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Title	Evaluation of peanut fermentation by gut microbes as a potential source of immunomodulatory metabolites
Authors	Ndwandwe, Cebile Samkelisiwe
Publication date	2026-03-02
Original Citation	Ndwandwe, C. S. 2026. Evaluation of peanut fermentation by gut microbes as a potential source of immunomodulatory metabolites. MRes Thesis, University College Cork.
Type of publication	Masters thesis (Research)
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Download date	2026-11-15 14:08:24
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Evaluation of peanut fermentation by gut microbes as a potential source of immunomodulatory metabolites

Thesis presented by

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for the degree of

Master of Science

University College Cork

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2026

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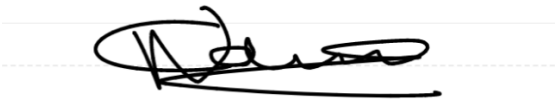
Abbreviations

AhR	aryl hydrocarbon receptor
BHI	Brain Heart Infusion
CFU	Colony forming units
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
FA	Fatty acid
FBS	Foetal bovine serum
FFM	Faecal fermentation medium
FS	Faecal slurry
FSI	Frozen standardised inoculum
GC-FID	Gas Chromatography-Flame Ionization Detection
GI	gastrointestinal tract
HDAC	histone deacetylases
IAld	indole-3-aldehyde
IBA	indole-3-butyric acid
IECs	intestinal epithelial cells
ILA	indole-3-lactic acid
LC-MS	Liquid Chromatography-Mass Spectrometry
NA	Nutrient agar
NMR	nuclear magnetic resonance
OA	oleic acid
OD	Optical density
PBS	Phosphate-buffered saline

RNA	Ribonucleic acid
SCFA	Short chain fatty acids
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SSF	simulated salivary fluid
TE	Tris-EDTA
TFC	total flavonoid content
Th2	T helper 2
TNF	tumour necrosis factor
TPC	total phenolic content
Trp	Tryptophan
TSB	Tryptic Soy Broth and
TSLP	Thymic Stromal Lymphopoietin
YCFA	Yeast Casitone Fatty Acids broth

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.



Cebile Samkelisiwe Ndwandwe

Acknowledgements

I would like to express my heartfelt appreciation to everyone that contributed to this study. First, a major thanks to my supervisor and co-supervisor Professor Liam O'Mahony and Professor Paul O'Toole for their good counsel, professional support and effort to help me improve as a researcher. I would like to thank Lu Yao, Songhiu Kim, Emer Shannon, and Hannah Devotta for their assistance during my research. I am also thankful to the Department of Microbiology, more especially to Dr David Wood, Dr John O'Callaghan, and Mary Jane Lynch for her technical and emotional support.

I would like to express my gratitude Professor Catherine Stanton for hosting and supporting my 6-month gut microbiome study at Teagasc, Moorepark. I am also grateful to Harsh Marthur, Fatma Koch, and Kevin Lenehan for their assistance with all aspects of my gut microbiome study.

Finally, to my mom and stepdad Bonsile and Emanuel, my brother and sister Mawena and Danny, my Cork friends Ciara, Cate, Shruti, and Somi, my childhood friends Juliet, Samu, Gcwa, and Buyi. I would like to thank my grandmother Esther who has no idea what I have been studying since my undergraduate degree but continues to be my biggest cheerleader. A special thank you to my husband Matt for all his love, encouragement, understanding, and unwavering support when I needed it most.

I dedicate this thesis to my late dad Gangi, whose love, wisdom, kindness, and encouragement has shaped the person I am today.

Abstract

The metabolism of dietary substrates by the gut microbiota is important for generation of metabolites that have effects on immune function. Peanut consumption in early life has been shown to protect from later life allergy. The aim of this study was to investigate the effects of peanut fermentation by gut microbes.

Blautia has been shown to be increased by peanut consumption so firstly *Blautia* utilization of potential substrates was assessed. *Blautia wexlerae* and *Blautia obeum* grew well on a variety of potential substrates, with some evidence of glycerol utilisation, indicating the existence of vitamin B12-dependent glycerol dehydratase genes. However, *Blautia faecis* was much more limited in its capacity to utilise these substrates with a much more selective carbohydrate utilisation profile.

Using human faecal microbiota bioreactors, peanut was fermented by the faecal microbiota with significant beneficial microbiological and metabolic outcomes. Peanut fermentation generated high levels of SCFAs (including acetate, propionate and butyrate), with enriched pathways for lipid and aromatic amino acid metabolism. Raw peanut fermentation was associated with increased relative abundance of SCFA producers such as *Bacteroidetes* and *Bifidobacteria*. However, fermentation also led to histamine production. Fermentation of digested peanut led to increased anti-inflammatory metabolites including jasmonic acid, while decreasing pathogenic *Escherichia-shigella* and increased SCFA producers such as *Bifidobacteria* and *Bacteroidetes*. Metabolomic patterns in general suggested increased production of potential immunomodulatory metabolites. On the other hand, defatted peanut produced the lowest levels of SCFAs and increased relative abundance of opportunistic pathogens *Escherichia-shigella*, indicating a trend towards less favourable gut ecological environments.

This work highlights the functional diversity of *Blautia* species, with species specific utilisation of a wide range of growth substrates. In addition, these findings illustrate how gut microbial fermentation of foods such as peanuts affects microbial composition

and metabolism, with production of immunomodulatory compounds. The impact of food processing methods was also demonstrated as many potential beneficial effects were lost for defatted peanut. While my research clearly demonstrates that peanut can be metabolized by gut microbes, future research should determine the effects of microbial peanut fermentation on host immune responses, such as animal models of food allergy.

CHAPTER 1: LITERATURE REVIEW

1.1 Background

The gut microbiome is the collective term for the rich and diverse microbial population found in the human gastrointestinal (GI) system. Microbial population consists of bacteria, eukaryotes, and archaea. They have co-evolved with the host over thousands of years, forming a symbiotic relationship (Bäckhed *et al.*, 2005a). This microbial ecosystem is essential for sustaining host physiology, which includes digestion, metabolism, immune regulation, and nutrition absorption (Lloyd-Price et al., 2016). The host's genetics, diet, and environmental exposures influence microbial community composition (seen in Figure 1.1 below). Microbial imbalance, also known as dysbiosis, has been linked to various conditions such as inflammatory bowel disease (IBD), obesity, and allergies (Shreiner, Kao and Young, 2015a).

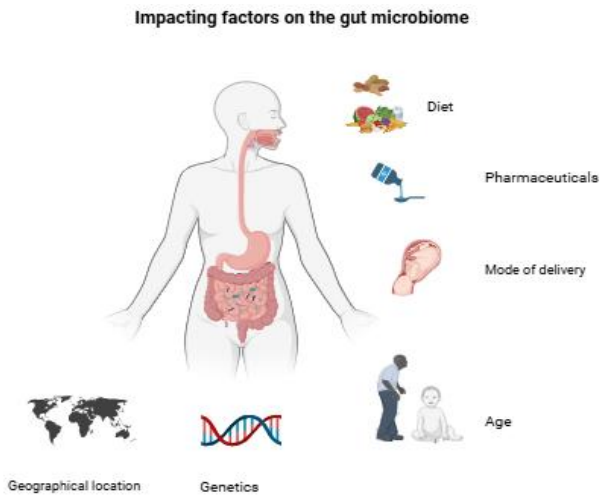


Figure 1.1 Key factors impacting the gut microbiome. The gut microbiome is a constantly changing ecosystem impacted by both intrinsic host factors and external environmental exposures. Diet is recognised as one of the most powerful modulators, with dietary fibre, and macronutrient composition influencing the microbial population. Pharmaceuticals, particularly antibiotics, can drastically affect microbiome diversity and stability. The mode of delivery at birth is an important early-life factor because vaginally delivered neonates acquire maternal vaginal and faecal microbiota, whereas caesarean-delivered infants are colonised by skin-associated and environmental organisms, which may have long-term immunological consequences. Age has an impact on microbiome composition throughout the life cycle, from the active building of the infant microbiome to the reduced diversity associated with late maturity. Although the heredity of certain microbial taxa is still low in comparison to environmental contributions, host genetics affects the makeup of the microbiome through processes such as immune gene variation (Morris and Bohannan, 2024). The impacts of sanitation infrastructure, regional nutrition, and ambient microbial reservoirs are further integrated by geographic location, resulting in the significant inter-population microbiome variations reported worldwide (Nadimpalli et al., 2020).

Diet is also an important regulator of gut microbial composition and metabolism. Proteins, polyphenols, lipids, and fibres are found in many plant foods including legumes and nuts, such as peanuts. They act as substrates for microbial fermentation (Gupta *et al.*, 2021). The fermentation of fibres and other complex substrates by the gut microbiome produces organic acids, gases, and short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate. Short-chain fatty acids are organic acids with 1 to 6 carbons. Nuts such as hazelnuts, almonds, pistachios, and walnuts are recommended as part of a healthy eating plan by the U.S. Department of Agriculture and U.S. Department of Health and Human Services (U.S. Department of Agriculture and U.S. Department of Health and Human Services., 2020). Nut consumption has been shown to increase gut microbial SCFA production (Parilli-Moser *et al.*, 2021). Wheat, oat bran, Inulin, and resistant starch are some of the common substrates for bacterial fermentation and the generation of SCFAs (Parada Venegas *et al.*, 2019). Dietary fibre fermentation is supported by various microbial enzymes. These microbial enzymes consist of 16 families of carbohydrate esterases, 22 polysaccharide lyases, and 130 glycoside hydrolases. This enables the gut microbiome to ferment a wide variety of substrates (Makki *et al.*, 2018). Most bacteria responsible for fibre fermentation are members of the *Actinomycetota* and *Bacillota* (formerly *Actinobacteria* and *Firmicutes*, respectively; Oren and Garrity, 2021) (Alfa *et al.*, 2018).

The immune system works closely with the microbiome to prevent pathogen invasion and colonisation. Studies have shown that SCFAs support host metabolism, intestinal barrier integrity, and inflammatory responses (Koh et al., 2016). The main butyrate-producing bacteria in the human gut belong to the phylum *Bacillota*, specifically *Faecalibacterium prausnitzii* and *Clostridium leptum* of the family *Ruminococcaceae.* A low-fibre, high-fat diet can substantially reduce gut microbiota diversity. In several cases of non-communicable diseases, high *Bacillota* and low *Bacteroidetes* abundances were observed in the gut. Gut microbiome balance can be enhanced by ingesting SCFA-producing bacteria (Thorburn, Macia and Mackay, 2014; Zmora, Suez and Elinav, 2019).

