in fermented foods can be selectively utilized by the probiotics, supporting their proliferation in the gut. Moreover, traditionally fermented foods are generally considered safe for consumption and do not raise safety concerns related to probiotic supplements, such as transfer of antibiotic resistance genes to pathogenic bacteria within the gut (M. Zheng et al., 2017). Therefore, fermented probiotic foods may be more advantageous than probiotic supplements in modulating the gut microbiota.

Poi has been traditionally used for therapeutic purposes, including the treatment of digestive disorders, food allergies, and failure-to-thrive syndrome (Brown & Valiere, 2004). Research has suggested the potential presence of probiotic LAB in poi that exhibit antimicrobial characteristics, bile tolerance, and the ability to produce beneficial enzymes like β-galactosidase (Q. Li, 2015; Pirazzini, 2008). An attempt has also been made to investigate the influence of poi consumption on gut microflora in healthy individuals, though no significant change was observed (Brown, Shovic, et al., 2005).

While these findings hint that poi has probiotic properties, a few questions remained unanswered: (1) what types of LAB are present in fermented poi, do they exhibit probiotic properties, and if so, what specific probiotic properties do they exhibit? (2) Can poi influence gut health and the composition and functionality of gut microbiota, and if so, how does poi affect the gut environment? What additional health benefits does poi provide by influencing the gut, and (3) What role does bacterial fermentation play in contributing to poi's probiotic and health-benefiting properties? Addressing these questions is crucial for bridging the research gaps related to poi's role as a fermented food in promoting gut health. Acquiring such information is invaluable in tailoring and promoting the consumption of fermented foods to improve overall wellness through the modulation of gut microbiota.

This dissertation aimed to address the above research gaps. The specific objectives of this dissertation are as follows (refer to Fig. 1.1 for a scheme of the dissertation framework):

Objective 1: Characterize the microflora profile of fermented poi before and after simulated in vitro digestion and evaluate the probiotic potential of LAB isolated from digested poi**.**

Objective 2: Investigate the effects of poi extracts (both fresh and fermented) on infectious properties of pathogenic bacteria, inflammation, and lipid metabolism using human Caco-2 cells as a model.

Objective 3: Assess the effects of poi (both fresh and fermented) on human gut microbiota, metabolic functional features, and SCFAs production in an in vitro model.

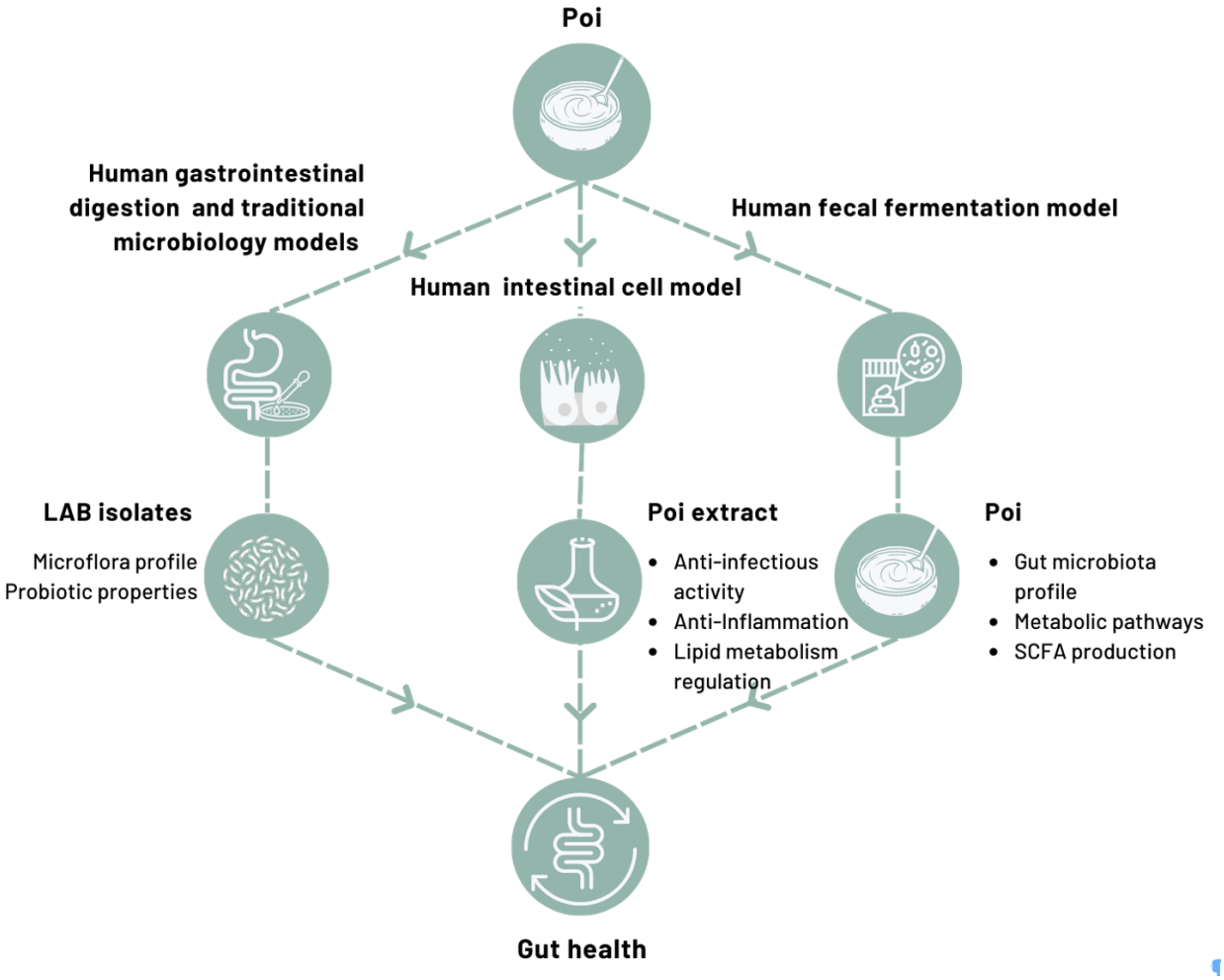

Figure 1.1 Scheme of the dissertation framework.

CHAPTER 2 LITERATURE REVIEW

2.1 Poi and the health benefits of poi

2.1.1 Taro

Taro (*Colocasia esculenta*) is an ancient root crop belonging to the *Araceae* family and has been cultivated across various tropical regions worldwide, including the Caribbean, Hawaii, the Solomons, American Samoa, Western Samoa, the Philippines, Fiji, Sri Lanka, India, Nigeria, Indonesia, New Hebrides, Tonga, Niue, Papua New Guinea, and Egypt (Maga, 1992). Its cultivation spans many centuries, with the earliest recorded dating back to 100 B.C. in China, and it has played a significant role in feeding approximately 10% of the global population (Maga, 1992; Whitney et al., 1939). In Hawaii, taro, known as “kalo” in Hawaiian, held a prominent position as the major staple food before the arrival of Captain Cook (Allen & Allen, 1933). Although no clear record exists, it is believed that vast expanses of land across the Hawaiian islands were dedicated to taro cultivation, sustaining an early population of 300,000 (Whitney et al., 1939). Taro cultivation in Hawaii involved remarkable engineering techniques, such as sophisticated systems of canals, locks, and ditches carved into stone cliffs, as well as underground water channels, all designed to address the crucial need for water in taro cultivation (Greenwell, 1947). Furthermore, taro holds immense cultural significance among native Hawaiians, as it is integrated in legends about Hawaiian gods, the origins of the Hawaiian people, and is utilized for ritual, ceremonial, and medicinal purposes (Cho et al., 2007). However, taro production has experienced a significant decline over time. From over 14 million pounds in 1948, taro production decreased to 6.8 million pounds in 2000 (Martin, 2001). The downward trend has persisted, with the total taro production in Hawaii in 2021 amounting to 4.8 million pounds (Hawaii Department of Agriculture, 2022). In Hawaii, two primary cultivation methods are practiced: lo’i culture or wetland culture, and dry culture or upland culture. In lo’i culture, taro is grown in flooded lands, predominantly producing varieties preferred for poi-making, such as the Lehua variety. In contrast, dry culture involves cultivating taro in irrigated lands near forests, where the soil is fertile and rainfall is abundant, resulting in varieties suitable for chip production, like the Chinese Bung-long variety (Greenwell, 1947; A. S. Huang et

al., 2000). Throughout history, over 300 Hawaiian taro variety names have been listed, representing approximately 170-175 distinct forms, with only 74 varieties currently preserved at the Waimānalo Research Station at the University of Hawaii (Whitney et al., 1939).

2.1.2 Nutrient composition and health benefits of taro

2.1.2.1 Macro-and micronutrients. Taro is a starchy food that offers various nutritional benefits. In a study by Allen & Allen (1933), the macronutrients of taro were compared with dasheen, potato, and sweet potato. Results showed that taro contained 18.2% (g/100 wet weight) starch, surpassing potato (14.7%) but lower than dasheen (21.8%) and sweet potato (20.2%). Taro also had higher total fiber content of 0.8% compared to dasheen (0.7%) and potato (0.4%). Additionally, taro exhibited 1.17% total ash and 2.0% protein, ranking second highest among the four crops examined. A 100 g serving of taro corm provided 95.8 calories, while potato, dasheen, and sweet potato provided 85.3, 129, and 125.1 calories per 100 g, respectively. These findings demonstrated that taro is relatively more nutrient-dense, offering greater fiber and mineral content while providing a lower calorie count compared to starchy root crops. It is worth noting that the nutrient composition can vary significantly between different taro varieties (Aboubakar et al., 2008; Derstine & Rada, 1952; A. S. Huang et al., 2000; Saxby, 2020). For instance, a study by Huang et al. (2000) compared the nutrient compositions of a wetland taro variety called Lehua and a dryland taro variety called Bun-Long. The fiber contents of these two taro verities were 3.6 and 3.8% (g/100g dry weight), respectively. Based on this calculation, the authors believed that when taro was consumed as a staple food by the Hawaiians, with an estimation of 1 lb per day, the fiber provided by taro reached the daily recommended amount of 25 g. A more recent study examined the nutrient and resistant starch contents of five taro varieties, including Bun-Long (dryland), Mana Ulu (dryland), Moi (dryland & wetland), Kauaʻi Lehua (wetland), and Tahitian (dryland) (Saxby, 2020). The total starch in Bun-Long, Moi, and Tahitian were 38.9, 34.1, and 40.8 % (g/100 g dry weight), respectively, while Mana Ulu and Lehua only contained 19.8 and 17.7% starch, respectively. The Hawaiian taro varieties exhibited resistant starch, a type of dietary fiber, ranging from 5.27 to 8.10%.

Variations in macro- and micronutrients were also observed among different taro varieties. Taro is a rich source of vitamins and minerals, including thiamin, riboflavin, niacin, vitamin C, phosphorus, potassium, calcium, magnesium, iron, and zinc (A. S. Huang et al., 2000; Saxby, 2020). Fresh poi, a commonly cooked form of taro, was found to contain more calcium, iron, vitamin A, thiamin, and vitamin C than both white rice and brown rice, as well as more phosphorus than white rice (Derstine & Rada, 1952).

2.1.2.2 Bioactive compounds. Taro corms and leaves contain various bioactive compounds that contribute to their potential health benefits. Studies have identified anthocyanins, flavonols, flavanols, and carotenoids in taro corm (Champagne et al., 2011; Ghan et al., 1977; Senga, 2021). Taro exhibited the highest amount of flavonols (up to 326.7 mg QGE/100 g dry weight) among other tropical root crops like yam and sweet potato (Champagne et al., 2011). Furthermore, 20 flavones, including glycosylated forms of apigenin, luteolin, and chrysoeriol have been identified in taro corms, with orange-flesh corms being particularly rich in β-carotene (Muñoz-Cuervo et al., 2016). It is important to note that cooking significantly reduces the total anthocyanin content in Hawaiian taro varieties, resulting in a decrease from 6.93–10.02 mg/g to 2.12–3.19 mg/g (Senga, 2021). Additionally, carotenoids such as β-carotene and lutein were completely lost after cooking except in the Mana Ulu variety (Senga, 2021). Taro leaves also contain a range of bioactive compounds, including orientin, vitexin, isovitexin, caffeic acid, p-coumaric acid, chrysoeriol derivatives, apigenin derivatives, luteolin derivatives, and isoorientin (Mitharwal et al., 2022). Moreover, taro exhibits other bioactive compounds such as tarin, taro-4-I polysaccharide, taro polysaccharides 1 and 2 (TPS-1 and TPS-2), A-1/B-2 α-amylase inhibitors, monogalactosyldiacylglycerols, digalactosyldiacylglycerols, polyphenols, and nonphenolic antioxidants, all of which contributed to the anticancer and immunomodulatory activities of taro (Ribeiro Pereira et al., 2021).

2.1.2.3 Antinutritional compounds. Raw taro contains antinutritional compounds, namely oxalate and phytic acids. Calcium oxalate crystals found in taro have needle-like structures that can irritate the skin and cause a burning sensation (Maga, 1992;

Pirazzini, 2008). Consumption of large amounts of oxalates can lead to lithiasis in the urinary system and hinder the absorption of minerals. Taro corms contain calcium oxalate ranging from 71 to 144 mg/100 g, and phytic acid ranging from 142 to 169 mg/100g (C.-C. Huang et al., 2007). These values were typically similar to those found in yam and cassava (C.-C. Huang et al., 2007). Insoluble oxalates are believed not be absorbed by the intestines (Catherwood et al., 2007). Cooking methods like soaking, boiling, and steaming can effectively reduce soluble oxalates in taro to an undetectable level, as these oxalates leach into the water during the process (Catherwood et al., 2007). Consequently, the oxalate level decreases after poi preparation (Brown et al., 2016).

2.1.3 Health benefits of taro

Taro has a long history of traditional use for therapeutic purposes in various cultures. It has been utilized for wound healing in India, as a tonic and treatment gastrointestinal disorders in China, during childbirth in the Philippines and Malaysia, and for complimentary food and healing purposes in Hawaii (Onwueme, 1999; Prajapati et al., 2011; Saxby, 2020). Notably, Polynesian populations, who incorporate taro as a significant component of their traditional diets, exhibit a lower incidence of colorectal cancer compared to Caucasian populations (Ferguson et al., 1992). This suggested that taro possesses anticancer, antimetastatic, and antimutagenic properties (Ribeiro Pereira et al., 2021). Studies have demonstrated the therapeutic potential of taro in cancer management. A water-soluble extract of taro was found to inhibit lung colonizing ability and spontaneous metastasis in a murine model with highly metastatic mammary gland-implanted tumors (Kundu et al., 2012). Additionally, taro-derived extracts have been shown to inhibit breast cancer stem cells, suppress tumor cell migration and metastasis through immune T-cell-dependent mechanisms (Kundu et al., 2021).

Moreover, bioactive compounds such as polyphenols in taro are antioxidants that can reduce oxidative stress and related inflammation in the body (Ribeiro Pereira et al., 2021).

Taro exhibits antimicrobial properties. Methanolic extracts from taro leaves have demonstrated effectiveness against pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Candida*

albicans, which may have contributed to the wound healing properties of taro leaves (Al-Kaf et al., 2019).

Bioactive compounds found in taro have been reported to have immunomodulation functions. For instance, a hemagglutinin (protein) isolated from taro tubers induced expression of the cytokines interleukin-1β (IL-1 β), IL-2, interferon-γ (IFN- γ) and tumor necrosis factor-α (TNF-α) in murine splenocytes (Chan et al., 2010). Two novel polysaccharides TPS-1 and TPS-2, isolated from taro, were found to promote the production of nitric oxide, TNF-α, and IL-6 in RAW 264.7 cells (H. Li et al., 2018).

Additionally, taro extracts have shown Inhibitory effects on pancreatic lipase, alpha-amylase, alpha-glucosidase, and human lanosterol synthase, as well as improved glucose tolerance and serum lipid levels (Ribeiro Pereira et al., 2021). These effects have been attributed to the presence of polyphenols, proteins, lipids, and polysaccharides in taro.

Furthermore, taro contains bioactive compounds and polysaccharides, such as resistant starch, that can modulate human gut microbiota, thereby influencing immune function and supporting the hyperglycemic and hypercholesterolemic effects of taro. In terms of prebiotic potential, taro has been found to exhibit comparable or higher prebiotic scores when paired with probiotic strains *L. plantarum*, *L. paracasei*, *L. rhamnosus*, and *L. acidophilus* compared with known prebiotics inulin and fructooligosaccharide (FOS) (Saxby, 2020). In addition, in vitro fecal fermentation of taro has been shown to significantly increase SCFA production and microbiome diversity, surpassing the effects of control and Inulin or FOS treatments (Saxby, 2020).

2.1.4 Poi

Taro is predominantly consumed in the form of poi in Hawaii. Poi hold great cultural significance and was a vital provision during The Hawaiian voyages due to its nutritional value and extended shelf life (Allen & Allen, 1933). It embodies important Hawaiian core cultural values, such as fostering strong familial relationships (`ohana) and honoring ancestors (`aumakua) (Brown et al., 2016). Commercial poi production involves steaming unpeeled taro corms, peeling and grinding the cooked taro, and blending it with water (Allen & Allen, 1933). Sour poi is created by allowing fresh poi to ferment at room temperature for a day or longer (Allen & Allen, 1933). Commercial poi

products come in various forms, including fresh poi, frozen poi, and poi powder. It is essential for commercially available poi to contain no less than 28% total solids, while ready-mixed poi should have no less than 15% total solids (Hawaii Department of Health, 2023). Poi itself possesses a mild flavor profile with subtle sweetness or sourness, and it is commonly enjoyed with meats or fish (Derstine & Rada, 1952).

2.1.5 Fermentation process of poi

2.1.5.1 Chemical process. Freshly made poi undergoes a natural fermentation process at room temperature. This fermentation is facilitated by lactic acid bacteria (LAB) present in the soil, taro, and the production environment (Brown & Valiere, 2004). During fermentation, the pH of poi decreases from neutral to approximately 5.0 within the first 24 h and gradually reduces further to around 4.0 (A. S. Huang et al., 1994). The fermentation process was chemically studied the earliest by Bilger and Young (Bilger & Young, 1935) who observed a rapid decrease in reducing sugars within the first 12 h, followed by stable levels. The starch content of poi (27%) decreased during the 2-8 days of fermentation. LAB in the fermentation process produced lactic acid, acetic acid, formic acid, alcohol, acetaldehyde, and carbon dioxide. Huang et al. (1994) found a decrease in sucrose but an increase in glucose during fermentation. They identified increased lactic acid and acetic acid levels, as well as decreased succinic acid and oxalic acid.

2.1.5.2 Microbiological process. The microbiological aspects of poi fermentation were investigated by Allen & Allen (1933) in their pioneering study. They described the fermentation process as a three-stage progression, starting with a diverse flora stage after corm crushing and water addition, followed by a stage dominated by acid-producing bacteria within six hours to three or four days of fermentation, and finally, a stage where yeasts and mold become more prevalent between five and six days of fermentation. Allen & Allen (1933) found similar quality and microorganisms in poi samples obtained from 13 factories. They identified three *Lactobacillus* species (*L. pastorianus*, *L. delbrucki*, and *L. pentoaceticus*) and two *Lactococcus* species (*L. lactis* and *L. kefir*) in fermented poi. Huang et al., (1994) identified *Lactococcus lactis* which

accounted for up to 85% of the total aerobic count (9.0 log CFU/g) in five-day fermented poi. He (2003) isolated *Weissella confusa*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Leuconostoc citreum*, and *Leuconostoc lactis* from fresh poi through phenotypical and genotypical analysis. Sour poi, on the other hand, was found predominantly to contain *Lactobacillus plantarum*. In a more recent study, Pirazzini (2008) used temperature gradient gel electrophoresis (culture-independent) and random amplified polymorphic DNA-PCR (RAPD-PCR; culture-dependent) to profile LAB isolated from four commercial poi brands. The study revealed 21 possible species using culture-independent methods and 19 unique isolates belonging to 10 species using culture-dependent methods. The most prevalent LAB species identified in poi were *Leuconostoc lactis*, *Weissella confusa*, *Enterococcus faecium*, and *Lactococcus lactis*. Pirazzini (2008) also found promising probiotic properties in the isolated LAB, including tolerance to low pH and bile salts, β- and α-galactosidase activities, and varying degrees of antibiotic resistance.

2.1.6 Limited survival of pathogens in poi

Although the available research on this topic may be outdated, it offers valuable insights into the potential antimicrobial properties of poi. Initial studies by Allen & Allen (1933) found that certain microorganisms, including *E. coli*, *Aerobacter aerogenes*, *Pseudomonas fluorescens*, chromogenic bacteria, and spore-forming rods, were present in poi at the beginning of fermentation but persisted for only 3 to 4 hours. Fung & Bushnell (Fung & Bushnell, 1948) observed that pathogenic microorganisms such as *E. coli*, *Eberthella typhosa*, *Salmonella schottmuelleri*, and *Shigella paradysenteriae* rapidly disappeared within about 3 days in poi kept at room temperature. However, when refrigerated at 13°C, these organisms persisted for up to 8 days. The authors suggested that fresh or refrigerated poi posed a higher potential risk from a microbiological safety standpoint compared to sour poi. Moreover, *Tubercle bacilli* (human tuberculosis bacteria) in fresh poi disappeared within 4 days when stored at room temperature, and enteric pathogens like *Salmonella* or *Shigella* were not detected among 100 poi samples (Ichiriu, 1949). In addition, taro corm has been found to serve as a suitable medium for the production of an antimicrobial bacterial protein called diplococcin when inoculated with its producer, *Streptococcus* sp. ATCC10035 (Muller et

al., 2002). The supernatant filtrate of a LAB strain belonging to *Leuconostoc mesenteroides*, isolated from fermented cooked taro skin, exhibited inhibitory effects against pathogenic bacteria such as *S.* Typhimurium and *Listeria monocytogenes*, suggesting the presence of antimicrobial-compound-producing bacteria in fermented poi (Yoshioka et al., 2015).

2.1.7 Nutrition aspects of poi

Poi, being made from taro, primarily consists of starch. Fresh poi, which contains approximately 71.6% water, is composed of 27% (g/100 g) starch and has a minimal protein content of 0.38% (USDA, 2023). The fiber content of fresh poi is 0.4% (USDA, 2023). Poi is rich in various vitamins and minerals such as calcium, iron, magnesium, potassium, phosphate, thiamine, vitamin A, vitamin C, and choline, etc. (USDA, 2023). The presence of small and easily digestible starch granules in poi, coupled with its low protein content, makes it a low-allergenic staple food alternative (Brown et al., 2006). Traditionally, poi has been used as a medicinal food for treating infants with food allergies or failure-to-thrive, as well as addressing digestive disorders like celiac disease and supporting cancer patients (Brown & Valiere, 2004; Roth et al., 1967). Even today, poi remains a popular choice as a first complementary food for infants among Native Hawaiians, other Pacific Islanders, and Filipinos in Hawaii (Mulville et al., 2022). Caregivers perceive poi to be ‘healthy’ and ‘hypoallergenic’ for infants.

2.1.8 Health aspects of poi

In vitro and clinical studies have been done to determine poi’s health effects. Poi soluble extracts have shown antiproliferative activity against the rat colon cancer cells by inducing apoptosis (Brown, Reitzenstein, et al., 2005). In contrast, poi extracts enhanced the growth of normal mouse splenocytes that were further identified to be mostly T cells, some B cells, and some Natural Killer cells (Brown, Reitzenstein, et al., 2005). This suggested poi extracts stimulated the immune system and activated lymphocytes by acting as mitogens. The anti-cancer properties of poi have found support in epidemiological studies, considering its role as a significant component of the traditional Hawaiian diet alongside other starchy vegetables (e.g., sweet potato, yams, breadfruit), greens, fruit, seaweed, fish, and chicken (Shintani et al., 1991). Notably, the

incidence of colorectal cancer is notably lower among Native Hawaiians compared to other ethnic groups except for the Chinese population in Hawaii (Tsark, 1998). In another study, Brown, Shovic, et al. (2005) explored the potential of poi to change human gut microflora as a non-dairy probiotic food. It was a cross-over clinical study where the participants were given fresh poi three times a day for four weeks. However, the study did not find significant changes in the total bacterial count or the concentration of individual bacterial species for *Escherichia coli*, *Enterobacter*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*. The study acknowledged the limitations of the microbiological methods used and the relatively short intervention period. Further research is warranted to comprehensively investigate the probiotic potential of poi, considering possible variations between fresh and sour poi. While evidence suggests that poi contains beneficial lactic acid bacteria (LAB) with probiotic properties, a systematic study on poi as a probiotic food is essential to provide comprehensive insights into its health benefits.

2.2 Gut microbiota

2.2.1 Introduction of gut microbiota

Human gut microbiota is a diverse community of microorganisms residing in the human gastrointestinal tract, consisting of approximately 100 trillion members encompassing 1,000–5,000 different species (Sender et al., 2016). The collective genetic material of the gut microbiota, known as gut microbiome, contains around 3.3 million, surpassing the human genome by 150-fold (Qin et al., 2010). The gut microbiota plays numerous essential roles in the host, including the synthesis of vital vitamins and energy supply, protection against pathogens, maintenance of the intestinal barrier integrity, production of metabolites such as SCFAs, tryptophan, and trimethylamine (TMA) that regulate various host functions, modulation of brain functions through the gut-brain axis, and even influence on genes related to fat accumulation in adipocytes (Eroğlu & Sanlier, 2022; Icaza-Chávez, 2013; Lazar et al., 2019).

The composition of gut microbiota varies greatly among individuals, even in healthy individuals, with Firmicutes (60%), Bacteroidetes (> 10%), Proteobacteria (> 1%), and Actinobacteria (> 10%) being the predominant phyla, with Firmicutes and

Bacteroidetes being the most abundant (de Vos & de Vos, 2012; Huttenhower et al., 2012). Several factors influence the composition of the gut microbiota, including host-intrinsic factors such as host genetics, sex, innate and adaptive immunity, and BMI; hot-extrinsic factors such as physical activity, cultural habits, diet, and medication; microbial intrinsic factors such as disease state, age-dependent state, compositional state, founder effects, and stochastic effects; and environmental factors such as regional strain pools, household and family, vertical transmission (maternal) and local environment (Schmidt et al., 2018). Understanding the intricate interplay between these factors and the gut microbiota holds the potential for targeted modulation of gut microbiota.

Diet interventions represent one strategy for modulating the gut microbiome, as diet can induce rapid changes in its composition. Short-term consumption (5 consecutive days) of diets exclusively consisting of animal or plant products has been shown to alter microbial community structure, SCFA production, and override inter-individual differences in gut microbiome (David et al., 2014). In David's study, an animal-based diet increased the abundance of species with high bile tolerance, such as *Alistioes*, *Bilophila*, and *Bacteroides*, while decreasing the abundance of species involved the metabolism of plant polysaccharides, including *Roseburia*, *Eubacterium rectale*, and *Ruminococcus bromii.*

2.2.2 Metabolites produced by the gut microbiota and their roles in energy and immune homeostasis

2.1.2.1 SCFAs. SCFAs are crucial products of gut microbiota fermentation of complex carbohydrates and play a significant role in regulating host energy metabolism, lipogenesis, hormone production, and immune function. SCFAs consist of fatty acids with fewer than six carbons, including formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4), and valeric acid (C5) (M. Sun et al., 2017). Among them, acetic acid, propionic acid, and butyric acid accounted for 95% of the gut SCFAs (M. Sun et al., 2017). In the gut, the molar ratio of acetic acid:propionic acid:butyric acid is approximately 3:1:1 (Parada Venegas et al., 2019). Firmicutes are the main producer of butyric acid and Bacteroidetes are the main producers of acetic acid and propionic acid

(Louis & Flint, 2009). Butyric acid and propionic acid are generated through glycolysis and organic acids and amino acids metabolism, while acetic acid is predominantly derived from acetyl-CoA derived via glycolysis (Parada Venegas et al., 2019).

In the gut, SCFAs can activate G-protein-coupled receptors (GPCRs), such as GPR41, GPR43, and GPR109a (M. Sun et al., 2017). Activation of these GPCRs, on one hand, stimulates enteroendocrine L cells to secrete hormones, such as peptide YY (PYY) and glucagon-like peptide 1 (GLP1), which regulates energy uptake and Vagus-nerve-mediated appetite control; on the other hand, triggers a range of regulatory activities, including the secretion of IL-18 and antimicrobial compounds by enterocytes, increased expression of tight junction protein, promotion of enterocytes proliferation and repair, modulation of almost all immune cells and the expression of pro- and anti-inflammatory cytokines (Canfora et al., 2019; Parada Venegas et al., 2019). SCFAs can also enter the gut barrier through passive diffusion and Na^+-coupled high-affinity transporter channels, particularly for butyrate (M. Sun et al., 2017). Once inside, SCFAs directly stimulate adipocytes to secrete the satiety hormone leptin, induce thermogenesis in adipose tissue, and promote adipose tissue browning (Canfora et al., 2019). SCFAs, particularly butyrate, can also directly inhibit the histone deacetylases (HDACs), which regulate T cell functions, macrophage production of pro-inflammatory cytokines, and IgA production of B cells (M. Sun et al., 2017). Furthermore, butyrate has anti-tumorigenic property through inhibition of HDACs, which activates histone acetylation, promotes tumor cell apoptosis, and prevents tumor cell proliferation (Garrett, 2015; Parada Venegas et al., 2019). Butyrate, as the main energy source for colonocytes, plays a crucial role in promoting the function of the epithelial gut barrier by stabilizing the hypoxia-inducible factor (a transcription factor coordinating barrier protection), inducing tight junction protein expression, and promoting the production of antimicrobial peptides by enterocytes (Parada Venegas et al., 2019). In addition, SCFAs are involved in gut-brain communications and have protective effects against neuroinflammation, alterations in brain function, and depressive and anxious behaviors caused by lipopolysaccharide (Eroğlu & Sanlier, 2022).

2.1.2.2 Other metabolites and molecules. Apart from producing vitamin K and vitamin B components, gut microbiota also metabolizes choline, carnitine, and betaine,

resulting in the formation of trimethylamine (TMA), which is further converted to trimethylamine-N-oxide (TMAO) in the liver (Lazar et al., 2019; J. Liu et al., 2022). Elevated circulating levels of TMAO have been associated with an increased risk of cardiovascular disease and mortality, while higher levels of its precursor betaine have been associated with type 2 diabetes mellitus (T2DM) (Heianza & Qi, 2017). Microbial metabolism of tryptophan leads to the production of tryptamine, skatole, indole, and indole derivatives, many of which serve as ligands of the aryl hydrocarbon receptor (AhR). AhR is a protein involved in organogenesis, the immune function of epithelial cells, and social behavior (J. Liu et al., 2022). Furthermore, gut microbiota produces a wide range of neurotransmitters, such as dopamine, norepinephrine and epinephrine, serotonin (5-hydroxytryptamine), and γ-aminobutyric acid which can influence host brain functions through the brain-gut axis (Lazar et al., 2019). Additionally, the gut microbiota is well-known for its ability to produce secondary bile acids by modifying primary bile acids synthesized by the liver. These secondary bile acids bind to specific receptors like G-protein-coupled bile acid receptor 1 (TGR5), the bile acid receptor farnesoid X receptor (FXR), and Liver X receptor (LXR), influencing cholesterol and lipid metabolisms as well as immune functions in the intestines, adipose tissues, and the liver (Bilotta et al., 2020; Ding et al., 2015). Patients with obesity and diabetes have been found to exhibit high levels of bacterial lipopolysaccharides (LPS), which may promote inflammatory response in the intestinal epithelial cells by activating Toll-like receptor 4 (TLR4) (Creely et al., 2007). Conversely, conjugated linoleic acid (CLA) has been shown to have anti-obesity effects by increasing energy expenditure, reducing adipogenesis and lipogenesis, and promoting lipolysis and adipocyte apoptosis (Gomes et al., 2018). The beneficial effects of CLA may be attributed to its ability to displace arachidonic acid from cell membrane phospholipids, leading to the reduction of inflammation, as well as its mediation of peroxisome proliferator-activated receptors (PPARs), which regulate cellular lipid metabolism, apoptosis, and immune function (Belury, 2002). Interestingly, individuals who regularly consume fermented foods have been found to have significantly higher levels of CLA and linoleic acids compared to those who do not (Taylor et al., 2020).

2.2.3 Gut microbiota and metabolic disorders

The human gut microbiota is established at birth and undergoes development during the first three years of life (Z. Xu & Knight, 2015). The diversity and community of gut microbiota changes with age and varies among individuals (Z. Xu & Knight, 2015). This diversity encompasses the taxonomy levels and the various functions the gut microbiota performs, allowing it to be resilient to normal stressors and to recover from unstable states (Lozupone et al., 2012). However, in the presence of acute disturbance such as antibiotic usage or persistent stressors like unhealthy diet, the gut microbiota may degrade to a secondary state. In this compromised state, the number of species may diminish, microbial metabolism may become simplified, and inflammatory response may be insensitive, resulting in resistance to colonization by beneficial bacteria (Lozupone et al., 2012). Disruptions in gut microbiota homeostasis and low microbial diversity are commonly observed in chronic diseases, including metabolic disorders such as non-alcoholic fatty liver diseases, obesity, and T2DM. These conditions are often associated with gut dysbiosis, which is accompanied by imbalanced in energy regulation, liver lipid accumulation, decreased adenosine 5′-monophosphate-activated protein kinase (AMPK) levels and β-oxidation in muscular tissues, inhibition of fasting-induced adipose factor (FIAF) promoting triglyceride deposition in the adipocytes, impaired SCFA production, disrupted hunger cues, and systemic inflammation (Lazar et al., 2019; Magne et al., 2020).

Firmicutes/Bacteroidetes ratio has been widely studied in relation to obesity, with an increased ratio often associated with the condition. The phylum *Firmicutes* has been shown to be negatively related with the resting energy expenditure and positively associated with fat mass percentage (Kocełak et al., 2013). *Firmicutes* are believed to be more efficient in extracting energy from food than *Bacteroidetes*, leading to enhanced energy absorption by the host (Krajmalnik-Brown et al., 2012). In obese and overweight Italian adults, *Bacteroidetes* were negatively correlated with body fatness and waist circumference, while *Firmicutes* showed positive associations with body fatness but negative correlations with muscle mass and physical activity levels (Palmas et al., 2021). Obese mice also demonstrated a 50% reduction in *Bacteroidetes* abundance and a proportional increase in *Firmicutes* compared to lean mice (Ley et al.,

2005). However, there is ongoing controversy surrounding the *Firmicutes*/*Bacteroidetes* ratio in obesity, as some studies have not consistently observed increased *Firmicutes/Bacteroidetes* levels in obese individuals (Magne et al., 2020). It is worth noting that the alterations at the family, genus, or species level within the gut microbiota may have more relevance than the *Firmicutes*/*Bacteroidetes* ratio (Magne et al., 2020). Increased abundance of the genus *Clostridium* and the species *Eubacterium rectale*, *Clostridium coccoides*, *Lactobacillus reuteri*, *Clostridium histolyticum*, and *Staphylococcus aureus* have been observed in obese subjects (Gomes et al., 2018). The higher abundance of gram-negative bacteria has been proposed as a potential explanation for the increase in serum endotoxin and low-grade systemic inflammation seen in obesity and T2DM (Gomes et al., 2018; Lau et al., 2021). In T2DM, reduced gut microbial diversity, along with decreased *Bifidobacterium*, *Firmicutes*, and *Clostridia*, and increased *Betaproteobacteria* have been observed (Lau et al., 2021). Genera *Akkermansia* and *Blautia* were found to be decreased in newly diagnosed diabetes patients but were restored after diabetic treatments. The consistent observation of depletion in *A. muciniphila* under conditions such as obesity, T2DM, and other metabolic disorders has illuminated the potential therapeutic applications of this bacterium in combatting obesity (Y. Xu et al., 2020). Additionally, *Sutterella* was found to be decreased in known diabetic patients undergoing treatment and was identified as a common driver taxon among newly diagnosed diabetics, known diabetics, and healthy subjects (Gaike et al., 2020).

2.2.4 Gut microbiota and inflammatory bowel diseases

2.2.4.1 Inflammatory bowel diseases (IBD). IBD encompasses ulcerative colitis (UC) and Crohn's disease (CD). While both UC and CD share similar symptoms, such as diarrhea, abdominal pain, fatigue, and loss of appetite and weight, as well as similar pathological features such like dysbiosis, impaired mucus layer, disrupted tight junction, and relapsing intestinal inflammation, they can be distinguished by their disease location and inflammation pathogenesis (Yeshi et al., 2020). UC primarily affects the colonic mucosa, whereas CD can manifest in any part of the gastrointestinal (GI) tract (Y.-Z. Zhang & Li, 2014). In UC, disruption of the intestinal epithelial barrier leads to the

activation of antigen presenting cells (APC), such as macrophages and dendritic cells, by gut commensal bacteria through TLRs. These activated APCs release pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12, IL-23, and TNF-α, which signal naïve CD4+ T cells to differentiate into Th2 and Th9 cells. Th2 cells release additional pro-inflammatory cytokines, such as TNF-α, IL-5, and IL-13. UC is considered a Th2-mediated immune disorder, and is characterized by the release of IL-9 by Th9 cells, which hinders mucosal wound healing. Furthermore, TNF- α and IL-1 activate nuclear factor κB (NF-κB) pathway, promoting the expression of pro-inflammatory and cell survival genes. Inflammatory chemokines, like IL-8, are upregulated, leading to the recruitment of circulating leucocytes and naïve T cells to the inflammatory, thereby exacerbating inflammation (Yeshi et al., 2020). On the other hand, CD is characterized as a Th1 (activated by IL-12) and Th17 (activated by IL-23) cell-mediated immune disorder, with elevated levels of IL-17 and IL-23 (Yeshi et al., 2020). However, precise etiology of IBD remains unclear, and effective non-invasive diagnosis and effective treatment strategies are limited (X. Guo et al., 2022).

2.2.4.2 Gut microbiota and IBD. Gut microbiota can serve as potential biomarkers in IBD. Depletion of gut butyrate-producing bacteria in the gut is commonly observed in patients with IBD (Parada Venegas et al., 2019). Many of these bacteria belong to the phylum *Firmicutes*, resulting in lower levels of *Firmicutes* in individuals with IBD compared to healthy individuals. Conversely, higher levels of *Actinobacteria* and *Proteobacteria* are often found in IBD patients (X. Guo et al., 2022). Notably, *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* are considered as hallmarks of active IBD. Reduced levels of *Akkermansia* have been consistently found in patients with IBD and other metabolic disorders. Genera *Akkermansia* and *Dorea* have been identified as causally associated with IBD (Z.-J. Zhang et al., 2021). *F. prausnitzii*, a well- known butyrate-producing bacterium, is negatively associated with IBD (X. Guo et al., 2022). The abundance of *F. prausnitzii* was significantly lower in patients with CD compared to healthy controls, and this decrease was accompanied by an increase in *E. coli* in active CD patients (Lopez-Siles et al., 2014). Moreover, other SCFA-producing bacteria, including *Roseburia inulinivorans, Bifidobacterium adolescentis*, *Dialister invisus*, *Blautia faecis, Clostridium lavalense*, and *Bacteroides uniformis* were reduced

in CD patients (Parada Venegas et al., 2019). In active UC, *Klebsiella*, *Enterococcus*, and *Haemophilus* were found to be significantly higher compared to individuals in remission, while *Roseburia*, *Lachnospira*, *Blautia*, and *Faecalibacterium* were enriched during remission (X. Guo et al., 2022). The level of *Bacteroides* was significantly lower in IBD patients compared to normal controls, and this reduction was even more pronounced in individuals with active IBD compared to those in remission (Zhou & Zhi, 2016).

2.2.5 Gut barrier integrity and dysbiosis

The intestinal barrier is a complex and dynamic system that serves as the first chemical and physical barrier to foreign chemicals and microbial antigens. The intestinal barrier consists of a single layer of epithelium cells joined by the tight junction (TJ) complexes and a protective mucus layer containing antimicrobial compounds, IgA, and commensal bacteria (Genua et al., 2021). The intestinal epithelial cells and the underlying lamina propria also contribute to the innate and adaptive immune responses, proving additional defense against antigens (Rohm et al., 2022). The TJ complex tightly seals the gaps between epithelial cells and regulates the permeability of the intestinal barrier, selectively allowing the passage of necessary molecules while preventing the entry of harmful substances from the gut lumen into the subepithelial tissues (Zuo et al., 2020). The TJ complex is composed of three families of transmembrane proteins including the claudin family, the Marvel domain-containing family (occludin, tricellulin, and MarvelD3), and the immunoglobulin superfamily (JAM, JAR). The transmembrane proteins are connected to the cytoskeleton by scaffold proteins such as zonula occludens 1 to 3, the cingulin and cingulin-like proteins, and the afadine, all of which work collectively or individually to regulate barrier permeability, mucosal immune function, and epithelium repairing (Paradis et al., 2021). Disruption of the intestinal barrier can result in bacterial translocation, entry of pro-inflammatory endotoxins such as LPS, localized inflammation, and the production of reactive oxygen species (ROS) (Genua et al., 2021). Increased ROS release can further damage the epithelial cells and contribute to neoplastic processes (Genua et al., 2021). On one hand, dysbiosis could result in a damaged gut barrier due to increased gut barrier permeability, microbial production of LPS, and pathogen invasion. For instance, in a high-fat diet (HFD),

excessive fatty acids and bile acids in the gut lumen can increase paracellular permeability by activating myosin light chain kinase and inducing conformational changes in TJ proteins (Usuda et al., 2021). Additionally, the enrichment of LPS-producing bacteria can trigger inflammation through TLR4, provoking further damage to the gut barrier (Usuda et al., 2021). Pathogenic bacteria can also compromise the intestinal barrier through mechanisms such as direct lytic or binding on TJ proteins to interfere with their connection to the TJ complex, activation of related signaling pathways in the epithelial cell leading to increasing the permeability, and pathogen-induced excessive pro-inflammatory cytokine and nitric oxide production (Paradis et al., 2021). Conversely, alterations of epithelial immune function can disrupt gut barrier function and contribute to dysbiosis. For example, immune cells producing IFN-γ and IL-1β have been found to increase gut barrier permeability in obesity, while IL-22- and IL-17-secreting intestinal group 3 innate lymphoid cells important for barrier integrity are reduced (Rohm et al., 2022). Impairment of autophagy in intestinal epithelial cells has been associated with an enrichment of pro-inflammatory and potentially pathogenic bacteria, including *Candidatus Arthromitus* and the *Pasteurellaceae* family, while bacteria associated with the control of inflammation, such as *Akkermansia muciniphila* and the *Lachnospiraceae* family were depleted (L. Yang et al., 2018).