Gut microbes convert amino acids such as tryptophan into bioactive metabolites. These molecules can activate host receptors, such as the aryl hydrocarbon receptor (AhR) and influence immune activity. (Agus et al., 2018). Tryptophan (Trp) is an essential aromatic amino acid with a β-carbon linked to the third position of an indole group. It has the highest molecular weight among the 20 common classical amino acids. It is also the least prevalent amino acid in proteins and cells. It is a metabolic precursor for numerous microbial and host metabolites (Alkhalaf and Ryan, 2015). Natural sources of Trp include peanuts, oats, poultry, cheese and chocolate. The World Health Organisation recommends a Trp intake of 4 mg/kg/day. No side effects have been reported from exceeding the recommended intake (Agus et al., 2018).

Food allergies are a growing concern in children and adults. The rate of peanut allergy among children has increased twofold over the last decade. It is the primary cause of anaphylaxis and fatalities related to food allergies (Cummings *et al.*, 2010). Peanut allergy is triggered by the major protein allergens Ara h1, Ara h2, Ara h3, and Ara h6, with Ara h6 and Ara h2 being the major inducers of anaphylaxis (Zhuang and Dreskin, 2013).

The genus *Blautia* has recently garnered attention for its potential to mitigate inflammatory and metabolic disorders (Kalyana Chakravarthy *et al.*, 2018). They are strict anaerobes, non-motile and cocci. Their optimum pH and temperature are 7 and 37°C respectively (Liu *et al.*, 2021). It has been found to be distributed in mammalian GI tract and faeces. A recent study by Holmberg et al. (2024) used human-to-mouse microbiota transplantation and *ex vivo* examination of colonic mucus function to examine fibre and the microbiome. They found that increased daily intake of dietary fibre enhanced the ability of gut microbiota to mitigate diet-induced mucus problems. The growth of colonic mucus correlated with the presence of *B. coccoides* promoted mucus development by generating acetate and propionate through the activation of the SCFA receptor Ffar2 (Holmberg *et al.*, 2024a).

This thesis aims to examine the relationships between peanuts, gut microbial communities, and host immunological responses. I also investigated the impact of peanut digestion and processing on microbial metabolism and SCFA synthesis. This study offers perspectives on how dietary changes in peanut composition can alter the interactions between the diet, microbes, and the immune system.

1.1.1 The Human Gut Microbiome: Composition and Function

The human gut microbiome is a complex community of viruses, bacteria, archaea, and fungi that inhabit the GI tract (Lloyd-Price, Abu-Ali and Huttenhower, 2016b). The microbial composition is influenced by host characteristics such as diet, age, and genetics (Shreiner, Kao and Young, 2015b). Acquisition of the gut microbiome begins at birth. The delivery mode is the primary determinant of early colonisation of the neonatal gut microbiome (Aagaard *et al.*, 2014). The microbiota of newborns delivered vaginally shows a high abundance of *Lactobacilli* in the first few days, indicative of exposure to the vaginal flora (DiGiulio *et al.*, 2008).On the contrary, children born via caesarean section are colonised by common skin and environmental microorganisms such as *Streptococcus* and *Staphylococcus* (Jiménez *et al.*, 2005). Early colonisation may provide a competitive advantage to bacterial communities associated with each delivery mode. Caesarean section delivery has been shown to delay the colonisation of the gut with specific bacterial species (Jakobsson *et al.*, 2014).

The adult gut microbiome is very diverse, characterised mainly by the dominance of bacterial phyla such as *Bacillota, Proteobacteria, Bacteroidetes*, and *Actinobacteria* (Thursby and Juge, 2017). In early life, this microbial environment undergoes continuous changes and forms a symbiotic relationship with its host, essential for maintaining overall health and enhancing disease resistance. (Gilbert *et al.*, 2018). The gut microbiota has a crucial role in host immunity and physiology. It aids in the synthesis of vitamins, particularly B-vitamins and vitamin K, the digestion of complex dietary fibres, and the synthesis of vital metabolites such as (SCFAs) (Koh et al., 2016). The gut microbiome also affects host metabolic pathways and neurological signalling through the gut–brain axis, regulates immune system development, and

confers resistance against colonisation by pathogens(Belkaid and Hand, 2014; Cryan *et al.*, 2019).

1.1.2 Factors influencing bacterial metabolism.

To survive in diverse environments, microorganisms adapt their metabolic pathways to changes in food availability, oxygen levels, and other host-associated changes and stresses. Understanding these factors helps in understanding disease, microbial ecology, and biotechnological applications. Nutrient availability is one of the main factors influencing bacterial metabolism. The availability of carbon, phosphorus, sulphur, and nitrogen determine specific microbial growth and metabolic pathways. For example, when nitrogen is limited, scavenging strategies are promoted, as well as the engagement of different metabolic pathways that drive microbial adaptation, as seen in Table 1 below. The availability of glucose, however, stimulates fermentation and glycolysis. Oxygen levels determines if the bacteria undergo fermentation, anaerobic respiration, or aerobic respiration. This is also intricately connected to nutritional regulation. For example, based on the availability of oxygen, facultative anaerobes like *Escherichia coli (E. coli)* switch between oxidative phosphorylation and mixed acid fermentation (Gottschalk, 1986; Racine *et al.*, 2021).

Temperature and pH are examples of environmental factors that further alter bacterial membrane fluidity and enzymatic activity. Lactic acid bacteria exhibit stress responses to acidic environments. They produce acid-shock proteins to maintain homeostasis. In psychrophilic and thermophilic bacteria, temperature changes also affect metabolic flux by altering membrane lipid composition and enzyme kinetics (Neidhardt, 1999; Lund et al., 2014). The selection of terminal electron acceptors is also significantly influenced by the redox potential (Eh) of the environment. In anoxic environments, and sulphate reducers diversify their energy-yielding routes by exploiting alternate electron acceptors (Thauer, 2007). The host environment significantly influences microbial metabolism. Microbial activity in the gut microbiome is shaped by host-derived substrates, such as bile acids and mucins, while microbial interactions affect nutrient competition. *Blautia* aids in the formation of SCFAs, a crucial metabolite that impacts host health (Rowland *et al.*, 2018). Also, bacteria depend on micronutrients such as

vitamins. For example, Propionibacterium uses vitamin B12 as a crucial cofactor in propionate metabolism (Degnan, Taga and Goodman, 2014a).

Table 1.2.1 Factors influencing bacterial metabolism

Factor	Mechanism	Impact	References
Nutrient Availability	Sources of carbon, phosphorus, nitrogen, and sulphur regulate metabolic flux.	Glucose induces the glycolytic pathway. Resource foraging mechanisms take over metabolism when nitrogen levels are low.	Rohmer et al., 2011 *Trends in Microbiology*
Oxygen Levels	Dictates whether bacteria utilise anaerobic respiration, aerobic respiration, or fermentation.	*E. coli* switches from TCA cycle to mixed acid fermentation when under anaerobic conditions.	Madigan, M.T., et al. (2021). *Brock Biology of Microorganisms*. Pearson.
pH	Affects enzyme activity and membrane transport.	Lactic acid bacteria tolerate acidic conditions by upregulating acid-shock proteins.	Lund, P., & Tramonti, A. (2020). Bacterial responses to acid stress. *Nat Rev Microbiol*, 18(9): 481–492.
Temperature	Alters membrane fluidity and influences the rate of metabolism.	To uphold cell and membrane function, psychrophiles produce more unsaturated fatty acids	Neidhardt, F.C. (1999). *Escherichia coli and Salmonella: Cellular and Molecular Biology*. ASM Press.
Redox Potential (Eh)	Determines if electron acceptors are accessible for respiration.	Sulfate reducers use SO_4^{2-}, methanogens use CO_2, and denitrifiers use NO_3^- as electron acceptors.	Thauer, R.K., et al. (2007). Energy conservation in anaerobic bacteria. *Phil Trans R Soc B*, 362: 1137–1152.
Host Environment (Microbiome Context)	Host elements such as bile acids and competition shape bacterial metabolism.	*Bacteroides* metabolise host glycans; *Blautia* produce short-chain fatty acids (SCFAs).	Rowland, I., et al. (2018). Gut microbiota functions. *Nutr Rev*, 76(6): 482–496.
Co-factors & Vitamins	Some bacteria require vitamins (e.g., B12, biotin) for metabolic pathways.	Propionate metabolism by *Propionibacterium* is dependent on vitamin B12.	Degnan, P.H., et al. (2014). Vitamin B12 as a modulator of gut microbial ecology. *Cell Metab*, 20(5): 769–778.
Stress Conditions (Osmotic, Oxidative)	Stress responses reprogram metabolism to maintain homeostasis.	*E. coli* activates the oxidative stress regulon (OxyR, SoxRS) under ROS.	Imlay, J.A. (2013). The molecular mechanisms and physiological consequences of oxidative stress. *Nat Rev Microbiol*, 11(7): 443–454.

Bacterial metabolism is further refined by regulatory mechanisms such as stress responses and quorum sensing. In response to cell density, quorum sensing enables the coordination of metabolic outputs, as well as the generation of secondary

metabolites (Miller and Bassler, 2001). Similarly, oxidative or osmotic stressors reprogram metabolic pathways to preserve redox equilibrium and sustain life. For example, the activation of the OxyR and SoxRS regulons in *E. coli* (van der Heijden and Finlay, 2015).

Collectively, these characteristics illustrate the adaptability of bacterial metabolism. This versatility not only allows bacteria to persist across many ecological niches but also shapes their role in disease, human health, and biotechnological innovation.

1.1.3 The Benefits of Peanut Consumption

Consuming foods that promote a healthy gut microbiome is important. The gut microbiota influences human health and illness. According to earlier studies, prebiotic fibres are an essential substrate that promotes alterations in the microbiota. Numerous studies have demonstrated the impact of various plant foods on the composition of the host microbiota, showing that diet is a major regulator of the gut microbiota. The primary ingredients of plants that give them their prebiotic qualities are dietary fibre (nondigestible polysaccharides) and polyphenols (polymerised chemicals) (Bamberger *et al.*, 2018).

One of the most popular oilseed crops worldwide is the peanut (*Arachis hypogaea* L.). It is used to make peanut butter and peanut oil and is high in lipids, proteins, and amino acids (Yu *et al.*, 2021). Eating peanuts high in oleic acid (OA), which are beneficial antioxidants, may reduce risk of diseases brought on by oxidative stress. In addition to lipids, oilseeds provide dietary fibre and high-quality protein. Edible seeds have a larger protein content (13 g/serving) than nuts, which have a protein value of 3 to 8 g per serving (Venkatachalam and Sathe, 2006). Peanuts' high-quality proteins (~25 g/100 g) contain a combination of essential amino acids (tryptophan, valine, isoleucine, lysine, methionine, threonine, phenylalanine, leucine, and histidine) that are more like the combination found in human tissues, which further contributes to their nutritional value (Waitzberg, 2004). A study performed by Abdualrahman (M.A.Y. Abdualrahman, 2013) reported that raw peanuts contain 19.68 g/100 g glutamic acid,

10.07 g/100 g aspartic acid, 1.01 g/100 g methionine, 1.00 g/100 g cystine, 4.44 g/100 g glycine, 4.55 g/100 g alanine, 5.17g/100 g valine, 317 g/100 g threonine, 3.82 g/100g lysine, 6.10 g/100 g phenylalanine, 7.31 g/100 g leucine, 4.22 g/100 g isoleucine, and 13.31 g/100 g arginine. A study by Asen et al. (2021) on the antioxidant properties of peanut flour (WPF) and defatted peanut protein meal (DPM) examined protein content, amino acid profile, total phenolic content (TPC), and total flavonoid content (TFC). The study showed that DPM had a TPC of 0.12 ± 0.02 mg gallic acid equivalent (GAE)/g, which was significantly ($p < 0.05$) higher than that found in WPF. However, WPF had a TFC of 0.21 ± 0.01 µg quercetin equivalent (QE)/g, which was significantly ($p < 0.05$) higher than DPM (0.16 ± 0.03 µg QE/g) and PPC (0.11 ± 0.05 µg QE/g). Defatted peanut protein concentration is much higher than raw peanut protein concentrations (Tu and Wu, 2019a; Asen *et al.*, 2021a).

Frequent nut consumption has been associated in both epidemiological and clinical trials with a lower risk of type 2 diabetes and coronary heart disease, as well as a lower risk of cardiovascular problems like hypertension and the development of a more advantageous lipid profile (Viguiliouk *et al.*, 2014). Nuts are rich in minerals, proteins, vitamins, unsaturated fatty acids, and other phytochemicals. They have the second-highest fibre content per 100 g of any food (Lamuel-Raventos and Onge, 2017).

1.1.4 Peanut Allergy

Food allergies are prevalent among children in their first three years of life. They affect approximately 6% to 8% of this age group. Long-term studies have shown that many food allergies resolve naturally as children grow older. By the age of 5, around 80% of infants who were allergic to eggs can safely eat them (Skolnick *et al.*, 2001). Severe peanut allergies can occur in both children and adults. Earlier studies indicated that factors such as family history of peanut allergy, maternal consumption of peanuts during pregnancy, and the early introduction of peanut products may lead to peanut sensitization (Lever, 1996; Tariq et al., 1996), which have been more recently demonstrated to be incorrect.

Food allergens are deemed major allergens if they are identified by serum IgE in over 50% of individuals with an allergy to that food. The primary allergens found in peanuts are Ara h 1 and Ara h 3, which belong to the cupin superfamily of proteins, as well as Ara h 2 and Ara h 6, which are part of the prolamin superfamily. Minor allergens are identified by serum IgE levels below 50% of individuals with an allergy to that food. The minor allergens present in peanuts consist of Ara h 5 from the profilin protein family and Ara h 8 from the Bet v 1-like superfamily (Mueller, Maleki and Pedersen, 2014).

The first exposure to a food allergen typically takes place at the mucosal surface of the GI tract. Food proteins are absorbed by specialized epithelial cells known as M cells and transported to antigen-presenting cells such as dendritic cells, where they are processed into peptide fragments that are displayed on the cell surface by class II MHC molecules. The peptides are subsequently presented to naive T helper (Th) cells through the interaction of MHC with the T cell receptor, leading to the priming and activation of Th cells. This process triggers both humoral and cellular responses related to peanut allergy. The activation of T helper cells prompts the release of specific cytokines that encourage B cells to produce IgE antibodies specific to peanuts during the sensitization phase of the immune response. T helper 2 (Th2) cells facilitate the secretion of several interleukins, including interleukin-4, interleukin-5, interleukin-9, and interleukin-13. During the effector phase of the immune response, the IgE antibodies generated specifically against peanuts bind to mast cells and basophils through high-affinity IgE receptors (FcεRI). Upon subsequent ingestion, the peanut protein binds to the specific IgE antibodies on mast cells and basophils, triggering the release of various inflammatory mediators, such as histamine, prostaglandins, leukotrienes, and platelet-activating factor. Furthermore, tumour necrosis factor (TNF), interleukin-5, and chemokines produced at the local site led to the activation and recruitment of eosinophils (Gleich, 2000).

1.1.5 Microbial Metabolites in Allergy

The gut microbiome's metabolic activity is crucial for preserving host health and homeostasis (Zhang *et al.*, 2023). Reduced gut microbiota diversity is frequently seen in patients with long-term inflammatory conditions like asthma and allergies (Kobayashi *et al.*, 2015). Increased epithelial damage and disease severity are associated with decreased microbial diversity and the loss of SCFA producing bacteria. Pathogenic *Moraxella, Streptococcus*, and *Haemophilus* are more prevalent in the airways of asthma patients. They have also been linked to type 2 immune responses and eosinophilic inflammation. The presence of *Staphylococcus aureus (S. aureus)* and the simultaneous decline of commensal species on the skin are features of dysbiosis linked to atopic dermatitis(Kobayashi et al., 2015; Son et al., 2020; McCauley et al., 2019).

Short-chain fatty acids can affect the cytolytic activity of immune cells, as well as their cytokine production and gene expression. The anti-inflammatory properties of SCFAs are primarily governed by two signalling pathways: 1) the inhibition of histone deacetylases. (HDAC), and 2) stimulation of GPCR signalling. SCFAs can cross the gut epithelium into the bloodstream, affecting distant organs. Exposing peripheral blood mononuclear cells and neutrophils to SCFAs *in vitro* can limit NF-κB activity, thereby decreasing TNF-α production through HDAC inhibition. Pregnancy-related maternal acetate has the ability to shield the foetus against allergic airway disease. Also, HDAC inhibitors such as sodium butyrate significantly reduce inflammation driven by brain microglia. The HDAC pathway actively supports the regulation of pro-inflammatory cytokines and the preservation of immunological tolerance (Kim *et al.*, 2007; Trompette *et al.*, 2014; Gray *et al.*, 2017).

Numerous disorders are closely associated with changes in Trp metabolism. Tryptophan and its metabolites work as an adaptive regulatory mechanism to limit excessive acute immune responses within tissues by modulating a variety of pro-inflammatory mediators and harmful T-cell activities (Xue *et al.*, 2023). Approximately 4–6% of dietary tryptophan is metabolised by gut microbes in the large intestine. L-tryptophan is an essential amino acid that the gut microbiome can convert to indole

and derivatives such as indole acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-acrylic acid (IA), indole-3-carbaldehyde and indole-3-lactic acid (ILA) (Agus, Planchais and Sokol, 2018b). Several bacteria have been shown to metabolise diet-derived L-tryptophan. *Escherichia coli* (*E. coli*) can convert dietary tryptophan into indole, and *Clostridium sporogenes (C. sporogenes)* produces tryptamine (Li *et al.*, 2021). Indole derivatives can influence the immune response via the AhR receptor, altering Th cell development and cytokine production towards anti-inflammatory responses. Furthermore, L-tryptophan derivatives can directly control macrophage activation, and treatment with indole-3-butyric acid (IBA) and indole-3-lactic acid (ILA) has been demonstrated to decrease LPS-induced cytokine production *in vitro*.

AhR is a helix-loop helix transcription factor. AhR signalling is essential for several immunological processes. When AhR binds an agonist, it is translocated to the nucleus where it forms an active heterodimer and stimulates transcription of genes. Immune cells such as B lymphocytes, innate lymphoid cells, myeloid cells, and specific T cell subtypes, express AhR. In both steady-state and inflammatory conditions, such as autoimmunity or infection, AhR activation plays an anti-inflammatory and immunoregulatory role in innate and adaptive immunity (Stockinger *et al.*, 2014). Gut immune cells and gut epithelium exhibit significant AhR expression. AhR has a role in the innate lymphoid cell development, Th17/Treg balance, and gut epithelium barrier integrity (Kiss *et al.*, 2011; Li *et al.*, 2011). There are many AhR ligands in the diet, such as plant compounds like glucosinolates and flavonoids, and in animal tissues, where xenobiotic ligands like dioxins accumulate. Food allergies, on the other hand, disrupt the epithelial barrier and cause food allergy in the gut. Gut epithelial cells' production of alarmins is also linked to the severity of food allergy. TSLP, produced by gut epithelial cells, enhances IL-4, IL-5, and IL-10 release from mesenteric lymph node lymphocytes (Frossard et al., 2015).

A mix of dietary fibres can alter the gut microbiota, increasing the synthesis of metabolites derived from tryptophan, including indole-3-lactic acid (ILA) and indole-3-aldehyde (IAld). Elevated amounts of these metabolites were found in the serum and faeces of mice with high fibre intake. Symptoms of atopic dermatitis significantly

improved when IAld and ILA levels increased. By activating the AhR ligands, these compounds are thought to alter immunological and barrier activities. These results demonstrate how specific dietary factors can influence microbial metabolism and have downstream effects on the immune system (Hammerschmidt-Kamper *et al.*, 2017).

1.1.6 The Role of *Blautia* Species in Gut Homeostasis

Blautia has been widely found in mammalian faeces and intestines. *B. hydrogenotrophica* and *B. stercoris* were first isolated from human faeces (Liu *et al.*, 2008). *B. wexlerae and B. luti* were found to be the most abundant of the *Blautia spp*. and are considered among the dominant species of the human intestine (Touyama *et al.*, 2015). The genus *Blautia* currently consists of 20 species with valid published names, including *B. producta, B. coccoides,* and *B. hansenii*, which were formerly misclassified as the genus *Clostridium* or *Ruminococcus* (Liu *et al.*, 2008).

Mucus and intestinal epithelial cells (IECs) act as a "barrier" to keep the microbiota separate from the host. The IECs serve as a barrier that helps maintain intestinal permeability, the capacity of fluids and solutes to permeate the gut tissue and lumen (Bäckhed *et al.*, 2005b). Increased gut permeability is associated with inflammation. A 2024 study indicates that *Blautia* plays a role in preserving gut mucus under nutritional stress. *Blautia*, mitigates the thinning of the gut barrier mainly through the secretion of SCFAs that enhance goblet-cell activity and mucin dynamics (Holmberg *et al.*, 2024b). These findings substantiate the hypothesis that *Blautia 's* metabolic output directly protects the barrier, which is essential for immunological tolerance and resistance to pathogen invasion.