2.3 Strategies to modulate gut microbiota

2.3.1 Prebiotics

2.3.1.1 Definition. Prebiotics are defined as ‘‘substrates that are selectively utilized by host microorganisms conferring a health benefit’’ (Gibson et al., 2017). Prebiotics are used to confer a selective advantage of beneficial microbiota (Schmidt et al., 2018). Prebiotics are typically fibers that are resistant to gastric acidity, enzymatic hydrolysis, and absorption in the upper gastrointestinal tract. Instead, they are fermented by the intestinal microflora, selectively stimulating the growth and activity of beneficial bacteria associated with health and well-being” (Slavin, 2013). Although bifidogenic, non-digestible oligosaccharides such as inulin and galactooligosaccharides are well-known

prebiotics that meet these criteria, prebiotics encompass a broader range of substances found in various foods (Pandey et al., 2015).

2.3.1.2 Health benefits. The health benefits of prebiotics include but are not limited to:

- alleviating infection and antibiotic-induced diarrhea.
- Reducing the inflammation and improving symptoms associated with IBD.
- Preventing colorectal cancer.
- Enhancing the bioavailability of minerals.
- Lowering the risk of cardiovascular disease.
- Reducing postprandial glucose and insulin concentrations.
- Promoting satiety and weight loss (Brownawell et al., 2012; Kellow et al., 2014).

The fermentation of prebiotics by gut microbiota leads to increased microbial diversity, changes in gut microbiota composition, and elevated production of SCFAs (Ho et al., 2016; Holscher et al., 2012; Leyrolle et al., 2021; Mayengbam et al., 2019; Moorthy et al., 2020; Müller et al., 2020). These alterations are associated with improved lipid profiles, increased insulin sensitivity, and ameliorated symptoms in obesity and related metabolic disorders (Basturk et al., 2016; Fujimori et al., 2009; Hume et al., 2017; Leyrolle et al., 2021; Mitchell et al., 2021; Neumer et al., 2021; Whelan, 2011).

2.3.2 Probiotics

2.3.1.1 Definition. Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”, as defined by FAO/WHO (2006). Lactic acid bacteria (LAB) such as *Bifidobacterium* (*adolescentis*, *animalis*, *bifidum*, *breve*, and *longum*) and *Lactobacillus* (*acidophilus*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*, *rhamnosus*, and *salivarius*) are well-established probiotics (Hill et al., 2014). However, it is important to note that not all LAB possess probiotic properties. Binda et al. (Binda et al., 2018) proposed criteria for selecting

probiotic strains, including (1) sufficiently characterized such as survival at relevant body sites, the production of lactic acid or other short chain fatty acids, adhesion to mucus or intestinal epithelial cells, interaction with human immune cells, resistance to digestive enzymes, bile or acid, antibacterial activity via competitive exclusion or production of bacteriocins or hydrogen peroxide; (2) the selection of probiotic strains much also consider safety for the human consumption, such as assessing strain- and species- specific virulence factors, antibiotic resistance, biogenic amines and D-lactate production, hemolytic and bile salt hydrolase activities, as well as in vivo safe tests and human intervention studies; (3) the efficacy of probiotics should be supported by at least one positive human clinical trial conducted according to generally accepted scientific standards or recommendations and provisions of local/national authorities when applicable; and (4) probiotics should be present in the product at an efficacious dose throughout shelf life, typically a daily viable dose of 10^8–10^{11} CFU.

2.3.1.2 Health benefits. Probiotics and probiotic foods offer numerous health benefits to the human body. Probiotics can be used to prevent or treat infectious diarrhea caused by pathogenic bacteria like *Salmonell*a, *Shigella*, *Listeria monocytogenes*, thanks to their ability to produce antimicrobial compounds such as organic acids, hydrogen peroxide, and antimicrobial proteins called bacteriocin (Vimont et al., 2017). In the gut, probiotics may inhibit the adhesion and invasion of pathogenic bacteria to the intestinal epithelial cells and reduce pathogenic bacteria cytotoxicity (Burkholder & Bhunia, 2009). In cases of gut dysbiosis caused by factors like stress or excessive use of antibiotics, probiotic foods can help restore the gut microflora (Beena Divya et al., 2012). Probiotics also exhibit anti-inflammatory and immunomodulatory properties, making them beneficial for managing symptoms associated with IBD and irritable bowel syndrome (IBS) (Beena Divya et al., 2012). Studies have shown that probiotics can reduce the gene expression of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (C. S. Lee & Kim, 2019; Shahbazi et al., 2021; S.-Y. Yang et al., 2021). Probiotics possess hypocholesterolemia by assimilating cholesterol from the gut, leading to reduced absorption of the lipid and non-metabolized cholesterol. They can alter the ratio of HDL to LDL cholesterol, thus decreasing the risk of atherosclerosis (R. Hassan et al., 2019). The mechanisms behind these effects include the precipitation of

cholesterol through deconjugation of bile salts by bile salt hydrolase (BSH), absorption of cholesterol into bacteria cell membranes, the release of cholesterol hydrolyzing enzymes, and the regulation of gene expressions involved in lipid and cholesterol metabolism (Liang et al., 2020; Romero-Luna et al., 2021). Bacterial metabolites like SCFAs further regulate metabolic pathways in the body (Romero-Luna et al., 2021). In particular, probiotics are known for their ability to modify intestinal bile composition and activate the LXR signaling pathway in enterocytes, which downregulates Niemann-Pick C1-like 1 (NPC1L1), a transmembrane protein responsible for cholesterol absorption, and upregulates adenosine triphosphate-binding cassette subfamily A members 5 and 8 (ABCG5/8) which are responsible for intestinal efflux of cholesterol (Vourakis et al., 2021). Probiotics can also activate Sterol regulatory element binding protein-2 (SREBP-2) to prevent cholesterol synthesis; meanwhile, the unconjugated bile acids produced by probiotics inhibit FXR signaling pathways that increase de-novo bile synthesis in the liver and suppress the reabsorption of bile acids by enterocytes (Y. Wang et al., 2022). Probiotics have shown regulatory activities in lipogenesis/lipolysis, primarily through AMPK pathway, where adipogenesis is inhibited by suppressing PPARγ and activating SREBP-1, and lipid oxidation is promoted by upregulation of PPARα (Y. Wang et al., 2022). Other beneficial properties of probiotics and probiotic foods include anti-cancer, anti-oxidant, anti-osteoporotic, anti-diabetic, and enzymatic functions (Ayyash et al., 2018; Khan et al., 2021; C. S. Lee & Kim, 2019).

In animal studies, modulation of gut microbiota has been observed following probiotic administration. For example, a *L. plantarum* strain isolated from kimchi reduced the mesenteric adipose depot and upregulated genes involved in lipid oxidation, such as acyl-coenzyme A oxidase, palmitoyltransferase1, PPARγ, and coactivator 1-alpha, in mice fed a HFD. The relative abundance of *Lachnospiraceae* was significantly higher in the probiotic-treated group compared to the control group (S. Park et al., 2017). *L. fermentum* reduced Th1-, Th2-, and Th17-related cytokine levels and increased IL-10 level in mice with UC, resulting in a higher diversity of the gut microbiota and increased abundance of beneficial *Lactobacillus* spp. and *Akkermansia* spp. (Jang et al., 2019). Although probiotics have been reported of positive outcomes in individuals with diseases such as IBD and obesity, the significant alteration of gut

microbiota has been inconsistent and poorly reported in some studies (León Aguilera et al., 2022; Štofilová et al., 2022). A systematic review of randomized controlled trials revealed no evidence of impact of probiotic supplementation on the gut microbiota in healthy adults (Kristensen et al., 2016). This indicated that gut microbiota colonization is resistant to both probiotics and pathogens (Schmidt et al., 2018). New strategies, or known as next-generation probiotics, may rely on the context of gut microbial ecology and the supplementation of complementary strains (Schmidt et al., 2018).

2.3.3 Postbiotics

2.3.3.1 Definition. Postbiotics refer to “any soluble factor resulting from the metabolic activity of a probiotic bacteria—living microorganisms which, when administered in adequate amounts, confer health benefits on the host—or any bacterial-released molecule capable of providing health benefits through a direct or indirect mechanism” (Mayorgas et al., 2021). Postbiotics consist of a mixture of microbial molecules rather than individual components. Various types of postbiotics exist, including (1) bacterial cell wall components such as muramyl dipeptide, surface layer proteins, lipoteichoic acid, exopolysaccharide; (2) bioconversion products meaning byproducts produced by bacterial fermentation of a substrate; (3) cell-free extracts (CFS); (4) Extracellular vesicles that are spherical, microbially derived entities containing bioactive compounds discharged into the extracellular environment; (5) SCFAs; (6) bacteriocins; (7) cell-free lysates usually referred as heat-killed bacteria or paraprobiotics (S.-J. Park et al., 2023).

2.3.3.2 Health benefits. Postbiotics offer a wide range of health benefits similar to probiotics, including antimicrobial, anti-inflammation, immunomodulation, anti-obesity, antioxidative properties, hypocholesterolemic effects, anticancerogenic effects, and antihypertensive effects (Aguilar-Toalá et al., 2018). Postbiotics are believed to overcome some disadvantages associated with the use of probiotics, such as safety concerns, antibiotic resistance, challenges in maintaining the viability of probiotics, and difficulties in the regulation and standardization of probiotic products (Aguilar-Toalá et al., 2018). CFS of *Bifidobacterium bifidum* and *Bifidobacterium longum* treatment in

HFD-induced obese mice reduced body weight and epididymal fat accumulation, improved insulin sensitivity and glucose tolerance, and lowered plasma triglyceride, LDL, and cholesterol levels (Rahman et al., 2021). The authors suggested that the CFS demonstrated anti-obesity effects via the Protein Kinase A/p38 MAPK signaling activation. CFS and heat-killed bacteria of *Lactobacillus plantarum* exerted inhibitory effects on the adhesion, invasion, and replication of *S.* Typhimurium, and modulated the inflammation induced by *S*. Typhimurium in porcine intestinal epithelium cell line (Wu et al., 2023). These postbiotics activated AMPK signaling pathway-mediated autophagy in intestinal epithelial cells and suppressed the activation of NOD-like receptor protein 3 inflammasomes induced by *S.* Typhimurium. Furthermore, postbiotics derived from kefir LAB bioconversion of whey and citrus pomace extract showed beneficial effects in HFD-induced obese mice (Youn et al., 2022). These postbiotics reduced body weight gain, adipose tissue weight/body weight ratio, hypertriglyceridemia, and adipocyte diameter. They also significantly increased the abundance of butyrate-producing bacteria *Anaerovorax odorimutans* while decreasing *Olsenella* spp. in the gut microbiota of the treated group.

2.3.4 Synbiotics

2.3.4.1 Definition. Synbiotics are defined as “a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host” (Swanson et al., 2020). There are two types of synbiotics: synergistic synbiotics, where “the substrate is designed to be selectively utilized by the co-administered microorganism(s)”, and complementary synbiotics, which compose of “a probiotic combined with a prebiotic, which is designed to target autochthonous microorganisms” (Swanson et al., 2020). For synergistic synbiotics, while neither the microorganism nor the substrate alone needs to meet the minimum criteria for probiotics or prebiotics, the selective utilization and a health benefits must be demonstrated (Swanson et al., 2020). In the case of complementary synbiotics, both the microorganisms and the substrates must meet the minimum criteria for probiotics and prebiotics when stand alone, and while a health benefit needs to be demonstrated in the targeted host, a selective utilization is not required (Swanson et al., 2020).

2.3.4.1 Health benefits. Synbiotics offer similar beneficial effects as probiotics and prebiotics, including amelioration of metabolic syndrome, systemic inflammation, constipation, and mental disorders (Duque et al., 2021; Núñez-Sánchez et al., 2021; Z. Yang et al., 2021). Synbiotics can synergistically restore dysbiosis of the gut microbiota, as probiotic supplementation alone has shown resistance in achieving significant alterations. In a placebo-controlled intervention trial, adults with overweight or obesity were provided with a synbiotic containing *L. acidophilus*, B. lactis, *B. longum*, *B. bifidum*, and prebiotic galactooligosaccharide for three months daily (Sergeev et al., 2020). While there were no significant changes in body mass, BMI, body fat mass, or body fat percentage, synbiotic supplementation increased the richness of the gut microbiota and the abundance of beneficial genera such as *Bifidobacterium* and *Lactobacillus*. Fecal *Prevotella* and *Gardnerella* were significantly decreased after the synbiotic intervention. In another study, healthy infants were fed with a standard formula supplemented with bovine milk fat globule membrane components, long-chain polyunsaturated fatty acids (arachidonic and docosahexaenoic acids), and synbiotics (fructooligosaccharides, inulin, sialic acid, *B.infantis* IM1, *Lactobacillus rhamnosus* LCS-742) for six months. This formula supported the growth of *Lactobacillus* and *Bifidobacterium* in the gut during infant development (Cerdó et al., 2022). In rats fed with a HFD, the administration of a synbiotic composed of probiotics *B. animalis* and *L. paracasei*, and the prebiotic oat β-glucan, significantly improved metabolic profiles (e.g. body weight, fasting blood glucose and insulin, serum cholesterol level) and suppressed inflammatory cytokines (e.g. TNF-α) in the adipose tissue, liver, and jejunum (X. Ke et al., 2019). These changes were more pronounced in the synbiotic-treated group compared to the individual components. In addition, the synbiotic significantly increased the richness of the gut microbiota in HFD-fed mice and increased the abundance of beneficial, such as *Faecalibaculum rodentium* and *Alistipes putredinis*, while promoting the production of SCFAs in the gut.

2.4 Fermented food

2.4.1 Definition

Food fermentation is a traditional preservation method in which microorganisms utilize food substrates, typically sugars, under anaerobic conditions, resulting in the

production of alcohols, carbon dioxide, and/or organic acids (Leeuwendaal et al., 2022). This process leads to the accumulation of antimicrobial metabolites like organic acids, alcohol, and bacteriocins, as well as reduction in pH levels, thereby reducing the risk of contamination by pathogenic and spoilage microorganisms. Additionally, fermentation enhances the flavor and sensory properties of foods and removes harmful inedible compounds (Dimidi et al., 2019). Food fermentation can be achieved through two predominant approaches: natural fermentation, also known as "spontaneous fermentation," and culture-dependent fermentation. Natural fermentation is facilitated by microorganisms inherently present in raw foods and the preparation environment (Dimidi et al., 2009). Foods such as poi, sauerkraut, and kimchi typically undergo this type of fermentation (Rezac et al., 2018). On the other hand, culture-dependent fermentation is initiated by introducing a specific starter culture or a small amount of a previously fermented batch into the food (Dimidi et al., 2019). This method is utilized in the fermentation of foods like kefir, kombucha, natto, and sourdough bread (Rezac et al., 2018). Fermented foods are widely recognized as “functional foods” due to their ability to provide numerous health benefits, such as anti-inflammatory and immunomodulatory effects, anti-obesity properties, regulation of blood sugar levels, reduction of blood pressure, and potential anti-cancer activities (Shahbazi et al., 2021). The mechanisms through which fermented foods contribute to improving health are multifaceted. Firstly, they provide direct nutritional values and bioactive compounds. Secondly, fermented foods act as a source of prebiotics or substrates that promote the growth of beneficial members of the gut microbiota. Lastly, the native microflora present in fermented foods can enter the intestine and either become a component of the gut microbiota or to compete with existing members of the gut microbiota (Leeuwendaal et al., 2022). Consequently, fermented foods contain a complex mixture of prebiotics, probiotics, and postbiotics, which may classify them as synbiotics, combining the benefits of all three components.

2.4.2 Microbiome in fermented food

Traditionally fermented foods contain a diverse group of LAB such as *Lactobacillus* spp., *Streptococcus thermophilus*, *Bifidobacteria* spp., *Lactococcus. lactis*, *Leuconostoc* spp., *Enterococcus* spp., and *Weissella* spp. (Stiemsma et al., 2020). Additionally, non-

LAB bacteria belonging to the *Enterobacteriaceae* family and *Klebsiella* species can also be present in fermented foods (Stiemsma et al., 2020). For example, in fermented soybean paste (Da-jiang), the main microbial species identified include *E. faecalis*, *L. mesenteroides*, *Acinetobacter baumannii*, and *Bacillus subtilis* (An et al., 2021). In fermented vegetables from Hainan, China, the most abundant genus was *Lactobacillus*, with *L. plantarum* being the predominant species, followed by *L. fermentum*, *L. pentosaceus*, and *W. cibaria* (Z. Li et al., 2023). Key LAB species found in kimchi include *Leuconostoc*, *Lactobacillus*, and *Weissella* (S. H. Lee et al., 2020). These examples showcase the diversity of LAB and non-LAB bacteria present in traditionally fermented foods.

Many LAB isolated from fermented foods exhibit probiotic properties. For instance, probiotic strains such as *E. durans* and *E. lactis* have been isolated from soft chhurpi, a traditionally fermented milk product from the Sikkim Himalayas (Ghatani et al., 2022). Bacteriocin-producing LAB, including *L. casei*, *L. lactis*, and *Leuconostoc lactis*, have been isolated from naturally fermented yak milk from the Qinghai-Tibet plateau (Luo et al., 2011). In addition, *L. plantarum* strains with bacteriocin production capabilities have been isolated from naturally fermented milk in Zimbabwe (Todorov et al., 2007). These findings highlight the presence of beneficial microorganisms in fermented foods, which can contribute to their probiotic properties and potential health benefits.

2.4.3 Health benefits and modulation of gut microbiota of fermented food

Consumption of fermented food has been found to modulate gut microbiota and offer various health benefits. A study by Taylor et al. (2020) involving 6,811 participants revealed significant differences in the microbiota of individuals who consumed fermented plant food compared to those who did not. Consumption of fermented products was associated with specific bacterial species such as *Bacteroides* spp., *Pseudomonas* spp., *Dorea* spp., *Lachnospiraceae*, *Prevotella* spp., *Alistipes putredinis*, *Oscillospira* spp., *Enterobacteriaceae*, *Fusobacterium* spp., *Actinomyces* spp., *Achromobacter* spp., *Clostridium clostridioforme, Faecalibacterium prausnitzii*, *Bacteroides uniformis*, *Clostridiales*, and *Delftia* spp. The differentially abundant microbiota found in fermented-food-consumers include microbes associated with fermented foods (e.g. *L. acidophilus*, *Levilactobacillus brevis*, *L. kefiranofaciens*,

Lentilactobacillus parabuchneri, *L. helveticus*, and *L. sakei*) as well as those independent from fermented foods (e.g. *Streptococcus dysgalactiae*, *Prevotella melaninogenica*, *Enorma massiliensis*, *Prevotella multiformis*, *Enterococcus cecorum*, and *Bacteroides paurosaccharolyticus*). In a 17-week randomized prospective study, participants were assigned a high-fiber-diet (n=18) or a high-fermented-food-diet (n=18). The high-fiber diet increased the production of SCFAs and glycan-degrading carbohydrate-active enzymes in the gut microbiome but did not alter the microbial community structure. On the other hand, the high-fermented-food diet led to a steady increase in microbiota diversity and reduced inflammatory signals and activity (Wastyk et al., 2021). Another study evaluated the intake of fermented dairy foods among 130 healthy adults using a food frequency questionnaire that included 26 fermented dairy foods (González et al., 2019). Consumption of natural yogurt was associated with increased fecal levels of *Akkermansia* and reduced serum levels of C-reactive protein, an indicator of inflammation.

Dietary interventions of fermented food exhibited beneficial effects on gut microbiota and human health. In mice with chronic kidney disease, consumption of a fermented soybean product significantly improved the disease state, reduced kidney levels of inflammatory cytokines, and reversed chronic-kidney-disease-associated reduction of *Clostridium leptum* in the cecum (L.-X. He et al., 2020). Consumption of a fermented milk product promoted SCFAs production, inhibited the abundance of potentially harmful *Bilophila wadsworthia* in individuals with IBS, and improved their IBS symptoms (Veiga et al., 2014). In patients with CD, kefir administration significantly reduced inflammatory markers such as erythrocyte sedimentation rate and C-reactive protein levels, and improved the bloating and the “feeling-good” scores among the patients (Yılmaz et al., 2019). Consumption of Camembert cheese by healthy volunteers significantly increased the population of *E. faecalis* in their feces (Firmesse et al., 2007). Furthermore, kimchi consumption significantly reduced the body weight, fat mass gain, levels of pro-inflammatory cytokines in serum, and improved the integrity of the blood-brain barrier in obese mice fed a HFD (N. Kim et al., 2022). In addition, the gut microbial abundance of *Akkermansia muciniphila* was increased along with the potentiated fecal acetate level in kimchi-treated mice. These results suggest that

fermented food may exert beneficial effects in preventing and ameliorating chronic disease such as chronic kidney diseases, IBS, IBD, obesity, and inflammation.

2.4.4 Contribution of microbial fermentation to the health benefits of fermented food

Some studies have investigated the role of fermentation in the health-promoting and gut microbiome modulating of fermented food. In a study involving obese women, the consumption of fresh or fermented kimchi for 8 weeks resulted in improved clinical parameters such as body weight, waist circumstances, BMI, body fat percentage, serum cholesterol level, HDL-cholesterol, fasting insulin, C-reactive protein, blood pressure, and insulin resistance (Han et al., 2015). Fresh kimchi specifically led to a significant reduction in waist circumstance, body fat percentage, and diastolic blood pressure, while fermented kimchi led to a significant reduction in HDL-cholesterol and systolic blood pressure. This suggests that the mode of action of fermented kimchi may differ at least partially from that of fresh kimchi. Moreover, only the fermented kimchi group showed changes in the expression levels of genes related to energy balance and immune functions. The gut microbiota composition was also different between the fresh ad fermented kimchi groups, with the fresh kimchi group showing a greater increase in *Bifidobacterium* abundance, while the fermented kimchi group showed a greater decrease of *Blautia* and increases of *Prevotella* and *Bacteroides*. In another study involving healthy adults, the effects of non-fermented soybean milk and fermented soybean milk on the fecal microbiota and metabolic activities were investigated (Inoguchi et al., 2012). Fermented soybean milk resulted in greater changes in microbial composition and fecal SCFAs. Specifically, ingestion of fermented soybean milk increased the number of *Bifidobacterium* and *lactobacillus* and decreased *clostridia* in the feces, whereas non-fermented soybean milk only slightly increased the population of *Bifidobacterium*. Treatment of pasteurized or unpasteurized fermented sauerkraut in patients with IBS both demonstrated improved IBS symptom scores and significant gut microbiota compositional changes. Sauerkraut-related *L. plantarum* and *L. brevis* were more frequently present in the unpasteurized sauerkraut group. However, since both treatments had equal effects on IBS, the observed effects may be predominantly attributed to the prebiotics and postbiotics present in fermented sauerkraut (Kristensen et al., 2016).

2.5 Conclusions

In conclusion, poi, a fermented taro product, has a long history of traditional use for therapeutic purposes, including the treatment of gastrointestinal disorders, digestive issues, infant allergies, and wound healing. Modern research has revealed that poi contains a diverse array of LAB, which may act as potential probiotics. Poi is also rich in dietary fiber, particularly resistant starch, which can modulate the gut microbiome and promote SCFA production. Furthermore, bioactive extracts derived from poi have demonstrated immunomodulatory and anti-cancer properties. The human gut microbiome plays a crucial role in both physical and mental health, and disruptions in its composition have been associated with various chronic diseases such as IBD and metabolic syndromes. Restoring a healthy gut microbiome and strengthening gut barrier integrity have been identified as potential strategies for mitigating and preventing these conditions. Diet is known to profoundly influence the community structure and functionality of the gut microbiome, making it a promising avenue for intervention. Fermented foods, including poi, have gained attention for their potential to promote health and modulate the gut microbiome due to their natural content of prebiotics, probiotics, and postbiotics. However, further evidence from larger longitudinal trials with longer follow-up periods is required to confirm these findings. Therefore, it is proposed that poi holds potential to impact the gut microbiome and contribute to positive health outcomes, particularly in relation to inflammation and factors associated with obesity. This dissertation sought to systematically explore the properties of poi and provide insights into the possible mechanisms linking fermented foods with human health.

CHAPTER 3 MICROFLORA COMPOSITION AND PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA IN DIGESTED POI

Abstract

Probiotics have been linked to various health advantages, such as the prevention of pathogenic bacterial infections, the maintenance of gut homeostasis, and the modulation of cholesterol and lipid metabolism. Poi, a significant traditional Hawaiian food, contains unknown bacteria that could potentially confer health benefits. This study aimed to profile the bacteria in fermented poi that survive simulated gastrointestinal digestion and investigate the probiotic properties of lactic acid bacteria (LAB) found in the digested product. Six commercially available brands of poi (A, B, C, D, E, and F) were naturally fermented and subjected to oral, gastric, and intestinal digestion processes. After this digestion, 30 LAB colonies were randomly isolated from each brand. DNA extraction and Next Generation Sequencing were conducted on both pre- and post-digestion poi samples, while the isolated LAB were differentiated and identified. This analysis revealed a shift in bacterial relative abundance and diversity in poi post-digestion. Among the 180 LAB isolates, 62 unique strains belonged to 13 different species. The top five identified species were *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Enterococcus lactis*, *Leuconostoc pseudomesenteroides*, and *Lactiplantibacillus plantarum*. Several LAB isolates (A3, A6, C1-C9, E5, E8, and D14) exhibited antimicrobial activities against *Listeria monocytogenes* and/or *Salmonella* Typhimurium, attributed to the production of hydrogen peroxide and bacteriocins. Additionally, 52 isolates showed bile salt hydrolase (BSH) activities, and all isolates demonstrated cholesterol assimilation ranging from 20% to 70%. The abilities of the isolates to adhere to human intestinal epithelial cells (Caco-2) ranged from 0.14% to 8.79%, while a commercial probiotic strain, *Lactobacillus rhamnosus* GG, exhibited 0.84% adhesion. No hemolysis or cytotoxicity was detected in any isolates. In conclusion, each isolated LAB strain demonstrated unique probiotic attributes, with noteworthy probiotic properties observed in isolates belonging to *L. lactis*, *L. plantarum*, *E. lactis*, and *Streptococcus lutetiensis* species. These findings suggest that poi is a

potential probiotic food and warrants further investigation into its specific benefits and underlying mechanisms.

Keywords: poi, lactic acid bacteria, probiotic, bile salt hydrolase, bacteriocin, cholesterol assimilation, adhesion, cytotoxicity

3.1 Introduction

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” by FAO/WHO (Food and Agriculture Organization of the United Nations & World Health Organization, 2006). Probiotics benefit the host through mechanisms such as production of organic acids and bioactive compounds, competitive exclusion of pathogens, bile salt metabolism, gut barrier reinforcement, and immunological and endocrinological effects (Hill et al., 2014). Many probiotics are lactic acid bacteria (LAB) including well-established species like *Bifidobacterium* spp. and *Lactobacillus* spp. and popularly researched species like *Enterococcus* spp., *Lactococcus* spp., *Leuconostoc* spp., and *Weissella* spp. (Ghatani et al., 2022; Hill et al., 2014; Jeong & Lee, 2015; K. W. Lee et al., 2012; M. Liu et al., 2019).

Probiotic LAB have therapeutic applications in preventing and treating infections caused by foodborne pathogenic bacteria like *Listeria monocytogenes* and *Salmonella*. spp., serving as an alternative to antibiotics (Wan et al., 2019). This is mainly due to their production of antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocins (Wan et al., 2019). Bacteriocins are peptides produced by bacteria that are active against other bacteria, against which the producer has a specific immunity mechanism (Dobson et al., 2012). Bacteriocins can facilitate bacterial colonization, inhibit competing bacteria, and function as signaling peptides to communicate with the bacterial community and the host (Dobson et al., 2012). Production of bacteriocins has been found in LAB species such as *Enterococcus faecium*, *Enterococcus thailandicus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Leuconostoc lactis*, *Lactococcus lactis*, *Weissella confusa*, etc. (Al-Madboly et al., 2020; Goh & Philip, 2015; Luo et al., 2011; Mao et al., 2001; Vimont et al., 2017). Furthermore, it has been suggested that probiotic LAB have hypo-cholesterolemic or hypolipidemic effects by producing bile salt hydrolase (BSH) that deconjugates and precipitates cholesterol, incorporating or binding cholesterol to the bacteria membrane, converting cholesterol into insoluble coprostanol, and inhibiting hepatic cholesterol and triglyceride synthesis through short chain fatty acids production (Romero-Luna et al., 2021). *E. faecium* strains showed BSH activities and reduced cholesterol in MRS medium by 57-92% (Singhal et al., 2019). Strains

belonging to the genera *Lactobacillus* and *Bifidobacterium* exhibited cholesterol assimilation in MRS broth and reduced lipid deposition in ox-LDL-induced macrophages (Liang et al., 2020). Co-incubation of *Lactobacillus rhamnosus* BFE 5264 or *Lactobacillus plantarum* NR74 with Caco-2 cells significantly increased the cholesterol efflux (Yoon et al., 2011). These studies have underscored the potential of probiotics as beneficial agents in the management of cholesterol-related conditions.

Poi is a traditional staple food among native Hawaiians. Commercially, poi is produced by steaming unpeeled taro corms, peeling and grinding the cooked taro, and mixing the ground taro with water (Allen & Allen, 1933). Fresh poi naturally ferments at room temperature. Fermented poi has been found to harbor a wide range of LAB species, including *Weissella confusa*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Leuconostoc citreum*, and *Leuconostoc lactis*, *Enterococcus faecium* (L. He, 2003; Pirazzini, 2008). Despite its cultural and economic significances in Hawaii, poi has not yet been characterized for potential probiotic properties. The FAO/WHO guidelines for evaluating probiotics instruct rigorous assessments of bacterial strain identities, bacterial survival and proliferation in the gastrointestinal tract, functional properties like antimicrobial properties and adhesion to the intestinal cells, as well as safety properties (FAO/WHO, 2006). Therefore, this study aimed to screen the probiotic properties of LAB in fermented poi. This process included determining the surviving patterns of the LAB in simulated gastrointestinal digestion using 16S rDNA Illumina Miseq sequencing, identifying the survived LAB, assessing functional properties including antimicrobial properties, BSH activities, cholesterol removal, and adhesion to human intestinal cells, and evaluating safety properties including hemolytic properties and cytotoxicity to human intestinal cells.

3.2 Methods

3.2.1 Sample collection and experimental design

Poi was collected from six commercial brands coded as Brands A-F. Fresh poi was purchased from local markets in Honolulu, Hawaii on the day of manufacturing and distribution and immediately transported in a cooler to the Food Microbiology Lab at the University of Hawaii at Manoa (Honolulu, USA). Fresh poi was left at room temperature

(RT, 21°C) for 48 h in a tightly sealed bag to allow natural fermentation, and the resultant poi was considered fermented. For *in vitro* digestion, each brand was collected three times in three independent occasions that were at least one week apart. Therefore, a total of 18 *in vitro* digestions were performed. Triplicated samples of fermented poi before and after digestion were collected, pooled, and subjected to DNA extraction and 16S rDNA Illumina sequencing. The digested poi was also spread on MRS agar for LAB isolation. Ten isolates were collected from each replicate, resulting in a total of 30 isolates collected from each brand and a total of 180 isolates collected from 6 brands. The isolates were identified and screened for probiotic properties. Each probiotic property analysis was replicated three independent times for each interested isolate. See Fig 2.1 for an illustration of the experimental flow.

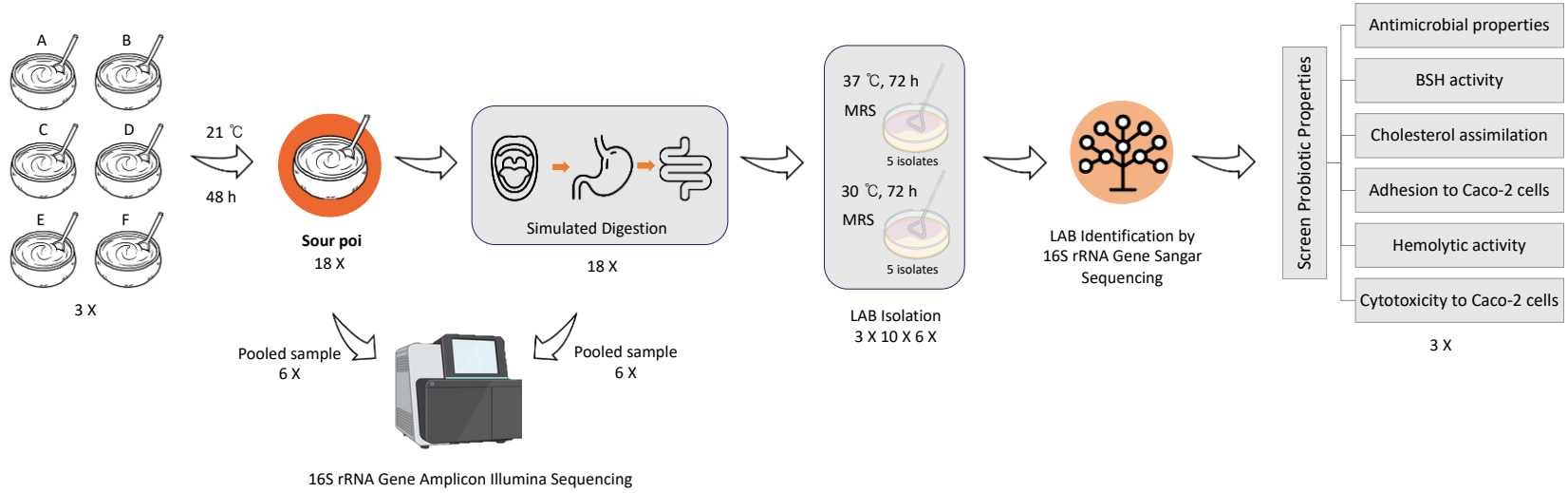

Figure 3.1 Illustration of the experimental flow. LAB: Lactic acid bacteria. MRS: de Man, Rogosa and Sharpe agar.

3.2.2 *In vitro* digestion

In vitro digestion was performed following the INFOGEST 2.0 protocol developed by Brodkorb et al. (2019), with some modifications. Briefly, 2.5 g of fermented poi was mixed with a simulated salivary fluid (SSF) containing human salivary amylase at 75 U/mL (Sigma-Aldrich, catalog number A1031, St. Louis, MO, USA) at 1:1 (w/w) ratio and incubated at 37°C for 2 min at pH 7.0. The oral bolus was then diluted 1:1 (v/v) with a simulated gastric fluid (SGF), pepsin at 2,000 U/mL (from porcine gastric mucosa, Sigma-Aldrich, catalog number P7012), and lipase at 60 U/mL (from *Rhizopus oryzae*, Sigma-Aldrich, catalog number 62305) and incubated under 100 rpm agitation at pH 2.5 (Tarrah et al., 2019) at 37°C for 2 h. Subsequently, the gastric chyme was diluted 1:1 (v/v) with a simulated intestinal fluid (SIF), 10 mM bile salts (Bile bovine, Millipore, catalog number B3883, Burlington, MA, USA), and pancreatin at 100 TAME trypsin U/mL (Pancreatin from porcine pancreas, Sigma-Aldrich, catalog number P7545; the trypsin activity in this product was 3.71 U/mg determined using the TAME method) and incubated at pH 7.0 for 2 h. The final compositions of SSF, SGF, and SIF are shown in Table 3.1.

All reagents and enzymes were sterilized by autoclaving or filter-sterilization using 0.22 µm cellulose acetate syringes filters. Enzyme solutions and 0.3 M $CaCl_2(H_2O)_2$ stock solution were prepared freshly before digestion. $NaHCO_3$ was replaced with NaCl based on the instructions given by INFOGEST 2.0. The whole digestion process was conducted aseptically.

The total MRS count was determined for poi before digestion, after oral digestion, after gastric digestion, and after intestinal digestion by serially diluting poi in 0.1% peptone water (Becton Dickinson, Franklin Lakes, NJ, USA) and plating on MRS agar (Hardy Diagnostics, Santa Maria, California and Springboro, Ohio, USA). The plates were incubated at 30°C anaerobically for 72 h, and the number of colony-forming units (CFU) was counted.

Table 3.1 Concentrations of buffering salts in simulated digestive fluids*.

Salt	SSF (mM)	SGF (mM)	SIF (mM)
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
NaCl	13.6	72.2	123.4
$MgCl_2(H_2O)_6$	0.15	0.12	0.33
$(NH_4)_2CO_3$	0.06	0.5	Not added
HCl	1.1	15.6	8.4
$CaCl_2(H_2O)_2$	1.5	0.15	0.6

*SSF: simulated salivary fluid. SGF: simulated gastric fluid. SIF: simulated intestinal fluid.

3.2.3 Isolation of lactic acid bacteria from digested poi

The LAB isolation process was modified according to Luo et al. (2011) and Zhan (2017). Poi digesta was diluted properly to a concentration of approximately 250-2,500 CFU/mL (25-250 colonies/plate) and plated on MRS agar to selectively grow LAB. The plates were incubated under anaerobic conditions at 30°C and 37°C for 72 h to favor the growth of different LAB (Abushelaibi et al., 2017; Luo et al., 2011; Russo et al., 2010; Singhal et al., 2019). A pre-isolation was performed by randomly picking and streaking 10 colonies from each culturing conditions and incubating for 48 h at 30 or 37°C to obtain purified isolates. A second round of selection was performed by staining the isolates with crystal violet (Aldon Corporation, Avon, NY, USA) and observing their morphologies under a microscope (Fisherbrand™ Micromaster™ II Microscope, Pittsburgh, PA, USA). Isolates of rods or cocci and those showed distinctive morphologies were further selected. Five isolates were selected from the 10 original selections. Their corresponding single colonies were picked and cultured in MRS broth at 30 or 37°C for 24-48 h. 50 µL of the purified bacterial culture was stored in 0.3 mL 50% (v/v) glycerol at -80°C. This process resulted in 30 isolates per brand from three independent replicates.

3.2.4 Differentiation and identification of LAB isolates

3.2.4.1 DNA extraction. Crude DNA was extracted from each LAB isolate using Prepman™ Ultra Sample Preparation Reagent (Applied Biosystems™, Waltham, MA, USA) according to the procedures provided by the manufacturer. Crude DNA was stored at -20°C before further analysis.

3.2.4.2 Random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR). RAPD-PCR randomly amplifies genomic sequences with an arbitrarily chosen short primer that results in different banding patterns from different DNA templates (Pirazzini, 2008). RAPD-PCR was performed on all 180 isolates using three separate reactions with primers P4 (5'-CCG CAG CGT T- 3'), P7 (5'-AGC AGC GTG G-3'), and M13 (5'-GAG GGT GGC GGT TCT-3') (Palla et al., 2020). The reaction mixtures (25 µL) contained 12.5 µL GoTaq® Hot Start Colorless Master Mix (Promega, Madison, WI, USA), 10 µL nuclease-free water, 0.5 µL primer (250 µM), and 2 µL crude DNA. The PCR conditions were: initial denaturation at 94°C for 1 min, 40 cycles of 1 min at 94°C, 20 s at 40°C, 2 min at 72°C, and a final extension at 72°C for 5 min (Palla et al., 2020).

Five microliters of the RAPD-PCR products were analyzed by gel electrophoresis in 1.8% agarose gels prepared in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 75 V for 4.5 h. The 100 bp Plus DNA ladder (Promega) was used as a molecular weight marker for reference. The gels were then stained with GelGreen Nucleic Acid Stain (Biotium, Fremont, CA, USA) and the gel banding patterns were photographed using FOTO/Analyst Investigator System (Fotodyne, Wisconsin, USA). Individual isolates displaying unique banding patterns among at least one of the three primers were considered unique.

3.2.4.3 16S rRNA gene amplification and Sanger sequencing. 16S rRNA genes of the unique isolates were amplified with primers 8F (5'-GGA GAG TTT GAT CCT GGC TCA G-3') and 518R (5'-TAT TAC CGC GGC TGC TGG CAC-3') targeting the V1-V3 region of the 16S rRNA (Vliegen et al., 2006). The reaction mixture (25 µL) contained 12 µl Master mix, 10 µl nuclease-free water, 0.5 µL forward primer, 0.5 µL reverse primers, and 2 µL DNA. The PCR reaction program included an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C for 30 s, 72°C for 1 min, and a final

extension step at 72°C for 10 min (Zhan, 2017). The amplification products of PCR reaction were checked by gel electrophoresis. After confirmation, the amplified DNA was cleaned of excess primers and dNTPs with ExoSAP-IT (Applied Biosystems™) according to the procedure provided by the manufacturer. The final products were stored at -20°C until Sanger sequenced at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) facility at the UH College of Natural Sciences (Honolulu, USA). Bacterial species were identified by comparing the obtained sequences with the nucleotide database using the National Center for Biotechnology Information (NCBI) BLAST Program.