SCFAs, such as butyrate and acetate, inhibit histone deacetylases and signal through GPCRs (e.g., GPR41/43) to promote regulatory T-cell differentiation and reduce NF-κB-mediated inflammation. Several *Blautia* strains produce additional immunoactive metabolites. *Blautia* produces less butyrate compared to other *Lachnospiraceae* but produce higher levels of acetate (Zhang et al., 2023; Salvadori and Rosso, 2024). Oral administration of *B. wexlerae* in mice showed reduced insulin resistance and obesity

induced by a high-fat diet, as well as metabolomic changes consistent with anti-inflammatory signalling. These results imply that *Blautia* generated metabolites may have both systemic and local effects to support homeostasis. *Blautia* is associated with better metabolic profiles in both observational and interventional studies. Improved glycaemic and lipid indicators were correlated with restored *Blautia*, especially *B. wexlerae* (Hosomi *et al.*, 2022). However, associations are not always consistent. *Blautia* may affect host metabolism mechanistically by (i) influencing hepatic gluconeogenesis and adipose lipolysis through SCFA-mediated effects; (ii) altering bile acid pools and FXR/TGR5 signalling (Chanda, Jiang and Liu, 2024).

The quantity and quality of dietary fibre play a crucial role in shaping *Blautia* ecology. Fermentable fibres, including resistant starch and inulin-type fructans, strengthen networks that promote the production of SCFAs in controlled studies. *Blautia* often acts as a source of acetate production, which in turn enhances the potential for butyrate production(Van-Wehle and Vital, 2024). *Blautia* development in the gut is largely influenced by early-life events such as birthing method, feeding practices, and antibiotic use. Several cohorts have linked higher levels of *Blautia* in infancy to better metabolic profiles later in life. However, the results vary and are still determined by dietary habits and the presence of other microbial taxa (Freitas *et al.*, 2022).

Blautia is being researched as a "next-generation" probiotic or live biotherapeutic because of its immunomodulatory, cross-feeding, and barrier-supporting properties. With preclinical proof of safety and effectiveness in metabolic and inflammatory contexts, *B. wexlerae* has made the most progress (Hosomi *et al.*, 2022). In animal models, several *Blautia* species have been reported to have anti-inflammatory or positive metabolic effects (Niu *et al.*, 2024). However, there are several obstacles to translation, including: (i) manufacturing limitations for strict anaerobes, (ii) reliance on host nutrition (iii) strain heterogeneity.

1.1.7 In vitro Digestion Models for Studying Gut Fermentation

Digestion models (*in vitro* and *in vivo*) have recently been developed for researching food nutrition. However, the majority of these require validation using human data. Comparing *in silico* modelling may help specify the parameters required for *in vitro* and *in vivo* models, making them more physiologically appropriate. Because digestive conditions fluctuate considerably between humans, it becomes critical to have multiple models for different populations (e.g. baby, adult, and old age) (Dupont *et al.*, 2011; Venema and van den Abbeele, 2013a).

In vitro digestion models are commonly employed to examine the gastrointestinal behaviour of foods and pharmaceuticals. They are based on physiological activities and conditions found *in vivo*. Even though human nutritional research remains the "gold standard" for addressing diet-related concerns, *in vitro* approaches offer advantages such as speed, labour efficiency, cost, and the absence of ethical constraints. This method enables simultaneous measurement of many samples for screening applications. These models are ideal for mechanistic investigations and hypothesis development due to their controllability, reproducibility, and ease of sampling at the region of interest (Minekus *et al.*, 2014a).

When modelling oral digestion, it is preferable to simulate chewing in the mouth for solid or semi-solid foods. To simulate gastric digestion, peristalsis and emptying are required to simulate contraction. When replicating the digestive function of the small intestine, the intestinal absorption of nutrients and the intestinal mucus environment must be simulated. Furthermore, for simulating fermentation in the large intestine, bacterial absorption and contact with epithelial cells are important (Guerra *et al.*, 2012; Ji *et al.*, 2022).

There are two types of *in vitro* fermentation models used to research gut microbiota: batch and continuous. The latter are more similar to the *in vivo* situation compared to batch fermentations (Venema and van den Abbeele, 2013b). COST Action INFOGEST is a worldwide network of over 200 scientists from 32 countries specialising in digestion. One goal of the network is to standardise conditions for simulated food digestion (Dupont *et al.*, 2011). Minekus et al. (2014) provided a detailed description

of the experimental parameters for the digestive process, including pH, digestion time, and enzyme activity. These variables were based on the physiological data that were available for the fed state of a typical meal (Minekus *et al.*, 2014b). Batch and continuous fermentation systems have gained popularity as models for simulating the microbiota of the human gastrointestinal tract, specifically the colon (Marzorati *et al.*, 2014; Tanner *et al.*, 2014). They use instrumented bioreactors to control pH, temperature, and dissolved oxygen (DO), creating physiological colonic conditions. However, they are labour-intensive (Rea *et al.*, 2011; Tanner *et al.*, 2014).

An *in vitro* benchtop fermentation system was developed that is similar to the *ex vivo* colon digestion model. Microbioreactors provide a fully controlled environment that bridges the gap between bioreactors and microwell systems, but on a smaller scale. Applikon Biotechnology developed the micro-Matrix^{TM}. The micro-Matrix^{TM} provides control over 24 separate bioreactors. Each of the 24 bioreactors on a plate offers independent control of pH, temperature, DO, and up to four distinct gas additions (each regulated separately) (O'Donnell *et al.*, 2016; Koc *et al.*, 2022). This technology enables scientists to simulate gut digestion of various substrates under strict anaerobic conditions.

1.1.8 Bacterial metabolism and metabolite analysis

Anaerobic respiration is the process of oxidising organic compounds in the absence of oxygen to generate energy for metabolism, growth and development. The oxidation reaction is carried out by a variety of bacterial species, which use terminal electron acceptors such as fumarate, NO_3, and CO_2, or SO_4^{2-}. For these organisms to grow, electron acceptor molecules must be introduced to the culture media. Natural anaerobes include methanogens, nitrate reducers, and archaea. These bacteria produce a variety of metabolites including methane, nitrate, enzymes and antibiotics (Singh and Christina, 2021). Bacteria produce metabolites to regulate their metabolic processes and promote growth. Some metabolites directly influence the organism's growth, while others indirectly support its survival. Bacterial metabolites can be beneficial or toxic to other species, affecting their growth and development (Rowland *et al.*, 2018).

Microbial metabolite levels change with gut microbiota dysbiosis. Host-sensing receptors can respond to changes in metabolite type and concentration. Metabolites can be analysed using several approaches, each with its own advantages and disadvantages. Metabolomics and metabonomics, the names for global metabolic profiling used to identify metabolic abnormalities (Deda *et al.*, 2015)and determine the functional state of host-microbial relationships in biological specimens. Most gut microbiome research focuses on investigating metabolites linked to disease or dysregulated metabolic pathways. The main advantage of metabolomics in systems biology lies in its capability to establish connectivity maps of pathways by linking metabolic networks to the foundational structure of reaction pathways. Recently, a reference map of the human serum metabolome has been developed, which allows for the prediction of potential factors influencing more than 800 metabolites (Chen *et al.*, 2019). The three most utilized analytical techniques are nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography mass spectrometry (LC-MS), and gas chromatography mass spectrometry (GC-MS).

1.1.9 Rationale for This Study

Peanut allergy is one of the most common and serious food allergies worldwide, often persisting into adulthood and leading to potentially fatal anaphylactic reactions. While peanut allergic patients maintain strict avoidance of peanuts, recent studies demonstrate that early life consumption of peanut is highly protective against the development of later life peanut allergy. Multiple studies indicate a link between gut microbiota and the development and severity of allergic reactions. Cases of dysbiosis, marked by a reduction in helpful microorganisms like *Blautia* and *Bifidobacterium*, have been observed in people with allergies. These microbes might play a role in immune tolerance by generating metabolites that influence T-cell responses and reduce inflammation. Investigating microbial changes during peanut fermentation could uncover taxa and metabolic pathways pertinent to the prevention of peanut allergies. Fermentation of peanuts, whether in their raw form, after digestion, or defatted, could modify the production of metabolites and the composition of microbes, which may, in turn, affect immunological pathways related to allergies. This study looks

at the potential role for peanut, in modifying gut microbial metabolism that may help protect against food allergy development.

We focused on two types of microbial metabolites: tryptophan derived metabolites and short-chain fatty acids (SCFAs), which enhance epithelial barrier integrity, promote the development of regulatory T cells (Treg), and activate pathways such as the AhR. With implications for allergy alleviation, this study explores whether peanut-driven microbial metabolism can increase the production of these immunoregulatory compounds. This study uses microbial sequencing, metabolomics, and immunoassays to investigate the functional repercussions of microbial peanut metabolism, extending beyond correlations to mechanistic insights.

Understanding how peanut substrates affect microbial metabolism and immunological responses could help improve dietary treatments, probiotic methods, and metabolite-based therapeutics for peanut allergy. Identifying microorganisms that produce protective compounds lays the groundwork for targeted manipulation of the microbiome. This work contributes not just to fundamental understanding but also to translational opportunities in food allergy management. Previous studies suggested that *Blautia* levels correlate with peanut consumption, so I examined the *in vitro* growth of *Blautia* species in detail.

Aim

This overall aim of this thesis was to investigate the potential of microbial peanut fermentation to generate metabolites with potential immune modifying effects.

Objectives

1. To identify the range of substrates that can be metabolised by *Blautia* species (*B. wexlerae*, *B. obeum* and *B. faecis*).
2. To investigate if *Blautia* species (*B. wexlerae*, *B. obeum* and *B. faecis*) ferment peanut.

3. To identify changes in taxa and metabolites following faecal fermentation of raw, digested, and defatted peanuts.

4. To determine if microbial fermentation of peanuts generates ligands for AhR activation.

2.0 Materials and methods

2.1 Bacterial growth

2.1.1 Bacterial Strains, Media and Cultivation Conditions

Three *Blautia* strains were studied: *Blautia wexlerae* (*B. wexlerae*), *Blautia obeum* (*B. obeum*), and *Blautia faecis* (*B. faecis*). They were obtained from the Microbial Culture Collection (MCC100), cultured by Perez et al. (2021). This culture catalogue was created through the anaerobic isolation of 692 commensal microorganisms from faecal samples of healthy donors (Perez *et al.*, 2021). All strains are strict anaerobes. They were obtained from glycerol stocks stored at −80°C by thawing them in the anaerobic chamber. Strains were prepared in the anaerobic gas chamber by first streaking them on Columbia blood agar and incubating at 37°C for 48 hours. Single colonies were picked and inoculated in 10 mL of media for 48 hours to generate additional working bacterial stock. All experiments were in biological triplicates and included a negative control. Optical density (OD_{600}) and pH readings were taken every 6 hours to create growth curves.

Three methods were employed to study bacterial growth: the traditional bacterial growth method that employs OD, pH, and CFU counts; the QUANTOM Tx™ Microbial Cell Counter (Logos Biosystems, Gyeonggi-do, South Korea); and the latest Biolog ODIN VIII system (Biolog, California, USA). Nutrient agar, Columbia blood agar, Tryptic Soy Broth (TSB) and Brain Heart Infusion (BHI) were made in accordance with the manufacturer's guidelines. Variations of Yeast Casitone Fatty Acids broth (YCFA) were made using a modified YCFA recipe by The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) (Koblitz *et al.*, 2023). YCFA was adjusted to the target pH of 7 ± 0.1. All media was autoclaved at 121°C for 15 minutes. All media (broth and agar plates) and pipette tips were flushed of oxygen in an anaerobic chamber with a gas mixture of $N_2/CO_2/H_2$ (composition 80:10:10) overnight, for at least 12 hours before use. The resazurin added in YCFA served as a redox indicator. It turned colourless once the media was flushed of oxygen.

2.1.2 Bacteria growth: OD_{600} and CFU counts

All growth curves were performed under strict anaerobic conditions. Strains were taken from stocks grown and stored at OD_{600} of 0.8. One hundred microliters of stock bacteria were pipetted into Falcon tubes with the three media broths. Growth was measured by monitoring pH and OD_{600} at 0, 24 and 48 hours. Colony-forming units (CFU) were then determined through the plating method. Dilution factors were prepared as illustrated in Figure 2.1 below.

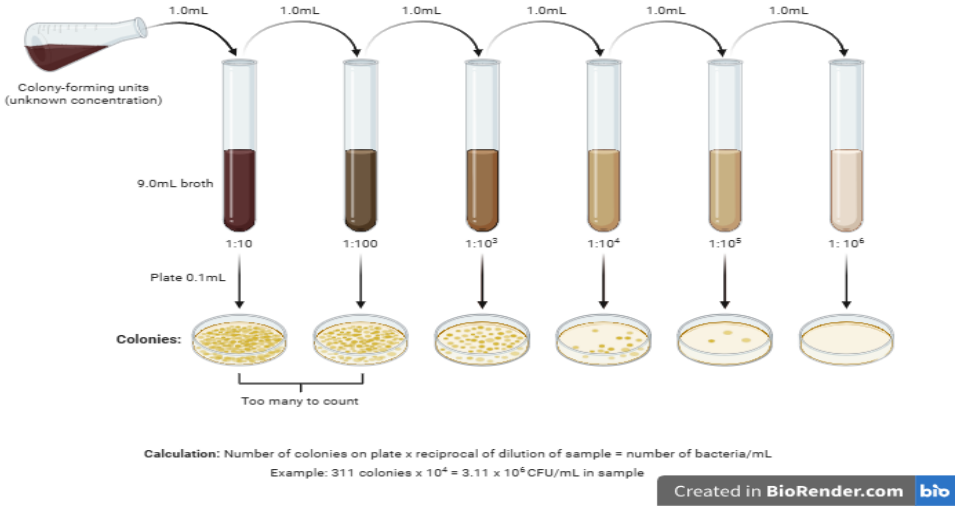


Figure 2.1.2. Serial dilutions illustration. A sample with an unknown colony-forming unit (CFU) concentration is ten-fold diluted in broth, and 0.1 mL of each dilution is plated to allow quantification of viable bacteria.

Microtubes were marked for the dilution series (10^{-1} to 10^{-6}). Nine hundred microlitres of sterile media was poured into each tube intended for 10-fold serial dilutions. Thereafter, 100 µL of bacterial culture was pipetted into the 900 µL sterile broth labelled 10^{-1}. This process was continued until the last tube, as seen in Figure 2.1 above. Sterile tips were used for each transfer. For each selected dilution, 100 µL from the dilution tube was pipetted and spread onto Columbia Blood Agar and nutrient agar plates. Plates were incubated for 48 hours. After incubation, colonies were counted and CFU subsequently deduced.

2.1.3 Bacterial Growth: QUANTOM Tx

The growth of the three *Blautia* strains was also examined with the QUANTOM Tx™. *Blautia* strains were grown under strict anaerobic conditions for 48 hours in complete YCFA media. After incubation, bacterial cells were stained by mixing 10 µL bacterial broth with an equal amount of QUANTOM Total Cell Staining dye in a sterile 0.5 mL microcentrifuge tube (Thermo Scientific™, Dublin, Ireland). The tube was then incubated at room temperature, wrapped in foil to avoid light exposure, and left to sit for 30 seconds. After incubation, 10 µL of the stained suspension was pipetted into a QUANTOM Cell Counting Slide and placed into the QUANTOM Tx device for automatic image acquisition and analysis. The equipment captured fluorescence images for each sample and automatically calculated the total bacterial cell concentration (cells/mL). All measurements were repeated twice.

2.1.4 Bacteria growth: Biolog ODIN VIII

Plates and reagents were purchased from Biolog: AN Microplates, Turbidimeter, and Inoculation fluid A (IF-A). Inoculation fluid A is a capped test tube containing 14 mL of sterile inoculating fluid: 0.03% of Pluronic F-68, 0.0015% of sodium thioglycolate, 0.084% of NaHCO, 0.40% of NaCl, 0.02% of Gellan Gum, and 0.000075% of Methylene Green. Sterile cotton swabs, sterile disposable reservoirs, and plate seals were purchased from Thermo Fisher.

AN microplate seals were broken, and plates were kept at room temperature (on lab bench) for 30 minutes. *Blautia* strains were cultured on Columbia blood agar and incubated under strict anaerobic conditions for 48 hours to facilitate extensive colony growth. Inoculation fluid and turbidimeter were put in the anaerobic chamber for 30 minutes. The turbidimeter was first adjusted to 100% with the IF-A tube placed in it. Colonies were scraped off agar plates with cotton swabs and transferred into IF-A tube; this was done until the turbidity reached 65%. One tube was used for each strain. After 30 minutes had elapsed, the Microplates were introduced into the anaerobic cabinet. Using a multichannel pipette, 100 µL of IF-A broth was dispensed into the AN microplate. One plate was used for each strain. Microplates were sealed and placed

into the pre-warmed ODIN VIII (37 ºC) and left to run for 24 hours. The machine took OD readings at 10-minute intervals for 24 hours. Metabolism was measured through the reduction of a tetrazolium dye. This is due to the strong hydrogenase activity of anaerobic bacteria, thereby producing a purple colour. The plate map is shown below.

AN Microplate Map

A-1 Water	A-2 N-Acetyl-D-Galactosamine	A-3 N-Acetyl-D-Glucosamine	A-4 N-Acetyl-β-D-Mannosamine	A-5 Adonitol	A-6 Amygdalin	A-7 D-Arabitol	A-8 Arbutin	A-9 D-Cellobiose	A-10 α-Cyclodextrin	A-11 β-Cyclodextrin	A-12 Dextrin
B-1 Dulcitol	B-2 i-Erythritol	B-3 D-Fructose	B-4 L-Fucose	B-5 D-Galactose	B-6 D-Galacturonic Acid	B-7 Gentiobiose	B-8 D-Gluconic Acid	B-9 D-Glucosaminic Acid	B-10 α-D-Glucose	B-11 α-D-Glucose-1-Phosphate	B-12 D-Glucose-6-Phosphate
C-1 Glycerol	C-2 D,L-α-Glycerol Phosphate	C-3 m-Inositol	C-4 α-D-Lactose	C-5 Lactulose	C-6 Maltose	C-7 Maltotriose	C-8 D-Mannitol	C-9 D-Mannose	C-10 D-Melezitose	C-11 D-Melibiose	C-12 3-Methyl-D-Glucose
D-1 α-Methyl-D-Galactoside	D-2 β-Methyl-D-Galactoside	D-3 α-Methyl-D-Glucoside	D-4 β-Methyl-D-Glucoside	D-5 Palatinose	D-6 D-Raffinose	D-7 L-Rhamnose	D-8 Salicin	D-9 D-Sorbitol	D-10 Stachyose	D-11 Sucrose	D-12 D-Trehalose
E-1 Turanose	E-2 Acetic Acid	E-3 Formic Acid	E-4 Fumaric Acid	E-5 Glyoxylic Acid	E-6 α-Hydroxybutyric Acid	E-7 β-Hydroxybutyric Acid	E-8 Itaconic Acid	E-9 α-Ketobutyric Acid	E-10 α-Ketovaleric Acid	E-11 D,L-Lactic Acid	E-12 L-Lactic Acid
F-1 D-Lactic Acid Methyl Ester	F-2 D-Malic Acid	F-3 L-Malic Acid	F-4 Propionic Acid	F-5 Pyruvic Acid	F-6 Pyruvic Acid Methyl Ester	F-7 D-Saccharic Acid	F-8 Succinamic Acid	F-9 Succinic Acid	F-10 Succinic Acid Mono-Methyl Ester	F-11 m-Tartaric Acid	F-12 Urocanic Acid
G-1 Alaninamide	G-2 L-Alanine	G-3 L-Alanyl-L-Glutamine	G-4 L-Alanyl-L-Histidine	G-5 L-Alanyl-L-Threonine	G-6 L-Asparagine	G-7 L-Glutamic Acid	G-8 L-Glutamine	G-9 Glycyl-L-Aspartic Acid	G-10 Glycyl-L-Glutamine	G-11 Glycyl-L-Methionine	G-12 Glycyl-L-Proline
H-1 L-Methionine	H-2 L-Phenylalanine	H-3 L-Serine	H-4 L-Threonine	H-5 L-Valine	H-6 L-Valine plus L-Aspartic Acid	H-7 2'-Deoxy Adenosine	H-8 Inosine	H-9 Thymidine	H-10 Uridine	H-11 Thymidine-5'-Mono-phosphate	H-12 Uridine-5'-Mono-phosphate

Figure 2.1.4.1 Layout of the Biolog™ AN MicroPlate. Reproduced from Biolog, Inc. (2024). AN MicroPlate Product Information. Retrieved from https://www.biolog.com/wp-content/uploads/2024/05/00P-003-Rev-E-AN-Microplate-IFU.pdf

2.2 Peanut substrate processing

2.2.1 Raw peanut

Whole peanuts with hulls were purchased at Dunnes Stores in Cork, Ireland. Peanuts were de-shelled, and the seed coat was removed. Raw peanut was ground into peanut powder with a pestle and mortar. Ultraviolet sterilisation would have been the preferred method of sterilisation because it preserves the organoleptic and nutritional qualities of raw peanuts. However, due to its inability to sterilise internal microbial reservoirs, peanut samples were autoclaved at 121ºC for 15 minutes.

2.2.2 Defatted peanut

Peanuts were defatted in accordance with Tu and Wu's (2019) method. A 50 mL Falcon tube containing 20 g of raw peanut powder was filled with *n*-hexane. The mixture was left on a mixer for 4 hours at a speed of 150 rpm. *N*-hexane was decanted, and the same process was repeated for another 4 hours. Defatted peanut was then left at room temperature to dry out and subsequently autoclaved for 15 minutes at 121ºC (Tu and Wu, 2019b).