3.2.4.4 Differentiation and identification between Enterococcus durans, Enterococcus faecium, and Enterococcus lactis. 16S rRNA gene sequencing was unable to differentiate the three Enterococcus species due to their close genetic relations. Isolates potentially belonging to *E. durans*, *E. faecium*, and *E. lactis* were further differentiated and identified using species specific primers listed in Table 3.2 (E. Kim et al., 2022). The identification was performed using quantitative PCR (qPCR) with a CFX96 Real-Time Detection Systems (Bio-Rad, Hercules, CA, USA). A total volume of 20 µL qPCR mixture was prepared with: 1 µL crude DNA, 1 µL of forward primer (50 µM), 1 µL of reverse primer (50 µM), 10 µL of 2× GoTaq qPCR Master Mix (SYBR green florescence, Promega), and 7 µL of Nuclease-free H_2O. The qPCR thermal cycle was carried out as the following: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s (E. Kim et al., 2022). Melting curves were also obtained at the end of the PCR cycle. Isolates showed florescence signals were identified as the species according to the corresponding primer sets.

Table 3.2 Primers used to identify Enterococcus species according to Kim et al. (2022).

Target species	Target gene	Forward primer (5'–3')	Reverse primer (5'–3')
E. durans	*FBPase_2*	CGA GGA ATG GTT CAG CAA GA	CCA TTG GCT CTG ATC GGT AA
E. faecium	*PurR*	ATA TCG GCT GTC TCC ATG CT	CCG CCG TCT ATA ATC CAT TC
E. lactis	*HTH_ARSR*	AGT CCG GTT AAA CCG ATC AA	ACA CCT ATC ACA CCG GCT AA

3.2.5 16S rRNA gene Illumina sequencing and bioinformatics

The three replicates of each un-digested and digested poi sample were pooled in equal amounts and thoroughly mixed. DNA was extracted from the pooled samples using a Qiagen Powerfecal Pro DNA kit (Hilden, Germany), following the manual provided by the manufacturer. 16S metagenomic sequencing library was prepared using the primer set (341F/785R) to amplify the V3-V4 region of the 16S rRNA genes. The library preparation and the metagenomic sequencing using an Illumina Miseq sequencer were performed at the ASGPB facility at the UH College of Natural Sciences.

Paired-end demultiplexed fastq sequencing data were processed using Qiime2 and Qiime2 plugins (Bolyen et al., 2019). The data were trimmed and denoised using Divisive Amplicon Denoising Algorithm 2 (DADA2) integrated in Qiime2 (Callahan et al., 2016). The feature table of the sequence variants (SVs) was generated in Qiime 2. Alpha-diversity of Shannon index, principle coordinates analysis (PcoA) plot based on weighted UniFrac distance, community composition analysis, and shared-core taxa analysis were performed using the Microbiome R package (Lahti & Sudarshan, 2017). Shared core-taxa was filtered at 1% compositional abundance threshold and a minimal prevalence of 1/12 (present in at least one sample).

3.2.6 Bacterial cultures

LAB isolates were activated by streaking the stock culture onto MRS and incubating at 37°C for 48 h. A single colony was inoculated into MRS broth, followed by culturing at 37°C overnight (16 h) and transferring twice before use for experimentation. A commercial probiotic strain *Lacticaseibacillus rhamnosus* GG (LGG, purchased from

Difco Laboratories, Sparks, MD, USA) was used in the screening of probiotic properties as a probiotic control.

Indicator pathogenic bacterial strains *Listeria monocytogenes* F2365 and *Salmonella enterica* Typhimurium ATCC 14028 were obtained from the Food Microbiology Lab at the University of Hawaii at Manoa. *L. monocytogenes* and *S.* Typhimurium were activated by striking the stock cultures onto modified Oxford agar (Becton Dickinson) and xylose lysine deoxycholate (Becton Dickinson) agar, respectively. The plates were incubated at 37°C for 48 h and a single colony of each bacterium was picked and grown in tryptic soy broth (TSB; Becton Dickinson) at 37°C overnight (16 h). Active cultures were transferred twice in TSB before use for experimentation.

3.2.7 Screening of antimicrobial activities

3.2.7.1 Modified deferred antagonism assay. A pre-screening of antimicrobial activity was performed using a modified deferred antagonism assay developed by Zhan (2017). This method was able to screen bacteriocin-producing bacteria while minimizing the interferences by organic acid. The base agar and the sandwich layer consisted of Elliker medium (Becton Dickinson), 1% Na_2HPO_4, 0.35% NaH_2PO_4, and 0.2% Tween 80 (pH 6.9). Elliker medium was selected for antimicrobial assay because it has been demonstrated to promote bacteriocin-production while limiting the production of organic acids (Zhan, 2017). The base agar contained 1.5% agar, while the sandwich layer and the indicator agar contained 0.75% agar. The indicator agar contained TS medium (TSA). Overnight LAB cultures were properly diluted by 10^7 times using 0.01 % peptone water to achieve a concentration of about 100 CFU/mL and plated on 10 mL base agar (1-10 colonies/plate). The inoculated base agar was dried in a biosafety hood for 1 h. Then 8 mL of the sandwich layer at about 50°C was poured over the base layer and incubated at 30°C for 48 h until visible colonies appeared. Next, 8 mL TSA was inoculated with about 10^7 CFU of indicator strains (*L. monocytogenes* and *S.* Typhimurium) and then poured over the sandwich layer. After solidifying, the plates were incubated at 37°C for 24 h. LAB isolates showed inhibition zones against *L. monocytogenes* or *S.* Typhimurium were selected for further confirmation using the microtitration assay.

3.2.7.2 Preparation of crude bacteriocin extract (CBE). 1 mL of LAB culture was incubated in 100 mL Elliker broth at 30°C for 48 h, after which cells were removed by centrifugation at 4431 *g* for 15 min. The cell-free supernatant (CFS) was adjusted pH to 6.5 with 5 M NaOH. Fifty-six gram of $(NH_4)_2SO_4$ was added into the cell-free supernatant to achieve 80% saturation and precipitate proteins (Yi et al., 2020). The mixture was left at RT for 1 h and centrifuged again at 4431 *g* for 15 min to obtain the protein precipitate. The precipitated proteins were re-dissolved in 8 mL of PBS (pH 6.5) and filter sterilized with 0.22 μm cellulose acetate syringe filters. The prepared CBE was stored at 4°C. Proteins in Elliker broth was extracted as control.

3.2.7.3 Microtitration assay. 50 μL of the CBE was mixed with 50 μL of indicator strain at 10^5 CFU/mL in 2× TSB (final concentration of 10^4 CFU/mL) in a 96-well plate. The mixtures were incubated at 37°C overnight and measured for absorbance at 600 nm using a microplate spectrophotometer (Bio-Rad, iMark, Hercules, CA, USA). Inhibition Efficiency (IE) was used to evaluate the inhibition of indicator strains by CBE (Mortezaei et al., 2020). IE = OD (CBE and indicator strains)/OD (Elliker protein extract and indicator strains). When IE > 1, CBE promoted the growth of the indicators; when IE = 1, CBE showed no inhibition nor promotion to the indicator growth; when IE < 1, CBE inhibited the growth of the indicators.

3.2.7.4 Treatment of CBE by catalase and proteases. CBE were treated with catalase and proteases to elucidate whether the antimicrobial activity was from H_2O_2 or bacteriocins. First, CBE was treated by 1 mg/mL sterile catalase (from bovine liver, Sigma-Aldrich, catalog number C9322; dissolved in 0.1 M Tris-HCl, pH 6.5) and incubated at 37°C for 1 h (Luo et al., 2011). The mixture was immediately placed on ice to stop the enzymatic reaction. Microtitration assay was performed. Next, catalase-treated CBE was further individually treated by 15 mg/mL of pepsin (dissolved in 50 mM sodium citrate, pH 3.0), trypsin (from porcine pancreas, Sigma-Aldrich, catalog number T4799; dissolved in PBS, pH 7.0), and proteinase K (Millipore, catalog number 70663; dissolved in 50 mM Tris-HCl, pH 7.0) (Yi et al., 2020). The enzymatic reactions were performed at 37°C for 3 h and stopped by immediately placing the mixtures on ice. The inhibitory effects of protease-treated CBE were evaluated using the microtitration assay.

3.2.8 Screening of BSH activities

The screening of BSH activities was conducted according to Liang et al. (Liang et al., 2020). MRS agar was prepared with 0.5% sodium taurodeoxycholic acid (TDCA) or sodium glycodeoxycholic acid (GDCA), and 0.37 % $CaCl_2$. A sterile blank disk with a diameter of 6 mm (Hardy Diagnostics) was placed into the bile-salt-containing MRS plate and 10 µL of overnight grown LAB culture was dropped on the disk. The plates were incubated at 37°C anaerobically for 48 h. The TDCA or GDCA enzyme activity scores were evaluated by the diameter size of the white halo.

3.2.9 Screening of cholesterol assimilation

Fifty microliter of overnight grown LAB culture was inoculated into 2 mL of MRS containing 100 ug/mL cholesterol with or without bile salts (2.8 mM GDCA and 1.2 mM TDCA) (Q. He et al., 2021; Singhal et al., 2019). The mixtures were incubated at 37°C for 24 h, and the supernatant was collected by centrifuging at 16,000 *g* for 2 min. Un-inoculated MRS broth with or without bile salts was also incubated as control. The concentration of cholesterol after incubation was determined by the *o*-phthalaldehyde method (Singhal et al., 2019). Briefly, 2 mL of 100% ethanol was added into 1 mL supernatant followed by the addition of 1 mL of 33% (w/v) KOH. The mixture was vortexed and incubated at 37°C for 15 min. Subsequently, 3 mL of n-hexane and 2 mL of distilled water were added into the mixture and briefly vortexed, and then sit at RT for 5 min to allow hexane separation. The top layer (hexane layer) was carefully transferred to a clean glass test tube, and the solvent was removed by heating at 90°C. The residue was re-dissolved in 2 ml of 5 mg/mL *o*-phthalaldehyde (TCI America, Portland, Oregon; dissolved in glacial acetic acid) by vertexing and incubating at RT for 10 min. Then, 1 mL of concentrated sulfuric acid was added and incubated at RT for 10 min, and the absorbance at 570 nm was measured using a spectrophotometer (Model 1600PC, VWR International LLC, PA, USA). The percentage of cholesterol assimilated was calculated as follows:

$$Cholesterol\ assimilated\ (\%) = \frac{\text{cholesterol in MRS control} - \text{cholesterol in MRS inoculated with LAB}}{\text{cholesterol in MRS control}} \times 100$$

3.2.10 Screening of hemolytic properties

Hemolytic activity was screened by streaking overnight LAB culture onto Columbia agar containing 5% (w/v) sheep blood (Hardy Diagnostics), and the plates were incubated at 37°C for 48 h (Yasmin et al., 2020). Isolates with clear zones around the colonies were considered β-hemolysis. Those with green zones were considered α-hemolysis. Those with no zone observed were not hemolysis (γ-hemolysis). *Staphylococcus aureus* (ATCC 25923) was the β-hemolysis-positive control. *Streptococcus pneumoniae* (VWR catalog number 470179-498) was the α-hemolysis control. LGG was the negative control.

3.2.11 Screening of LAB adhesion and cytotoxicity in Caco-2 cells

3.2.11.1 Caco-2 cell culture. Human colorectal adenocarcinoma cell line 2 (Caco-2) was purchased from MilliporeSigma (catalog number: 86010202, Lot. 21G005; Burlington, MA, USA). All reagents used in the cell medium were purchased from Gibco™ (Thermo Fisher Scientific, Waltham, MA, USA). The cells were maintained in fresh medium containing Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) fetal bovine serum (FBS), 0.03% L-glutamine (200 mM, 100×), 1% non-essential amino acids (NEAA), and 1% penicillin-streptomycin at 37°C in 5% CO_2 air. The cells were sub-cultured at 80-90% confluence every 3-4 days. Passages 20-30 were used. For the adhesion assay, the cells were seeded at 10^5 cells/well in 12-well plates and cultured for 21 days to initiate spontaneous differentiation. The culture medium was changed every 48 h. The cell culture medium was changed to a cell medium without antibiotics (containing 10% FBS, 0.03% L-glutamine, and 1% NEAA) one day before the adhesion assay. For the cytotoxicity assay, the cells were suspended in cell medium without FBS or antibiotics (containing 0.03% L-Glutamin and 1% NEAA), seeded at 10^4 cells/well, and cultured for 24 h to obtain a monolayer.

3.2.11.2 Adhesion. The adhesion assay was carried out following Fonseca et al. (2021) with modifications. Overnight grown LAB isolates and LGG pellets were collected by centrifuging at 16,000 *g* for 2 min and then washed twice with Dulbecco's phosphate-buffered saline (DPBS, Gibco™). The pellets were resuspended and diluted to $1x10^7$ CFU/mL in fresh cell medium without FBS or antibiotics. 1 mL of the bacterial suspension was added per well to achieve a ratio of bacteria to eukaryotic cells of 100:1

(Multiplicity of infection [MOI=100]) (Vimont et al., 2017). Inoculated Caco-2 cells were then incubated at 37°C for 1 h in 5% CO_2 atmosphere. The un-adherent cells were removed by removing the bacterial suspension and washing three times with DPBS. The adhered bacteria were detached from Caco-2 cells by adding 1 mL of 0.1% (v/v) Triton X-100 and incubating at RT for 10 min. The CFU number of bacteria adhered was determined by enumerating on MRS agar incubated at 37°C for 48 h. The percentage of adherence was calculated using the following formula:

$$Adherence\ (\%) = \frac{CFU\ of\ adhered\ bacteria}{CFU\ of\ initial\ added\ bacteria} \times 100$$

Where CFU means colony forming unit.

3.2.11.3 Cytotoxicity. Cytotoxicity was evaluated using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Yasmin et al., 2020). The effects of 1% MRS broth on the viability and morphology of Caco-2 cells were determined. 1 % MRS caused no significant cytotoxicity or alterations of cell morphology in Caco-2 cells (Data not shown). Bacterial CFS was used instead of bacteria cells to minimize bacterial interference in this assay. One-hundred µL per well of 10^5 cells/mL Caco-2 cells were seeded in a 96-well plate and cultured for 24 h (Urbizo-Reyes et al., 2022). The culture medium was removed and washed with fresh DMEM twice. The cells were then treated with 1% (v/v) filter-sterilized CFS (equivalent to MOI=100) diluted in fresh cell medium without FBS or antibiotics (pH=7.0-7.5). Caco-2 treated with 1% (v/v) MRS broth in fresh cell medium without FBS or antibiotics was used as the negative control A (Dowdell et al., 2020). The untreated Caco-2 cell monolayer was the negative control B. The 96-well plates were incubated at 37°C, 5% CO_2 for 24 h followed by removing the CFS and washing the cells with fresh DMEM twice. One-hundred µL of 0.5 mg/ml MTT solution in fresh cell culture medium without FBS or antibiotics was added to each well. After 4 h of incubation at 37°C in 5% CO_2, the medium was removed and replaced with 100 µL of dimethyl sulfoxide. The mixture was incubated at RT for 10 min to dissolve the blue crystals, and absorbance at 570 nm was recorded. Each isolate was tested for three independent replicates with three technical replicates. Cytotoxicity was expressed by

the percent ratio of the viability of cells treated with LAB CFS to the viability of cells treated with MRS.

3.2.12 Statistical analysis

In vitro digestion and isolation of LAB were conducted in triplicates using poi purchased on three different occasions that were at least one week apart. Probiotic properties of each unique LAB isolate were analyzed in independent triplicates. Data were expressed as mean ± standard deviation (SD). Phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis (MEGA) version 11 program (Tamura et al., 2021). Maximum likelihood method was used to construct the tree and bootstrap analyses based on 1,000 random re-samplings to determine the confidence values of each branch. Kimura 2-parameter model was used to ensure the quality of the phylogenetic analysis. Clustering of isolates based on their probiotic properties was generated using Euclidean distance and complete linkage methods in R (Nami et al., 2020). For microbiome data, differences between groups were analyzed using non-parametric methods including Kruskal-Wallis test for multiple comparisons and Wilcoxon test for paired comparisons. For other data, parametric Analysis of Variance (ANOVA) and Tukey's HSD tests were performed to compare means. Data visualization was achieved in R.

3.3. Results and discussion

3.3.1 *In vitro* digestion altered the microflora dynamics in poi

3.3.1.1 Total LAB count. *In vitro* digestion conditions significantly reduced the total number of LAB in poi, particularly during gastric and intestinal digestion stages (Fig 3.2). This was expected as bacterial survival in the gastrointestinal tract is challenged by low pH, digestive enzymes, and bile salts. The average total LAB (evaluated by total MRS count) among the six brands before digestion, after oral digestion, after gastric digestion, and after intestinal digestion were 10.17±0.54, 9.07±0.49, 6.07±2.08, and 6.62±0.88 log CFU, respectively. Notably, Brand C poi had lower total LAB than other brands and was particularly affected by the gastric digestion conditions, resulting in a reduction of 6.281 log CFU (1.91×10^6 folds). Although the LAB population in Brand C

recovered to 4.93 log CFU after intestinal digestion, it still had the lowest LAB count among all brands.

3.3.1.2 Alpha- and beta- diversity. The impact of *in vitro* digestion on the diversity and composition of the microbiome in poi was further investigated using 16S rRNA metagenomic sequencing. The Shannon index, an alpha-diversity measure for both species richness and phylogenetic diversity, revealed similarities and dissimilarities among brands (Fig 3.3 A) (Roswell et al., 2021). Brand D had the greatest diversity of all groups, and digestion showed minimal effect on its microbial diversity. On the other hand, Brand C exhibited the lowest Shannon index among all brands, which dropped to zero after digestion. Unlike brands A, E, and F, the alpha diversity of Brand B slightly increased after digestion. However, overall, digestion led to a reduction in microbiome diversity in poi, as shown in Fig 3.3 B. The PCoA plot was constructed based on the beta-diversity of weighted UniFrac distances between the samples (Fig 3.3 C), which measures both species abundance and the branch length with abundance difference. This means weighted UniFrac is more sensitive in detecting the differences among more abundant species compared to rare species (J. Chen et al., 2012). The PCoA plot revealed interesting nuances about the microbiome structures in each sample: (1) digestion altered the beta-diversity of the microflora in Brand D even though no significant difference was observed in alpha-diversity; (2) Brands A and B exhibited similar microbial community structures; (3) Brands E and F initially displayed similar microbial community structures, however, after digestion, the microflora community shifted more towards Brands A and B.

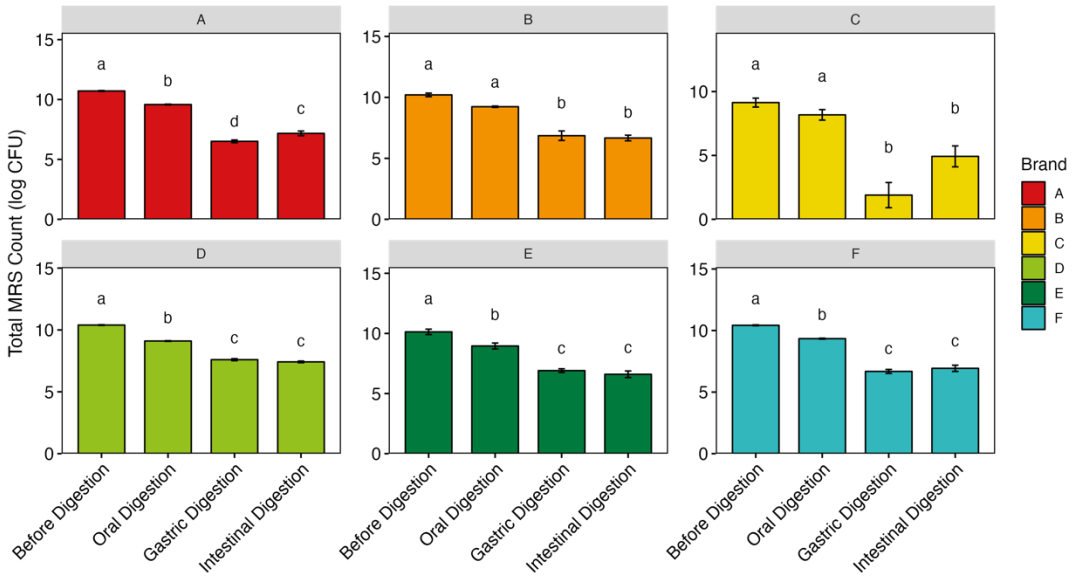

Figure 3.2 The dynamics of the total MRS count of poi of Brands A-F during digestion. Error bars are SD (n=3). Bars labeled with different letters in each plot mean they are significantly different ($P < 0.05$).

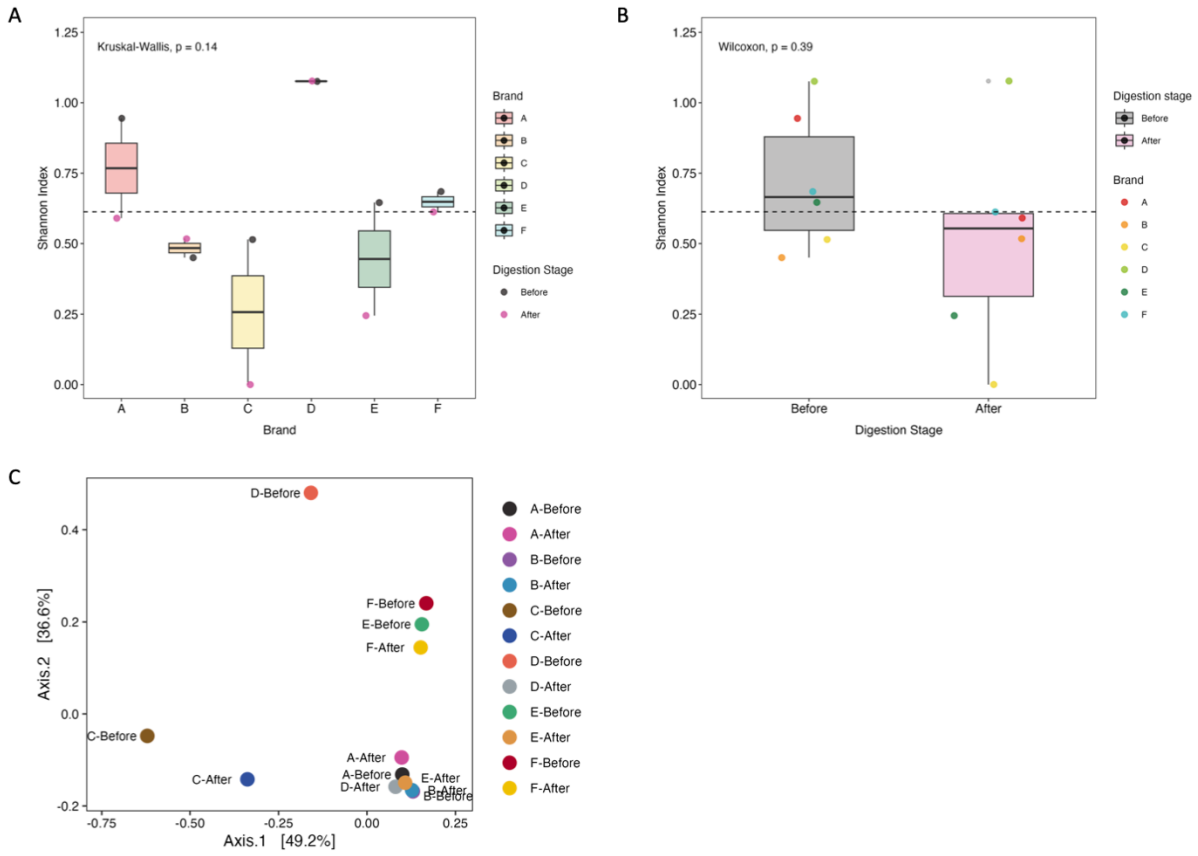

Figure 3.3 Alpha- and beta-diversities of microbiome compositions in poi before and after digestion. (A) Shannon index grouped by brand. Dissimilarities between groups were analyzed by the Kruskal-Wallis test. The dashed line represents the average Shannon index of all brands. (B) Shannon index grouped by digestion stage. The dissimilarity between the two groups was analyzed by the Wilcoxon test. (C) PCoA plot based on weighted UniFrac.

3.3.1.3 Community composition. The relative abundance of taxa at the species level in each pooled sample is shown in Fig 3.4 A. A total of 18 taxa belonging to 17 unique species and 11 unique genera were identified. The diversity of species in poi is considerably lower and simpler compared to other traditionally fermented foods, such as *kinema* (fermented soybean food), kimchi (fermented cabbage), Rubing milk cake, Yan-cai vegetable pickles, and Tibetan kefir grains (Kharnaior & Tamang, 2021; X. Liu et al., 2018; S.-E. Park et al., 2019; Zeng et al., 2022).

In fermented poi, lactic acid bacteria made up the majority of the microbial community, including *Leuconostoc* spp., *Lactococcus* spp., *Weissella* spp., and *Lactobacillus* spp. *Bacillus* spp. are not lactic acid bacteria, however, they are commonly found in fermented foods and many possess probiotic properties (Shivangi et al., 2020). In addition, *Acetobacter* spp., an acetic-acid-producing species, is also prevalent in fermented foods and drives vinegar production (Kaashyap et al., 2021). Other environmental bacteria, including *Paenibacillus humicus*, *Klebsiella* spp., and *Pantoea septica,* were found in fermented poi.

Bacterial species in all brands were altered by the digestive conditions. These alterations were most possibly driven by the acid, bile, enzyme tolerances of the species as well as competitions for nutrients. For instance, some *Weissella* species have been shown to be less bile tolerant than *Lactococcus* spp. (Mortezaei et al., 2020). This was observed in Brand A where *Weissella* spp. were outcompeted by *Lactococcus lactis*. In Brand C, the species was completely taken over by *Enterococcus* spp. The disappearance of *Bacillus* spp. and *Paenibacillus humicus* may be attributed to their low-acid and low-bile tolerances (Hyronimus et al., 2000). In Brand D, *Acetobacter* spp. were outcompeted by LAB which may be due to their competition for fermentation substrates. A similar trend was observed in Kombucha when LAB was added (Bueno et al., 2021). In Brand E, *Pantoea septica* diminished while *Exiguobacterium* spp. rose. *Pantoea septica* has a low acid-tolerance and *Exiguobacterium* spp. are frequently found in harsh environments (Fancello et al., 2020; Ordoñez et al., 2013). Microbial communities in poi before and after digestion shared five common taxa including *Leuconostoc Lactis*, *Leuconostoc mesenteroides*, *Weissella* spp., *Lactococcus lactis*, and *Klebsiella* spp. (Fig 3.4 B). The Venn diagram in Fig 3.4 C showed that the

community compositions of Brands A and B shared a common taxa, *Leuconostoc Lactis*, while they shared a common taxa, *Weissella* spp., with Brand D. Brands E and F shared a common taxa, *Leuconostoc mesenteroides*. Brand C stood out alone. These results corroborate the previous results regarding the similarities and differences between the brands.

To summarize, *in vitro* digestion altered the microbial community diversity and relative abundance in fermented poi. This alteration generally favored the survival of acid- and bile- tolerant lactic acid bacteria. Among the six brands, some showed similar microflora structures, while others displayed unique features, such as the *Enterococcus* spp. found only in Brand C poi.

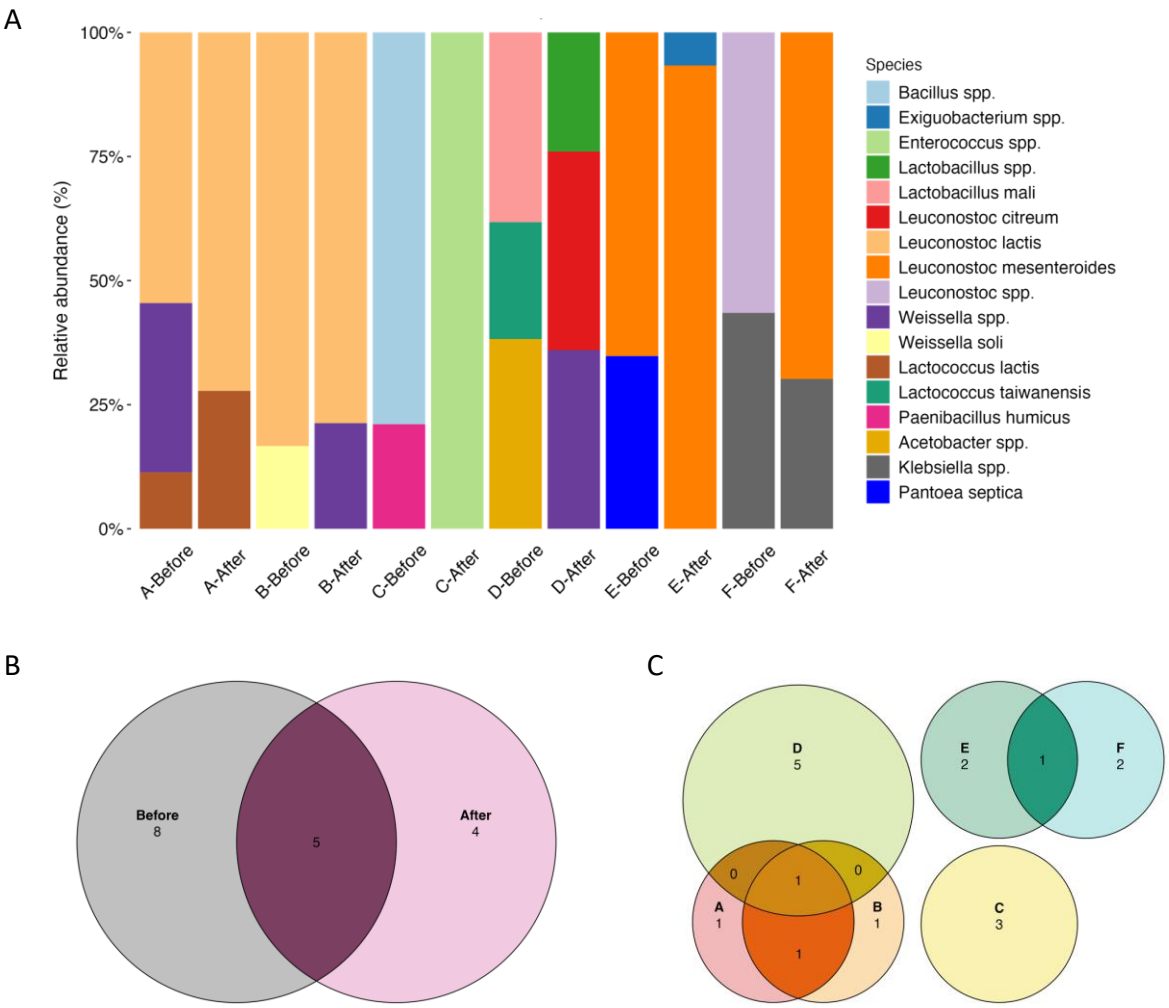

Figure 3.4 Relative abundance and shared core taxa in poi before and after digestion. (A) Relative abundance of taxa at species level in poi before and after digestion. Un-identified species were named by their genus names. (B) Venn diagram of shared core-taxa between digestion stages. (C) Venn diagram of shared core-taxa between brands. Shared core-taxa was filtered at 1% compositional abundance threshold and a minimal prevalence of 1/12.

3.3.2 Identification of LAB isolated from digested poi

Sixty-two out of the 180 LAB isolates in digested poi showed unique banding patterns in RAPD-PCR (Fig 3.5). These isolates belonged to 13 bacterial species, including *Leuconostoc lactis* (17 isolates), *Leuconostoc mesenteroides* (12 isolates), *Enterococcus lactis* (6 isolates), *Leuconostoc pseudomesenteroides* (5 isolates), *Lactiplantibacillus plantarum* (5 isolates), *Streptococcus lutetiensis* (4 isolates), *Weissella confusa* (4 isolates), *Liquorilactobacillus hordei* (3 isolates), *Lactococcus lactis* (2 isolates), *Enterococcus casseliflavus* (1 isolate), *Lactococcus taiwanensis* (1 isolate), *Leuconostoc holzapfelii* (1 isolate), and *Limosilactobacillus fermentum* (1 isolate). Similarities and dissimilarities were found between this study and previous reports. Certain species identified in this study were previously recognized in fresh poi, including *Weissella confusa*, *Lactobacillus plantarum, Lactococcus lactis*, *Leuconostoc mesenteroides*, *Leuconostoc pesudomesenteroids*, and *Leuconostoc lactis* (L. He, 2003; Pirazzini, 2008). On the other hand, some species previously identified were not found in fermented poi in this study; however, their belonging genera were present, such as , *Leuconostoc citreum*, , *Streptococcus macedonicus*, *Streptococcus bovis*, and *Enterococcus faecium*. Thirdly, the species *Lactobacillus delbrueckii* ssp. *Lactis*, identified by He (2003), was not detected in this study. According to Pirazzini (2008), the three most abundant species in fresh poi were *Leuconostoc lactis*, *Weissella confusa* , and *Enterococcus faecium*. While *Leuconostoc lactis* was also found to be the most abundant in this study, *Weissella confusa* was less prevalent in fermented poi. In addition, this study identified a greater number of species compared to previous reports. Interestingly, *Lactobacillus lactis*, a bacterium commonly found in fermented foods and previously detected in fresh poi, was absent in fermented poi.

Moreover, those LAB identified by metagenomic sequencing, were also identified by conventional culture-based methods. Brand D was found to be the most diverse brand with 16 unique isolates, which agreed with its greatest alpha-diversity among samples. *Enterococcus lactis* was primarily detected in Brand C poi, aligning with its distinctive features of microbiome structure discussed above.

Fig 3.6 depicts the phylogenetic relationship of the 62 isolates, illustrating the degree of gene similarity of the LAB strains based on their partial 16S rRNA gene

sequences. All of these isolates fall under the *Lactobacillales* order. The phylogenetic tree exhibits two primary clusters: one comprising isolates from the *Leuconostoc* and *Weissella* species of the *Leuconostocaceae* family, which are genetically more closely related, and the other cluster containing the remaining isolates.

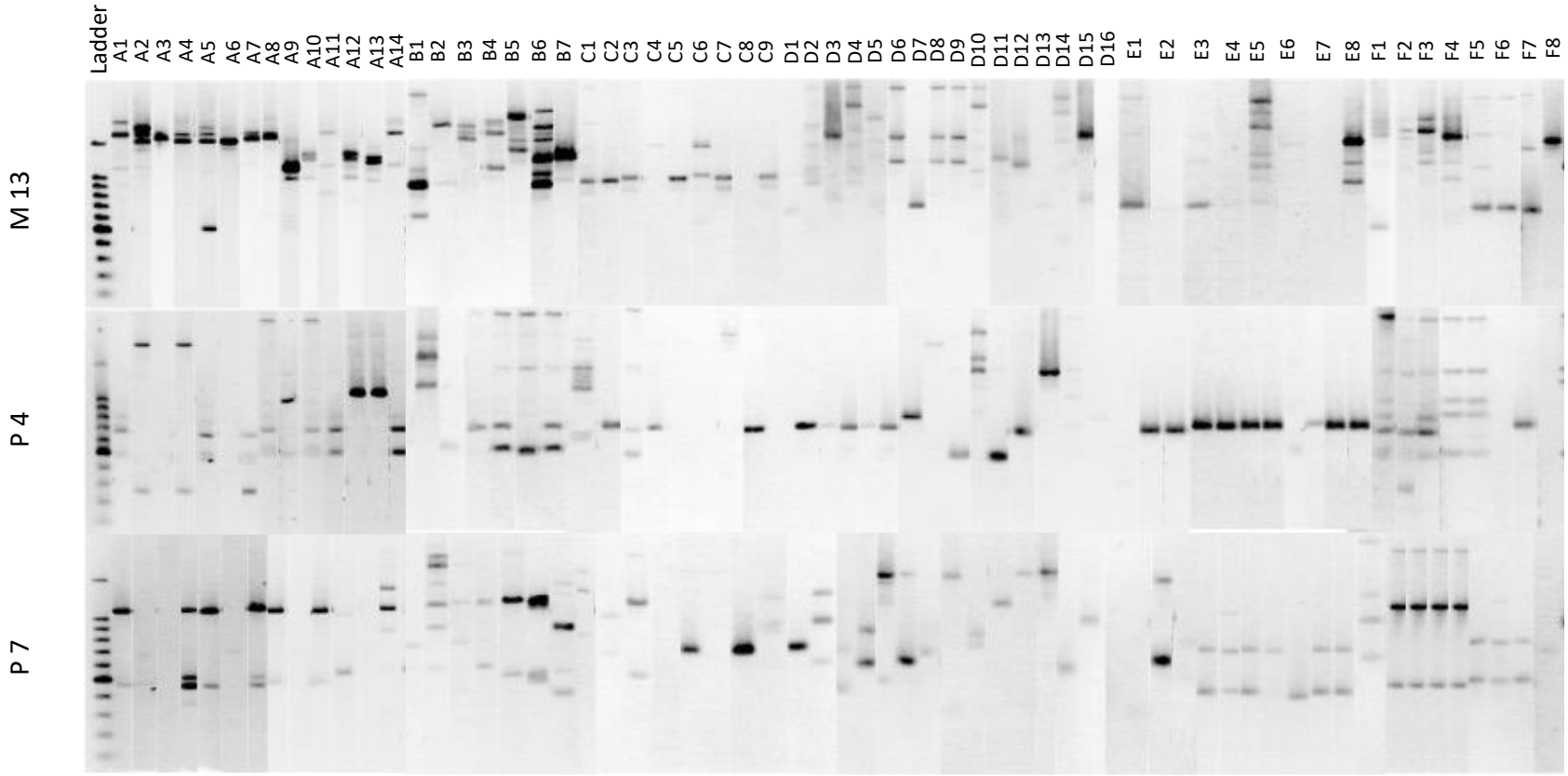

Figure 3.5 Banding patterns of the 62 unique LAB isolates in digested poi differentiated by random amplification of polymorphic DNA-PCR (RAPD-PCR). All isolates had unique patterns that were different from each other when amplified by at least one of the three primers (M13, P4, and P7).

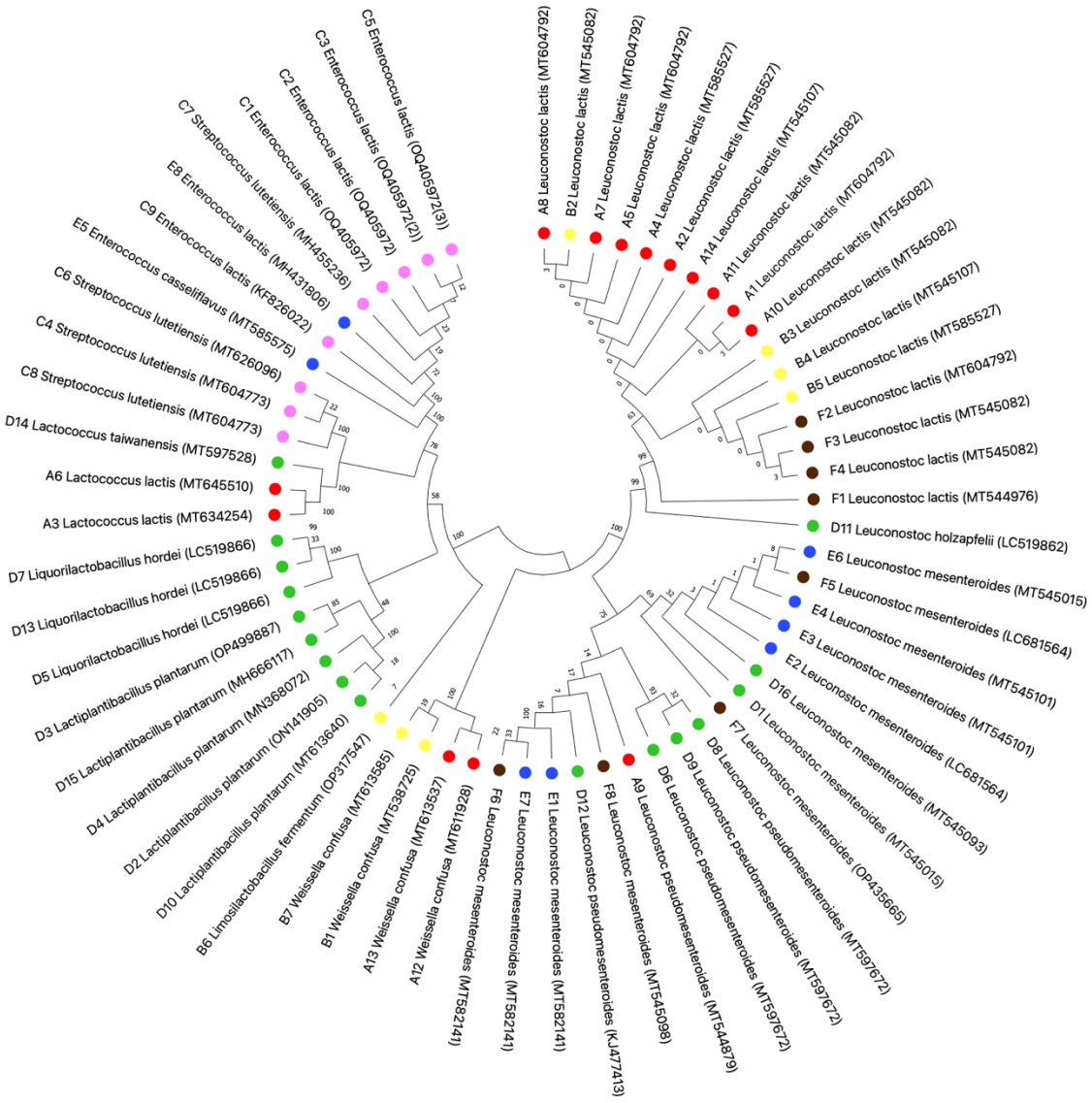

Figure 3.6 Phylogenetic tree of the unique LAB isolated from digested poi based on partial 16S rRNA gene sequences. The tree was constructed using the Maximum likelihood method, 1,000 bootstrap re-samplings, and Kimura 2-parameter distance correction. LAB isolated from different brands were coded with different colors: red—Brand A, yellow—Brand B, Pink—Brand C, Green—Brand D, Blue—Brand E, and Brown—Brand F.