2.2.3 Digested Peanut

The simulated gastrointestinal digestion of peanuts followed the standardised INFOGEST static digestion protocol created by Minekus et al. (2014). This method mimics the oral, gastric, and small intestinal phases of human digestion under similar digestive conditions. All reagents were of analytical grade and prepared fresh before use. Simulated digestive fluid ingredients were purchased from Thermo Fisher Scientific (Ireland). Simulated fluids: simulated salivary fluid (SSF), pH 7, simulated gastric fluid (SGF), pH 3, and simulated intestinal fluid (SIF); pH 7, were made according to the INFOGEST recipe as shown in Table 2 below:

Table 2.2.3.1 INFOGEST stock recipes

	Stock concentration (g L^{-1})	**SSF Stock volume (mL)**	**SGF Stock volume (mL)**	**SIF Stock volume (mL)**
KH_2PO_4	68	3.7	0.9	0.8
$NaHCO_3$	84	6.8	12.5	42.5
NaCl	117	0	11.8	9.6
KCl	37.3	15.1	6.9	6.8
$MgCl_2(H2O)_6$	30.5	0.5	0.4	1.1
$(NH4)_2CO_3$	48	0.06	0.5	0

Enzyme stock solutions were prepared as seen below:

Enzyme	**Concentration**	**Solvent**
Amylase	41.6 mg/mL	Water
Pepsin	18.7 mg/mL	SGF
Pancreatin	0.1176 g/mL	SIF
Bile	22.8 mg/mL	SIF

All enzyme solutions were prepared just before the experiment. Peanut digestion began with the oral phase, where 10 g of the raw peanut sample was added to a 100mL glass bottle containing 8 mL of SSF. Thereafter, 50 µL of $CaCl_2(H_2O)_2$ (0.3 M), 1.45 mL of water, and 500 µL (41.6 mg/mL) of amylase were added to achieve 75 U/mL in the final volume of 10 mL digestion mix, with pH 7.0. The mixture was incubated for 2 minutes at 37°C in a water bath with gentle agitation to mimic chewing. Next, the gastric phase began with the addition of 16 mL of SGF to the oral bolus. To the mixture, 10 µL of $CaCl_2(H_2O)_2$ (0.3 M), 1 mL of pepsin (2000 U mL^{-1}, final activity), and 30 µL of 25% HCl were added. The pH was adjusted to 3, and water was added to a total volume of 20 mL. The mixture was incubated at 37°C for 2 hours under continuous agitation (150 rpm) to mimic stomach churning.

To the 20 mL SIF mixture, the intestinal phase began with the addition of 17 mL of SIF, 80 µL of $CaCl_2(H_2O)_2$ (0.3 M), 5 mL of fresh bile solution (10 mM), and 10 mL of pancreatin solution (trypsin activity, 100 U mL^{-1}). The pH was adjusted to 7.0 with 7.9 mL of NaOH, and water was added to bring the solution up to 40 mL. The mixture was incubated in a shaker incubator for another 2 hours at 37°C. All incubations were done under static conditions without pH regulation to reflect normal digestion kinetics. At the end of the digestion, enzyme activity was halted by cooling the mixture on ice. The digested peanut was separated from the liquid using nylon mesh. (Minekus *et al.*, 2014a).

2.3 Microbial fermentation of peanuts

To investigate *Blautia* 's potential to ferment peanut, minimal YCFA broth was used. The Leibniz Institute DSMZ recipe was modified to omit most carbohydrate and complex nutritional sources (glucose, yeast extract, VFA mix, and vitamin solution). Fermentation studies were performed with *B. faecis, B. wexlerae,* and *B. obeum* MCC100 to assess their growth and metabolic activity on various peanut preparations. YCFA broth and all peanut preparations (raw, digested, and defatted) were autoclaved and flushed in an anaerobic chamber overnight. Peanut was used as a carbon source in a low-nutrient environment. Peanut samples weighing 0.25g were added to 5 mL aliquots of sterile YCFA medium, along with two negative controls. The two negative

controls with no bacterial inoculation were YCFA broth with peanuts and YCFA broth. All treatments were prepared in triplicate. One hundred microlitres of bacterial stock was then added to the Falcon tubes. Cell suspension was calculated from stock solutions taken at OD 0.8 to be $8 \times 10^8 \times 0.1 = = 8 \times 10^7$ CFU/mL (80 million bacterial cells). Tubes were incubated at 37°C for 24 hours under strict anaerobic conditions. Bacterial growth was monitored by measuring the OD_{600} at 0 and 24 hours. The pH of each culture was monitored at the same time intervals to determine acidification. To account for background growth, control tubes with YCFA medium and no peanut substrate were included in all readings. After incubation, the fermentation broths were filtered through a 0.2 µm syringe filter (Cytiva Whatman™, Sigma-Aldrich® Solutions, Ireland) to separate the supernatant from the bacterial cells. The filtered supernatants were used immediately for AhR activation assays, while the remaining supernatants were kept at -80°C for further metabolomic and biochemical analyses.

2.4 Maintenance and passaging of HT-29 Cells

HT-29 cells are a cell line derived from a human colon adenocarcinoma (large intestine cancer) (Martínez-Maqueda, Miralles and Recio, 2015). The cell line was procured from an already established laboratory supply at the School of Microbiology, University College Cork. Cells were grown and maintained in T75 flasks with Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Dublin, Ireland), containing 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, Dublin, Ireland), and 1% (v/v) 10,000 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Dublin, Ireland). Cell cultures were kept in a humidified incubator with 5% CO_2, at 37°C. Media was changed every 2 to 3 days, and the cells were subcultured at 90% confluence.

At 90% confluence, discoloured medium was discarded, and the cell monolayer was rinsed with 3 mL of sterile phosphate-buffered saline (PBS, pH 7.4). This was done to remove any remaining serum that could impair trypsin function. Cells were detached by adding 5 mL of 0.25% trypsin-EDTA solution (Thermo Fisher Scientific, USA) and incubated at 37°C for 7 minutes. An equal volume (5 mL) of sterile, complete growth DMEM media was added to the cells to inhibit trypsinization. Cells were then centrifuged at 300 × g for 5 minutes to pellet cells. Media supernatant was discarded,

and cells were reconstituted with warm, complete DMEM media and counted using the Scepter™ 3.0 Handheld Cell Counter (Millipore Sigma, Ireland). To avoid microbiological contamination, all passaging steps were conducted aseptically. To ensure phenotypic stability, cells were maintained for no more than 25 passages and routinely tested for vitality with the trypan blue exclusion assay.

2.5 Gut Microbiome Fermentation Study

2.5.1 Faecal Donor recruitment

Ethical approval for faecal donation was obtained from the Research Ethics Committee of the Cork Teaching Hospitals (protocol no. APC092). Permission was sought from 6 donors. They were asked to complete consent forms, affirming their eligibility and desire to participate in the study. All participants were healthy adults who had not taken antibiotics for at least 6 months. Donors were provided with resealable collection pots, spoons and gloves for the collection. All faecal donations were coordinated to facilitate same-day collection and subsequent sample processing within an hour.

2.5.2 Frozen Standardised Inoculum (FSI) and Fermentation Medium

Frozen standardised inoculum (FSI) was prepared in accordance with Marthur et al. (2023). Sample pots with faecal samples were collected and stored in anaerobic gas jars with anaerobic gas packs (Anaerocult ^{R} A gas packs) at 4°C until they were ready for processing. This was done to attenuate *E. coli* blooms. Once all samples were collected, they were immediately transferred to a 37°C anaerobic chamber for processing and pooling. Equal quantities (67 g) of each donor sample were weighed into a sterile stomacher bag with a 0.5 mm pore size filter (Seward Stomacher^{R} bags) for a total volume of 400 g. To this bag, 400 mL of 50 mM PBS, with 0.05% w/v L-cysteine HCl was added, hand mixed until homogenous. The internal sieve in the stomacher separated the solid matter from the liquid slurry. The slurry was poured into 50 mL conical Falcon tubes. All tubes were processed in unison. Tubes were centrifuged at 4000 × g at room temperature for 25 minutes. In an anaerobic gas chamber, pellets were reconstituted in a sterile solution of PBS with 0.05% w/v L-

cysteine HCl and 25% (v/v) glycerol. The reconstituted faecal slurry was pooled into a sterile bottle and mixed by inverting 4 to 5 times. The faecal slurry was subsequently divided into 10 mL and 12 mL Falcon tube aliquots and stored at -80 ºC (Mathur *et al.*, 2023).

Faecal fermentation medium (FFM) was prepared in accordance with Fooks and Gibson's (2003) protocol. Basal FFM ingredients (g L^{-1}) were yeast extract (2), peptone water (2), NaCl (0.1), Tween 80 (2), $CaCl_2$ $6H_2O$, $NaHCO_3$ (2.0), K_2HPO_4 (0.04), $MgSO_4$ $7H_2O$ (0.01), KH_2PO_4 (0.04), bile salts (0.5), cysteine HCL (0.5), Resazurin (0.5), Vitamin K1 (10 µL), and hemin dissolved in 3 drops of NaOH (0.05). PH was then adjusted to 7.0. The FFM was then autoclaved at 121°C for 15 minutes. Media was stored at -4 ºC for further use.

2.5.3 Micro-Matrix™ Cassette Set-Up

The installation of the mini bioreactor cassette and the attachment of the relevant components of the fermenter were carried out in accordance with the micro-Matrix manual. First, 20% (v/v) liquid ammonia solution was prepared by transferring 125 mL of 32% liquid ammonia solution (Sigma Aldrich, Dublin, Ireland) to a Duran bottle, adding 75 mL of distilled water and mixing gently. Ammonium solution was filled into the Ammonia pressure vessel. Nitrogen tanks were monitored to ensure an adequate gas supply for 24-hour runs. Pressure was maintained at 2 bar. All components (except the 24-well cassette) and FFM were autoclaved and left in the anaerobic chamber overnight.

Faecal fermentation slurry was prepared in accordance with O'Donnell et al., 2018.Ten percent 10% (v/v) of the prepared faecal slurry was added to sterile FFM in the anaerobic gas chamber. In a Duran bottle, defrosted 11.52 mL of faecal slurry was added to 132.48 mL of FFM and mixed gently. To each of the 24 wells, 6 mL of prepared fermentation slurry was added. In duplicate, 0.25 g of peanut substrates were added to wells. A negative control of fermentation slurry (no peanut added) was also made in duplicate. From each well, 1 mL of sample (T0) was pipetted into a 1.5 mL, sterile screw-cap tube and stored at -80 ºC for further analysis. All these steps were performed quickly to mitigate *E. coli* blooms (O'Donnell *et al.*, 2018).

The plate was assembled by attaching the top plate and transport clamps to the cassette. It was then transported onto the orbital platform and connected to the device. Parameters were set on the micro-Matrix software (HMI). Nitrogen gas release into wells was set at 40%, the orbiter was set to 250 rpm, and 4 M NaOH was supplied to control pH levels. Predetermined set-points were pH 6.8 (minimum 6.6 and maximum 7.2), temperature at 37°C (minimum 35°C and maximum 38°C), and DO at 0% (with a low alarm at 0% and maximum 20%). If either parameter were out of range, alarms would sound. The machine was run for 24 hours and stopped at exactly 24 hours of incubation. After fermentation, 1.5 mL of each sample (T24) were stored in sterile 2 mL tubes for DNA extraction, SCFA analysis, and metabolomic analysis. Cassettes with remaining fermentate were autoclaved and discarded.

2.6 16S rRNA gene amplicon sequencing

2.6.1 DNA extraction

Bead and reagent preparation

Faecal DNA extraction was performed in accordance with a protocol modified by Yu et al. (2004). The two-day faecal extraction protocol encompassed repeated bead beating plus column (RBB+C) method with the QIAGEN QIAamp Fast DNA Stool Mini Kit (QIAGEN Life Science, Ireland). Bead-beating tubes were prepared in 2 mL screw-cap tubes by adding 1 scoop (0.2 mL PCR tube) of 0.1 mm zirconia/silica lysis beads (BioSpec Products, Bartlesville, USA), 1 scoop of 1.0 mm zirconia/silica lysis beads, and a single 2.3 mm zirconia/silica bead. Each tube was used for 1 sample. Tubes were closed and autoclaved.

Faecal lysis buffer was prepared in a laminar flow to maintain sterility by adding sterile water (27.5 mL), 5M NaCl (5 mL),1M Tris-HCl (2.5 mL), 0.5M EDTA (5 mL), and 20% SDS (10 mL). The final volume was 50 mL, with a final concentration of 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% SDS. Lysis buffer was stored at 4 ℃ and used within 24 hours.

Other reagents used were molecular grade 70% ethanol, QIAamp Fast DNA Stool Mini Kit, Invitrogen™ Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kit Qubit (Fisher Scientific, Dublin, Ireland), 7.5 M ammonium acetate, Tris-EDTA buffer solution, 96% molecular grade ethanol, isopropanol (also known as 2-propanol), DNase-free RNase A (10 mg/mL).

Day 1

Cell lysis:

A centrifuge was pre-cooled to 4 ºC for 20 minutes. DNA lysis buffer was preheated in a water bath to 45 ºC to dissolve precipitates. Five hundred microlitres of each faecal slurry (T0 and T24) were transferred into the sterile 2 mL screw-cap tube containing sterile zirconia beads. A beat beater was run at the highest speed for 1.5 minutes per cycle. At the end of the cycle, samples were left to cool on ice at one-minute cooling intervals. In the same tube, 1 mL of lysis buffer was added. The second cycle of bead beating was run for three minutes with no cooling interval. Tubes were then incubated at 70°C for 15 minutes with gentle agitation. Tubes were centrifuged at 4°C for 5 minutes at 16,000 × g. Three hundred and fifty microlitres of 7.5 M ammonium acetate was added to each lysate tube, mixed and incubated for 5 minutes. Tubes were then centrifuged at 4°C for 10 minutes at 16,000 × g. The supernatant was transferred to two 1.5 mL Eppendorf tubes (600 µL in each tube). An equal volume of isopropanol was added to the tubes and mixed gently. Eppendorf tubes were then incubated in the fridge overnight.

Day 2

Precipitation of nucleic acids:

After overnight incubation, Eppendorf tubes were centrifuged at 4°C for 15 minutes at 16,000 × g, and the supernatant was discarded. The nucleic acid pellet was washed with 200 µL of 70% ethanol, vortexed, and centrifuged at 4°C for 1 minute at 16,000 × g. Supernatant was discarded. The pellet was left in the laminar flow to dry for 15

minutes. One hundred microlitres of TE (Tris-EDTA) buffer was used to dissolve the pellets, and aliquots were pooled.

To purify samples and remove proteins and RNA, 2 µL of DNase-free RNase was added to the nucleic acid samples and incubated at 37°C for 15 minutes. Thereafter, 15 µL of proteinase K (Qiagen) and 200 µL of Buffer AL (Qiagen) were added, mixed and incubated at 70°C for 10 minutes. Two hundred microlitres of molecular grade ethanol (96%) was added to the mixture, transferred to a QIAamp column and centrifuged at 16,000 × g for 1 minute at room temperature. The collection tube was discarded with flow-through. To the column, 500 µL of Buffer AW1 (Qiagen) was added and centrifuged for 1 min at room temperature. The collection tube was discarded. To the column, 500 µL of Buffer AW2 (Qiagen) was added, and centrifuged for 1 min at room temperature. The column was transferred to a fresh collection tube and dried by centrifugation at room temperature for 1 min. The column was transferred to a sterile 1.5 mL Eppendorf tube (this is the final storage tube), to which 100 µL of Buffer AE (Qiagen) was added and incubated at room temperature for 2 minutes. The column was centrifuged at room temperature for 1 min to elute the DNA. DNA concentration was quantified using the Qubit 1X dsDNA HS Assay Kit. Standard 1 and Standard 2 were prepared in accordance with the manufacturer's instructions. For each 2 µL DNA sample, 198 µL of Qubit 1X dsDNA HS working solution was added, vortexed and incubated at room temperature for 2 minutes. Standards and samples were read immediately using the Qubit™ Flex Fluorometer. DNA samples were sent to Edinburgh Genetics (Midlothian, UK) for 16S rRNA gene amplicon sequencing of the V4 region sequencing and data analysis. A subset of samples was validated using the QIAxcel DNA Screening Kit (929004, Qiagen) on a QIAxcel Advanced instrument, ensuring DNA quality and fragment integrity.

2.6.2 Amplicon Sequencing

First PCR reaction was performed using a Mastermix Composition, the egSEQ Amplicon Library Prep Kit (EG1400, Edinburgh Genetics). Each 30 µL reaction included DNA samples of up to 40 ng per reaction, 5 µL (10 µM) of a pooled primer 16S-515F-806R, 10 µL DNA polymerase (EG140101, Edinburgh Genetics), 3.5 µL

buffer NB (EG140102, Edinburgh Genetics), 2.5 µL DMSO (EG140103, Edinburgh Genetics), and nuclease-free water adjusted to a total volume of 30 µL. Cycling conditions are shown below:

Table 2.6.2.1 Fist PCR cycle

Temperature	Time	PCR Cycle
95 °C	3 mins 30 secs	Hold
98 °C	20 secs	
55 °C	1 min	
60 °C	1 min	22 cycles
65 °C	1 min	
72 °C	5 mins	Hold

Thereafter, post-PCR purification was performed by magnetic bead-based cleanup using egSEQ Pure Beads (EG1200, Edinburgh Genetics) on a magnetic stand. Two ethanol washes were performed with freshly prepared 80% ethanol to remove primers and small molecular contaminants. PCR product was then eluted with 24 µL nuclease-free water. A second PCR reaction was performed using Mastermix composition (egSEQ Amplicon Library Prep Kit, per 30 µL reaction) containing 10 10 µL of high-fidelity DNA polymerase (EG140101, Edinburgh Genetics), 2.5 µL of DMSO, 2 µL of nuclease-free water, 2 µL (5 µM) CDI Index Primers (EG1403/1404/1405/1406, Edinburgh Genetics), and 13.5 µL of purified first PCR product. Second PCR cycle is shown below:

Table 2.6.2.2. Second PCR cycle

Temperature	Time	PCR Cycle
95 °C	3 mins 30 secs	1 cycle
98 °C	20 secs	
58 °C	1 min	9 cycles
72 °C	30 secs	
72 °C	5 mins	Hold

Second PCR purification was performed using magnetic bead-based cleanup with the egSEQ Pure Beads (EG1200, Edinburgh Genetics) on a magnetic stand. Two ethanol washes were performed with freshly prepared 80% ethanol to remove primers and small molecular contaminants. Thereafter, PCR product was eluted in 24 µL nuclease-free water.

Library quality control was done by measuring the final library concentration using the Qubit HS DNA Kit (Q32854, Life Technologies) on a Qubit 4. For a subset of samples, library size distribution and integrity were confirmed using the LabChip GX Touch Nucleic Acid Analyzer with HT DNA NGS 3K Reagent Kit (CLS960013, Revvity). Based on the concentration of each library, libraries were pooled and sequenced with 20-30% PhiX Control using the Illumina NovaSeq 6000 and NovaSeq 6000 SP Reagent Kit v1.5 (500 cycles) (20028402, Illumina). Sequencing was performed as paired end reads (2 × 250 bp).

2.7 Short-chain fatty acid analysis

Reagents prepared:

Acidified water was prepared by adding 50 mL of Milli-Q® Millipore Water to 4 µL of 25% HCl (pH was confirmed to be 3). It was stored at room temperature until required. Internal standard (IS) with stock concentration of 100 mM was prepared by adding 3 mL of formic acid, 63 µL of 2-ETBA (2-ethyl-butyric acid), and the volume was adjusted to 5 mL with formic acid. The IS was stored at -20°C until required.

SCFA: Day 1

Fecal fermentate was prepared for SCFA analysis by adding 200 µL of sample and 1 mL of acidified water into a sterile 2 mL microcentrifuge tube. The mixture was vortexed for 5 minutes and centrifuged at 16000 × g for 30 minutes at 4°C. The supernatant was transferred to a new 2 mL Eppendorf tube. The pellet was dissolved with 1 mL of acidified water and centrifuged for 30 minutes with the same parameters. Both supernatants were pooled and centrifuged again with the same parameters. The flow-through supernatant was then double-filtered. The first filtration was performed by adding 550 µL of supernatant to a 5 µm filter tube (Ultrafree-MC-SV Centrifugal filters), centrifuging at 12000 × g for 10 minutes. The flow-through was then transferred to a 0.2 µm filter tube (SpinX Centrifuge Tube Filter 0.22 µm Cellulose Acetate Certified RNase/DNase Free 2.0 mL Tube) and centrifuged at 12000 × g for 10 minutes. The filtrate (270 µL) was transferred to two 1.5 mL tubes. Thirty microlitres of the IS was added to each duplicate sample, vortexed, and centrifuged at 15,000 × g for 10 minutes at 4°C (final concentration of internal standard in each sample was 10 mM).

Each duplicate sample was transferred to 250 µl amber vials with inserts and stored at -20°C for GC-FID analysis.

SCFA: Day 2

To run standards for fatty acids on the GC-FID, standard solutions were prepared as demonstrated in Table 2.1.3 below.