2.3.3 Antimicrobial activities

The pre-screening of antimicrobial activities using the deferred antagonism assay yielded 14 isolates active against *L. monocytogenes* and four isolates active against *S.* Typhimurium (Table 3.3). Notably, the probiotic control LGG had no antimicrobial activity. All nine isolates from Brand C showed antimicrobial activities, with C1, C2, C3, C5, and C9 identified as *E. lactis* and C4, C6, C7, and C8 as *S. lutetiensis*. Isolates A3 and A6 were *L. lactis* and D14 was *L. taiwanensis*. E5 and E8 were *E. casseliflavus* and *E. lactis*, respectively. Isolate A6 was also found in Brands B and F (data not shown). All these isolates were previously reported to possess bacteriocin-producing abilities (de Oliveira et al., 2022; Lei et al., 2022; X. Li et al., 2021; Todorov et al., 2023; Uymaz Tezel, 2019). These isolates were further confirmed for their antimicrobial activities using the microtitration assay.

Table 3.3 Inhibition zones of LAB isolated from poi against *L. monocytogenes* and S. Typhimurium determined by deferred antagonism assay*.

Isolate	Inhibition zone against *L. monocytogenes* (mm)	Inhibition zone against *S.* Typhimurium (mm)
A3	4.0±0.4^{b}	–
A6	6.2±1.1^{ab}	–
C1	8.0±1.4^{a}	3.1±0.75
C2	8.4±1.5^{a}	–
C3	8.1±0.5^{a}	–
C4	4.7±0.1^{b}	3.6±0.73
C5	8.0±1.1^{a}	–
C6	4.6±0.4^{b}	3.5±0.78
C7	8.7±0.9^{a}	–
C8	4.4±0.4^{b}	3.4±0.61
C9	8.6±1.1^{a}	–
D14	8.8±1.0^{a}	–
E5	3.9±0.1^{b}	–
E8	4.0±0.1^{b}	–
LGG	–	–

*Data are mean±SD (n=3). Numbers in the same column with different superscript letters are significantly different ($P < 0.05$). "–" means no inhibition. LGG: *Lacticaseibacillus rhamnosus* GG.

The growth of *L. monocytogenes* was inhibited by neutralized CBE from all 14 isolates screened from the deferred antagonism assay (Table 3.4). CBE of C9 completely inhibited *L. monocytogenes,* resulting in a negative IE. CBE of C7 and C8 also resulted in IE values close to zero. When the CBE were treated with catalase to remove residual H_2O_2, an antimicrobial compound, IE values increased but remained less than 1 for all 14 isolates, indicating the presence of non-H_2O_2 antimicrobial compounds in the extracts (Luo et al., 2011). Catalase-treated CBE of D14 exhibited the lowest IE value of 0.25±0.094 while the IE value of C9 treatment significantly increased to 0.425±0.082, suggesting that C9 may be a strong H_2O_2 producer. Catalase-treated CBE were further treated with proteases to evaluate the presence of antimicrobial proteins (bacteriocins) produced by the isolates. Broad-spectrum proteases trypsin, proteinase K, and pepsin were applied due to their unique binding sites and functioning conditions, providing implications on the structures and chemical properties of these bacteriocins. Trypsin and proteinase K are active at neutral pH whereas pepsin is active at acidic pH. Trypsin targets peptide bonds at the carboxy side of lysine or arginine, whereases proteinase K and pepsin cleave between hydrophobic amino acid residues such as phenylalanine, tryptophan, and tyrosine (Berg, 2007; Lodish & Darnell, 1995; Tang et al., 2019). CBE of all isolates lost most of their anti-*Listeria* activities after being treated by at least one of the proteases, suggesting all isolates produced bacteriocins against *L. monocytogenes*. CBE of the isolates reacted differently upon protease treatments. For example, CBE of A3 was somewhat resistant to proteinase K whereas A6 belonging to the same species was hydrolyzed by this enzyme. This suggested that different types of bacteriocins were produced. Further purification and physiochemical analysis are needed to find out what they are.

Neutralized CBE of all isolates showed some inhibition against *S*. Typhimurium, which was inconsistent with the pre-screening results. This might be due to the excessive H_2O_2 produced by many isolates, except for C7, C8, and C9 which maintained their inhibitory actions after catalase treatment (Table 3.5). These three isolates consistently showed remarkable inhibitory activities against the two indicator strains, with their antimicrobial activities nearly eliminated by proteases.

In summary, antimicrobial LAB were discovered in all six poi brands, with all LAB isolated from Brand C displaying antimicrobial activities. Fourteen isolates produced antimicrobial compounds, including H_2O_2 and bacteriocins. Moreover, these 14 LAB isolates produced bacteriocins effective against *L. monocytogenes,* and C7, C8, and C9 produced bacteriocins effective against both *L. monocytogenes* and *S*. Typhimurium.

Table 3.4 Inhibition efficiency (IE) of neutralized- and enzyme-treated crude bacteriocin extracts (CBE) against *L. monocytogenes**.

Isolate	Neutralized CBE	Catalase-treated CBE	Trypsin-treated CBE	Proteinase-K-treated CBE	Pepsin-treated CBE
A3	0.445 ± 0.043^{abC}	0.677 ± 0.024^{abB}	1.133 ± 0.015^{aA}	0.637 ± 0.054^{eB}	1.151 ± 0.078^{aA}
A6	0.509 ± 0.026^{aC}	0.76 ± 0.095^{aB}	1.083 ± 0.097^{aA}	1.025 ± 0.035^{abcA}	1.157 ± 0.059^{aA}
C1	0.143 ± 0.02^{defD}	0.458 ± 0.007^{cC}	0.674 ± 0.089^{efB}	0.715 ± 0.096^{deB}	1.047 ± 0.056^{abcA}
C2	0.352 ± 0.082^{abcB}	0.454 ± 0.011^{cB}	0.77 ± 0.075^{defA}	0.867 ± 0.023^{bcdA}	0.934 ± 0.079^{bcA}
C3	0.26 ± 0.045^{cdeC}	0.376 ± 0.015^{cdC}	0.749 ± 0.029^{defB}	0.889 ± 0.089^{bcdAB}	1.01 ± 0.061^{abcA}
C4	0.4 ± 0.094^{abcB}	0.479 ± 0.034^{cB}	1.046 ± 0.113^{abA}	1.063 ± 0.09^{abA}	0.979 ± 0.087^{abcA}
C5	0.126 ± 0.094^{efC}	0.366 ± 0.039^{cdB}	0.817 ± 0.03^{cdefA}	0.902 ± 0.063^{abcdA}	0.863 ± 0.038^{cA}
C6	0.444 ± 0.052^{abC}	0.509 ± 0.025^{bcC}	1.008 ± 0.015^{abcAB}	0.965 ± 0.01^{abcB}	1.082 ± 0.048^{abA}
C7	0.058 ± 0.079^{fC}	0.418 ± 0.103^{cdB}	1.036 ± 0.08^{abA}	0.859 ± 0.048^{cdA}	0.985 ± 0.072^{abcA}
C8	0.078 ± 0.027^{fD}	0.36 ± 0.002^{cdC}	0.934 ± 0.012^{abcdB}	1.092 ± 0.025^{aA}	0.977 ± 0.02^{abcB}
C9	-0.017 ± 0.051^{fC}	0.425 ± 0.082^{cB}	0.811 ± 0.031^{cdefA}	0.901 ± 0.056^{abcdA}	0.941 ± 0.061^{bcA}
D14	0.282 ± 0.037^{bcdeC}	0.25 ± 0.094^{dC}	0.786 ± 0.067^{defB}	0.961 ± 0.054^{abcAB}	0.938 ± 0.053^{bcA}
E5	0.305 ± 0.063^{bcdC}	0.442 ± 0.066^{cC}	0.86 ± 0.114^{bcdeB}	1.037 ± 0.054^{abcA}	1.095 ± 0.096^{abA}
E8	0.283 ± 0.048^{bcdeC}	0.349 ± 0.046^{cdC}	0.648 ± 0.038^{fB}	0.873 ± 0.123^{bcdA}	0.86 ± 0.099^{cAB}

*IE < 1, CBE inhibited bacteria growth; IE > 1, CBE promoted bacteria growth; IE = 1, CBE did not affect bacteria growth. Data are mean±SD (n=3). Different lowercase superscripts indicate significant differences across each column ($P < 0.05$). Different capitalized superscripts indicate significant differences across each row ($P < 0.05$).

Table 3.5 Inhibition efficiency (IE) of neutralized- and enzyme-treated crude bacteriocin extracts (CBE) against *S.* Typhimurium*.

Isolate	Neutralized CBE	Catalase-treated CBE	Trypsin-treated CBE	Proteinase-K-treated CBE	Pepsin-treated CBE
A3	$0.637+0.011^{bcdeB}$	$1.173+0.074^{aA}$	$1.012+0.11^{bA}$	$1.029+0.036^{bcA}$	$1.151+0.078^{aA}$
A6	$0.902+0.107^{aB}$	$1.114+0.11^{abAB}$	$0.995+0.04^{bAB}$	$1.083+0.096^{abAB}$	$1.157+0.059^{aA}$
C1	$0.832+0.112^{abB}$	$1.178+0.032^{aA}$	$1.017+0.076^{bAB}$	$1.053+0.048^{abcA}$	$1.064+0.048^{abA}$
C2	$0.802+0.01^{abcC}$	$1.079+0.015^{abA}$	$0.955+0.086^{bAB}$	$0.995+0.018^{bcAB}$	$0.934+0.079^{bBC}$
C3	$0.695+0.077^{abcdB}$	$1.036+0.086^{abA}$	$0.91+0.069^{bcA}$	$1.019+0.044^{bcA}$	$1.01+0.061^{abA}$
C4	$0.67+0.088^{bcdC}$	$0.939+0.046^{bcB}$	$1+0.04^{bB}$	$1.199+0.02^{aA}$	$1.048+0.1^{abAB}$
C5	$0.585+0.036^{deB}$	$1.031+0.053^{abA}$	$0.943+0.148^{bA}$	$1.071+0.058^{abA}$	$1.001+0.087^{abA}$
C6	$0.736+0.031^{abcdC}$	$1.212+0.043^{aA}$	$1.238+0.051^{aA}$	$1.02+0.052^{bcB}$	$1.082+0.048^{abB}$
C7	$0.462+0.123^{efC}$	$0.736+0.108^{cdB}$	$0.847+0.046^{bcAB}$	$0.919+0.04^{cAB}$	$0.985+0.072^{abA}$
C8	$0.156+0.016^{gD}$	$0.498+0.039^{eC}$	$0.811+0.021^{bcB}$	$0.729+0.047^{dB}$	$0.978+0.019^{abA}$
C9	$0.26+0.051^{fgD}$	$0.538+0.016^{deC}$	$0.721+0.055^{cB}$	$0.761+0.065^{dB}$	$0.941+0.061^{bA}$
D14	$0.622+0.053^{cdeD}$	$1.057+0.05^{abAB}$	$0.987+0.029^{bBC}$	$1.131+0.011^{abA}$	$0.938+0.053^{bC}$
E5	$0.302+0.021^{fgB}$	$1.076+0.052^{abA}$	$0.987+0.036^{bA}$	$1.004+0.064^{bcA}$	$1.095+0.096^{abA}$
E8	$0.581+0.074^{deC}$	$1.14+0.116^{abA}$	$1.003+0.05^{bAB}$	$1.005+0.04^{bcAB}$	$0.929+0.021^{bB}$

*IE < 1, CBE inhibited bacteria growth; IE > 1, CBE promoted bacteria growth; IE = 1, CBE did not affect bacteria growth. Data are mean±SD (n=3). Different lowercase superscripts indicate significant differences across each column ($P < 0.05$). Different capitalized superscripts indicate significant differences across each row ($P < 0.05$).

3.3.4 BSH activities and cholesterol assimilation

Screening yielded 52 LAB isolates that were positive for TDCA hydrolase and 15 LAB isolates that were both TDCA and GDCA hydrolase positive, whereas LGG was only TDCA positive (Table 3.6). E5 belonging to *E. casseliflavus* showed strong TDCA hydrolase activity with an inhibition zone diameter of 17.7±0.5 mm. The 15 isolates with GDCA hydrolase activities strictly belonged to *E. lactis*, *L. plantarum*, and *Lactococcus* spp. Moreover, those showing GDCA hydrolase activities displayed equal or stronger inhibitory scores for GDCA compared with TDCA. This could be because GDCA was the preferred substrate for bacteria rather than TDCA (Begley et al., 2006). The ratio of glycolconjugated to tauroconjugated bile salts in human bile is about 3:1 (Begley et al., 2006). Bile salt hydrolase produced by bacteria can remove or unconjugate the amino acid group from primary bile salts, resulting in the precipitation of unconjugated primary bile salts and their subsequent transformation into secondary bile salts by gut bacteria (Begley et al., 2006). BSH activity not only has crucial implications for bacteria bile resistance and persistence in the gut, but also plays a role in cholesterol removal in the intestines. This is achieved by preventing the reabsorption of biliary cholesterol and co-precipitating dietary cholesterol with the unconjugated bile salts (Jia et al., 2021).

All isolates assimilated cholesterol to some extent, with many displaying assimilation levels comparable to LGG (52.9±6.8% without bile salts, 63.0±7.5% with bile salts). As shown in Fig 3.7, the cholesterol assimilation percentages (without bile salts) of most isolates ranged from 40% to 68 %, while a few isolates in Brand C showed relatively low cholesterol removal levels of about 7-30%. However, when bile salts were added, isolates from Brand C significantly assimilated more cholesterol by 20-38% ($P < 0.05$). Similar trends of increased cholesterol assimilation in the presence of bile salts were observed among isolates from Brands A and B. Brand D displayed mixed results with bile salts, which may be due to the readily high cholesterol assimilation levels of those isolates (e.g. D4). Interestingly, contrasting effects of bile salts were observed in isolates from Brands E and F, compared to the other brands, where bile salts reduced the level of assimilated cholesterol. The majority of the isolates from Brands E and F belonged to *L. mesenteroides* and lacked GDCA hydrolase activities (Table 3.6). These isolates might have been inhibited by GDCA, resulting in

lowered cholesterol removal. However, the physiological processes of bacterial cholesterol assimilation are rather complex and are not only affected by BSH activities. Certain probiotics are able to incorporate or attract gut cholesterol to bacterial cell membranes, while others produce enzymes that convert cholesterol into a less soluble form, corprostanol (Romero-Luna et al., 2021). Comprehensive investigations are needed to fully understand the modes of action of each isolates. Regardless, LAB isolated from poi showed cholesterol-removing abilities, highlighting their potential application in alleviating and preventing conditions like hyperlipidemia and atherosclerosis.

Table 3.6 Bile salt hydrolase (BSH) activities of unique LAB isolated from poi*.

Isolate	TDCA hydrolase activity score	GDCA hydrolase activity score	Isolate	TDCA hydrolase activity score	GDCA hydrolase activity score
A1	+	–	D1	+	–
A2	–	–	D2	+	+++
A3	+	++	D3	++	+++
A4	+	–	D4	++	+++
A5	–	–	D5	++	–
A6	+	+	D6	–	–
A7	+	–	D7	++	–
A8	++	–	D8	++	–
A9	+	–	D9	–	–
A10	–	–	D10	++	+++
A11	–	–	D11	++	–
A12	+	–	D12	++	–
A13	+	–	D13	+	–
A14	–	–	D14	+	++
B1	++	–	D15	++	+++
B2	–	–	D16	++	–
B3	++	–	E1	++	–
B4	++	–	E2	+	–
B5	++	–	E3	++	–
B6	++	–	E4	+	–
B7	–	–	E5	++++	–
C1	++	++	E6	+	–
C2	++	++	E7	+	–
C3	++	+++	E8	++	++++
C4	++	–	F1	–	–
C5	++	+++	F2	+	–
C6	+++	–	F3	++	–
C7	++	+++	F4	+	–
C8	+++	–	F5	++	–
C9	++	+++	F6	+	–
LGG	++	–	F7	++	–
			F8	+	–

*Hydrolase activity scores were determined on the size of the white precipitation zone (diameter): –, no activity; +, < 8.5 mm; ++, 8.6–10 mm; +++, > 10 mm; ++++, > 15 mm. LGG: *Lacticaseibacillus rhamnosus* GG.

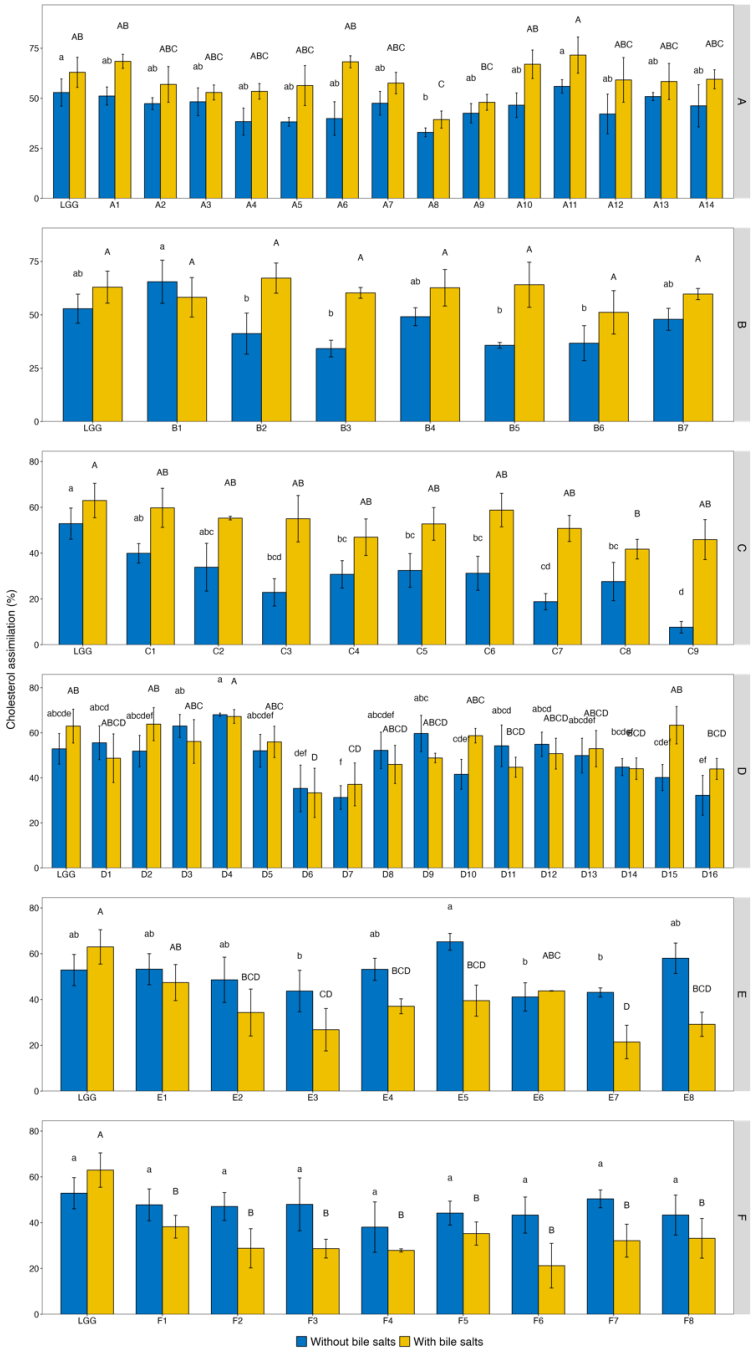

Figure 3.7 Cholesterol assimilation (%) of LAB isolated from poi with or without the presence of bile salts (2.8 mM GDCA and 1.2 mM TDCA). Error bars are SD (n=3). Lowercased letters indicate statistics between isolates analyzed within the “Without bile salts” group in each plot. Uppercased letters indicate statistics between isolates analyzed within the “With bile salts” group in each plot. Significance threshold $P < 0.05$. LGG: *Lacticaseibacillus rhamnosus* GG.

3.3.5 Adhesion to Caco-2 cells

Adhesion to the intestinal epithelium is the first step in exerting probiotic properties, preventing pathogenic bacteria, and modulating the immunomodulation functions (Zhou et al., 2021). Strong adhesion also increases the probability of bacterial colonization in the gut and provides long-lasting benefits, and hence is a favorable probiotic trait (Krausova et al., 2019). The average adhesion of LGG was 0.84±0.15%. The adhesion capacity of the isolates from poi varied from 0.14–8.14% (Fig 3.8). It is worth pointing out that many isolates displayed extraordinary adhesion to Caco-2 cells that were much higher than LGG ($P< 0.05$). For example, isolates C9 and D15 adhered 8.7 and 8.4 folds more than LGG, respectively. *Enterococcus* have been found to possess excellent adhesion properties. For example, a probiotic *E. durans* strain exhibited 2.29 folds more adhesion to Caco-2 cells than LGG (Zhou et al., 2021). Isolates belonging to *E. lactis,* including C3, C5, C9, and E8, all had higher adhesion than LGG. Isolates D3, D4, D10, and D15, identified as *L. plantarum,* also showed higher adhesion levels than LGG. Interestingly, the same isolates also exerted excellent BSH activities with both TDCA and GDCA hydrolase activities, and high cholesterol removal abilities ranging from 56-67% in the presence of bile salts. Moreover, although isolates A2, A4, A5, A7, A8, A9, and A11 all belonged to *Leuconoctoc lactis* species, their adhesion abilities varied, indicating the versatility of these isolates. Isolates from Brands E and F mostly showed comparable adhesion as LGG, with F4 and F8 demonstrating the lowest levels of adhesion.

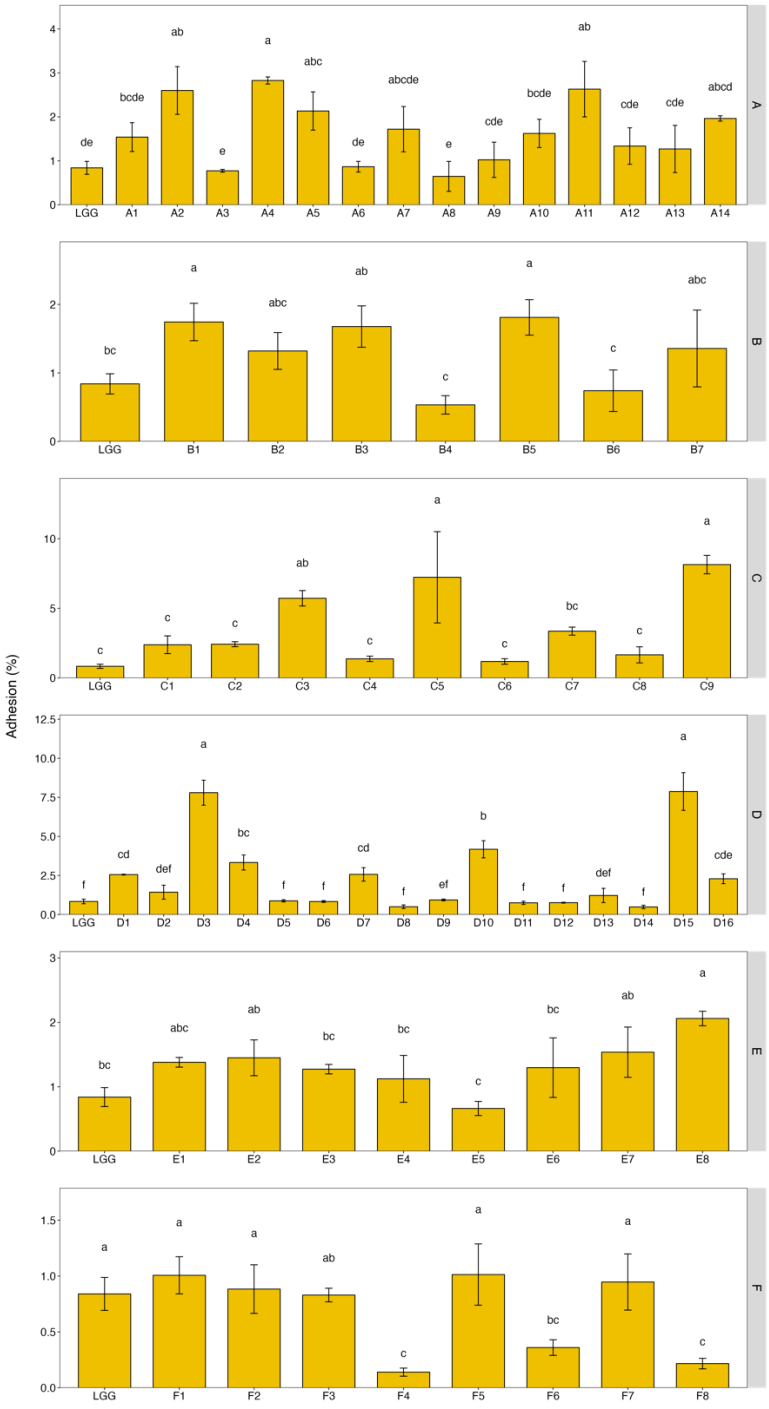

Figure 3.8 Adhesion (%) of LAB isolated from poi on Caco-2 cells. The percentage of adhesion was calculated by dividing the total number of adhered LAB by the total number of inoculated LAB. Error bars are SD (n=3). Bars labeled with different letters mean they are significantly different in each plot ($P < 0.05$). LGG: *Lacticaseibacillus rhamnosus* GG.

3.3.6 Safety properties

Bacteria exert three types of hemolytic activities: α-, β-, and γ-hemolysis. Only β-hemolysis is the true hemolysis where red blood cells are hydrolyzed by hemolysins produced by the bacteria (Buxton, 2005). α-hemolysis refers to the reduction of hemoglobin to methemoglobin caused by hydrogen peroxide. γ-hemolysis means no hemolysis (Buxton, 2005). Hemolytic activities are not inherently dangerous, however, certain pathogenic bacteria species are associated with beta- or alpha- hemolysis, for instance, *Streptococcus* spp. (McDevitt et al., 2020). As shown in Table 3.7, 12 isolates were not hemolytic, while the others were α-hemolytic meaning that they produced hydrogen peroxide. No β-hemolytic activity was detected in any isolate. Hemolytic streptococci strains could be pathogenic. However, *Streptococcus lutetiensis* identified in poi belongs to the Group D streptococci that are indigenous bacteria in human gastrointestinal tract and are usually not pathogenic (Chayakulkeeree et al., 2015).

Moreover, probiotics should be able to adhere to intestinal barriers without causing toxicity to the epithelial cells. This study demonstrated that at a MOI equivalent to 100:1, which was also used in the adhesion assay, the CFS of all isolates did not induce any toxicity in Caco-2 cells (Figure 3.9). In fact, some isolates promoted the cell proliferation, resulting in viabilities higher than 100%, such as A7 (149.5±10.9%), C1 (119.6±7.8%), D15 (121.2±7.0%), E3 (139.1±5.6%), and F7 (139.4±7.5%). This type of promotive effects of probiotic CFS have been observed in *L. lactis* and *E. faecium* (Dowdell et al., 2020).

Table 3.7 Hemolytic activities of LAB isolated from poi*.

Isolate	Hemolysis	Isolate	Hemolysis
A1	α	D1	α
A2	α	D2	α
A3	γ	D3	α
A4	α	D4	α
A5	α	D5	γ
A6	γ	D6	α
A7	α	D7	γ
A8	α	D8	α
A9	α	D9	α
A10	α	D10	α
A11	α	D11	γ
A12	γ	D12	α
A13	α	D13	γ
A14	α	D14	α
B1	α	D15	α
B2	α	D16	α
B3	α	E1	α
B4	α	E2	α
B5	α	E3	α
B6	α	E4	α
B7	γ	E5	α
C1	α	E6	α
C2	γ	E7	α
C3	α	E8	α
C4	γ	F1	α
C5	α	F2	α
C6	γ	F3	α
C7	α	F4	α
C8	γ	F5	α
C9	α	F6	α
LGG	γ	F7	α
		F8	α

*α, α-hemolysis; γ, no hemolysis. n=3.

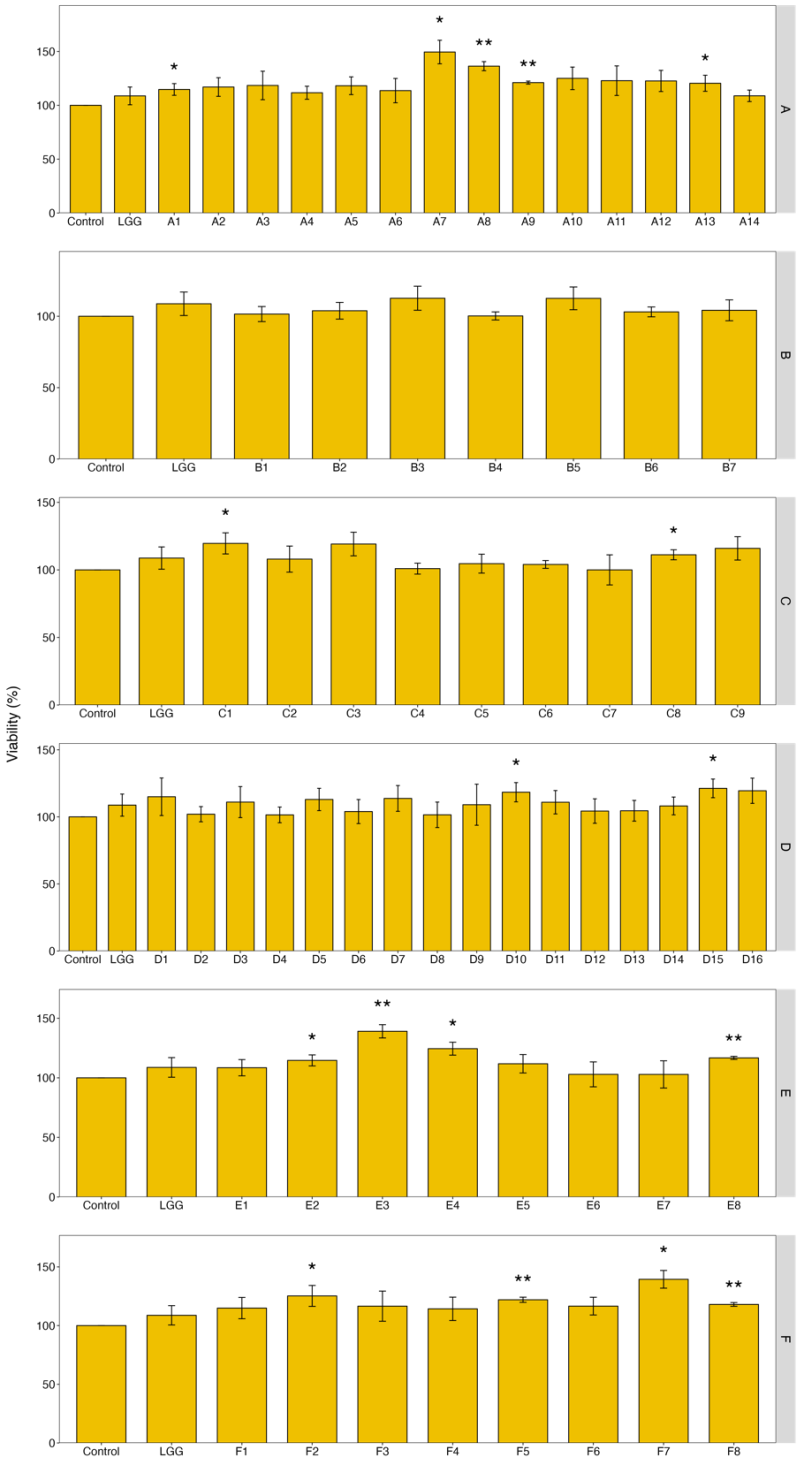

Figure 3.9 Cytotoxicity of 1% of the cell free supernatant of LAB isolates in Caco-2 cells. Error bars are SD (n=3). Bars labeled with "*" mean they are significantly different from the viability of Caco-2 cells treated with 1% MRS (control, 100%; "*", $P < 0.05$; "**", $P < 0.01$). LGG: *Lacticaseibacillus rhamnosus* GG.

3.3.7 Overall probiotic properties of the isolates

At last, a heatmap of clustering was constructed based on the probiotic properties of the isolates, including TDCA and GDCA hydrolase activities, antimicrobial activities against *L. monocytogenes* and *S.* Typhimurium, cholesterol assimilation, adhesion, and cytotoxicity (Fig 3.10). This heatmap provides an overview of the probiotic property distribution among the isolates. The 10 isolates (C7, C5, D4, D2, C3, A3, D10, C1, D3, and C2) in sub-cluster *a*, cluster 1 exhibited strong overall probiotic attributes. They belonged to the species: *S. lutetiensis* (C7), *E. lactis* (C5, C3, C1, and C2), *L. plantarum* (D4, D2, D10, and D3), and *L. lactis* (A3). In addition to the isolates showing overall good probiotic characteristics, some isolates stood out due to their specialized traits. For example, C9, E5, C8, D14, and C4 in cluster 2 had high antimicrobial activities. F7, E3, A8, and A8 in cluster 5 were excellent at enhancing Caco-2 cell viability. Overall, all LAB isolated from poi showed probiotic attributes to some extent, indicating that fermented poi is a health-promoting probiotic food.

3.4 Conclusions

In conclusion, *in vitro* digestion reduced the LAB load and altered the microbiome community structure in fermented poi. This study identified 62 unique LAB strains belonging to 13 species. The five most frequently identified species were *Leuconostoc lactis*, *Leuconostoc mesenteroides, Enterococcus lactis*, *Leuconostoc pseudomesenteroides*, and *Lactiplantibacillus plantarum*. Fourteen isolates showed antimicrobial properties, 52 isolates had BSH activities, all isolates were able to assimilate cholesterol, and 17 isolates adhered to Caco-2 cells more effectively than the commercial probiotic strain LGG. No isolate exhibited hemolytic or toxic effects on Caco-2 cells. Isolates belonging to *S. lutetiensis*, *E. lactis*, *L. plantarum*, and *L. lactis* stood out due to their extraordinary overall probiotic properties. However, all LAB isolates in poi showed characteristics beneficial to health. The results obtained from this study provide evidence suggesting the probiotic potential of fermented poi and warrant further *in vivo* and human study analysis to understand their effects.

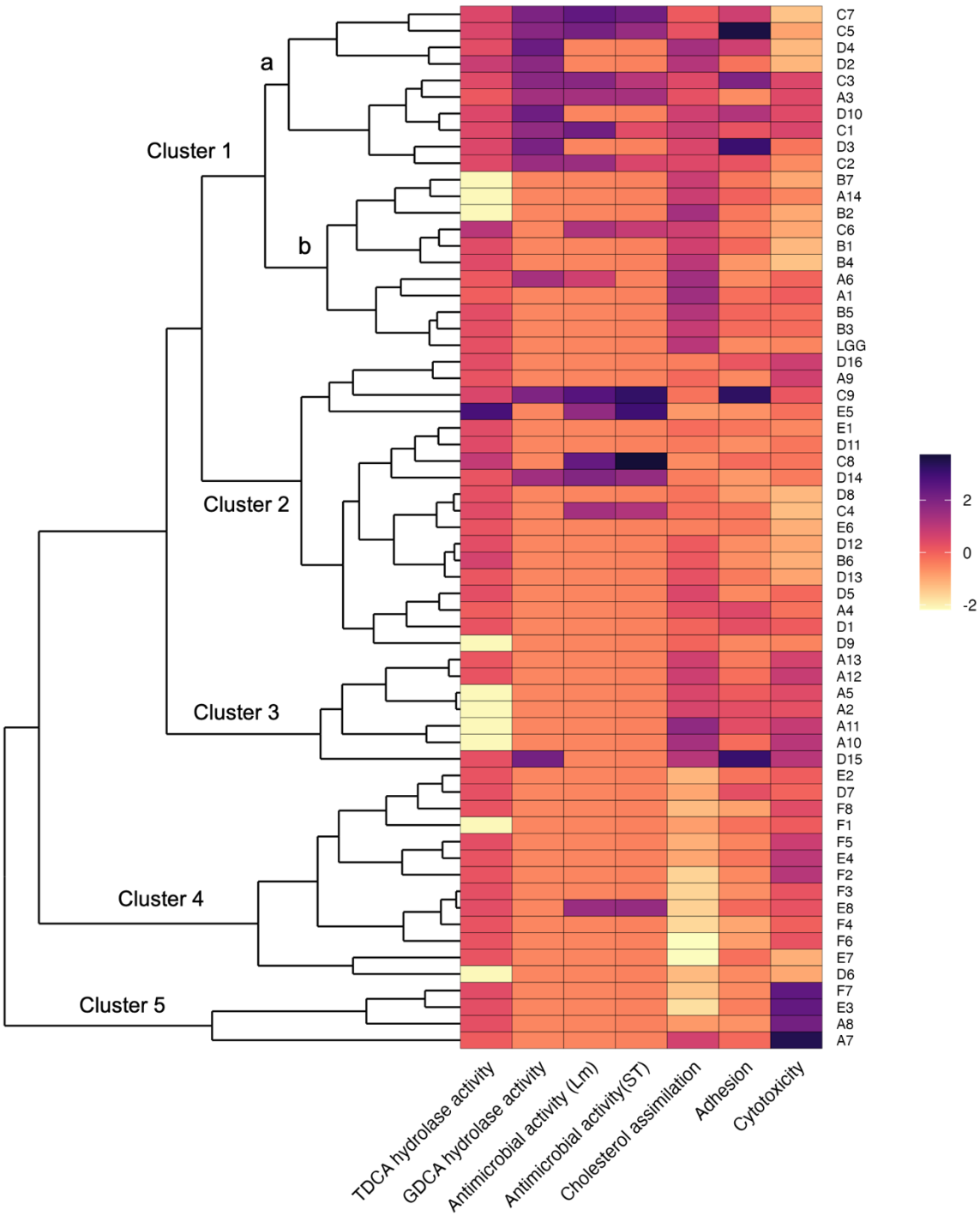

Figure 3.10 Heatmap clustering of LAB isolates based on the Euclidean distance of the probiotic property values. Dark color means stronger activity. Cytotoxicity was expressed as viability in Caco-2 cells.

CHAPTER 4 EFFECTS OF FRESH AND FERMENTED POI ON INFECTION, INFLAMMATION, AND LIPID METABOLISM IN CACO-2 CELLS

Abstract

Dysbiosis, defined as an imbalance in the gut microflora, has been linked to diseases such as inflammatory bowel diseases, metabolic disorders, and colorectal cancer. The intestinal epithelium acts as a barrier, but damage to the mucus layer and epithelium can trigger inflammation and facilitate pathogen infiltration. Fermented plant-based foods, rich in nutrients, bioactive compounds, probiotic bacteria, and postbiotics, have gained attention for their health-promoting properties. Postbiotics, which are soluble metabolites produced by live bacteria, offer similar benefits without requiring the administration of live bacteria. Poi has been demonstrated to harbor probiotics that produce antimicrobial compounds and reduce cholesterol levels. This study aimed to investigate the effects of both fresh and fermented poi extracts on intestinal epithelial cells in various scenarios involving bacterial infection, inflammation, oxidative stress, and gene expression related to lipid metabolism. Water-soluble extracts (50%) were prepared from two brands of fresh and fermented poi (Brand A and Brand C). Caco-2 cells were pre-treated with neutralized poi extracts for 24 h and then used to study bacterial infection, inflammation, and regulation of lipid metabolism. The adhesion and invasion of *Listeria monocytogenes* and *Salmonella* Typhimurium were assessed in Caco-2 cells treated with poi at a multiplicity of infection (MOI) of 100:1. Furthermore, inflammation was induced in Caco-2 cells using 500 μM H_2O_2 for 24 h, 10 μg/mL lipopolysaccharides for 24 h, *L. monocytogenes* (MOI=100:1) for 24 h, and *S.* Typhimurium (MOI=100:1) for 4 h. The relative expressions of pro-inflammatory, anti-inflammatory, and tight junction protein genes were determined. Expression of eight genes related to cholesterol and lipid homeostasis were also evaluated in poi-treated Caco-2 cells. Results demonstrated that fermented poi extracts A and C inhibited the adhesion of *L. monocytogenes* to Caco-2 cells by 30 and 55%, respectively. Extracts from fresh and fermented brand C poi reduced *L. monocytogenes* invasion by 20 and 36%, respectively. Only extract from fermented brand C poi had a significant effect on the adhesion of S. Typhimurium, resulting in a reduction to 26%. The extracts exhibited

anti-inflammatory properties by suppressing pro-inflammatory genes (IL-6, IL-8, and TNF-α) and promoting the expression of anti-inflammatory genes (IL-4, IL-10, TGF-β, and TLR2). Poi extracts also enhanced the gene expression of tight junction proteins, including claudin-1, occludin, and ZO-1, which are vital for maintaining gut barrier integrity, as well as epithelial cell proliferation and survival. Moreover, the extracts displayed significant potential in modulating lipid metabolism by upregulating the expression of PPARs, FIAF, and SREBP-2 in Caco-2 cells, which may favor lipolysis in different tissues and promote gastrointestinal immunoregulation. However, genes involved in cholesterol homeostasis, such as LXR and NPC1L1, were not affected by poi. Notably, fermented poi extracts exhibited more potent overall effects compared to fresh poi extracts, highlighting the beneficial role of postbiotics. In conclusion, these findings suggest that poi extracts possess promising anti-infection, anti-inflammatory, and lipid metabolism modulation properties in Caco-2 cells.

Keywords: poi, Caco-2 cell, anti-inflammation, pro-inflammation, gene expression, PPAR, FIAF, LXR, SREBPs, NPC1L1, adhesion, invasion, tight junction

4.1 Introduction

The human gut is a complex ecological system. Disturbances in the balance of the indigenous gut microflora, known as dysbiosis, have been associated with a range of diseases including inflammatory bowel diseases (IBD) like Crohn's disease (CD) and ulcerative colitis (UC), metabolic disorders like obesity and type 2 diabetes, mental disorders like autism and depression, and colorectal cancer (Mayorgas et al., 2021). These diseases exhibit complex bi-directional interactions with low-grade inflammation and oxidative stress in the intestines (Agarwala et al., 2021; Genua et al., 2021; Gregor & Hotamisligil, 2011; Lau et al., 2021).

The intestinal epithelium serves as a barrier that prevents the free diffusion and entry of harmful chemicals, antigens, and pathogens from the gut lumen to the underlying lamina propria (Zuo et al., 2020). However, damaged mucus layer and epithelium allows the influx of pathogens, triggering the activation of antigen-presenting cells (APC) like macrophages and dendritic cells. This activation leads to the production of inflammatory cytokine (e.g. tumor necrosis factor- α [TNF-α], Interleukin [IL]-1, IL-6, IL-8, etc.) and the release of reactive oxygen species (ROS) (Yeshi et al., 2020). Under pathologic conditions, the uncontrolled chronic inflammatory response and oxidative stress further damage the tissue, creating a detrimental feedback loop (Agarwala et al., 2021). In a healthy state, inflammation is modulated by immunoregulatory T cells like Th2 and Treg. These cells release anti-inflammatory factors such as IL-4, IL-10, and transforming growth factor- β (TGF-β) that inhibit the production of anti-inflammatory cytokines and play important roles in immune tolerance (Azad et al., 2018; Däbritz, 2015). Moreover, the integrity of tightly sealed intercellular junctions prevents the free entry of gut pathogens and antigens, thus limiting inflammatory responses in the subepithelial tissues. The complex structure of tight junctions comprises over 40 proteins, primarily including claudins, occludin, and ZO-1 (Paradis et al., 2021). Claudin forms an irreplicable transmembrane barrier, whereas occludin and ZO-1 contribute to barrier function, although they are not essential in the absence of external stressors (Kuo et al., 2022). However, occludin and ZO-1 play essential roles in regulating epithelial apoptosis, proliferation, and repair processes (Kuo et al., 2022). Together,

these components play vital roles in regulating gut Immunological homeostasis and epithelial cell proliferation and survival.

Fermented plant-based foods have garnered attention due to their health-promoting properties, including anti-oxidative, anti-inflammatory, anti-obesity, anti-diabetic, anti-hyperlipidemia, and anti-carcinogenic effects (Shahbazi et al., 2021). These beneficial properties can be attributed to the presence of nutrients and natural bioactive compounds in plant foods, such as anthocyanins, flavonols, proanthocyanidins, as well as probiotic lactic acid bacteria (LAB) and their metabolites produced during fermentation (Mayorgas et al., 2021; Shahbazi et al., 2021). Postbiotics, the soluble metabolites produced by live bacteria or released after bacterial lysis, have beneficial effects on the host . Postbiotics, commonly understood as a set of bacterial metabolites instead of a single compound, include short chain fatty acids (SCFAs), exopolysaccharides (EPS), bacteriocins, enzymes (e.g. glutathione peroxidase), Indole derivatives and polyamines derived from amino acid metabolism, and so on (Mayorgas et al., 2021; S.-J. Park et al., 2023). Postbiotics are considered advantageous to probiotics as they can provide similar effects to the body without the associated risk of administrating live bacteria.

It has been reported that postbiotics possess anti-inflammatory and immunomodulatory effects, promoting the functions of intestinal epithelial cells. These effects include the activation of the adenosine 5′-monophosphate-activated protein kinase -activated protein kinase (AMPK) signaling pathway, leading to the induction of autophagy and the inhibition of adhesion, invasion, and proliferation of pathogenic bacteria in intestinal cells (Wu et al., 2023). Moreover, postbiotics have been observed to reduce pro-inflammatory cytokines such as IL-8, IL-6 and TNF-α, and reactive oxygen species (ROS), while restoring the morphology of tight junction and preserving the integrity of the gut barrier (Algieri et al., 2023; Filannino et al., 2021).

In addition to their anti-inflammatory properties, probiotics and postbiotics have shown potential in combating obesity, cardiovascular diseases, and obesity-related physiological factors (Beena Divya et al., 2012; A. Hassan et al., 2019; S.-J. Park et al., 2023). Research has furnished compelling evidence for the beneficial effects of probiotics on the regulation of intestinal levels of fasting-induced adipose factor (FIAF)

through the release of ROS or SCFAs (Aron-Wisnewsky et al., 2021; Jacouton et al., 2015). FIAF can inhibit the activity of lipoprotein lipase and promote lipolysis, resulting in reduced triglyceride accumulation and cholesterol uptake in various tissues (Y. Wang et al., 2022). FIAF is regulated by upstream genes known as peroxisome proliferator-activated receptors (PPARs; Jacouton et al., 2015). Elevated serum FIAF protein levels and decreased fat storage were observed in mice fed with a high-fat diet supplemented with probiotics (Aronsson et al., 2010). Furthermore, increased expression of intestinal FIAF and a corresponding rise in circulating FIAF were noted in mice treated with probiotics, and this upregulation of FIAF was found to be regulated by PPAR-α (Jacouton et al., 2015). Moreover, probiotics and postbiotics downregulate genes involved in cholesterol uptake and synthesis such as Niemann–Pick C1-like 1 (NPC1L1), liver X receptor (LXR) pathway (LXR), and ATP-binding cassette sub-family G members 5 and 8 (ABCG5/8) in the intestines (Liang et al., 2020; Yoon et al., 2011). Overall, the growing body of evidence suggests the potential of probiotics to exert positive effects on serum lipid profiles, lipogenesis, and lipolysis. These findings offer promising avenues for the management of lipid-related disorders through the modulation of gut microbiota.

Poi, made from cooked mashed taro, is rich in dietary fiber such as resistant starch and bioactive compounds like phenolic compounds and flavonoids (Ghan et al., 1977; Saxby, 2020; Senga, 2021). In Chapter 2, it was discovered that fermented poi contains probiotic LAB with antimicrobial and cholesterol-lowering properties. Based on this finding, we hypothesized that the postbiotics produced by LAB in poi may exert similar anti-infection, anti-inflammation, and anti-hyperlipidemia properties. The differentiated human colorectal adenocarcinoma cell line-2 (Caco-2) develops structures and functionalities similar to human enterocytes, making it a suitable model for investigating the physiological responses of the intestinal epithelium to various stimuli (Lea, 2015). Therefore, this study aimed to explore and compare the effects of water-soluble extracts from fresh and fermented poi on Caco-2 cells under three scenarios: (1) infection by pathogenic bacteria, including gram-positive *Listeria monocytogenes* and gram-negative *Salmonella* Typhimurium; (2) inflammation induced by lipopolysaccharides (LPS), live

bacteria (*L. monocytogenes* and *S.* Typhimurium), and oxidative stress induced by H_2O_2; and (3) expression of genes related to cholesterol and lipid metabolism.

4.2 Methods

4.2.1 Cell culture

Human Caco-2 cell line was purchased from MilliporeSigma (catalog number: 86010202, Lot. 21G005; Burlington, MA, USA). All reagents used in cell culture medium were purchased from Gibco™ (Thermo Fisher Scientific, Waltham, MA, USA). Caco-2 cells were maintained in a complete medium containing Dulbecco's modified Eagle'smedium(DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 0.03% L-glutamine (200 mM, 100×), 1% non-essential amino acids (NEAA), and 1% penicillin-streptomycin at 37°C in the presence of 5% CO_2. The cells were sub-cultured at 80-90% confluence in 250 mL Falcon® flasks every 3-4 days and were used between passages 30-40 for all experiments. For the MTT assay, 100 µL of 10^5 cells/mL exponentially growing Caco-2 cells were seeded in 96-well plates (10^4 cells/well) and cultured for 24 h to obtain a monolayer. To observe the effects of poi extract on cell morphology, the cells were seeded at 10^5 cells/well in 12-well plates and cultured for 24-72 h to achieve monolayer. For other experiments, the cells were seeded at 10^5 cells/well in 12-well plates. After confluence, the cells were incubated for 21 days to allow spontaneous differentiation. The culture medium was changed on alternate days with the complete medium. The cell culture medium was changed to a cell medium without antibiotics (containing 10% FBS, 0.03% L-glutamine, 1% NEAA) one day before any treatment.

4.2.2 Preparation of poi extract

Poi Brands A and C were selected for this study according to their superior probiotic properties observed in Chapter 2 and convenient market availability. Fresh poi was purchased from local markets on the day of distribution and transferred in a cooler immediately to the lab. Fermented poi was prepared by allowing natural fermentation of fresh poi at room temperature (RT, 21°C) for 48 h. Water-soluble extract was prepared from poi according to Brown et al. (2005), with modifications. Poi was incubated in

Dulbecco's phosphate-buffered saline (DPBS, Gibco™) at 0.1 g/mL in a sterile 50 mL centrifuge tube at 37°C for 2 h with agitation at 100 rpm, followed by centrifugation at 4,000 × *g* for 10 min. The supernatant was neutralized with 1 M NaOH to pH 7.0, and then sterilized by filtering through 0.22 μm cellulose acetate syringe filters. The filter-sterilized extract was diluted to the desired concentration in cell medium without FBS or antibiotics (containing 0.03% L-glutamine, 1% NEAA).

4.2.3 MTT assay and cell morphology

The cytotoxicity of poi extracts in Caco-2 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay (Choi et al., 2021; Yasmin et al., 2020). At confluence, cell medium was removed and washed with fresh DMEM twice. 100 μL of poi extract at 5, 10, 20, 25, 50, 60, and 70% was added to the cells and incubated at 37°C in 5% CO_2. After 24 h, the extract was discarded. The treated cells were washed with DME twice and added with 100 μL of 0.5 mg/mL MTT solution (Invitrogen™, Thermo Fisher Scientific) diluted in fresh cell medium without FBS or antibiotics. After 4 h of incubation at 37°C, the MTT solution was removed and replaced with 100 μL dimethyl sulfoxide (Thermo Fisher Scientific). The formazan crystals were allowed to dissolve for 10 min at RT, and then absorbance at 570 nm was obtained using a spectrophotometer (Bio-Rad, iMark, Hercules, CA, USA). The cell viability was calculated as follows (Filannino et al., 2021).

Viability (%)= the absorbance of poi-treated cells/the absorbance of un-treated cells ×100

Another set of cells were seeded in 12-well plates as described above. At confluence, the cells were treated with different concentrations of poi extract for 24 h. Images of Caco-2 cell monolayer were taken using a ZOE Fluorescent Cell Imager (Bio-Rad). Three independent replicates of images were collected. Only representative images are shown in this chapter.

4.2.4 Adhesion and invasion assays

Poi extract treatments. Differentiated Caco-2 cells were washed with DPBS twice and added with 1 mL of 50% different poi extracts, followed by incubation at 37°C in 5%

CO_2 for 24 h. The control was incubated in 1 mL of cell medium without FBS or antibiotics.

Bacterial culture. Foodborne pathogenic bacterial strains *Listeria monocytogenes* F2365 and *Salmonella enterica* Typhimurium ATCC 14028 were obtained from the Food Microbiology Lab at the University of Hawaii at Manoa (Honolulu, USA). Stock cultures of *L. monocytogenes* and *S.* Typhimurium were activated by striking onto selective agar of modified oxford agar and xylose lysine deoxycholate agar (Becton Dickinson, Franklin Lakes, NJ, USA), respectively, and incubating at 37°C for 48 h. Single colony of each bacterium was picked and grown in tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C overnight (16 h). Active culture was transferred twice in TSB before the use for experimentation. The bacterial cell pellets were collected by centrifugation at 16,000 × *g* for 2 minutes and then washed with DPBS twice. The final pellets were resuspended and diluted in Caco-2 cell medium without FBS or antibiotics at desired concentrations.

Adhesion assay. The bacterial adhesion assay was performed following the protocol described by Jayashree et al. (2018), with modifications. Poi-extract-containing medium was remove from Caco-2 cells after the treatment. The cells were washed with DPBS twice, followed by adding 1 mL of bacterial suspension at a multiplicity of infection (MOI) ratio of 10:1 meaning the number of bacteria cells to Caco-2 cells ratio was 10 to 1. The Caco-2 cells were then incubated at 37°C in 5% CO_2 for 2 h. Subsequently, the bacterial suspension was removed, and the Caco-2 cells were washed with DPBS three times to remove un-adherent bacterial cells. 1 mL of 0.1% Triton X-100 (Thermo Fisher Scientific) was added to each well and incubated at RT for 5 min to lyse the Caco-2 cells and release the adhered bacteria. The number of adhered bacteria was determined by serially diluting the lysate in 0.1% peptone water (Becton Dickinson) and culturing on plate count agar (PCA, Becton Dickinson) at 37°C for 48 h.

Invasion assay. The bacterial invasion assay followed the same procedure as the adhesion assay except before lysing the Caco-2 cells with Triton, the extracellular bacteria were killed by incubating in 1 mL of 100 µg/mL gentamicin (Thermo Fisher

Scientific) at 37°C for 1 h in 5% CO_2 (Aljasir & D'Amico, 2021). The internalized bacteria were also enumerated on PCA.

The relative percentage of adhesion or invasion was calculated by dividing the total number of adhered or invaded bacteria in poi-extract-treated Caco-2 cells by the total number of adhered or invaded bacteria in untreated Caco-2 cells.

4.2.5 Induction of oxidative stress and inflammatory response in Caco-2 cells

Experimental design. One set of the differentiated Caco-2 cells was treated separately with 50% fresh and fermented poi extracts from Brands A and C for 24 h. Another set of the cells was incubated in cell medium without FBS or antibiotics for 24 h as the control. Both treated and un-treated cells were divided into five sets. Four sets were induced with H_2O_2, LPS, *L. monocytogenes*, and S. Typhimurium, while the fifth set was incubated in cell medium without FBS or antibiotics. The fifth set was designated to observe any effects of poi extracts on the expression of interested genes and served as negative controls to the induction groups. All treatments were performed in three independent replicates.

Oxidative stress. Caco-2 cells were washed with DPBS twice and then exposed to 1 mL of 500 µM H_2O_2 (VWR Chemicals BDH®) diluted in fresh DMEM per well for 24 h to induce oxidative stress.

LPS-induced inflammation. The inflammatory response was induced by adding lipopolysaccharide (LPS) from *S.* Typhimurium (Sigma- Aldrich, catalog number: L6143, St. Louis, MO, USA). Caco-2 cells were washed with DPBS twice and then exposed to 1 mL of 10 ug/mL LPS diluted in cell medium without FBS or antibiotics for 48 h.

Bacterial infection-induced inflammation. Caco-2 cells were washed with DPBS twice and infected with *L. monocytogenes* at MOI of 100:1 for 24 h or *S*. Typhimurium at MOI of 10:1 for 4 h.

4.2.6 Relative gene expression of inflammatory cytokines and tight junction proteins

The treated and control Caco-2 cells were washed three times with DPBS. One milliliter DPBS was added in the well, and then the cells were detached by scraping from the tissue culture plate and collected into sterile micro-centrifuge tubes. Immediately, the cell suspension was centrifuged at 300 *g* for 5 min at RT and the

supernatant was replaced with 1 mL RNALater (Thermal Fisher Scientific). The collected cells were stored at 4°C and RNA was extracted within one week. RNA was extracted using the Qiagen RNeasy Mini Kit, and potential DNA contamination was removed following an on-column digestion protocol with Qiagen RNase-Free DNase Set (Qiagen, Hilden, Germany). The concentration of RNA extracts was determined using a NanoDrop™ spectrophotometer (Thermo Scientific™ Invitrogen™). To assess the quality of the extracted RNA, the ratios A_{260}/A_{280} and A_{260}/A_{230} were made sure to be higher than 2.0. Two micrograms (10 µL) of extracted RNA was immediately reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) and stored at -20°C until qPCR analysis.

Quantitative PCR was performed using CFX96 Real-Time Detection System (Bio-Rad) with the FAM/SYBR® green channel. The quantified target genes, the endogenous control gene (human glyceraldehyde 3-phosphate dehydrogenase [GAPDH]), and their corresponding primers are listed in Table 4.1. PCR amplifications were performed in a 20 µL mixture of: 1 µL of cDNA, 1 µL of forward primer (50 µM), 1 µL of reverse primer (50 µM), 10 µL of 2× GoTaq qPCR Master Mix (Promega, Madison, WI, USA), and 7 µL of Nuclease-free H_2O. The PCR program was set with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The relative expression level of target genes was estimated by calculating the $2^{-\Delta\Delta Ct}$ values against the endogenous control gene GAPDH. The $2^{-\Delta\Delta Ct}$ fold change was calculated by the ratio of treatment or induction groups to un-treated un-induced groups.

4.2.7 Relative gene expression of proteins involved in lipid metabolism

Caco-2 cells were treated with the poi extracts for 24 h for the investigation of lipid metabolism. The control group was incubated with fresh cell medium without FBS or antibiotics for 24 h. RNA in the treatment and control groups were extracted and subjected to RT-qPCR targeting genes listed in Table 4.1 against GAPDH, following the same procedures above. The $2^{-\Delta\Delta Ct}$ fold change was calculated by the ratio of treatment to control.

4.2.8 Statistical analysis

All experiments were performed in three independent replicates, where poi from different batches were obtained on different occasions. The means between groups were compared using the Analysis of Variance (ANOVA) and Tukey's HSD tests. Data analysis and visualization were achieved using R.

Table 4.1 List of primers used for the qPCR amplification of target and reference genes in Caco-2 cells.

Function	Gene	Primer sequences	Reference
Pro-inflammatory	TNF-α	F: 5'- CCCAGGGACCTCTCTCTAATC -3' R: 5'- ATGGGCTACAGGCTTGTCACT -3'	(Gao et al., 2012)
	IL-6	F: 5'- GCTGAAAAAGATGGATGCTTCC -3' R: 5'- AACTCCAAAAGACCAGTGATGATTT -3'	(Gao et al., 2012)
	IL-8	F: 5'- AGAGTGATTGAGAGTGGACC -3' R: 5'- ACTTCTCCACAACCCTCTG -3'	(Son et al., 2005)
Anti-inflammatory	IL-10	F: 5'- TCAGGGTGGCGACTCTAT -3' R: 5'- TGGGCTTCTTCTAAATCGTTC -3'	(Bahrami et al., 2011)
	TGF-β1	F: 5'- GCTGCTGTGGCTACTGGTGC -3' R: 5'- CATAGATTTCGTTGTGGGTTTC -3'	(Bahrami et al., 2011)
	TLR2	F: 5'- GGCCAGCAAATTACCTGTGTG -3' R: 5'- AGGCGGACATCCTGAACCT -3'	(Gao et al., 2012)
	IL-4	F: 5'- TCATTTTCCCTCGGTTTCAG -3' R: 5'- AGAACAGAGGGGGAAGCAGT -3'	(Bahrami et al., 2011)
Tight junction proteins	Claudin1	F: 5'- ATGCAAAGATGTTTTGCCACAG -3' R: 5'- TACAAATTCCCATTGCAGCCC -3'	(Yan et al., 2020)
	Occludin	F: 5'- TCACTTTTCCTGCGGTGACT -3' R: 5'- GGGAACGTGGCCGATATAATG -3'	(Zhao et al., 2021)
	ZO-1	F: 5'- GCTGCCTCGAACCTCTACTC -3' R: 5'- TTGCTCATAACTTCGCGGGT -3'	(Yan et al., 2020)
Lipid metabolism	PPARα	F: 5'- CTGGAAGC TTTGGCTTTACG -3' R: 5'- TGTCCCCG CAGATTCTACAT -3'	(Moosavi et al., 2020)
	PPARβ	F: 5'- ACAGCATG CACTTCCTTCCA -3' R: 5'- TCACATGC ATGAACACCGTA -3'	(Moosavi et al., 2020)
	PPARγ	F: 5'- GAGCCCAAGTTTGAGTTTGC -3' R: 5'- CAGGGCTTGTAGCAGGTTGT -3'	(Moosavi et al., 2020)
	FIAF	F: 5'- CGTACCCTTCTCCACTTGGG -3' R: 5'- GCTCTTGGCGCAGTTCTTG -3'	(Moosavi et al., 2020)
	SREBP-1	F: 5'- ACCGCTCCTCCATCAATGAC -3' R: 5'- CAGGCCGACACCAGATCCTTC-3'	(Le & Yang, 2019)
	SREBP-2	F: 5'- ATCGCTCCTCCATCAATGAC -3' R: 5'- TCGATGCCCTTTAGAAGCTTG-3'	(Le & Yang, 2019)
	NPC1L1	F: 5'- TCTTCCCCTTCCTTGCCATT -3' R: 5'- CGGCAGGGTAATTGTTGAGG -3'	(Liang et al., 2020)
	LXR	F-: 5'- AGACATCGCGGAGGTACAAC -3' R-: 5'- GGCTCACCAGCTTCATTAGC -3'	(Liang et al., 2020)
Reference gene	GAPDH	F: 5'- AACGGATTTGGTCGTATTG -3' R: 5'- GCTCCTGGAAGATGGTGAT -3'	(Devi et al., 2018)

4.3 Results and discussion

4.3.1 Effects of poi extracts on Caco-2 cell viability

As shown in Fig. 4.1, a mild dose-dependent effect was observed in Brand A groups, with a decrease in viability observed at concentrations of 60% and 70%, while cells treated with 70% fresh poi extract still achieved over 90% viability. Brand C groups showed a similar trend, with a few outliners which displayed viabilities over 80%. Seventy percent fresh and fermented Brand C poi extracts resulted in the lowest Caco-2 cell viability values of 77% and 71%, respectively. The 50% concentration was chosen because no significant reduction in cell viability was observed in all treatments at this level.

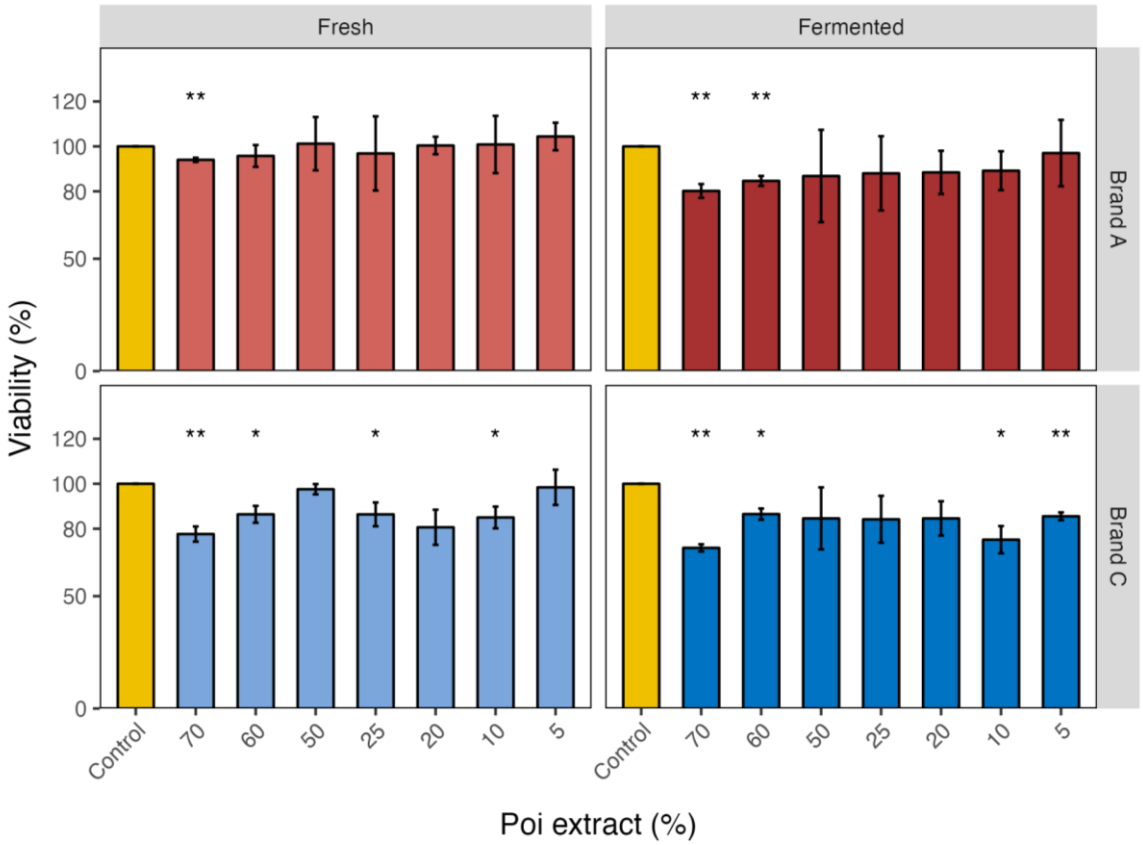

Figure 4.1 Effects of 5, 10, 20, 25, 50, 60, and 70% of poi extract on Caco-2 cell viability evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Error bars are standard deviations (SD, n=3). Differences between groups were analyzed by ANOVA. "*" indicates significant differences between poi-treated cells to the control (100%; * $P < 0.05$; ** $P < 0.01$). Different colors represent treatments: yellow—Control, light red—Fresh Brand A poi extract, dark red—Fermented Brand A poi extract, light blue—Fresh Brand C poi extract, and dark blue—Fermented Brand C poi extract.

4.3.2 Poi extracts inhibited the adhesion and invasion of pathogenic bacteria to Caco-2 cells

Fermented poi extracts showed stronger inhibitory effects on bacterial adhesion and invasion than fresh poi extracts (Fig. 4.2). Poi extracts demonstrated more potent effects against *L. monocytogenes* than *S.* Typhimurium, with a greater inhibition of bacterial adhesion than invasion. Fermented poi extracts from Brands A and C reduced the adhesion of *L. monocytogenes* by 30 and 55 %, respectively ($P < 0.05$). Fresh and fermented Brand C poi extracts also reduced the invasion of *L. monocytogenes* by 20 and 36% ($P < 0.05$), respectively, whereas fermented Brand A poi extract inhibited the internalization of *L. monocytogenes* by 13% ($P > 0.05$). Only poi extracts from Brand C showed inhibition on *S.* Typhimurium adhesion, with adhesion of 92% in the fresh poi group ($P > 0.05$) and 74% in the fermented poi group ($P < 0.05$). However, no inhibition on the invasion of *S.* Typhimurium to Caco-2 cells was observed in all treatments, except for a slight decrease by Brand C poi extracts (94 and 86% respectively in fresh and fermented groups).

Listeria and *Salmonella,* being gram-positive and gram-negative bacteria, respectively, adhere to and enter host cells through distinct mechanisms–Both types of bacteria possess adhesive hair-like structures called pili and/or fimbria, whose primary role is to attach bacteria to a surface (Pizarro-Cerdá & Cossart, 2006). In addition, various adhesins such cadherins and integrins have been found to assist bacteria attach to the host cell surface structures (Pizarro-Cerdá & Cossart, 2006). Upon adhesion, *Listeria* induces the Zipper mechanism through the internalin (Inl) A and/or InlB pathways that interact with the surface E-cadherin and the Met receptors, and activate host cell actin cytoskeletal remodeling and bacterial engulfment (da Silva et al., 2012). On the other hand, *Salmonella* activates actin cytoskeletal rearrangement through the Trigger mechanism by injecting a series of bacterial proteins that favor actin polymerization with a type III secretion system (da Silva et al., 2012). Fermented poi extracts consistently demonstrated stronger bacterial inhibition, suggesting the role of postbiotics in preventing pathogenic bacteria infection.

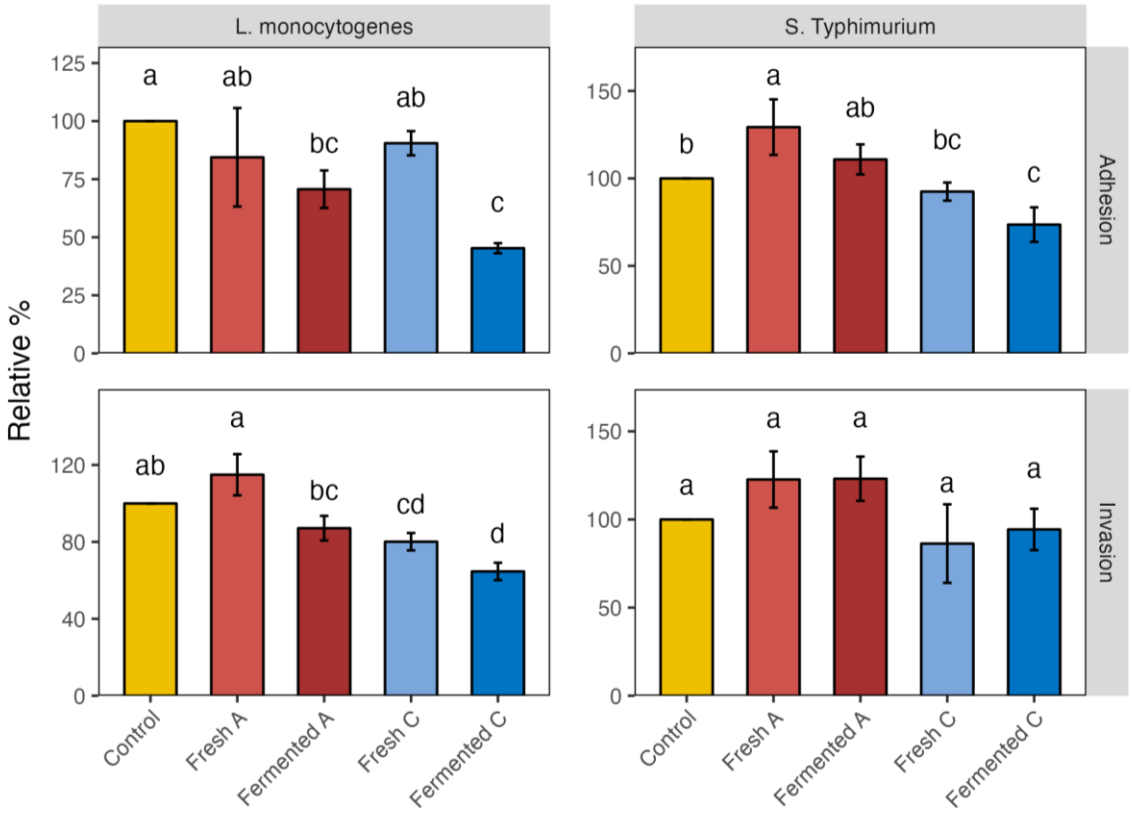

Figure 4.2 Effects of pre-treatment of Caco-2 cells with poi extracts on the adhesion and invasion of Listeria monocytogenes and Salmonella Typhimurium. Error bars are SD (n=3). Differences between groups were analyzed by the ANOVA and Tukey's HSD tests. Bars labeled with different letters in individual plot mean they are significantly different ($P < 0.05$). Relative % was evaluated by comparing the treatment groups to the control. Different colors represent treatments: yellow—Control, light red—Fresh Brand A poi extract, dark red—Fermented Brand A poi extract, light blue—Fresh Brand C poi extract, and dark blue—Fermented Brand C poi extract.

4.3.3 Poi extracts suppressed inflammation induced by oxidative stress, LPS, and pathogenic bacteria

As shown in Fig 4.3, H_2O_2, *L. monocytogenes*, and *S.* Typhimurium induced the expression of pro-inflammatory genes IL-6, IL-8, and TNF-α. LPS triggered the upregulation of the IL-8 gene. Treatment with poi extracts alone did not significantly affect the expression of these pro-inflammatory genes as shown in the "Blank" column (Fig 4.3, $P < 0.05$). However, when inflammation was induced by oxidative stress, the cells treated with poi extracts showed reduced expression levels of IL-6 and IL-8 ($P < 0.05$). Moreover, slightly lower expression of IL-8 was observed in cells pre-treated with fresh poi extracts from Brand A and Brand C. When inflammation was induced by LPS, poi extracts lowered the expression level of IL-8. Notably, fermented poi extracts exerted strong inhibition on IL-8 mRNA expression ($P < 0.05$). On the other hand, fresh poi extracts significantly inhibited the expression of IL-6 in *L. monocytogenes*-treated cells ($P < 0.05$). A decrease in IL-8 gene expression was found in the fresh Brand A poi group ($P < 0.05$). However, fermented poi extracts reduced the expression of TNF-α in *L. monocytogenes*-treated cells and IL-6 in *S.* Typhimurium-treated cells ($P < 0.05$). Moreover, fermented Brand A poi extracts significantly lowered IL-8 expression in *S.* Typhimurium-treated cells ($P < 0.05$). Contrary to these observations, poi promoted the gene expression of TNF-α when Caco-2 cells were exposed to *S.* Typhimurium. This might be related to the previously observed weaker effectiveness of poi in inhibiting *S.* Typhimurium compared to *L. monocytogenes*. Nonetheless, poi extracts demonstrated their ability to lower the expression of pro-inflammatory genes under different inflammatory conditions. Fresh and fermented poi showed unique effects on different genes in different scenarios, highlighting the need for further research to understand the mechanisms underlying the immunoregulatory effects of poi.

Exposure to H_2O_2, LPS, *L. monocytogenes*, and *S.* Typhimurium led to a decrease in the expressions of anti-inflammatory genes including IL-4, IL-10, and TGF-β1 in Caco-2 cells (Fig. 4.4). In H_2O_2-induced groups, IL-4 mRNA expression was significantly promoted by fresh and fermented poi extracts from Brand A and fermented poi extracts from Brand C ($P < 0.05$). There was also a significant increase in IL-10 mRNA expression in the Fresh Brand A and Fermented Brand C poi groups. The expression of

TGF-β1 showed no significant changes in cells pre-treated with poi extracts ($P > 0.05$). When inflammation was induced by LPS, fermented poi extracts dramatically augmented the expressions of IL-4 and IL-10 ($P < 0.05$). Similar trends in IL-4 and IL-10 expressions levels were found in cells induced with *L. monocytogenes* ($P < 0.05$). All poi treatments promoted the expression of TGF-β1 in both inflammatory scenarios, with Fresh Brand A poi group showing the highest expression when induced with LPS and the Fermented Brand C poi group having the highest expression when induced with L. monocytogenes. When inflammation was induced by *S.* Typhimurium, Caco-2 cells pre-treated by poi extracts demonstrated significantly higher IL-4 expression levels than control. Furthermore, poi raised the expression levels of IL-10 in Caco-2 cells, with higher values seen in cells treated with fermented poi compared to fresh poi. Last but not the least, pre-treatment by fermented Brand C poi extracts resulted in the highest TGF-β1 expression level when inflammation was induced by *S.* Typhimurium.

Although TLRs as members of pattern recognition receptors (PRR) can be activated by pathogens to produce APCs and pro-inflammatory factors, they have the potential to regulate T cells through influencing APCs, and hence modulate the level of inflammatory cytokines (Cristofori et al., 2021). An upregulation of TLR leads to the differentiation of native T cells into Treg and increases the production of IL-10 and TGF-β (Thakur et al., 2016). Probiotics are known to influence TLR-mediated pathways (Cristofori et al., 2021). TLRs, particularly TLR2 and TLR4, are integral components of the innate immune system, playing a role in the recognition of pathogen-associated molecule patterns from Gram-positive and Gram-negative bacteria, respectively (Rose et al., 2021). While TLR4 is commonly associated with pro-inflammatory responses, TLR2-mediated pathways have been linked to immunoregulation in specific contexts. For example, *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *Lactis* reduced IL-8 levels through activating TLR2-mediated nuclear factor-kappa B (NF-κB) and mitogen-activated-protein kinase (MAPK) signaling pathways in inflammatory HT-29 cells (S.-C. Li et al., 2019). In addition, the activation of TLR2 is known to promote structural modifications of ZO-1 by enhancing intracellular protein kinase C activities. Depletion of TLR2 stimulation is believed to be correlated with a compromised gut barrier (Rose et al., 2021). For these reasons, this study investigated the effects of poi

extracts on the expression of TLR2 as one of the immunomodulation mechanisms. TLR2 expression in Caco-2 cells was drastically reduced under inflammatory state (Fig. 4.4). However, pre-treatment by poi extracts elevated the expression level of TLR2 in all scenarios, except in the Fresh Brand C poi group when inflammation was induced by *L. monocytogenes*. In H_2O_2-induced cells, fresh poi was more effective in elevating the TLR2 expression level than fermented poi. In cells induced with LPS, TLR2 mRNA level reached the highest in cells treated with fermented Brand C poi extract. When inflammation was triggered by pathogenic bacteria, cells treated with fermented poi extracts presented the highest TLR2 expression level.

Overall, poi extracts modulated inflammatory responses under the above four scenarios by suppressing pro-inflammatory genes, upregulating anti-inflammatory genes, and promoting the expression of TLR2. Additionally, fermented poi, especially from Brand C, presented strong modulatory effects, supporting our hypothesis of the beneficial role of postbiotics produced during bacterial fermentation. The contrary effects of fresh and fermented poi extracts on certain genes under certain scenarios (e.g. IL-6—*L. monocytogenes* versus IL-6—S. Typhimurium) implied that they might act complimentarily to alleviate inflammation triggered by different conditions.

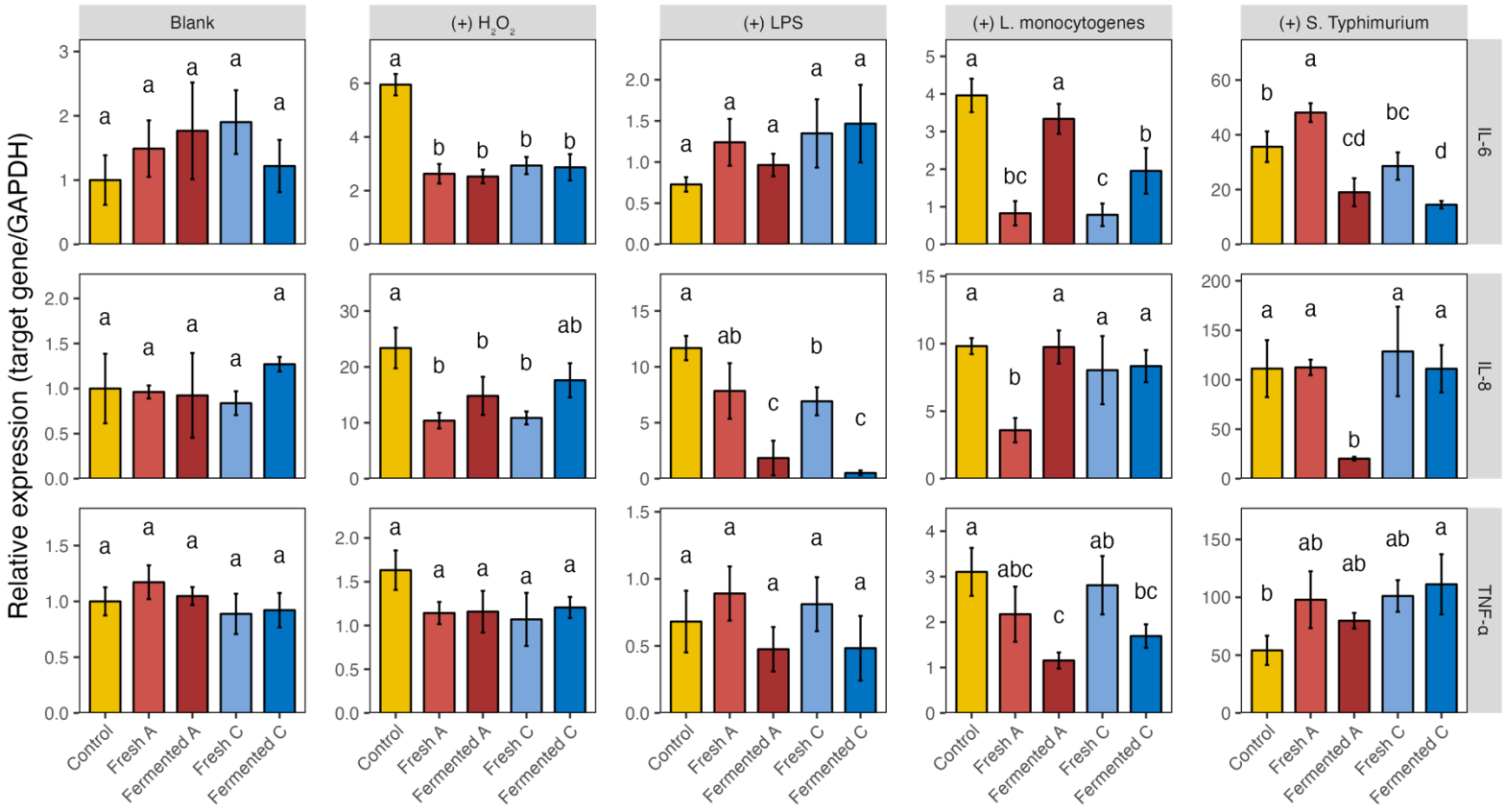

Figure 4.3 Effects of pre-treatment by poi extracts on the relative expression of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) in Caco-2 cells. Inflammation was induced by H_2O_2 (500 μM, 24 h), LPS (10 μg/mL, 48 h), *L. monocytogenes* (MOI=100:1, 24 h), and *S.* Typhimurium (MOI=10:1, 4 h). Error bars are SD (n=3). Data were analyzed by the ANOVA and Tukey's HSD tests. Bars labeled with different letters in individual plots mean they are significantly different ($P < 0.05$). Relative expression was evaluated by comparing the $2^{-\Delta\Delta Ct}$ values of the treatment groups to the control. Different colors represent treatments: yellow—Control, light red—Fresh Brand A poi extract, dark red—Fermented Brand A poi extract, light blue—Fresh Brand C poi extract, and dark blue—Fermented Brand C poi extract.

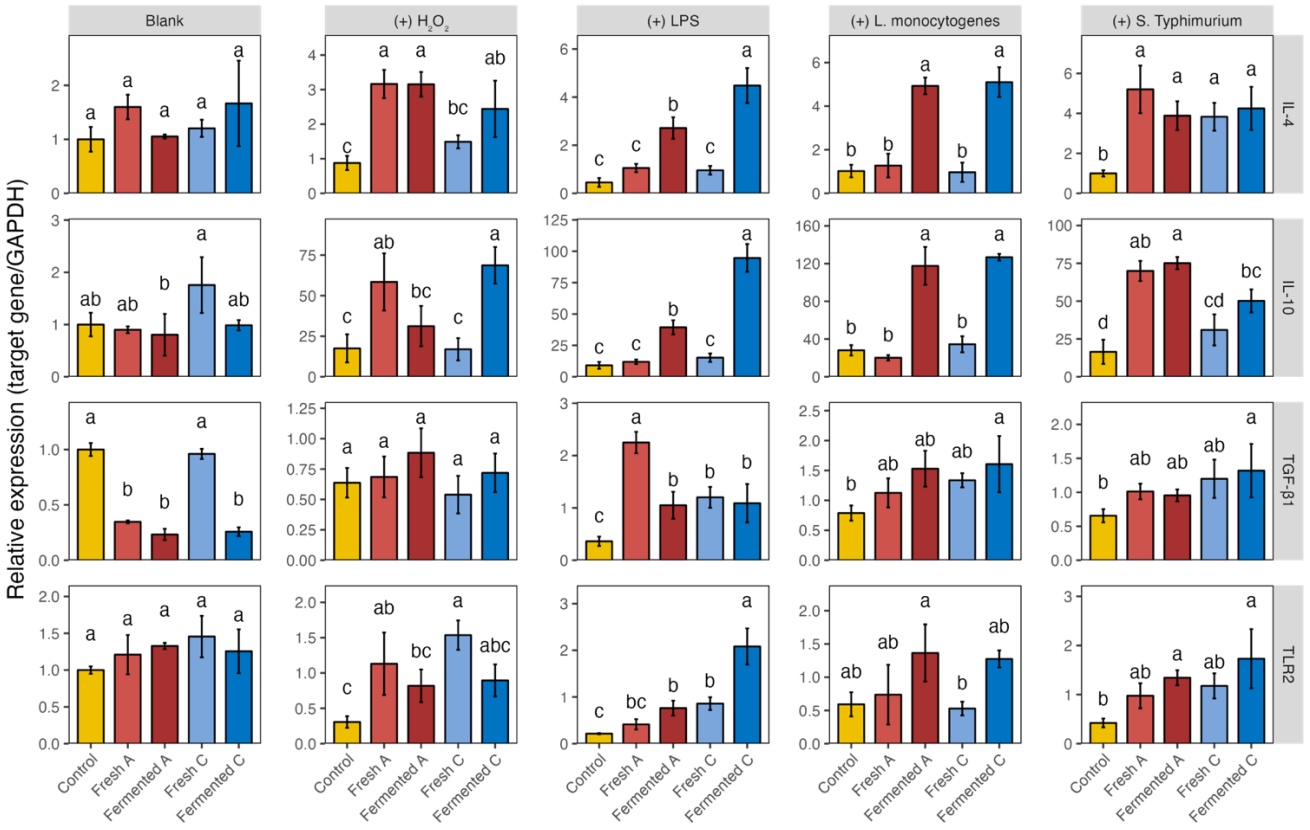

Figure 4.4 Effects of pre-treatment by poi extracts on the relative expression of anti-inflammatory cytokines (IL-10, IL-4, and TGF-β1) and TLR2 in Caco-2 cells. Inflammation was induced by H_2O_2 (500 μM, 24 h), LPS (10 μg/mL, 48 h), *L. monocytogenes* (MOI=100:1, 24 h), and *S.* Typhimurium (MOI=10:1, 4 h). Error bars are SD (n=3). Data were analyzed by the ANOVA and Tukey's HSD tests. Bars labeled with different letters in individual plots mean they are significantly different ($P < 0.05$). Relative expression was evaluated by comparing the $2^{-\Delta\Delta Ct}$ values of the treatment groups to the control. Different colors represent treatments: yellow—Control, light red—Fresh Brand A poi extract, dark red—Fermented Brand A poi extract, light blue—Fresh Brand C poi extract, and dark blue—Fermented Brand C poi extract.

4.3.4 Poi extracts potentially preserved intestinal barrier integrity during inflammation by promoting the expression of tight junction protein genes

As shown in Fig. 4.5, inflammation diminished the expression of claudin-1 and ZO-1 while elevating expression of occludin in the control group. Remarkably, pre-treatment with poi extracts enhanced the expression of all tight junction protein genes in Caco-2 cells, regardless of the inflammatory inducing factors used. In most cases, fermented poi extracts demonstrated superior stimulation on the expression of tight junction proteins in inflamed Caco-2 cells. These cases include: claudin and occludin in H_2O_2-induced inflammation; claudin, occludin, and ZO-1 in LPS-induced inflammation; claudin, occludin, and ZO-1 in *L. monocytogenes*-induced inflammation; and claudin in *S.* Typhimurium-induced inflammation. In Caco-2 cells induced with H_2O_2, fresh Brand A poi extracts led to the highest expression of ZO-1. All poi treatments were equally promotive to the expression occludin in *S.* Typhimurium-induced cells. These results suggest that poi extracts can help preserve intestinal barrier integrity by upregulating the gene expression of tight junction proteins, with postbiotics produced during bacterial fermentation magnifying this effect.

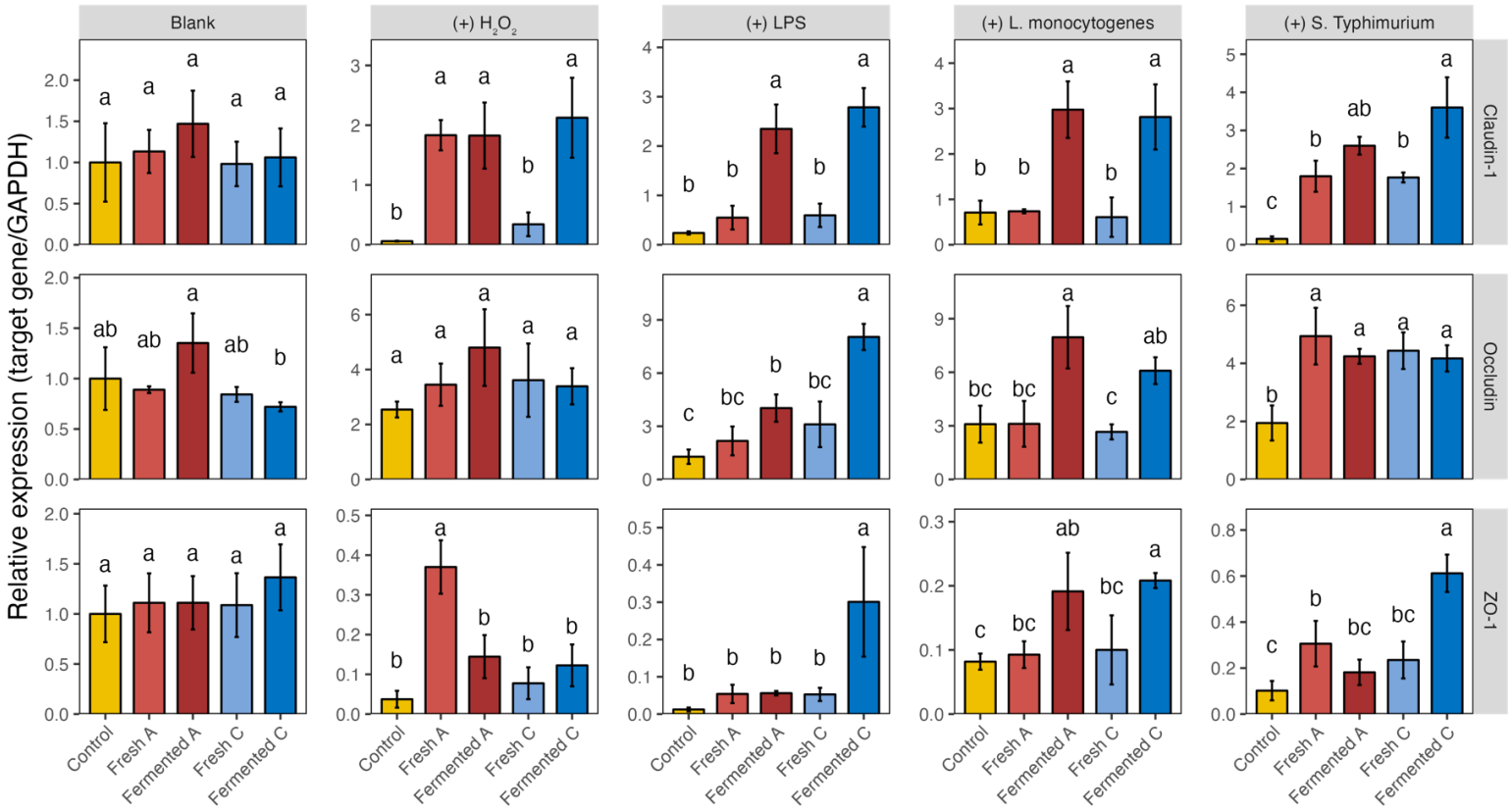

Figure 4.5 Effects of pre-treatment by poi extracts on the relative expression of tight junction protein genes (Claudin-1, Occludin, and ZO-1) in Caco-2 cells. Inflammation was induced by H_2O_2 (500 µM, 24 h), LPS (10 µg/mL, 48 h), *L. monocytogenes* (MOI=100:1, 24 h), and *S.* Typhimurium (MOI=10:1, 4 h). Error bars are SD (n=3). Data were analyzed by the ANOVA and Tukey's HSD tests. Bars labeled with different letters in individual plots mean they are significantly different ($P < 0.05$). Relative expression was evaluated by comparing the $2^{-\Delta\Delta Ct}$ values of the treatment groups to the control. Different colors represent treatments: yellow—Control, light red—Fresh Brand A poi extract, dark red—Fermented Brand A poi extract, light blue—Fresh Brand C poi extract, and dark blue—Fermented Brand C poi extract.

4.3.5 Poi extracts altered the expression of genes involved in the regulation of lipid metabolism in Caco-2 cells.

As shown in Fig. 4.6, the expression of PPARα, PPARβ, and PPARγ were significantly upregulated by pre-exposure of Caco-2 cells to poi extracts ($P < 0.05$). Fermented Brand A and Brand C poi extracts upregulated PPARα gene. Fermented Brand A and fresh and fermented Brand C poi extracts significantly elevated the expression of PPARβ. Brand C poi extracts induced the upregulation of PPARγ in Caco-2 cells. The expression of FIAF in Caco-2 cells was promoted by fermented Brand A and Brand C poi extracts ($P < 0.05$). Sterol regulatory element binding protein-2 (SREBP-2) was significantly downregulated in the Fresh Brand A group but upregulated in the Fresh Brand C group ($P < 0.05$). However, cholesterol regulatory genes LXR and NPC1L1 were not altered by poi extracts in Caco-2 cells ($P > 0.05$).

PPARα has been found to be a main target of gut microbial metabolites in the intestines and can influence gut immune functions and permeability, the production of antimicrobials by colonocytes, and indirectly modulate gut microbiota composition (Grabacka et al., 2022). Similarly, PPARβ regulates epithelial cell proliferation and modulates intestinal inflammation associated with IBD or colorectal cancer (Peters et al., 2008). Interests in the role of PPARβ in preventing colorectal cancer have also been growing in recent years (Peters et al., 2019). Although PPARγ activation is part of the process during late adipogenesis in the liver and adipocytes, adequate expression of PPARγ in intestinal epithelial cells is essential for maintaining mucosal immune function and preventing chronic inflammation in the gut (Mohapatra et al., 2010). PPARs function as upstream regulators of APCs and inflammasome like NF-κB (Grabacka et al., 2022). Therefore, the effects of poi extract on modulating the intestinal inflammatory response might be mediated through PPARs-mediated pathways.

Moreover, PPARs are also the upstream regulators of FIAF. In this study, the increased PPARs expression levels were found to be consistent with the elevated expression levels of the FIAF gene. As mentioned previously, the upregulation of intestinal FIAF could result in enhanced circulating FIAF protein and may lead to reduced lipid accumulation in other tissues (Aron-Wisnewsky et al., 2021).

SREBP-1 and -2, abundantly expressed in the liver, are critical for lipogenesis and cholesterol synthesis. However, their functions in the intestines are quite different (Y. Wang et al., 2022). First of all, SREBP-1 has little or no role in regulating intestinal cholesterol synthesis, while SREBP-2 is the main contributor to this function (Field et al., 2001). Secondly, SREBP-2 controls downstream genes involved in cholesterol homeostasis, including NPC1L1, which is responsible for dietary and biliary cholesterol absorption (Ishimwe et al., 2015; Miyata et al., 2014). Activation of intestinal SREBP-2 may suppress the transcription of hormone fibroblast growth factor (FGF19) mediated by the farnesoid X receptor (FXR), thereby regulating bile acid homeostasis.(Miyata et al., 2014). FXR and LXR, both activated by bile acids, are negatively associated with each other (Ishimwe et al., 2015). In this study, factors involved in intestinal cholesterol and bile acid homeostasis such as NPC1L1 and LXR were not altered by poi extracts (Fig. 4.6). It is well-know that probiotics can reduce cholesterol uptake in enterocytes by upregulating LXR, which further activates cholesterol efflux proteins ABCG5/ABCG8, and downregulating NPC1L1 to reduce cholesterol cellular uptake (Ishimwe et al., 2015; Le & Yang, 2019; Liang et al., 2020). The unchanged expressions of LXR and NPC1L1 in Caco-2 cells following treatment with poi extracts may be due to probiotic triggering mechanism. First, LXR and NPC1L1 modulations are mainly triggered by the shifts of bile salts or cholesterol levels and/or bacterial modification of bile salts (Ishimwe et al., 2015; H. Zhang et al., 2018). Since there was no bile acids or cholesterol added in the cell culture medium, there was no trigger. Additionally, bacterial membrane components instead of bacterial metabolites might be the triggers for the regulation of LXR expressions in Caco-2 cells. Previous research has found that metabolites extracted from *Lactobacillus rhamnosus* and *Lactobacillus plantarum* did not alter the expression of LXR and its downstream genes ABCG5/ABCG8, while heat killed bacteria and bacterial cell wall components did exhibit regulatory effects (Yoon et al., 2011). In this chapter, Caco-2 cells were only treated with bacterial metabolites, which may not be effective in influencing the expression of LXR or NPC1L1.

Therefore, poi extracts appear to modulate PPARs-mediated lipogenesis/lipolysis pathways and SREBP-2 mediated cholesterol metabolism.

4.3.6 Limitations

Poi has demonstrated significant effects on the regulation of gene expression involved in mucosal immune function and lipid metabolism, highlighting its promising potential. However, further studies are necessary to validate these findings. Chemical analysis of protein production and cytokine levels is required to authenticate the hypothesis regarding its immunomodulatory functions. Physiochemical assessments, such as Transepithelial Electrical Resistance measurement and advanced imaging techniques, are needed to verify the ability of poi to preserve intestinal barrier integrity. Extensive in vitro, in vivo, and clinical studies are required to consolidate the role of poi in the modulation of intestinal immune response and lipid metabolism in humans.

4.4 Conclusion

In conclusion, water-soluble extracts of poi inhibited *L. monocytogenes* and *S*. Typhimurium infections in Caco-2 cells by restraining bacterial adhesion and invasion. These extracts ameliorated the inflammation response in Caco-2 cells by downregulating pro-inflammatory genes and upregulating anti-inflammatory genes. The expressions of tight junction proteins essential to maintaining gut permeability and barrier integrity were upregulated by poi extracts. Poi extracts also showed notable potential in regulating lipid metabolism through the activation of PPAR/FIAF and SREBPs-mediated pathways, thereby potentially limiting lipogenesis in other tissues, reducing cholesterol uptake by the intestines, and promoting gastrointestinal immunoregulation. Moreover, fermented poi extracts showed overall stronger effects than their fresh counterparts in most cases, indicating the health-promoting property of postbiotics. Distinct effects were observed for each poi treatment, suggesting that fresh poi and fermented poi, as well as poi from different brands, may be complimentary depending on the pathogenic context.

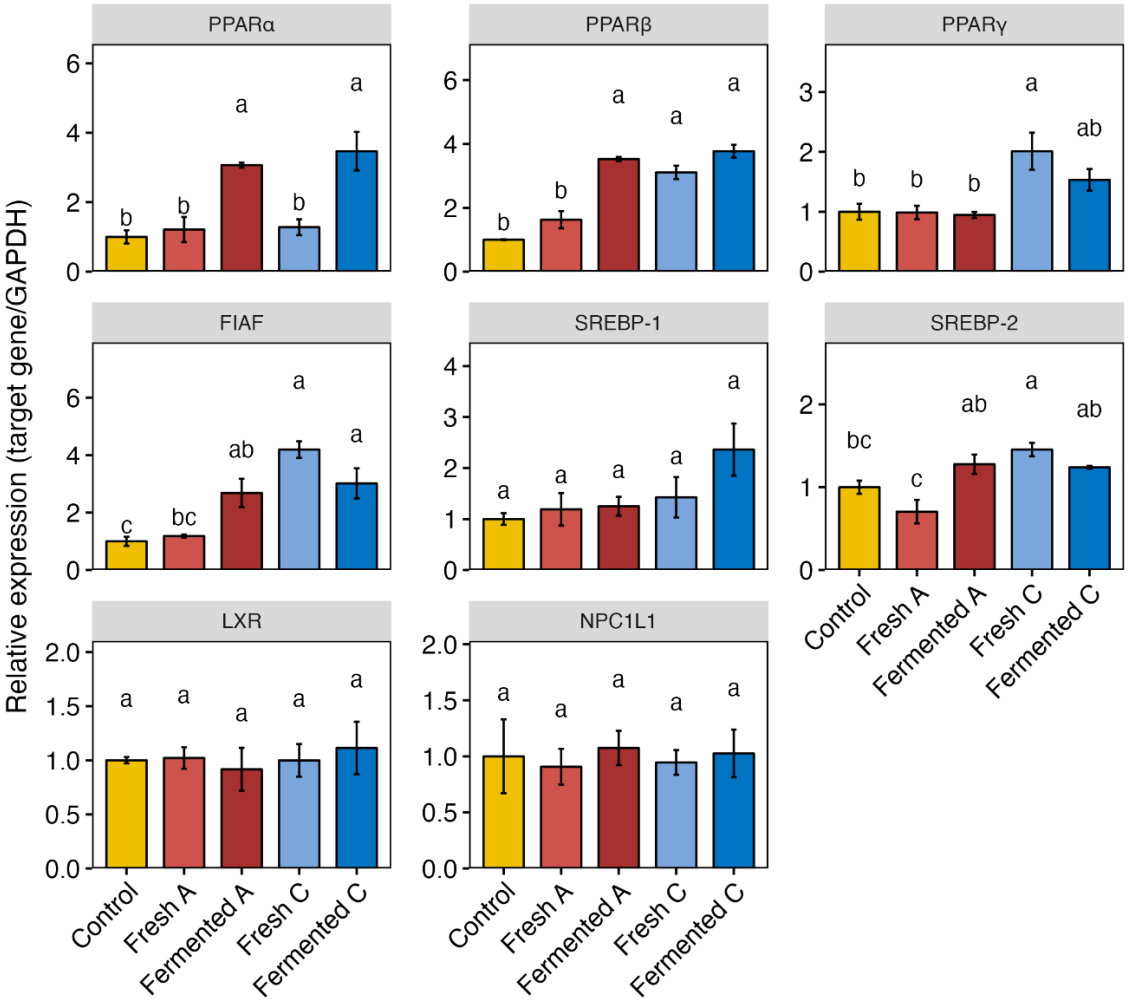

Figure 4.6 Effects of pre-treatment by poi extracts on the relative expression of genes involved in the regulation of cholesterol and lipid metabolism (PPARα, PPARβ, PPARγ, FIAF, SREBP-1, SREBP-2, LXR, and NPC1L1) in Caco-2 cells. Error bars are SD (n=3). Data were analyzed by the ANOVA and Tukey's HSD tests. Bars labeled with different letters in individual plots mean they are significantly different ($P < 0.05$). Relative expression was evaluated by comparing the $2^{-\Delta\Delta Ct}$ values of the treatment groups to the control. Different colors represent treatments: yellow—Control, light red—Fresh Brand A poi extract, dark red—Fermented Brand A poi extract, light blue—Fresh Brand C poi extract, and dark blue—Fermented Brand C poi extract.

CHAPTER 5 FERMENTATION OF POI ENHANCES ITS ABILITY TO MODULATE HUMAN GUT MICROBIOTA AND SHORT-CHAIN FATTY ACID PRODUCTION DURING *IN VITRO* FECAL FERMENTATION

Abstract

The gut microbiota plays a pivotal role in various physiological and psychological processes within the human body. Fermented foods, such as poi, may contain prebiotics, probiotics, and many other bioactive compounds that can regulate the composition of the gut microbiota and enhance the production of short-chain fatty acids (SCFAs). This study investigated the effects of both fresh and fermented poi on human gut microbiota during *in vitro* fecal fermentation. Fecal samples were collected from six healthy donors and pooled for analysis. Two types of poi were subjected to *in vitro* digestion prior to being inoculated with human feces for fermentation. Subsequently, the resultant microbiota was analyzed for microbial diversity and relative abundance, SCFA production, and Kyoto Encyclopedia of Genes and Genomes (KEGG) predicted metabolic pathways. The results showed that fermented poi significantly increased the alpha-diversity indices of the microbiota. Beta-diversity demonstrated distinctive separation between the control, fresh poi, and fermented poi groups. Moreover, the structure of the gut microbial community was notably influenced by the introduction of poi. At the phylum level, differential abundances of *Actinobacteriota*, *Fusobacteriota*, *Firmicutes*, *Bacteroidetes, Desulfobactrota, and Verrucomicrobiota* were observed in samples treated with poi. In the fermented poi group, the *Firmicutes* to *Bacteroidetes* (F/B) ratio exhibited a slight decrease, a phenomenon that has been associated with an increase in obesity. In total, 20 genera were significantly altered by the poi treatments. The abundances of beneficial genera such as *Allisonella*, *Bacteroides*, *Bifidobacterium*, *Catenibacterium*, *Enteroccous*, *Megasphaera*, *and Roseburia* increased in the poi groups, while harmful genera such as *Fusobacterium* and *Klebsiella* were suppressed by poi. Both fresh and fermented poi promoted microbial production of SCFAs including butyrate (1.83- and 1.88-folds to the control, respectively) and valerate (3.45- and 3.58-folds to the control, respectively). In addition, fermented poi significantly increased fecal levels of isobutyrate and isovalerate (1.57- and 1.31-folds to the control, respectively).

Fresh and fermented poi significantly altered the abundances of 57 predicted KEGG pathways, such as genetic information processing, environmental information processing, amino acid metabolism, glycan biosynthesis and metabolism, lipid metabolism, PPAR signaling, and RIG-I-like receptor signaling. A more profound effect was observed in the fermented poi group compared to the fresh poi group, indicating the important role of natural fermentation in the probiotic effect of poi. Furthermore, distinct effects were observed in the fresh and fermented poi groups, suggesting the multifaceted nature of poi in modulating human gut microbiota and its potential impact on health. In conclusion, both fresh and fermented poi increased the gut microbiota diversity, altered the gut microbiota community structure and functionality, and amplified SCFA production. Thus, poi holds great potential for influencing human health through the modulation of the gut microbiota.

Keywords: poi, fermentation, gut microbiota, short-chain fatty acids, 16S rRNA sequencing

5.1 Introduction

The role of the human gut microbiota as a crucial determinant influencing human health has gained substantial recognition. Disruption of gut microbiota homeostasis is associated with various metabolic syndromes including obesity, non-alcoholic fatty liver disease, insulin sensitivity, type 2 diabetes mellitus, and low-grade chronic inflammation (Canfora et al., 2019). In-depth research has revealed covariates linked to the gut microbiota, opening up possibilities for tailored interventions aimed at promoting a healthier gut microbiota compositional balance. Extrinsic factors such as lifestyle, physical activity, diet, and medication, have been identified as influential contributors to the gut microbiome (Schmidt et al., 2018). Dietary intervention has become popular as an effective strategy for modulating the gut microbiota due to its rapid and reproducible outcomes (David et al., 2014).

Dietary interventions mainly operate through two modes of actions. The first involves the provision of indigestible carbohydrates known as prebiotics. The fermentation of prebiotics by gut microbiota produces short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, along with other metabolites like succinate (Canfora et al., 2019). These SCFAs interact with G-protein-coupled receptors (GPCRs) like GPR41, GPR43, and GPCR109A, thereby regulating various physiological processes including inflammation, enteroendocrine functionality, junction protein synthesis, and the maintenance of intestinal pH (Lazar et al., 2019). SCFAs stimulate the release of peptide YY and glucagon-like neuropeptides GLP-1 and GLP-2 from local L-cells, impacting mucus secretion, digestion, satiety signals through the central nerves systems, and liver lipid metabolism (Lazar et al., 2019; Y. Yu et al., 2019). Reduced intestinal SCFAs levels have been documented in metabolic syndromes and inflammatory bowel diseases (IBD), indicating their importance in maintaining gut health (Parada Venegas et al., 2019; Sanna et al., 2019). In addition, prebiotics selectively nourish beneficial bacteria, thereby adjusting the gut microbiota composition in a desired direction (Schmidt et al., 2018). For example, conventional prebiotics such as inulin-type fructans have been shown to increase the relative abundances of butyrate-producing genus *Anaerostipes* and SCFA-producing *Bifidobacterium*, while reducing *Bilophila* that is negatively associated with softer stools (Vandeputte et al., 2017). New-

generation prebiotics, like polyphenols, have also exhibited beneficial effects in modifying gut microbiota by increasing bacterial families such as *Bifidobacteriaceae* and *Lactobacillaceae* while decreasing pathogenic bacteria like *Escherichia coli*, *Clostridium perfringens*, and *Helicobacter pylori* (Plamada & Vodnar, 2022).

The second mode of intervention involves the administration of live beneficial microorganisms, known as probiotics, to the host. This approach has demonstrated efficacy in subjects with underlying pathologies, although its impact on healthy individuals has not been consistent (Nagpal et al., 2018). The colonization resistance nature of the gut microbiota and the degree of complementation between the probiotic strains and the individual's indigenous microbiota contribute to this inconsistency (Schmidt et al., 2018). Using a probiotic cocktail consisting of multiple complementary probiotic strains may enhance the success of gut microbiota modulation. For instance, administrating a 1×10^{11} CFU probiotic cocktail of 10 *Lactobacillus* and *Enterococcus* strains with a single dose or for five consecutive days significantly increased gut microbiota diversity and SCFAs production in healthy mice, with the degree of increases surpassing treatments by individual *Lactobacillus* or *Enterococcus* strains (Nagpal et al., 2018). Supplementing probiotics with their substrate prebiotics, known as synbiotics, can further enhance probiotic colonization and synergistic effects. For instance, the supplementation of bovine milk-derived oligosaccharides strengthened the colonization of probiotic *Bifidobacterium animalis* ssp. *Lactis* in the gut of healthy infants (Marsaux et al., 2020).

Fermented foods possess great potential to exert a positive impact on the gut microbiota through several mechanisms. They may provide substrates that promote the proliferation of beneficial gut microbes, inhibit the growth of harmful bacteria, and increase SCFA production. Additionally, fermented foods harbor abundant and diverse microbiota, many of which exhibit probiotic characteristics. Last but not the least, fermented foods contain metabolites such as lactic acid, vitamins, and exopolysaccharides that are beneficial to health (Leeuwendaal et al., 2022). Consequently, fermented foods may be naturally synbiotic. The consumption of fermented foods has been associated with an array of gut bacteria including *Bacteroides* spp., *Pseudomonas* spp., *Dorea* spp., *Lachnospiraceae*, *Prevotella* spp.,

Alistipes putredinis, *Oscillospira* spp., *Enterobacteriaceae*, *Fusobacterium* spp., *Actinomyces* spp., *Achromobacter* spp., *Clostridium clostridioforme*, *Faecalibacterium prausnitzii*, *Bacteroides uniformis*, *Clostridiales*, and *Delftia* spp. (Taylor et al., 2020). Current research on fermented food primarily focuses on isolating probiotics, highlighting a research gap in investigating the comprehensive impact of fermented food on the gut microbiota. Popularly studied fermented foods are limited to kefir, kombucha, fermented cabbage (like sauerkraut and kimchi), fermented soy (like tempeh, natto, and miso), and sourdough bread (Dimidi et al., 2019). Fermented root vegetables such as sweet potato or taro have not yet been explored.

A previous investigation evaluated the nutritional value and prebiotic potential of five taro varieties (Bun-Long, Tahitian, Moi, Mana Ulu, and Lehua) (Saxby, 2020). The study found that these taro varieties contained dietary fiber and resistance starch (6.1-8.4 g/100 g). Taro changed the gut microbial profile after 24 h of *in vitro* fecal fermentation, with an increased ratio of *Bacteroidetes*/*Firmicutes* and a decreased presence of *Proteobacteria*. Moreover, taro significantly increased the production of SCFAs. Among these taro varieties, Moi and Lehua are commonly used in poi production, suggesting that poi may also have prebiotic properties and the ability to modulate the gut microbiota (Whitney et al., 1939). Furthermore, certain lactic acid bacteria (LAB) present in fermented poi, such as *Enterococcus* spp., *Streptococcus lutetiensis*, *Weissella confusa*, *Leuconostoc lactis*, and *Lactiplantibacillus plantarum*, were found to survive gastrointestinal digestion and show probiotic characteristics in the previous chapters. Hence, fermented poi can be considered synbiotic. We hypothesize that fermented poi can alter the composition and activity of human gut microbiota. To evaluate the effect of natural fermentation on the ability of poi to modulate the gut microbiota, a simulated colonic fermentation study was conducted using both fresh poi and fermented poi inoculated with human feces from healthy donors.

5.2 Methods

5.2.1 Poi samples and *in vitro* digestion

One poi brand was selected for this study based on its superior probiotic properties found in the previous chapters. Fresh poi was purchased on the day of distribution,

transported in a cooler, and used immediately. Another set of fresh poi was left at 20°C for 48 h to allow natural fermentation. Fresh and fermented poi were subjected to simulated gastrointestinal digestion conditions following the INFOGEST 2.0 protocol with minor modifications (Brodkorb et al., 2019). The protocol consisted of oral, gastric, and intestinal phases. Briefly, in the oral phase, 2.5 g of poi was mixed with simulated salivary fluid (SSF, pH 7.0) at 1:1 ratio. The final concentration of the salts in SSF were: 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM NaCl, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 1.1 mM HCl, and 1.5 mM $CaCl_2(H_2O_2)_2$. The final concentration of α-Amylase (from human saliva, Sigma-Aldrich, catalog number A1031, St. Louis, MO, USA) was 75 U/mL. The mixture was incubated at 37°C with agitation at 100 rpm for 2 min. After the oral phase, the mixture was immediately added with simulated gastric fluid (SGF) at 1:1 ratio and adjusted to pH 2.5 with 1 M HCl. The volume of HCl to be added was determined in a preliminary test. The final concentration of the salts in SGF were: 6.9 mM KCl, 0.9 mM KH_2PO_4, 72.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 15.6 mM HCl, and 0.15 mM $CaCl_2(H_2O_2)_2$. SGF contained 60 U/mL lipase (from *Rhizopus oryzae*, Sigma-Aldrich, catalog number 62305) and 2000 U/mL pepsin (from porcine gastric mucosa, Sigma-Aldrich, catalog number P7012). The gastric mixture was incubated at 37°C with agitation at 100 rpm for 2 h. Gastric digestion was stopped by immediately adding simulated intestinal fluid (SIF) at 1:1 ratio and adjusting pH to 7.0 with 1 M NaOH. The volume of NaOH to be added was determined in a preliminary test. The final concentrations of the salts in SIF were: 6.8 mM KCl, 0.8 mM KH_2PO_4, 123.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 8.4 mM HCl, and 0.6 mM $CaCl_2(H_2O_2)_2$. SIF contained pancreatin at 100 TAME trypsin U/mL (Pancreatin from porcine pancreas, Sigma-Aldrich, catalog number P7545; the trypsin activity in this product was 3.71 U/mg determined using the TAME method) and bile at 10 mM (Bile bovine, Millipore, catalog number B3883, Burlington, MA, USA). The intestinal mixture was incubated at 37°C with agitation at 100 rpm for 2 h.

The digested poi samples were placed on ice immediately to stop the reaction and processed for *in vitro* fermentation. 0.3 M $CaCl_2(H_2O_2)_2$ stock solution was prepared freshly before digestion to prevent precipitation. Enzymes and buffer solutions used in the digestion experiment were filter sterilized using 0.22 μm cellulose acetate syringe

filters and polyether sulfone syringe filters, respectively. The digestion was conducted aseptically. Fresh and fermented poi replicates were digested right before *in vitro* fermentation. A control sample containing 2.5 mL PBS was digested with each replicate to assess the effects of digestive enzymes on the fermentation.

5.2.2 Collection and storage of fecal samples

The fecal collection protocol was approved by the University of Hawai'i Institutional Review Board (IRB). Six fecal donors were recruited through a circulating flyer (Appendix A1) and word of mouth. Their health status and poi consumption habits were screened using a survey questionnaire (Appendix A2). Three male and three female donors with racial identities of Asian or Pacific Islander, Black or African American, Native American or Alaska American, White or Caucasian, and Multiracial or Biracial, aged 19-30, BMI 22.7-25.0, were selected based on the following criteria: not on a special diet, consumed poi less than once per week, no smoking, had not taken antibiotics in the past 3 months, were not pregnant or lactating, had no history of bowel conditions (e.g. irritable bowel syndrome, Crohn's diseases, ulcerative colitis, etc.), were not diabetic or obese, had no cardiovascular diseases (e.g. atherosclerosis, heart diseases, etc.) or other health issues. The donors were educated on the collection protocol and signed consent forms (Appendix A3) before proceeding. All fecal samples were collected using a Fisherbrand™ Commode Specimen Collection System (Fisher Scientific, Waltham, MA). The storage of fecal samples followed an *in vitro* batch fermentation protocol (Pérez-Burillo et al., 2021). The collected fecal samples were kept under anaerobic conditions and stored at 4°C until being pooled together in equal parts within 24 h. The pooled fecal sample was mixed with 20% (w/v) glycerol at 1:1 ratio, frozen in anaerobic bags at -80°C, and used within 2 months.

5.2.3 *In vitro* fecal fermentation

The *in vitro* fecal fermentation was conducted following the protocol by Pérez-Burillo et al. with minor modifications (2021). The frozen fecal sample was thawed at room temperature and centrifuged at 4,000 x *g* for 10 min to remove glycerol. The supernatant was discarded. The fecal pellet was resuspended in 0.1 M NaH_2PO_4 buffer (pH 7.0) at a concentration of 32% (w/v). The fecal suspension was vortexed and

centrifuged at 550 g for 5 min to remove large particles. The supernatant was collected as the fecal slurry. A reductive solution was freshly prepared by dissolving 312 mg of L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) and 312 mg of sodium sulfide in 2 mL of 1 M NaOH, the final volume was made up to 50 mL with distilled water. Fermentation medium was prepared by mixing 1 L of 1.5% (w/v) peptone solution (VWR Life Science, Radnor, PA, USA) with 50 mL of the reductive solution and 1.25 mL of freshly prepared 0.1% (w/v) resazurin solution (Sigma-Aldrich, St. Louis, MO, USA). The digesta from section 5.2.1 was centrifuged at 4,000 x *g* for 10 min. In a 50 mL sterile centrifuge tube, 0.5 g of the solid digestion residue and 10% of the corresponding digestion supernatant were mixed with 7.5 mL fermentation medium and 2 mL fecal slurry. The mixture was vortexed and bubbled with nitrogen for 1 min to remove oxygen. The tightly sealed centrifuge tubes were incubated at 37°C for 20 h with agitation at 20 rpm. At the end of the fermentation, aliquots of fermented slurry were collected and placed on ice to stop microbial activities. One set of the samples was used for determining total solids, and two other sets of the samples were stored at -80°C until DNA extraction for 16s rDNA Miseq sequencing and SCFA measurements. All procedures were conducted under aseptic conditions. Fermented samples included digested fresh poi, digested fermented poi, and a sample containing only digestive enzymes as control for fermentation. All fermentation procedures were performed in three independent replicates.

5.2.4 SCFA analysis

SCFAs in fermented fecal samples were determined according to a Metabolite Array Technology developed by Xie et al. and analyzed at the Metabolomics Shared Resource at the University of Hawaii Cancer Center (G. Xie et al., 2021). The sample preparation followed their protocol for serum samples. Briefly, the thawed fermented fecal suspension was well mixed, and a volume of 20 µL was mixed with 120 µL of internal standards solution for SCFAs (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid). The mixture was centrifuged at 13,500 x *g* for 10 min at 4°C. A volume of 30 µL of the supernatant was transferred to a 96 well plate for derivatization and analysis. SCFAs were quantified using a Waters ACQUITY ultraperformance LC system coupled with a Waters XEVO TQ-S mass spectrometry with an ESI source controlled by MassLynx 4.1 software (Waters, Milford, MA) following

the protocol described by Xie et al. (2021). SCFAs concentrations (ng/mL) were converted into nanogram per gram total solids in the fecal suspension.

5.2.5 16s rDNA sequencing

DNA of the fermented fecal samples was extracted using Qiagen Powerfecal Pro DNA kit (Hilden, Germany) following the instructions provided by the manufacturer. 16s rDNA V3-V4 region was amplified using a set of primers (341 F/785 R) and sequenced using an Illumina MiSeq sequencer at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) facility at the University of Hawaii College of Natural Sciences.

5.2.6 Bioinformatics

Microbial sequencing data were analyzed using Qiime2 and Qiime2 plugins (Bolyen et al., 2019). Paired-end demultiplexed fastq files were trimmed and denoised using DADA2 integrated in Qiime2 (Callahan et al., 2016). The feature table of the sequence variants (SVs) was generated in Qiime 2. The Alpha-diversity indexes, beta-diversity indexes, and principal coordinate analysis (PCoA) of the SVs were also evaluated in Qiime2. Classification and phylogenetic information visualization were performed in Qiime2 using Silva database version 138 (99% sequence identity). Analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2) multiple pairwise comparison was employed to identify differentially abundant taxa in microbiome data using ANCOM R package (Lin et al., 2022; Lin & Peddada, 2020).

Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) metagenome functional features were predicted with SVs more than 100 frequencies using Qiime2 plugin Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Douglas et al., 2020; Kanehisa et al., 2016). Alpha- and beta-diversity analyses and PCoA of the KO features were also conducted in Qiime2. KO features were annotated with KEGG pathways and analyzed for differential abundance with ANOVA-Like Differential Expression Analysis 2 (ALDEx2) using ggpicrust2 R package (Fernandes et al., 2013, 2014; Gloor et al., 2016; C. Yang et al., 2023).

5.2.7 Statistical analysis

In vitro fermentation was conducted in three independent replicates. For SCFA analysis, differences were assessed by Analysis of Variance (ANOVA) with student's t-test for pairwise comparisons. Spearman's rank correlation coefficient was used to analyze the correlations between SCFA production and bacterial taxa. P-values were adjusted based on taxa using the Benjamini-Hochberg method.

Differences in the microbiome and KO metagenome data were analyzed using non-parametric tests. Differences in Alpha-diversity analysis between groups were determined using the Kruskal-Wallis pairwise test in the following indices: Shannon index, Simpson Index, Chao-1, and evenness. Beta-diversity analysis was established with weighted UniFrac distances for microbiome data and Bray-Curtis distances for KO metagenome data. The differences in beta-diversity between groups were analyzed with the permutational multivariate analysis of variance (PERMANOVA) test using 999 permutations. In the ANCOM-BC2 analysis, raw p values were adjusted with the Holm-Bonferroni method, and pseudo-count sensitivity analysis was used to confirm outlying taxa. In the ALDEx2 analysis, Kruskal-Wallis test, generalized linear model (GLM), and Welch's t-test were employed to assess differences between groups. ALDEx2 p-values were corrected using the Benjamini-Hochberg method. Data visualization was performed with R.

5.3 Results and discussion

5.3.1 Poi treatment increased gut microbiome diversity

As shown in Fig. 5.1 A, although gut microbiome supplemented with fresh poi showed somewhat reduced alpha-diversity of Shannon and Simpson indexes compared with control, treatment with fermented poi greatly increased microbiome Shannon, Simpson, and Chao1 indexes compared with control (statistically significant for Shannon index) and fresh poi treatment (statistically significant for all three indexes). Treatment with fermented poi also increased the evenness of the microbiome, indicating the species in this community were more equally abundant than the control and fresh poi treatment.

Moreover, the gut microbiome composition beta-diversity weighted UniFrac, which considered both phylogenetic distances and species abundances, between control, fresh poi, and fermented poi treated groups was significantly different ($p = 0.005$). The PCoA plot showed a clear separation between the three groups, indicating dissimilarity of the gut microbiota composition among the groups (Fig. 5.1 B).

Overall, the diversity indices suggested an increase in the microbiome diversity with fermented poi supplementation, which indicated that fermented poi had greater impact on gut microbiome diversity than fresh poi. A more diverse gut microbiome is a favorable characteristic associated with health. Low gut microbial diversity was found in individuals with obesity traits such as adiposity, insulin sensitivity, dyslipidemia, and low-grade systemic inflammation (Le Chatelier et al., 2013). Decreased gut microbial diversity has also been reported in individuals with type 2 diabetes mellitus, non-alcoholic fatty liver disease, and IBD (Di Ciaula et al., 2022; X. Guo et al., 2022; Lau et al., 2021).

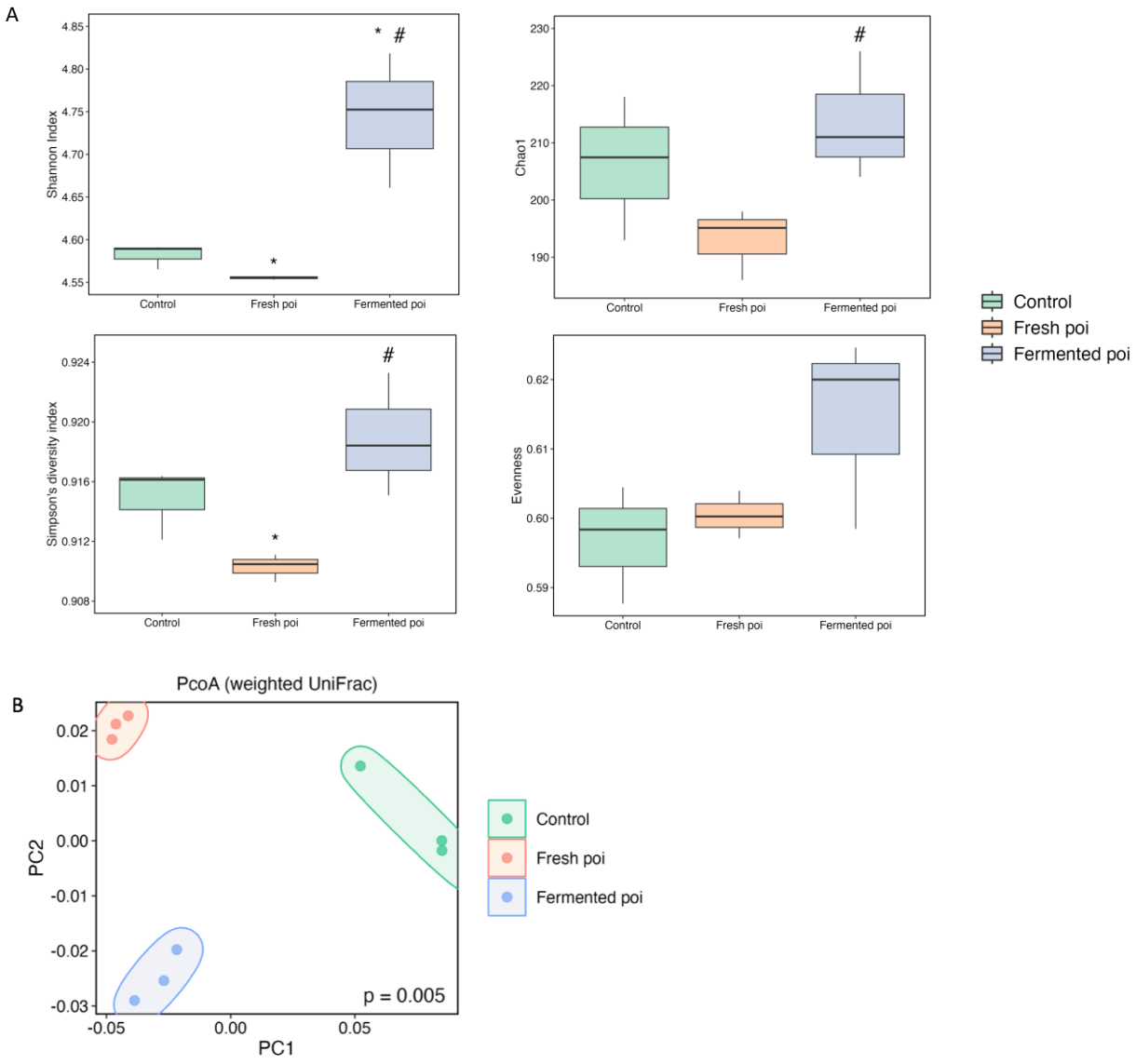

Figure 5.1 Diversity of human gut microbiome treated by fresh and fermented poi *in vitro*. (A) Alpha-diversity indexes: Shannon, Simpson, Chao1, and Evenness. Differences in alpha-diversity between groups were analyzed by the Kruskal-Wallis pairwise test. "*" indicates significant differences between the control and treatment groups ($P < 0.05$). "#" indicates significant differences between fresh poi treatment and fermented poi treatment ($P < 0.05$). (B) PCoA of beta-diversity based on weighted UniFrac distance. Pairwise comparisons were analyzed using PERMANOVA test.

5.3.2 Poi shifted the relative abundance of gut microbiota at the phylum level

The relative abundance and normalized abundance of taxa at the phylum level for each sample are shown in Fig. 5.2 A & B. Four and six out of the eight known phyla found in fecal samples treated with fresh and fermented poi, respectively, were differentially abundant according to the ANCOM-BC2 analysis (Fig. 5.2 D). The relative abundance of *Actinobacteriota* (also known as *Actinobacteria*) increased in samples treated with poi, and the increase was more pronounced in samples treated with fermented poi than fresh poi. In contrast, the abundance of *Fusobacteriota* (also known as *Fusobacteria*) underwent a substantial reduction in fecal samples treated with poi, with fresh poi showing a more significant reduction than fermented poi. The relative abundances of both *Firmicutes* and *Bacteroidetes* were elevated in the poi-treated fecal samples, and fermented poi demonstrated a more significant increase (Fig. 5.2 C & D). This study also noted a slight decrease in the *Firmicutes*/*Bacteroidetes* (F/B) ratio in the fresh poi group, whereas more reduction was observed in the fermented poi group (Fig. 5.2 C). The abundances of fecal *Desulfobactrota* and *Verrucomicrobiota* were reduced by fresh poi but were increased by fermented poi.

The human gut microbiota is predominantly composed of bacteria from the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidota* (also known as *Bacteroidetes*), and *Actinobacteriota* (Huttenhower et al., 2012). The increased *Actinobacteriota* and *Verrucomicrobiota* include probiotic genera such as *Bifidobacterium* and *Akkermansia* (Binda et al., 2018; Gryaznova et al., 2022) *Actinobacteria* are known for their ability to degrade complex carbohydrates and produce SCFAs (Law et al., 2020). *Actinobacteria*, a phylum that was negatively correlated with body fat percentage, reportedly were found increased in women with obesity after administration of kimchi over eight weeks (Han et al., 2015). *Verrucomicrobiota* have been associated with mucin degradation, which may stimulate the secretion of colonic mucin, subsequently benefiting the regulation of immune and metabolic functions (Gryaznova et al., 2022; B. Wang et al., 2017). The contrasting effects observed between the *Lactobacillaceae* family and the *Bifidobacteria* family on the abundance of *Verrucomicrobiota* were noted in healthy mice, where the former increased the phylum and the latter decreased it (Gryaznova et al., 2022). In this study, the contrasting effects on *Verrucomicrobiota* observed between the fresh and

fermented poi treatment groups might be attributed to the growth of certain unique LAB in poi during natural fermentation.

Fusobacteria and *Desulfobactrota* have been associated with health conditions. *Fusobacteria* have been implicated in certain disease conditions such as colorectal cancer (Kelly et al., 2018) and cirrhosis (Y. Chen et al., 2011). In addition, *Fusobacteria* have also been shown to suppress the immune system by inducing cell death in human lymphocytes (C. W. Kaplan et al., 2010). Whereas the sulfate-reducing bacteria *Desulfobactrota* have been associated with meat consumption, obesity development, and an increased inflammatory response in the gut (David et al., 2014; M. Li et al., 2022; Rajput et al., 2023; Vandeputte et al., 2017).

Firmicutes and Bacteroidetes are the most abundant bacterial phyla in the human gut (Huttenhower et al., 2012). While controversy exists over whether the *Firmicutes*/*Bacteroidetes* ratio is a determinant of health status, it is generally believed abnormally high F/B ratio is associated with dysbiosis and dysbiosis-related diseases (Han et al., 2015; Magne et al., 2020; Nagpal et al., 2018). A decrease in F/B ratio in healthy individuals is often considered a favorable characteristic in studying gut microbiota. As such, the reduction of F/B ratio observed in the fermented poi group indicated a positive shift of the gut microbiota.

In summary, fresh and fermented poi showed the ability to alter the gut microbiota composition at the phylum level *in vitro*. The trend of alteration favored the prevention of obesity and metabolic syndromes, the regulation of inflammation, and other quality-of-life metrics. In regards to the phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Verrucomicrobiota*, fermented poi demonstrated more advantages. Regarding the phyla *Fusobacteria* and *Desulfobactrota*, fresh poi demonstrated more advantages. This suggests the versatile functions of poi in modulating gut microbiota.

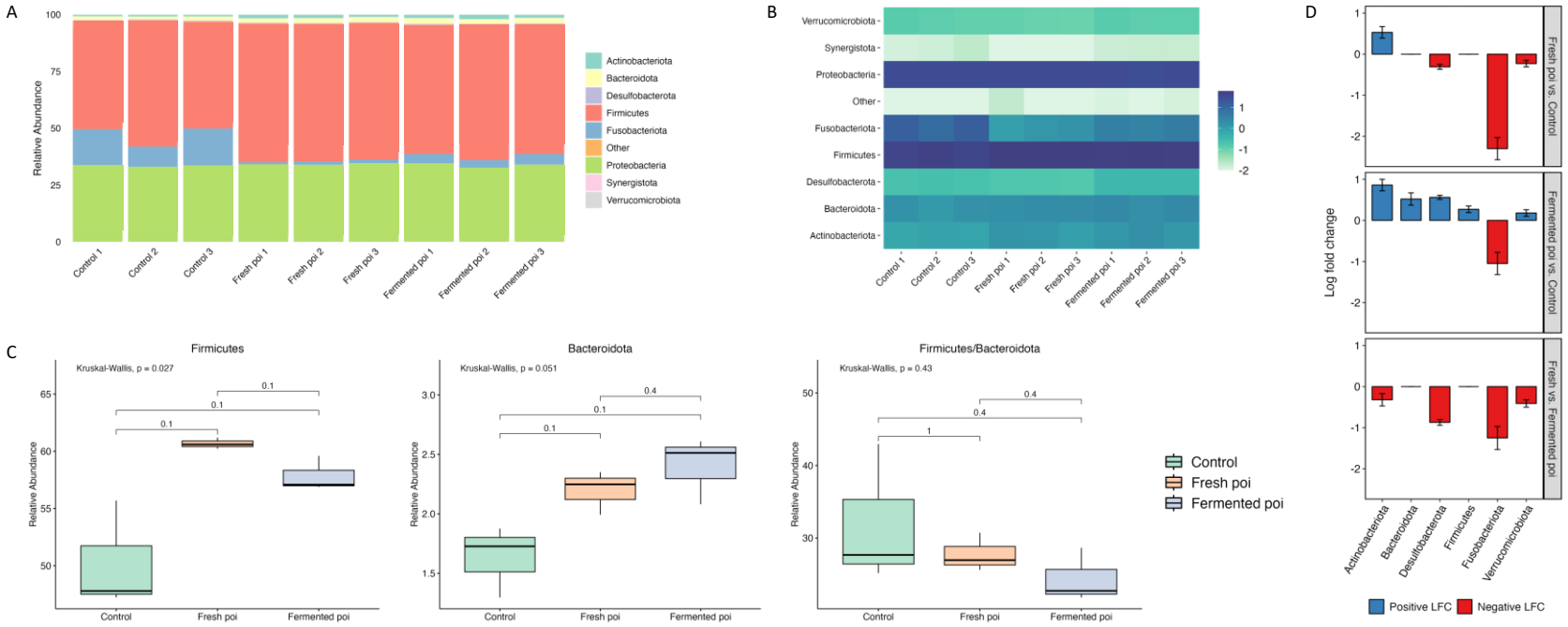

Figure 5.2 Effects of fresh and fermented poi on gut microbiota at the phylum level. (A) Relative abundance of taxa in each sample at the phylum level. (B) Heatmap of normalized relative abundance of taxa in each sample at the phylum level. (C) The relative abundance of Firmicutes, Bacteroidetes, and the ratio of Firmicutes to Bacteroidetes. Similarities between the groups were analyzed using the Kruskal-Wallis method, p values between all groups are labeled on the top-left corners, and p values of pairwise comparisons are labeled with connectors. (D) Differentially abundant phyla analyzed by ANCOM-BC2 multiple pairwise comparison.

5.3.3 Poi shifted the gut microbiota relative abundance at the genus level

A total of 157 unique genera were identify in the fecal microbiota. The relative abundances of the top 30 genera are shown in Fig. 5.3 A & B. Among all the genera, 20 genera were differentially abundant (Fig. 5.3 C). The genera *Allisonella*, *Bacteroides*, *Bifidobacterium*, *Catenibacterium*, *Enterococcus*, *Escherichia Shigella*, *Megasphaera* and *Roseburia* became more abundant in fecal samples treated with fresh and fermented poi than control. Except for *Enteroccous*, the log fold changes of these genera were slightly higher in fecal samples treated with fermented poi than those treated with fresh poi, although no statistical significance was found.

Bacteroides species are among the most abundant and prevalent bacteria in the human gut, and shifts in their abundance represent important health implications. These bacteria are well-known for their ability to ferment complex carbohydrates such as glucan, and they are recognized as SCFA producers (Fernandez-Julia et al., 2021). *Bacteroides* can interact with the host immune system and influence immune responses. They have been shown to interact with immune cells, modulate cytokine production, and contribute to the development and maintenance of immune homeostasis in the gut (Zafar & Saier, 2021). High *Bacteroides* abundance has been linked to a healthy gut microbiota composition while low Bacteroides abundance has been linked to conditions like obesity, metabolic disorders, and IBD (Zafar & Saier, 2021; Zhou & Zhi, 2016). Bacteroidetes was discovered to modulate succinate-activated intestinal gluconeogenesis in the liver through secondary bile acid-activated Farnesoid X Receptor (FXR) signaling in an animal model (Palmas et al., 2021). A notable correlation has been established between *Bacteroides* and consumption of fermented foods like kimchi and wine (Leeuwendaal et al., 2022).

Bifidobacterium species are normal anaerobic commensal bacteria found in the human gut, and they have been established as SFCA producers and probiotics (Leeuwendaal et al., 2022; Sivan et al., 2015). *Bifidobacterium* species can help maintain gut health by producing antimicrobial compounds, competing with potential pathogens for resources and adhesion sites, modulating immune responses, promoting the production of anti-inflammatory cytokines, enhancing gut barrier function, alleviating allergies, and other functions (Álvarez-Mercado et al., 2022; Sivan et al., 2015; Turroni

et al., 2014). An increased *Bifidobacterium* level in gut microbiota has been linked to various types of prebiotics, probiotics, synbiotics, and fermented foods (Inoguchi et al., 2012; C. Liu et al., 2022; Singh et al., 2021; S. Xu et al., 2022; Z. Yang et al., 2021). In addition, the altered abundance of *Bifidobacterium* was in agreement with the effect of taro on this genus (Saxby, 2020).

Catenibacterium, a polysaccharide-depredating bacterium, has the ability to produce SCFAs. A significantly higher abundance of *Catenibacterium* was found in individuals who consumed Mediterranean diet than those who did not (Garcia-Mantrana et al., 2018). A similar trend was noted in healthy individuals than individuals with colorectal cancer (Ai et al., 2019). The increased abundance of this genus corresponds with the prebiotics present in poi and suggests that poi has the potential to enhance gut health as a beneficial and nutritious dietary option.

Enterococcus species are opportunists. While some are probiotic, others are linked to infections. *Enterococcus faecalis* and *Enterococcus faecium* are the most common *Enterococcus* species found in the human gut (Firmesse et al., 2007). Enterococcus species, including *E. casseliflavus* and *E. lactis,* were found in fermented poi after simulated digestion in Chapter 2. This may contribute to its increased abundance after fecal fermentation. Interestingly, fresh poi slightly induced more growth of *Enterococcus* than fermented poi, suggesting the potential presence of substrates that selectively support the growth of *Enterococcus* in poi. In fermented poi, since such substrates may have been utilized during fermentation, the promotion of *Enterococcus* growth was weakened. *Enterococcus cecorum* has been associated with the consumption of fermented foods (Taylor et al., 2020). *Enterococcus* population significantly increased in healthy individuals who consumed Camembert cheese (Firmesse et al., 2008).

Roseburia, a high-butyrate-producing species, is associated with healthy guts (Holscher et al., 2018; Pryde et al., 2002). SCFA butyrate plays an important role in maintaining a healthy colonic epithelium, such as providing energy, exerting anti-inflammatory effects, controlling gene expression, and inducing apoptosis in tumorous cells (Pryde et al., 2002). Low butyrate levels have been linked to cancer and IBD (Pryde et al., 2002). An increased *Roseburia* population in the feces of healthy individuals was observed after almonds (dietary fiber) consumption (Holscher et al.,

2018). This evidence indicates that poi, as a dietary source, can promote the growth of *Roseburia*, contributing to improved gut health by fostering the production of beneficial butyrate.

Echerichia Shigella species are also common commensal gut residents that possess opportunistic pathogenic capabilities (Loo et al., 2022). These species are pro-inflammatory species, and an increase of this genus is associated with IBD gut dysbiosis (D. Chen et al., 2020). However, the roles of *Echerichia Shigella* can vary depending on specific strains and context. Certain strains may cause infections and pose a health risk, whereas others are important constituents of the normal gut microbiota. The promotion of these genera in poi-treated feces may indicate that poi has the potential to increase the abundance of commensal bacteria.

The roles of the genera *Allisonella* and *Megasphaera* in the human gut and their implications in health have not been extensively reported. However, *Allisonella* appears to be negatively associated with psychological stress and depression (Geng et al., 2020; Yamaoka et al., 2022). *In vitro* fermentation of human gut microbiota with anthocyanins from *Lycium ruthenicum* Murray significantly increased the relative abundance of *Allisonella*. *Megasphaera*, on the other hand, appears to be linked to positive health outcomes, with a higher abundance in healthy individuals compared to patients with colon cancer or IBD (Bamola et al., 2022; D. Chen et al., 2020). As a result, poi's role in increasing the abundance of these two genera seems health-promoting.

Fermentation of poi led to reduced *Fusobacterium* and *Klebsiella* populations in human feces. Fresh poi resulted in a higher log fold change in both genera than fermented poi, with the reduction of *Fusobacterium* being statistically significant (Fig. 5.3 C). *Fusobacterium* belonging to the phylum *Fusobacteria* as mentioned in section 4.4.2 are generally considered commensal bacteria, however, some strains can exhibit pathogenic potential under certain circumstances. In particular, *Fusobacterium nucleatum* has been widely associated with inflammatory diseases and colorectal cancer through various mechanisms such as invading human epithelial cells, stimulating human inflammatory response, suppressing human immune response to cancer cells, inducing oncogenic gene expression, and promoting colorectal cancer cell growth (D.

Chen et al., 2020; C. W. Kaplan et al., 2010; Kelly et al., 2018). Interestingly, taro consumption has been significantly associated with a decreased risk of colorectal cancer (Saxby, 2020), suggesting the ability of poi to reduce gut *Fusobacterium* abundance could be linked to the protective role of taro against colorectal cancer. *Klebsiella* species are part of the normal human gut microbiota, yet certain strains are pathogenic. *Klebsiella* possess various virulent factors such as genes encoding adhesins, siderophores, lipopolysaccharides, and invasins (Amaretti et al., 2020). The abundance of *Klebsiella* was higher in patients with ulcerative colitis and colorectal cancer (X. Guo et al., 2022; T. Wang et al., 2012). Consequently, a reduction in *Klebsiella* abundance in the gut microbiota is generally considered beneficial.

The abundances of several genera in the feces were only altered by fresh poi. The abundance of *Acidaminococcus* was higher after fecal fermentation with fresh poi, while the abundance of *Tyzzerella* and “uncultured_4” belonging to the *Lachnospiraceae* family were lowered by fresh poi. Conversely, the abundances of the genera *Dorea*, *Lachnoclostridium*, *Lachnospira*, and *Sutterella* were only increased with fermented poi treatment. *Acidaminococcus* is a fermentative bacterium that uses amino acids to produce acetate and butyrate (L. Sun et al., 2020). The variations observed between fresh and fermented poi can be attributed to the differing presence of LAB and bacterial substrates. These differences likely lead to distinct competitions and feedback loops within the microbial communities present in poi and the gut. Higher *Acidaminococcus* abundance and lower *Sutterella* abundance were observed in diabetic individuals than non-diabetic individuals (Gaike et al., 2020). Increased *Acidaminococcus* abundance was also observed in overweight/obese Italian adults and in individuals who consume a more pro-inflammatory diet (Palmas et al., 2021; J. Zheng et al., 2020). *Tyzzerella* has been positively associated with health outcomes such as cardiovascular diseases, Crohn’s disease, and hyperlipidemic (A. A. Xu et al., 2022). A reduction in the *Tyzzerella* population was observed in healthy mice fed with *bifidobacteria* (Gryaznova et al., 2022). The genera “unculture_4”, *Lachnoclostridium*, and *Lachnospira* are members of the *Lachnospiraceae* family. This family is known for its ability to break down complex carbohydrates and produce SCFAs. *Lachnoclostridium* species are butyrate producers and *Lachnospira* species are acetate and butyrate producers

(Mayengbam et al., 2019; Shrode et al., 2023). *Lachnospiraceae* has been associated with the consumption of yogurt and other fermented foods (Le Roy et al., 2022; Taylor et al., 2020). Supplementing pea fiber to adults with obesity significantly increased the abundance of *Lachnospira* (Shrode et al., 2023). *Dorea,* also belonging to the *Lachnospiraceae* family*,* may have a negative causal relationship with IBD (Z.-J. Zhang et al., 2021). Besides being abnormally highly abundant in cases of autism spectrum disorder, the *Sutterella* species exhibits controversial associations with health outcomes (Agarwala et al., 2021; Telle-Hansen et al., 2022; Zhou et al., 2023). *Sutterella* was negatively associated with SCFA production and positively associated with pro-inflammatory markers in patients with irritable bowel syndrome (Zhou et al., 2023). However, in healthy individuals, a three-day beta-glucan intervention significantly improved the glycemic response and the abundance of *Sutterella* (Telle-Hansen et al., 2022). Further research is needed to understand the roles of *Sutterella* in the gut microbiota and its impact on human health.

Two genera showed contrary log fold changes between fresh poi and fermented poi treated fecal samples: ‘bacteria_4’ belonging to the *Enterobacteriaceae* family, and *Bilophila*. The abundance of both genera significantly decreased in fresh poi treated feces, but significantly increased in fermented poi treated feces. *Enterobacteriaceae,* an abundant and prevalent bacterial family in the human gut, plays a crucial role in maintaining gut health, with *Escherichia coli* being one of its major members. *Enterobacteriaceae* are opportunists that could be pro-inflammatory and pose infectious risks in dysbiosis (Amaretti et al., 2020). An increased prevalence of *Enterobacteriaceae* was observed in patients with cirrhosis and those who are obese (Y. Chen et al., 2011; Palmas et al., 2021). Members of the *Enterobacteriaceae* family are frequently found in fermented foods, and have also been observed prevalent in individuals who consume fermented foods regularly (Leeuwendaal et al., 2022). Notably, the health implications of *Enterobacteriaceae* can vary in specific context. *Bilophila* belongs to the phylum *Desulfobactrota* as mentioned in section 4.4.2. *Bilophila*, particularly *Bilophila wadsworthia,* is associated with an animal-based diet due to its high bile-tolerance (David et al., 2014). *Bilophila wadsworthia* is also involved in promoting the development of obesity through the promotion of inflammatory

responses, intestinal barrier dysfunction, and bile acid dysmetabolism (Natividad et al., 2018). Supplementations with eugenol reduced the abundance of *Bilophila* in mice fed a high fat diet (M. Li et al., 2022). Additionally, Reduced *Bilophila* in the gut has been associated with alleviated symptoms and improved quality of life indices in constipation (Vandeputte et al., 2017). According to Chapter 3, many isolates found in digested poi showed bile salt hydrolase activities and the ability to process bile. Deconjugated bile could be easily converted into secondary bile salts, which could potentially advantage the growth of *Bilophila* (So et al., 2021).

Although the abundance of *Akkermansia* did not significantly increase at the genus level, the phylum it belongs to, *Verrucomicrobiota,* was significantly increased by fermented poi in the gut microbiota (Fig. 5.2.D). Moreover, the abundance of *Akkermansia* was significantly lower in feces treated with fresh poi compared to those treated with fermented poi. *Akkermansia*, particularly *Akkermansia muciniphila,* is a mucin-degrading bacteria that resides in the mucus layer and plays an important role in gut barrier function (Y. Yu et al., 2020). Lower abundance of *Akkermansia* was found in patients with IBD and metabolic disorders, with a causal relationship between *Akkermansia* and IBD being established (Z.-J. Zhang et al., 2021). A higher level of *Akkermansia muciniphila* has been linked to improved metabolic biomarkers and body composition in obese adults (Dao et al., 2016). Supplementation of beta-glucan increased the abundance of *Akkermansia* in the gut of healthy individuals (Telle-Hansen et al., 2022).

In summary, poi induced significant changes in the human gut microbiota. These changes are overall beneficial, with the majority of differentially increased genera being associated with improved metabolic status, inflammatory response, and overall quality of life measurements. Conversely, the majority of differentially decreased genera were linked to obesity and related metabolic syndromes, chronic inflammation, cancer, and infections. In addition, unique effects of both fresh poi and fermented poi on fecal bacterial communities were observed, suggesting the versatile function of poi in altering gut microbiota and the potential for tailoring poi to meet personalized nutritional needs. Among the 20 genera, some, such as the *Enterococcus* genus, may reflect the

microbial content found in poi, while others may not. This observation agrees with previous findings (Taylor et al., 2020).

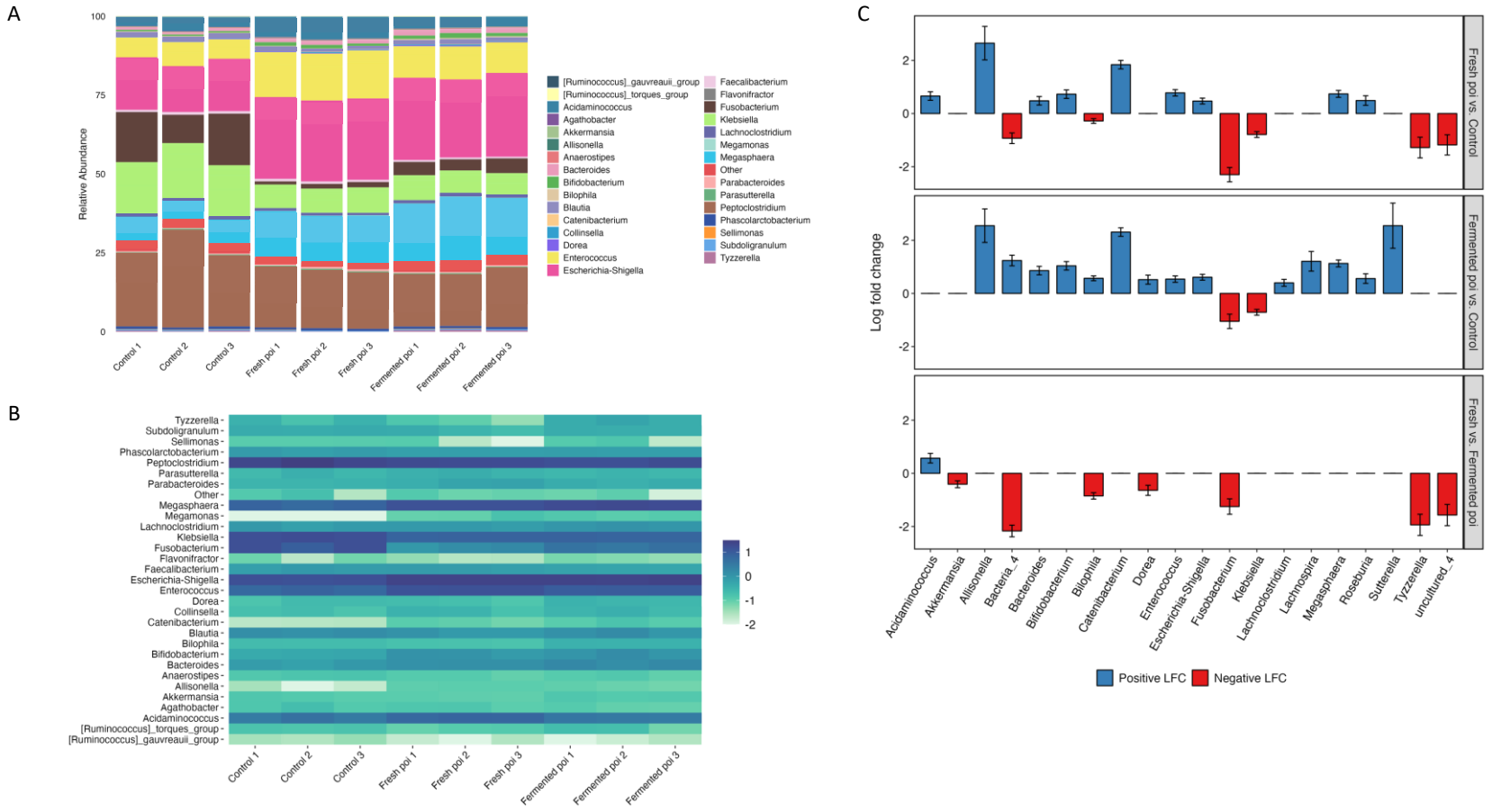

Figure 5.3 Effects of fresh and fermented poi on gut microbiota at the genus level. (A) Relative abundance of the top 30 most abundant genera in each sample. (B) Heatmap of normalized relative abundance of the top 30 most abundant genera in each sample. (C) Differentially abundant genera analyzed with ANCOM-BC2 multiple pairwise comparison. “Bacteria_4” belongs to the *Enterobacteriaceae* family and “uncultured_4” belongs to the *Lachnospiraceae* family.

5.3.4 Poi increased SCFA production during *in vitro* fermentation

Acetate, propionate, and butyrate constitute about 95% of SCFAs in the human intestine, with the remainder being made up of others like valerate and isovalerate (Feng et al., 2018). Despite no statistical significance, a slight increase in fecal acetate levels was observed with poi treatments (Fig. 5.4). Both fresh and fermented poi significantly elevated the levels of butyrate and valerate, whereas only fermented poi significantly raised isobutyrate levels in the feces ($P < 0.05$). Fresh and fermented poi increased the fecal levels of butyrate and valerate by 0.83- and 0.88-folds and 2.45- and 2.58-folds, respectively. The fecal levels of isobutyrate in the fermented poi group were 1.57-folds relative to the control group. The level of isovalerate was significantly higher in fermented-poi-treated samples (436.58±11.76 μg/g) than those treated with fresh poi (219.70±44.26 μg/g; $P < 0.05$). The level of propionate was not affected by poi. SCFAs are produced during bacteria fermentation of dietary fibers, amino acids, organic acids, and other substrates available in the gut. They play crucial roles in modulating gut activities and in regulating host metabolism and the immune system. Butyrate, the primary energy source of colonocytes, is essential for maintaining gut barrier integrity by promoting mucosa secretion, tight junction zone protein synthesis, cell proliferation, and the production of antimicrobial peptides (Parada Venegas et al., 2019). Acetate, propionate, and butyrate activate GPCRs that regulate various physiological functions including glucose and lipid metabolism, inflammation, and oxidative stress. They also inhibit histone deacetylases (HDACs) thus reducing NF-κB-induced pro-inflammatory mediators such as TNF-α, IL-6, IL-12, and inducible nitric oxide synthase and promoting the expression of anti-inflammatory mediators like IL-10 (Feng et al., 2018; Parada Venegas et al., 2019). In addition, SCFAs stimulate the production of GLP-1 and PYY, which are involved in glucose tolerance, insulin response, lipogenesis, and regulation of appetite via the vagus nerve (Lazar et al., 2019). Therefore, high SCFA production is typically found in healthy individuals, whereas disrupted or low SCFA production is associated with diseases such as IBD, obesity, and type-2 diabetes (D. Chen et al., 2020; Mayengbam et al., 2019; Telle-Hansen et al., 2022; Z. Yang et al., 2021). Increased fecal acetate and butyrate levels were found in diabetic mice treated with *Lactobacillus paracasei* IMC 502, and the increased SCFAs were positively correlated

with increased PYY and GLP-1 production and decreased pro-inflammatory cytokines IL-6 and TNF-α (Gu et al., 2023). Increased butyrate production is causally associated with improved insulin response, while abnormal propionate production is causally related to an increased risk of type 2 diabetes (Lau & Vaziri, 2019). The trends of slightly increased acetate, unaffected propionate, and significantly increased butyrate levels through the modulation of gut microbiota observed in this study are consistent with the above reports, indicating a potential beneficial effect of poi on insulin and inflammatory responses via gut SCFA mediation.

A Spearman's correlation analysis was conducted to investigate the contribution of differentially abundant genera to SCFA production for each treatment (Fig. 5.5). In the gut, acetate and propionate are primarily produced by *Bacteroidetes,* while butyrate is primarily produced by *Firmicutes* (Feng et al., 2018). Fewer genera were positively correlated with acetate, propionate, and butyrate in the control group compared with the treatment groups. *Allisonella*, *Bifidobacterium*, *Catenibacterium*, *Enterococcus*, *Megasphaera*, *Roseburia*, *Sutterella*, *Dorea*, *Lachnoclostridium*, *Lachnospira*, *Acidaminococcus*, and *Klebsiella* were positively associated with butyrate production in feces treated with both fresh and fermented poi. Among these bacterial genera, *Bifidobacterium*, *Catenibacterium*, *Acidaminococcus*, and *Lachnoclostridium* are known SCFA producers, and *Roseburia* is a well-known butyrate-producing bacterium. The positive correlation between butyrate and *Roseburia*, *Sutterella*, and *Bacteroides* was reported in healthy individuals who consumed beta-glucan (Telle-Hansen et al., 2022). Genera that potentially contained harmful species such as *Fusobacterium, Echerichia Shigella*, and *Bilophila* were negatively associated with butyrate production in fermented-poi-treated feces. Meanwhile, these three genera showed strong positive correlations with isovalerate in the fermented poi group, consistent with the higher abundance of the corresponding genera in these samples compared to the fresh poi group. *Akkermansia,* an acetate and propionate producing bacterium, was only positively associated with butyrate in the fresh poi group, which agreed with the higher abundance of this genus in fresh-poi-treated feces and the slightly higher levels of acetate and propionate in the fresh poi group. Moreover, *Roseburia*, *Sutterella*, *Lachnoclostridium*, *Bilophila*, *Akkermansia*, and *Acidaminococcus* exhibited strongly

positive correlations with all SCFA levels in fresh-poi-treated feces, which aligns with their SCFA-producing nature.

Overall, poi promoted the production of SCFAs by fecal microbiota, resulting in significantly increased levels of butyrate, isobutyrate, valerate, and isovalerate. These increased SCFA levels were positively correlated with several well-known SCFA-producing bacterial genera.

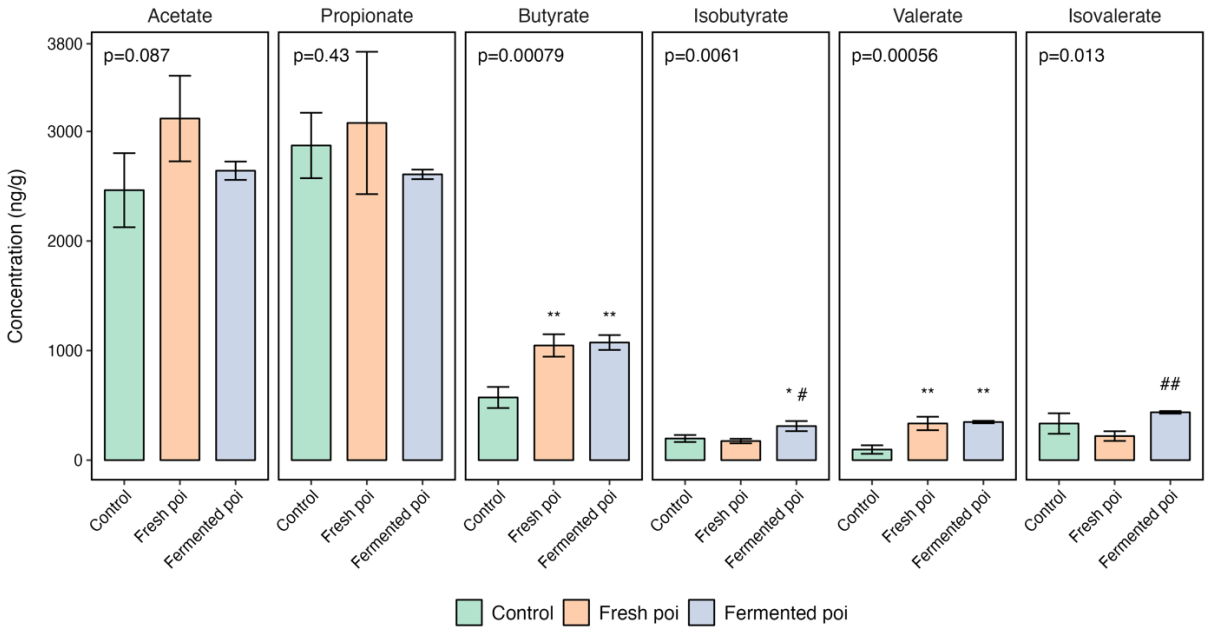

Figure 5.4 Short chain fatty acid (SCFA) production during *in vitro* fecal fermentation. Differences between groups were analyzed by ANOVA. "*" indicates significant differences between the control and treatment groups (* P < 0.05; ** P < 0.01). "#" indicates significant differences between fresh poi treatment and fermented poi treatment (#: P < 0.05; ##: P < 0.01).

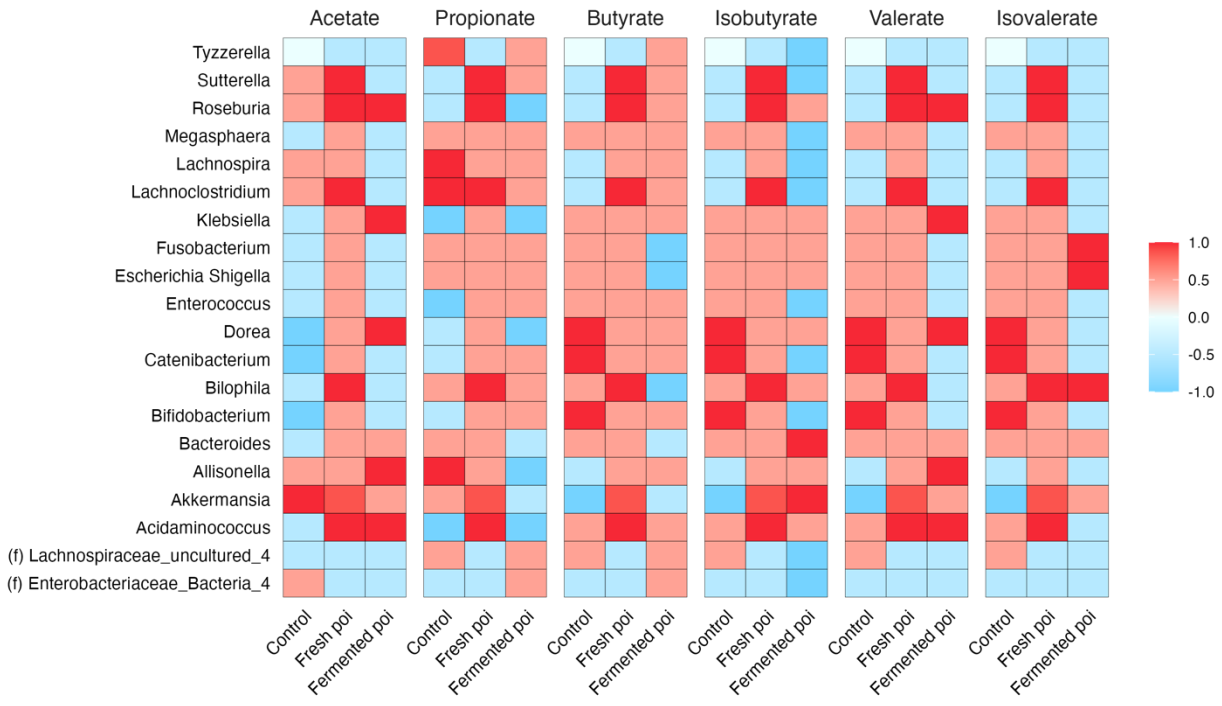

Figure 5.5 Correlation analysis between differential genera and SCFAs. Correlation analysis was performed using Spearman's correlation coefficient. Differential genera were obtained using ANCOM-BC analysis. Red tiles mean positive correlation and blue tiles mean negative correlation. No significant relationship was found (p-adjusted > 0.05). Two unknown genera "(f) Lachnospiraceae_uncultured_4" and "(f) Enterobacteriaceae_Bacteria_4" belonged to the families *Lanchnospiraceae* and *Enterobacteriaceae*, respectively.

5.3.5 Poi significantly altered the metabolic functional features of the gut microbiome

Predicted metabolic features or pathways derived from microbiome data like KEGG pathways offer insight into the potential biological processes that are active within the microbiome (Kanehisa et al., 2016). This information helps researchers understand the functional capabilities of the microorganisms, their interaction with host systems, and potentially identify biomarkers associated with diseases and health conditions. Ultimately, understanding the functional pathways opens up possibilities for developing personalized nutrition and medicine. KEGG pathway analysis provides a valuable framework; however, it is important to keep in mind that this research field is still evolving. KEGG pathway data only provides predictions of the potential functions of the microbial community. The interpretation of the results is context-specific and requires supporting evidence from chemical analyses to draw definitive conclusions. Caution is warranted in assuming that poi will lead to the health consequences listed in this section. The alpha-diversities, as measured by Shannon index and Evenness, were lowered by poi treatments (Fig. 5.6 A). Statistical significances were found in both treatments for Shannon index, and in fresh poi treatment for Evenness. Beta-diversity analysis using the Bray-Curtis metric showed distinct clusters for the control, fresh poi, and fermented poi groups, indicating the dissimilarity among the three groups (P=0.005, Fig. 5.6 B). However, in pairwise comparisons of beta-diversity, no significant difference was found between the poi groups. Nevertheless, the diversity of the KO features was significantly altered by poi treatments, although no significant difference was found between fresh and fermented poi groups.

From the three treatment groups, 170 unique KO features were predicted, with 165 being successfully annotated with KEGG pathway names and classes. A differential abundance analysis was performed using ALDEx2 to identify the metabolic pathways that underwent significant alterations. Both Kruskal-Wallis and glm tests were employed to compare between multiple groups (control, fresh and fermented poi). While both methods have strengths for analyzing abundance, they have some differences and the choice between them depends on the specific data distribution and the research objectives. The Kruskal-Wallis test analyzes non-parametric data while the glm test makes certain assumptions on the data distribution and fits statistical models on the

data (Wallen, 2021). In group comparisons, the Kruskal-Wallis test is more commonly used to compare the abundance of features, while the glm test allows for more flexible comparisons and includes the effects of any covariates. The main purpose of this study was to compare features between groups, hence the Kruskal-Wallis test was more suitable. Wallen (2021) compared several differential abundance analysis methods on two large Parkinson's disease gut microbiome data sets and found similar pairwise concordances and similar calls on average between the Kruskal-Wallis and the glm tests. In this study, all 165 annotated KEGG pathways were found significant with the glm test, with adjusted p values less than 0.05, and 139 pathways had adjusted p values less than 0.01 (data not shown). However, the ALDEx Kruskal-Wallis test only revealed 57 pathways with a marginal adjusted p value of 0.05 (data not shown). Since this study aimed to identify differentially abundant pathways between the groups, and there was no significant difference between the fresh and fermented poi groups based on alpha- and beta-diversities, a more conservative approach was chosen to compare only two groups at a time (fresh poi vs. control and fermented poi vs. control) using Welch's t-test. With this approach, 54 differentially abundant KEGG pathways were identified when comparing the fresh poi group with the control (Fig. 5.6). The 54 pathways were also identified through the ALDEx Kruskal-Wallis test, and the other three pathways were recognized when comparing the fermented poi group to the control. Thirty-one differentially abundant KEGG pathways were identified in comparison between the fermented poi group and the control (Fig. 5.8). The 31 pathways overlapped with the 57 pathways identified using the ALDEx Kruskal-Wallis test. In other words, pairwise comparisons recognized all differentially abundant pathways found in the group comparisons, ensuring the analysis captured the full spectrum of microbial changes associated with the poi treatments.

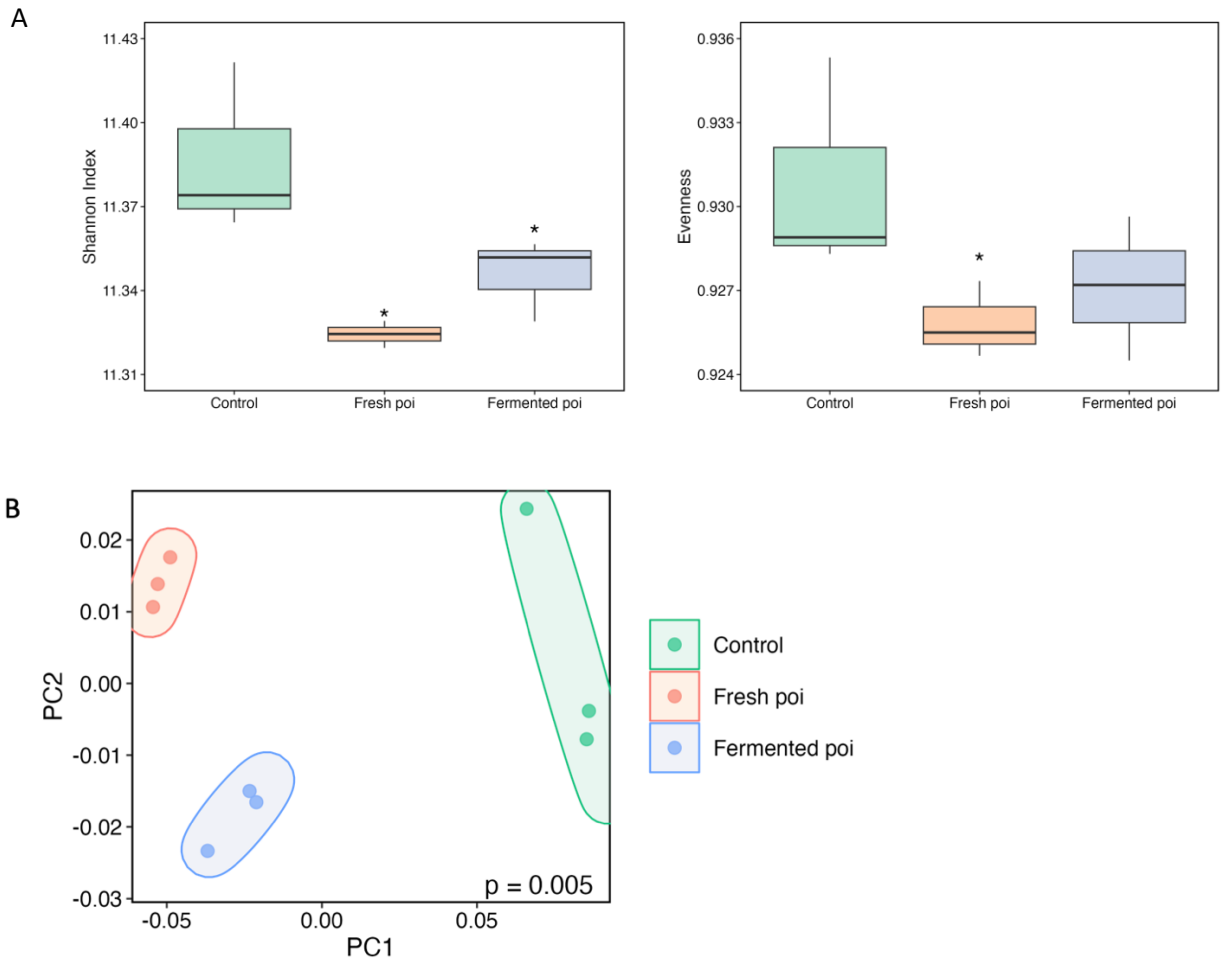

Figure 5.6 Diversity of KO features predicted from microbiome treated by fresh and fermented poi. (A) Alpha-diversity indexes: Shannon and Evenness. Differences in alpha-diversity between groups were analyzed by the Kruskal–Wallis pairwise test. "*" indicates significant differences between the control and treatment groups ($P < 0.05$). (B) PCoA of beta-diversity based on Bray-Curtis distance. Pairwise comparisons were analyzed using the PERMANOVA test.

5.3.6 KEGG pathways altered by fresh poi and their functional implications in the gut

Fresh poi treatment resulted in increased abundances of metabolic pathway classes involved in bacterial cell cycle, transport and catabolism (lysosome pathways), genetic information processing, biosynthesis of secondary metabolites including phenylpropanoid, flavone and flavonol, carbohydrate metabolism (pentose phosphate pathway), energy metabolism, glycan biosynthesis and metabolism, nucleotide metabolism, protein and carbohydrate digestion and absorption, and insulin signaling pathway (Fig. 5.7). First of all, lysosome is involved in one of the major defense mechanisms, autophagy, which breaks down cytoplasmic materials to remove harmful substances or pathogens, recycle nutrients, and regulate the turnover of tight junction zone proteins and preserve intestinal apterium integrity (Y. Zhang et al., 2023). Impaired lysosome activity is associated with diabetes and IBD (Su et al., 2020; L. Yang et al., 2018). Alterations in the KEGG insulin signaling pathway and pathways involved in carbohydrate metabolism of the gut microbiota were observed in rats with a decreased gut F/B ratio, increased relative abundance of *Akkermansia*, improved insulin sensitivity, better lipid profiles, and attenuated bodyweight gain (T. Li et al., 2022). In poi-treated samples, the alteration of microbial insulin signaling pathway was also accompanied by changes in carbohydrate metabolism, including the pentose phosphate pathway. Moreover, the elevated metabolism in the pentose phosphate pathway agreed with the extensively increased genetic information processing that consisted of nine significantly altered pathways. These pathways have multifaceted implications that seem to relate to high cellular biological activities such as microbial metabolism, immune regulation, and adaptation activities through communications between the host and the gut microbiota, and so on (S. Liu et al., 2020; Ma et al., 2019). In mice with circadian rhythm disorder, oolong tea polyphenols supplementation induced increased protein expression involved in genetic information processing (T. Guo et al., 2019). *Phascolarctobacterium*, a genus abundant in patients with Hashimoto's thyroiditis with hypothyroidism, was found to negatively regulate many metabolic pathways, including cellular processes, environmental information processing, genetic information processing, and metabolism (S. Liu et al., 2020). Phenolic compounds such as phenylpropanoid and flavonoids such as flavone and flavonol are bioactive compounds. Such compounds have antioxidant

and anti-inflammatory properties and may reduce the risk of chronic diseases while contributing to overall gut health (Korkina, 2007; Z. Li et al., 2023). Given the low bioaccessibility of flavonoids, the increased biosynthesis process may indicate the production of readily available flavonoids by the gut microbiota. Intestinal glycans consist of dietary-derived glycans and host-originated glycans such as mucin glycans. These glycans serve diverse functions, including cell recognition, cell adhesion, signaling, immune response, and energy storage (Kudelka et al., 2020). Disrupted glycans metabolism is linked to imbalances in carbohydrate metabolism and weight gain (Hou et al., 2017). Depleted glycan biosynthesis was found in obese children, adolescents, and adults (Hou et al., 2017; Palmas et al., 2021). Epithelial glycan and glycosylation play important roles in maintaining gut epithelium integrity, mediating interactions with gut microbes, and regulating immune responses (Kudelka et al., 2020). Understanding the interactions between epithelial glycan and gut microbiota allows to develop strategies for the prevention and treatment of IBD.

Cellular activities related to cell motility (bacterial chemotaxis), xenobiotics biodegradations metabolism, and retinoic acid-inducible Gene I (RIG-I)-like receptor pathways were reduced by fresh poi treatment. Bacterial cell motility activities like chemotaxis enables bacteria to sense and migrate to nutrient-rich favorable living conditions (Keegstra et al., 2022). The impact of the enriched bacterial chemotaxis depends on whether the increased activity is from commensal bacteria or pathogenic bacteria. In healthy elderly who consumed probiotics, enriched genes coding bacterial chemotaxis were associated with beneficial genera *Bifidobacterium*, *Eubacterium*, and *Roseburia*. On the contrary, a more active bacterial chemotaxis was found in obese children and mice fed a high fat diet (Hildebrandt et al., 2009; Hou et al., 2017). Xenobiotics are potentially toxic chemicals originated from industrial manufacturing, motor emissions, and environmental contamination. These substances are usually carcinogenic, mutagenic, and can have detrimental effects on human reproductive and cardiovascular systems (Rampelli et al., 2020). Increased gene expression related to xenobiotics degradation was found in individuals with exceptionally long lifespans (Rampelli et al., 2020). Regrettably, fresh poi reduced the xenobiotic degradation activities in the gut., which could be partially explained by the decreased F/B ratio in poi

treated feces. Firmicutes possess more ATP-binding cassette (ABC) membrane transporters responsible for transporting a wide range of substrates like nutrients and xenobiotics (Palmas et al., 2021). Microbial activities involved in xenobiotic degradation are rather complex and are affected by a number of factors including shifts in gut microbiota composition (Maurice et al., 2013). Increased abundance of xenobiotic degradation was also found in children with obesity (Hou et al., 2017). One solution to this disadvantage could be to consume complementary foods that favor xenobiotic degradation, for instance, low bush wild berries and traditional herbs (Lacombe et al., 2013; Song et al., 2020). Additionally, RIG-like receptors (RLRs) are part of the innate immune systems specifically involved in detecting viral infections. Upon recognition of viral RNA, RLRs signal the production of type I interferons (IFN-α and IFN-β) and further induce the release of other pro-inflammatory markers like IL-6 and TNF-α (Ramos & Gale, 2011; R. Yang et al., 2020). Under certain conditions, RLRs could induce cell apoptosis and be manipulated in cancer treatment (R. Yang et al., 2020). However, overexpression of RLR signaling could lead to autoimmune diseases like osteoporosis (N. Wang et al., 2022). Nevertheless, the ability of poi to alternate intestinal immune response was found to be consistent with the results obtained in Chapter 4.

Observed trends in amino acid metabolism, lipid metabolism, metabolism of cofactors and vitamins, and metabolism of terpenoids and polyketides showed a degree of variability. In the amino acid metabolism, an increase in the biosynthesis of phenylalanine, tyrosine and tryptophan was accompanied by a reduction in the corresponding metabolism processes. Besides functioning as building blocks, phenylalanine, tyrosine and tryptophan are precursor of neurotransmitters like dopamine, norepinephrine, epinephrine, and serotonin. The gut microbiota can communicate with the host via the gut-brain axis by producing these neuroactive compounds that regulate brain function, behavior, mood, circadian rhythm, and appetite (Lazar et al., 2019). These aromatic amino acids and their metabolites also regulate essential physiological processes, such as the homeostasis of intestinal epithelial cells, the response of immune cells, and the metabolism of energy within the liver (D. J. Kim et al., 2018; Y. Liu et al., 2020). Interestingly, β-glucan significantly elevated this pathway in mice with ulcerative colitis, suggesting an association between prebiotic

treatment, neurotransmitter precursors, and gut health (C. Liu et al., 2023). These findings underscore the potential of poi to exert a positive influence on the gut microbiome. This influence may enhance overall well-being through the modulation of neurotransmitter precursors and their related key physiological processes. In lipid metabolism, primary and secondary bile biosynthesis were promoted accompanied by a decrease in glycerolipid and arachidonic acid metabolism. Bacterial modifications of primary and secondary bile salts are one of the most important actions of gut microbiota-host communication. The modified bile salts not only serve as substrates for other symbiotics but also act as signaling molecules that can interact with host receptors, such as FXR and the G protein-coupled bile acid receptor 1 (TGR5). These interactions regulate various physiological processes, including bile acid synthesis, lipid and glucose metabolism, inflammation, and gut barrier integrity (Mullish et al., 2018). This finding was in agreement with the previously observed abundant BSH activities among LAB in digested poi and the altered expression levels of genes related to cholesterol uptake and synthesis in poi treated Caco-2 cells (Chapter 3 and Chapter 4). Metabolic pathways in thiamin, retinol, and pantothenate CoA biosynthesis were found to be more abundant in the fresh poi group, while porphyrin metabolism was more abundant in the control group. Thiamin, retinol, and pantothenate are essential micronutrients. The positive shift in their biosynthesis processes fits the role of gut microbiota in providing vitamins to the host. Zeatin and terpenoid backbone biosynthesis increased, while tetracycline biosynthesis and limonene degradation decreased. Terpenoids and polyketides are plant material that can be broken down into bioactive compounds that could have antibiotic, anti-inflammatory, and anti-oxidant properties and considerably impact gut microbiota dynamics (L. Liu et al., 2020). This KEGG pathway was among the most differentially abundant pathways induced by conjugated linoleic acid, a popular health-beneficial compound in obese mice (L. Liu et al., 2020).

In summary, the majority of the changes in KEGG pathways induced by fresh poi appear to be beneficial to the host in aspects of regulating carbohydrate and lipid metabolisms, immune and inflammatory responses, and brain-gut communications. Many of these changes are consistent with each other and with the previous findings.

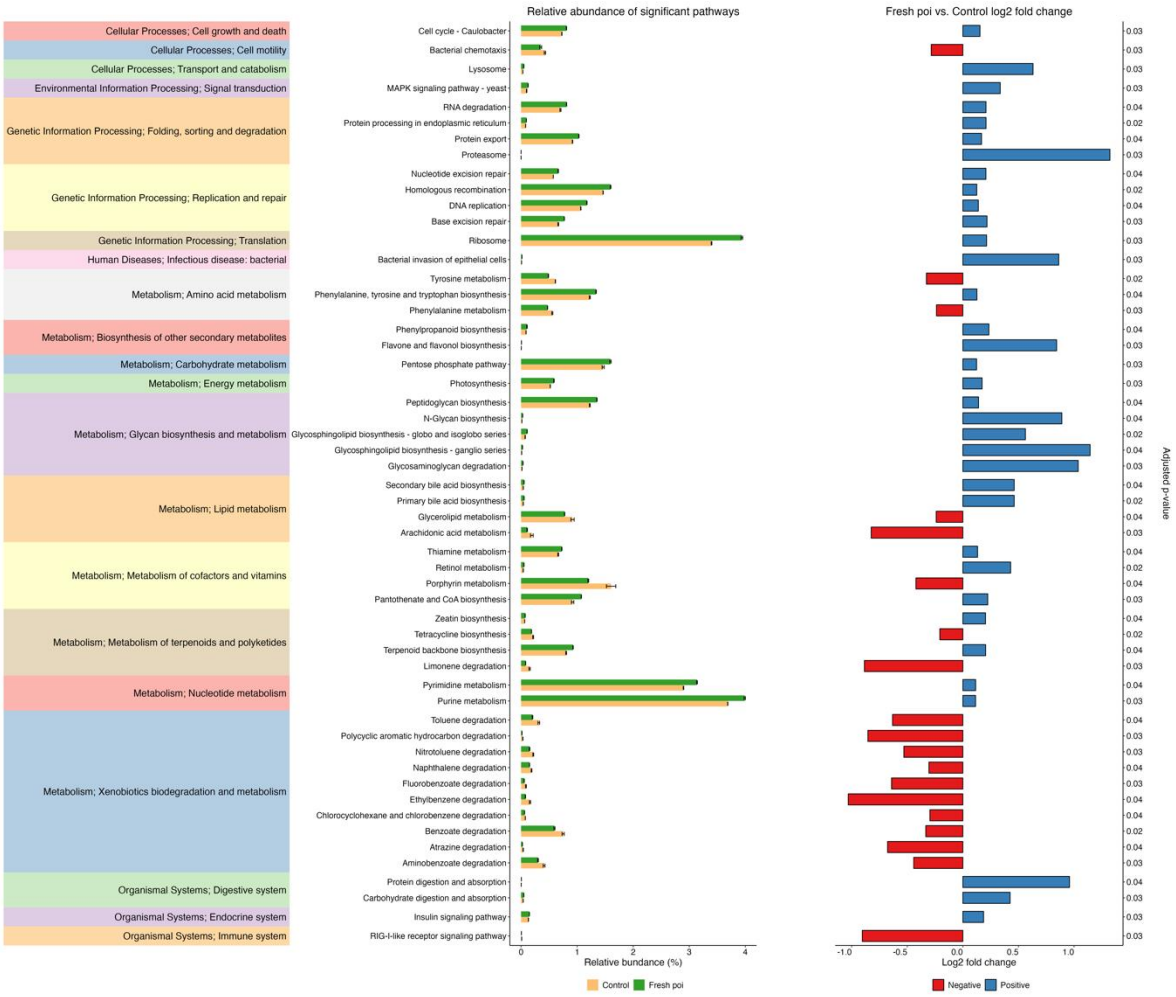

Figure 5.7 Differentially abundant KEGG metabolic pathways predicted from the microbiome data analyzed using the ALDEx2 method (fresh poi vs. control). The pathways were grouped into their belonging classes listed in the left-most column. Positive $\log_2$ fold change means the pathway feature was more abundant in fresh-poi-treated samples (colored in blue). Negative $\log_2$ fold change means the pathway was more abundant in the control samples (colored in red). Statistical significance was determined using the Welch's t-test. P-values were adjusted with the Benjamini-Hochberg method. $P < 0.05$ was considered statistically significant.

5.3.7 KEGG pathways altered by fermented poi and their functional implications in the gut

The trends of differentially abundant KEGG pathways in the fermented poi group shared some similarities with the fresh poi group, including lysosome pathway, bacterial invasion to epithelial cells, genetic information processing, glycan biosynthesis and metabolism, lipid metabolism, terpenoids and polyketides metabolism, xenobiotics biodegradation and metabolism, digestive systems, and immune systems. However, a decrease in the biosynthesis of amino acids was noted (Fig. 5.8). Meanwhile, several unique features stood out in the fermented poi group. The pathways involved in environmental information processing including ABC transporters and the two-component system were significantly reduced. These pathways are important cellular processes involved in the exchange of nutrients, ions, and other bioactive molecules (Kulbacka & Satkauskas, 2017). The reduced abundance of these pathways could be indicative of the more pronounced effects of fermented poi on the gut microbiota than fresh poi, since shifts in these pathways were only observed in the fermented poi group, bearing significant implications. Upregulated ABC transporter pathways of the gut microbiota were associated with obesity (Hildebrandt et al., 2009; Hou et al., 2017; Palmas et al., 2021). An unexpected result was found within the Citrate cycle (TCA cycle) pathway, revealing its potential link to obesity. Specifically, a decreased abundance of this pathway was observed in the fermented poi group, a phenomenon that was positively correlated with obesity. This might be counterbalanced by the increased photosynthesis activities found in the fresh poi group (Fig. 5.7). This could be a limitation of the *in vitro* settings over *in vivo*, as bacteria exposed to light may favor photosynthesis over cellular respiration for energy acquisition. The phosphonate and phosphinate metabolism pathway was altered by fermented poi. These compounds are natural products synthesized by gut microbes, many of which are believed to have antimicrobial activities (Peck & van der Donk, 2013). Interestingly, fermented poi altered the abundance of peroxisome proliferator-activated receptors (PPAR) signaling pathway. PPAR signaling in the gut microbiome has been positively associated with obesity induced by high fat diets and negatively associated with the control group in mice. However, when the obese group was treated with an anti-obesity drug, Orlistat,

the treatment became more positively associated with KEGG PPAR signaling, with improvements in obesity-related factors (J. Ke et al., 2020). This suggests that obesity might be related to imbalanced gut microbiome PPAR signaling. The alterations observed in the PPAR signaling pathway, as induced by fermented poi, signify its potential impact on lipid metabolism through the modulation of the gut microbiome by fermented poi. Lastly, the abundance of proximal tubule bicarbonate reclamation was reduced. This process is essential for maintaining the acid-base-balance in the body. Enrichment of this gut microbiome function has been found in patients with kidney stones, type 2 diabetes, and mice with colorectal cancer (Inoue et al., 2017; J. Xie et al., 2020; C. Yang et al., 2021). Overall, fresh poi and fermented poi had similar influences on the predicted KEGG functions of the gut microbiota. Although fresh poi affected more pathways than fermented poi, both demonstrated unique potential benefits to the host.

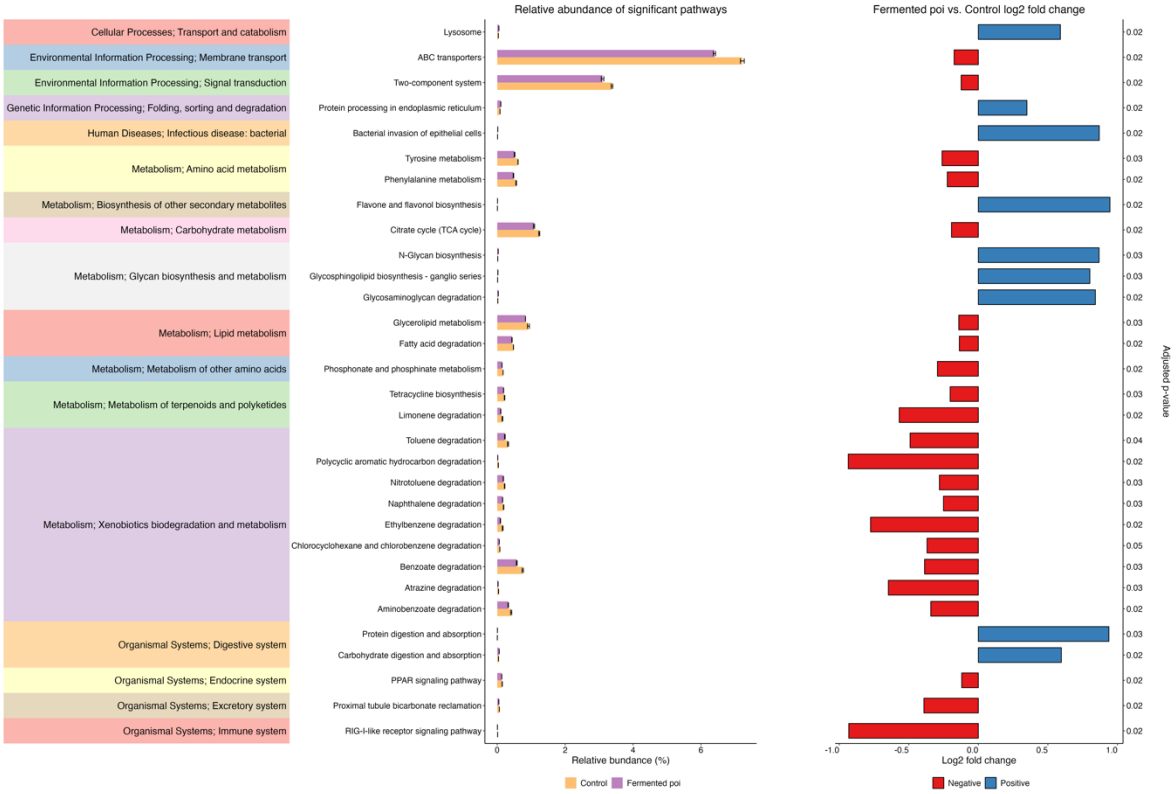

Figure 5.8 Differentially abundant KEGG metabolic pathways predicted from the microbiome data analyzed using the ALDEx2 method (fermented poi vs. control). The pathways were grouped into their belonging classes listed in the leftmost column. Positive $\log_2$ fold change means the pathway feature was more abundant in fermented-poi-treated samples (colored in blue). Negative $\log_2$ fold change means the pathway was more abundant in the control samples (colored in red). Statistical significance was determined using the Welch's t-test. P-values were adjusted with the Benjamini-Hochberg method. $P < 0.05$ was considered statistically significant.

5.3.8 Limitations

Firstly, the effects of poi on human gut microbiota were evaluated using an *in vitro* model instead of an *in vivo* model. The *in vitro* digestion and fermentation protocol employed in this study has been well developed and tested. It allows fast, convenient investigations on the effects of food on human gut microbiota under controlled conditions. This protocol has generated reproducible data in a number of studies involving different foods (Pérez-Burillo et al., 2021). The results of SFCAs and other metabolites obtained using this protocol was consistent with what was observed in *in vivo* and human intervention data (Pérez-Burillo et al., 2021). The microbial communities structures obtained from this protocol was able to reflect different effects between various food groups (Pérez-Burillo et al., 2021). Therefore, it is an excellent protocol that preserves the functionality of human fecal microbes and allows to identify the effects of food on fecal microbiota. However, *in vitro* models cannot fully replicate the exact conditions present in the human gut. Such conditions include continuous material and buffer exchange with the host, interactions with other foods and chemicals, and the absence of light. In this study, it appeared that bacterial photosynthesis was enriched, which would not happen in *in vivo* models (Fig. 5.7). Furthermore, the transfer and handling of fecal samples may also influence the microbial community structure. This could account for some differences in microbial community structures, typically observed between *in vitro* and human studies. In humans, *Bacteroidetes* and *Firmicutes* usually make up the majority of the gut microbiota, along with smaller proportions of *Proteobacteria* and *Actinobacteria* (Han et al., 2015; Unno et al., 2015). The four bacterial phyla constitute about 93% of the human gut microbiota (Lazar et al., 2019). In this study, *Proteobacteria* made up approximately 30% of the microbial community (Fig. 5.2). This kind of differences in the relative abundance of the four main gut microbial phyla was also observed in other *in vitro* studies (Cheng et al., 2020; Fu et al., 2019; Nagpal et al., 2018; L. Yu et al., 2021). In addition, the *in vitro* model used pooled fecal samples to minimize inter-individual variations. However, gut microbiota is in fact different between individuals, making individual-level study expansion worthwhile. The second limitation of this study was that only one brand of poi was tested and just three replicates were conducted. As reported in the previous chapters, poi from different

brands harbor different LAB, showed different probiotic properties, and had different effects on Caco-2 cells. We choose a brand that exhibited the most promising probiotic potentials for the *in vitro* fermentation study. But it would be more representative if various brands are investigated. Furthermore, a sample size smaller than five may affect variance estimation in the ANCOM-BC2 analysis and other differential abundance analyses.

5.4 Conclusions

In conclusion, poi significantly altered the gut microbiota diversity, composition, and functional metabolic features, and promoted SCFA production during *in vitro* fermentation with feces collected from healthy individuals. Poi shifted the relative abundance of six phyla and decreased the *Firmicutes*/*Bacteroidetes* ratio. At the genus level, 20 genera, including *Bacteroides* and *Bifidobacterium,* demonstrated differential abundance in poi groups. Poi increased the levels of butyrate and valerate in the fecal samples. The significantly influenced KEGG pathways showed trends supporting the microbial and chemical changes. Moreover, differences were identified between fresh and fermented poi groups. Fermented poi significantly increased alpha diversity indices compared to control and fresh poi groups. Fermented poi also showed slightly more contributions to the log fold change of most of the differentially abundant genera. Fecal samples treated with fermented poi also contained higher levels of isobutyrate and isovalerate than the fresh poi group. Such enhancement by fermented poi was also found in fermented kimchi compared to fresh kimchi, and fermented soy milk compared to fresh soy milk (Han et al., 2015; Inoguchi et al., 2012), highlighting the crucial role of fermentation in the health effects of fermented foods. Additionally, unique properties of fresh and fermented poi-treated gut microbiota were observed, suggesting that fresh and fermented poi could influence different health aspects in the human body and could be tailored to individual needs. Ultimately, the results and predictions obtained from the *in vitro* study will require further validation through *in vivo* and human studies.

CHAPTER 6 CONCLUSION AND FUTURE DIRECTIONS

The primary objective of this dissertation was to investigate the health benefits of poi, focusing specifically on its probiotic properties.

The first study (Chapter 3) involved characterizing the microflora profiles of poi before and after in vitro digestion. Additionally, LAB in the digested fermented poi were isolated and screened for potential probiotic properties. The results of this chapter indicated that 99.97% of the LAB in poi were killed during digestion. The overall diversity of the microbiome in poi decreased, while the abundance of acid-tolerant and bile-tolerant bacterial species increased. A total of 62 unique LAB isolates from the digested poi were identified, belonging to 13 species. Many of these isolates demonstrated superior probiotic properties compared to a commercial probiotic strain, *Lacticaseibacillus rhamnosus* GG. Cluster heatmap analysis of all the unique isolates based on their probiotic properties revealed that 10 isolates from *S. lutetiensis*, *E. lactis*, *L. plantarum*, and *L. lactis* species exhibited superior overall probiotic potential. Additionally, similarities and differences were observed in the microflora community structure and probiotic properties among poi produced by six local factories. The differences between various brands of poi may be attributed to factors such as the source of taro used, specific manufacturing techniques, geographical location of manufacturing, and handling workers, etc. For instance, brands A and B shared similar taxa, brand C stood out with unique microbial taxa and superior antimicrobial activities, brand D had the most diverse microbiome and excellent probiotic properties, while brands E and F shared similar taxa, and isolates from both brands enhanced the viability of Caco-2 cells.

Based on the findings of the first study, a hypothesis was formulated that poi might possess anti-infectious and anti-inflammatory properties due to the presence of antimicrobial LAB, LAB that increased Caco-2 cell viabilities, and the prevalence of probiotic LAB isolated from poi. This hypothesis was tested in the second study (Chapter 4). In Chapter 4, water-soluble extracts of poi, which include bioactive compounds, antimicrobial substances, bacteria-derived metabolites and molecules, soluble fibers, etc., have demonstrated inhibitory effects on the adhesion and invasion

of pathogenic bacteria such as *Listeria monocytogenes* and *Salmonella Typhimurium* to Caco-2 cells. Poi extracts also reduced the expression levels of pro-inflammatory genes (IL-6, IL-8, and TNF-α) under conditions of oxidative stress or infection-induced inflammation, while increasing the expression levels of anti-inflammatory genes (IL-4, IL-10, TGF-1β, and TLR2). The extracts showed the potential to enhance the integrity of the intestinal barrier by upregulating the expression levels of tight junction proteins (claudin-1, occludin, and ZO-1). These results indicated that poi extract could help improve the immune function of the gut. Furthermore, it was discovered that poi extract might regulate lipid and cholesterol metabolism in Caco-2 cells through the PPAR/FIAF and SREBP-2 signaling pathways. Interestingly, the overall regulatory activities of poi extracts were more pronounced in fermented poi compared to fresh poi, Brand C demonstrated greater effects compared to Brand A. However, this was not always the case, as fresh poi or Brand A poi extracts showed more pronounced effects in certain instances, such as the reduction of IL-8 when inflammation was induced by H_2O_2 or *L. monocytogenes*.

Finally, Chapter 5 presented the effects of fresh and fermented poi on the modulation of the gut microbiome during in vitro fermentation. This study revealed that only fermented poi increased the diversity of the gut microbiome. Both fresh and fermented poi magnified the abundance of beneficial genera such as *Allisonella*, *Bacteroides*, *Bifidobacterium*, *Catenibacterium*, *Enteroccous*, *Megasphaera*, and *Roseburia*, while reducing the abundance of potentially obesogenic and pro-inflammatory genera such as *Fusobacterium* and *Klebsiella*. Poi significantly increased the fecal levels of short-chain fatty acids (SCFAs), especially butyrate. The analysis of microbiome functionality was consistent with the findings reported in the previous chapters. For example, the regulation of bile acid biosynthesis aligned with the results observed in Chapter 3, and the regulation of immune functions (RIG-I-like receptor signaling pathway) and lipid metabolism (PPAR-γ) aligned with the results found in Chapter 4. Overall, the diversity and community structure of the gut microbiome were more significantly altered by fermented poi. However, unique beneficial properties of fresh poi were also observed, such as the reduction of *Bilophila*, an obesity-associated genus.

In conclusion, the three studies conducted herein have successfully demonstrated the beneficial effects of poi on gut health and overall well-being. Poi was shown to regulate immune function and metabolism in intestinal epithelial cells while also modulating the gut microbiome. The findings corroborated the hypothesis that microbial fermentation substantially enhances poi's health-promoting properties, with fermented poi exhibiting even greater benefits. Interestingly, fresh poi displayed unique context-dependent beneficial properties, and different brands of poi showcased their distinct LAB profiles and advantages.

The cumulative evidence presented in this dissertation reinforces poi's stature as a health-beneficial food. Promoting poi, its cultural significance, and the traditional Hawaiian diet based on these discoveries could not only benefit local communities but also have a global impact. The valuable insights provided by this study significantly contribute to the literature on the modulatory effects of fermented foods on gut microbiota, a critical determinant in human health. These findings underscore the versatility of poi in promoting gut health, making it advisable to consume poi regularly across a variety of brands and fermentation stages. However, as a starchy food, poi’s overconsumption may lead to an increased calorie intake. Therefore, moderation is advised, and it could be considered a suitable replacement for daily carbohydrate sources. To exploit specific health benefits, such as alleviating bacterial infections, inflammatory bowel disease (IBD), hypercholesterolemia, or metabolic disorders, selecting the appropriate poi based on its LAB community, probiotic properties and targeted effects on these conditions is essential. Personalized needs may require further exploration of the bioactive compounds and bacteria in poi, along with understanding the underlying mechanisms responsible for these beneficial effects. Lastly, since the beneficial properties discovered in this dissertation were demonstrated using *in vitro* models, supplementary validation through *in vivo* methodologies, such as animal studies under high-fat diet or disease conditions, and human studies like randomized control trials, is necessary before making definitive claims about poi's health implications.

In summary, the research on poi has unearthed substantial insights into its positive impact on gut health and holistic well-being. By understanding its unique properties, poi

can be strategically incorporated into a balanced diet to promote health effectively. However, further investigations are indispensable to fully uncover the potential health benefits and to ensure evidence-based recommendations for poi consumption.

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APPENDIX

A1 Recruitment flyer for healthy fecal donors.

(Recruitment Flyer for a Poi Study)

The University of Hawai`i is conducting a study:

Uncovering the probiotic potential of poi, a naturally fermented Hawaiian food

Are you a healthy individual who would like to contribute to local poi research?

If the answer is **YES**…

Lianger Dong Researcher would like to invite you to participate in a research study.

The purpose of this study is to learn more about the health benefits of poi on the human gut. The study is being funded by the U.S. Department of Agriculture. This study will be Lianger researcher's dissertation for her doctorate degree in nutrition sciences.

- If you meet our criteria, You will be asked to donate your fecal samples to the study.
- Study visit will take place at your home.
- Your fecal samples will be pooled with a few others and used in a simulated colonic fermentation experiment.

To learn more about the study,

please call/text/email Lianger Researcher, MS

at 865.306.6440 or ldong@hawaii.edu

UH IRB Approval Date January 31, 2023

A2 Questionnaire for screening fecal donors.

Participant screening questionnaire
Study title: Uncovering the probiotic potential of poi, a naturally fermented Hawaii food

Questionnaire for participating in a poi study

Name:_____ Age:_____

Contact Info

Phone number:_____ Email:_____

Sex:

☐ Male ☐ Female

Ethnicity:

- ☐ Asian or Pacific Islander
- ☐ Black or African American
- ☐ Hispanic or Latino
- ☐ Native American or Alaskan Native
- ☐ White or Caucasian
- ☐ Multiracial or Biracial
- ☐ A race/ethnicity not listed here

Measurements:

Body weight:_____ lbs Height:____feet ____inches

Does the following apply to you?			
1. On a special diet	☐ Yes	☐ No	
2. Consume poi	<1 time/week	2-3 times/week	>3 times/week
2. Smoking	☐ Yes	☐ No	
3. Have taken antibiotics in the past 3 months	☐ Yes	☐ No	
4. Pregnant or lactating	☐ Yes	☐ No	
5. History of bowel conditions (irritable bowel syndrome, Crohn's disease, ulcerative colitis, etc.)	☐ Yes	☐ No	
6. Diabetes or obesity	☐ Yes	☐ No	
7. Cardiovascular diseases (atherosclerosis, heart disease, etc.)	☐ Yes	☐ No	
8. Other health issues (what is it?_____)	☐ Yes	☐ No	

A3 Consent form for fecal collection.

University of Hawai'i
Consent to Participate in a Research Project
Dr. Yong Li, Principal Investigator
Study title: Uncovering the probiotic potentials of poi, a naturally fermented Hawaiian food

Consent Form

Aloha! You are being asked to participate in a research study conducted by Dr. Yong Li (Ph.D.) and Lianger Dong (M.S.) from the Department of Human Nutrition, Food and Animal Sciences at the University of Hawaii. The results obtained from this study will contribute to Lianger Dong's dissertation for her doctorate.

What am I being asked to do?
If you participate in this study, you will be asked to provide your fecal samples.

Taking part in this study is your choice.
You can choose to take part or you can choose not to take part in this study. You also can change your mind at any time. If you stop being in the study, there will be no penalty or loss to you.

Why is this study being done?
The purpose of my study is to identify potential health benefits of poi on the human gut. I am asking you to participate because this study will be done in a simulated colonic fermentation condition, which requires the use of human gut microbes from feces.

What will happen if I decide to take part in this study?
If you decide to participate in this study, you will be asked to do the following: (1) pick up the fecal sample collection kit from the Agriculture Science building on campus, and receive a short instruction on how to use the kit at home properly, which will take about 10 minutes. (2) When notified by Lianger, collect your feces with the fecal sample collection kit on that day, once, at home, and wait for Lianger to collect your fecal sample from your home. This step will take no more than one hour.

What are the risks and benefits of taking part in this study?
There are minimal risks with the study procedures. Gloves and alcohol wipes will be provided to you. You will be wearing gloves when collecting the stool sample. You will be asked to wash your hands before and after the sample is collected to protect from spreading infection.

You will contribute to the research that may potentially benefit the health of the community. Poi is a major component of the traditional Hawaiian diet that contributes to the overall well-being of the native communities. The results from this study will bring more awareness to this dish and the Hawaiian culture to the public. It will help promote poi as a functional food and raise its popularity among health-conscious people, which essentially, may reduce obesity and cardiovascular disease. The benefits greatly overweigh the risks in this study.

Results of Research:
No test will be done on your fecal sample alone. Hence, there will be no result regarding your fecal sample. The results obtained from the fecal fermentation using the pooled fecal materials from all participants will be published in Lianger's dissertation.

Privacy and Confidentiality:
Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law. Confidentiality will be maintained by means of assigning a number to the participant. The information of the participants will be kept in the database that will only be accessible to Dr. Yong Li and Lianger Dong.

When I report the results of my research study, I will not use your name. I will not use any other personal identifying information that can identify you. I will use pseudonyms (fake names) and report my findings in a way that protects your privacy and confidentiality to the extent allowed by law.

Future Research Studies:
Even after removing identifiers, the feces collected for this study will not be used or distributed for future research studies.

Compensation:
You will not receive any compensation. The research may lead to new tests, drugs, or other products for sale. If it does, you will not get any payment.

Questions: If you have any questions about this study, please call or email Lianger Dong at (865)-306-6440 & ldong@hawaii.edu. You may also contact my advisor, Dr. Yong Li at (808) 956-6408 & liyong@hawaii.edu.

If you agree to participate in this project, please sign and date the following signature page and return it to:

Keep a copy of the informed consent for your records and reference.

Signature(s) for Consent:

I give permission to join the research project entitled, Uncovering the probiotic potentials of poi, a naturally fermented Hawaiian food.

Name of Participant (Print): _____

Participant's Signature: _____

Signature of the Person Obtaining Consent: _____

Date: _____