Table 2.7.1 Standard solution reagents

Standard curve	mM	1. Acidified water	2. Fatty acid stock	3. Internal standard stock
1	0.1	268.5 µL	1.5 µL	30 µL
2	0.5	262.5 µL	7.5 µL	30 µL
3	1	255 µL	15 µL	30 µL
4	2	240 µL	30 µL	30 µL
5	4	210 µL	60 µL	30 µL
6	8	150 µL	120 µL	30 µL

To a 5 mL volumetric flask, fatty acid (FA) 100 mM stock was prepared by adding 3 mL of acidified Milli-Q water, 5.48 µL of isovaleric acid, 54.4 µL of valeric acid, 46.2 µL of butyric acid, 4.64 µL of isobutyric acid, 37.5 µL of propionic acid, and 28.7 µL of acetic acid. The flask was brought up to 5 mL with acidified Milli-Q water. This mixture was stored at -20°C.

2.8 Metabolomics

Samples did not require any prior preparation in the lab before being sent for metabolomic analysis. Faecal fermentates were stored at -80ºC until shipment. Samples were kept in a 100-place hinged laboratory vial box. A total of 50 samples, single replicates of human faecal samples, were shipped on 10 kg dry ice to The Metabolomics Innovation Centre (TMIC), Alberta, Canada, for untargeted metabolomic profiling. Samples were sectioned as follows:

Table 2.8.1 Sample information

Group	Sample name	Description
Group 1	FS	Faecal slurry only
Group 2	FSRP	Faecal slurry + raw peanut
Group 3	FSDG	Faecal slurry + digested peanut
Group 4	FSDF	Faecal slurry + defatted peanut

The methodology used for metabolomics analysis was provided by TMIC. Before any processing, samples were randomised to reduce any potential technical variance caused by sample preparation and equipment drift. Total concentrations of the samples were obtained using the NovaMT Sample Normalisation kit. Based on the quantification results, different quantities of water were added to get the samples to the same concentration of 8 mM.

Aliquoting and Generation of the Pooled Sample

Each sample was vortexed and centrifuged at 15000 × g for one minute. Supernatant was separated into four aliquots for various labelling procedures, backup, and pooled sample processing. To generate the pooled sample, 15 µL of each sample was combined and carefully mixed. This pooled sample served as the reference.

Chemical Isotope Labelling

For amine/phenol sample labelling, 19 µL of LC-MS grade water was added before labelling. First,12.5 µL of buffer reagent (Reagent A) and 37.5 µL of 12C2-labelling (for individual and pooled samples) or 13C2-labelling (for pooled samples) reagent (Reagent B) were added to the samples. Samples were vortexed and centrifuged. The mixtures were incubated at 40 ºC for 45 minutes. Next, 7.5 µL of quenching reagent (Reagent C) was used to remove excess labelling reagent. The mixes were incubated at 40°C for an additional 10 minutes. Finally, 30 µL of pH-adjusting reagent (Reagent D) was added. Before the carboxyl labelling, 19 µL of LC-MS grade ACN was applied to a single sample aliquot. The labelling protocol adhered precisely to the SOP supplied in the package. Ten microlitres of catalysing reagent (Reagent A) and 25 µL of 12C2-labelling (for individual and pooled samples) or 13C2-labelling (for the pooled sample) reagent (Reagent B) to the samples. The samples were vortexed, then spun down. The mixtures were incubated at 80ºC for 60 minutes. Next, 40 µL of quenching reagent (Reagent C) was used to remove excess labelling reagent.

Mixing

The 12C2-labelled individual samples were mixed with an equivalent volume of 13C2-labelled reference sample. The combination was ready to be analysed using LC-MS. Before LC-MS analysis, a quality control (QC) sample was created using an equal volume mix of a 12C-labelled and a 13C-labelled pooled sample. The LC-MS analysis adhered exactly to the SOP (Rapid LC-MS Analysis for HP-CIL Metabolomics Platform). To check instrument performance, quality control samples were injected once per ten sample runs. Instrument specifications are shown below in table 2.1.3.

Table 2.8.2 LC/MS specifications for faecal fermentation sample analysis

Instrument	Thermo Scientific Vanquish LC linked to Bruker Impact II QTOF Mass Spectrometer
Column	Agilent eclipse plus reversed phase C18 column (150 x 2.1 mm, 1.8 µm particle size)
MPA	: 0.1% (v/v) formic acid in water
MPB	0.1% (v/v) formic acid in acetonitrile
Gradient	0 min, 25% B; t = 10 min, 99% B; t = 15 min, 99% B; t = 15.1 min, 25%
Flow Rate	400 µL/min
Column Oven Temperature	40°C
Mass Range	m/z 220-1000
Acquisition Rate	1 Hz

Data was processed using Bruker DataAnalysis 4.4 software. A 2-channel analysis was performed, generating 110 LC-MS data (55 LC-MS data, including 5 QC, in each channel). This data was exported to a .csv file.

2.9 AhR activation of HT-29 epithelial cells

Following passaging, HT-29 cells were seeded onto 96-Well Microtiter™ Microplates (Thermo Fisher, Dublin, Ireland), with each well containing 50,000 cells. Cells were incubated overnight to facilitate adherence to the plate. Cells were exposed to 10, 5 or 1% (v/v) of each bacterial fermentation supernatant (samples taken from bacterial fermentation of peanuts and gut microbiome study fermentates). A positive control was cells treated with 100 µM l-kynurenine (a known AhR agonist), while the negative

control was cells in medium only. Cells were incubated for 24 hours. AhR activity in HT-29 cells was measured using an inducible luciferase reporter. The assessment of luciferase activity was performed with the QUANTI-Luc™ 4 Lucia/Gaussia, a coelenterazine-based luminescence assay reagent (Invivogen, USA). QUANTI-Luc™ 4 Lucia/Gaussia for Glow detection was prepared by mixing 1.25 ml of QUANTI-Luc™ 4 Reagent (20X) and 1 ml of QUANTI-Luc™ 4 Stabilizer (25X) in 22.75 mL of sterile water. To measure luciferase activity, 20 µL of HT-29 cell supernatant from each well was pipetted into a white Nunc™ FluoroNunc™/LumiNunc™ 96-Well plate (Thermo Fisher, Dublin, Ireland). Thereafter, 50 µL of QUANTI-Luc™ 4 Lucia/Gaussia solution was added to each well, and luminescence was read immediately using the SpectraMax® iD3s reader (Molecular Devices, California, USA). Excess QUANTI-Luc™ solution was wrapped in foil and preserved at -20°C for a month. To ensure reproducibility, all treatments were carried out in biological triplicates and repeated in at least three independent trials. The resulting data were analysed to evaluate the relative fold-induction of AhR activity, which indicates the presence of bioactive compounds in bacterial fermentation supernatants that can modulate AhR signalling.

2.10 Statistical analyses

IBM SPSS (v20.0), GraphPad Prism and Microsoft Excel were used. One-way analysis of variance (ANOVA) was used to evaluate differences between experimental groups (control, raw peanut, digested peanut, and defatted peanut). A significance threshold of $p < 0.05$ was used. Multivariate methods, including PCA and PLS-DA, were used to visualise global metabolic variance and discover differences between peanut preparations. Peak intensities in metabolomics data were normalised and log-transformed before multivariate analysis. MetaboAnalyst was used to show clustering patterns and find unique metabolites between groups. Metabolite differences were evaluated using fold-change analysis. KEGG reference libraries were used to conduct pathway enrichment analyses.

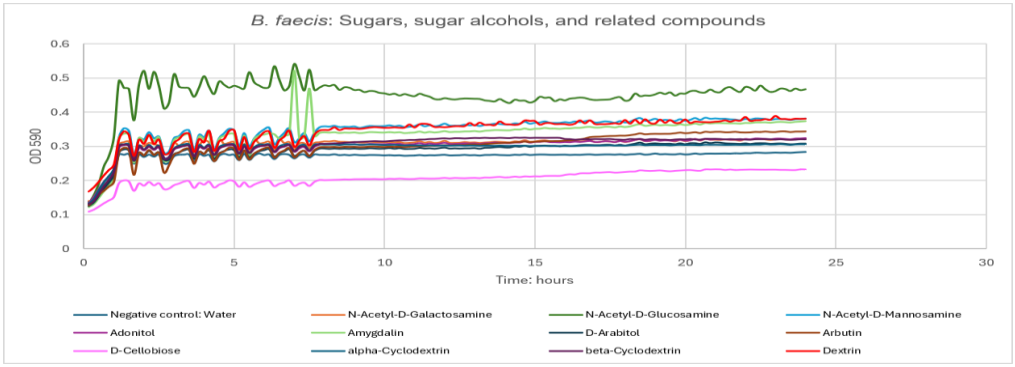
3.0 RESULTS

3.1 Bacteria growth

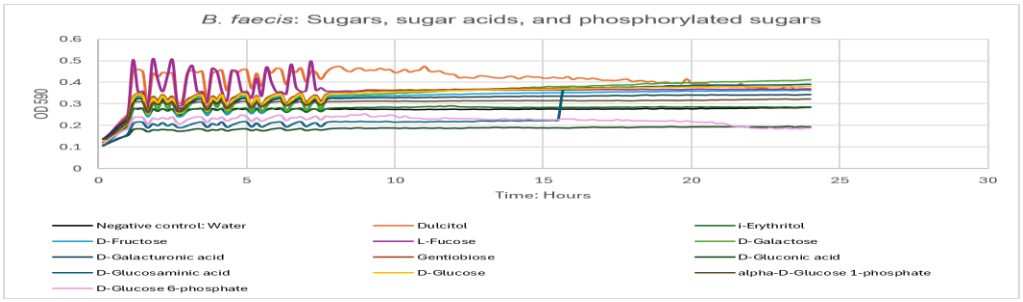
The substrate dependent growth patterns of the *Blautia* strains were assessed using microbial growth assay Biolog ODION VIII. To differentiate between baseline survival and active metabolic utilisation, growth responses were evaluated across water and specified carbon sources in a 92-well plate. *Blautia* strain growth was measured every 10 minutes over 24 hours (OD590).

B. faecis substrate utilization:

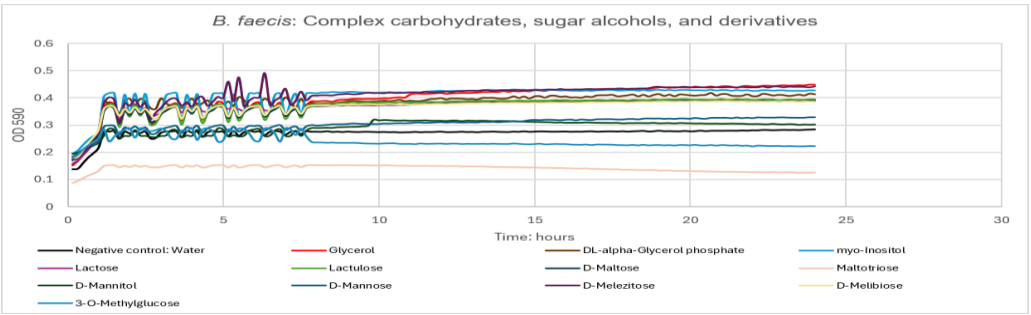
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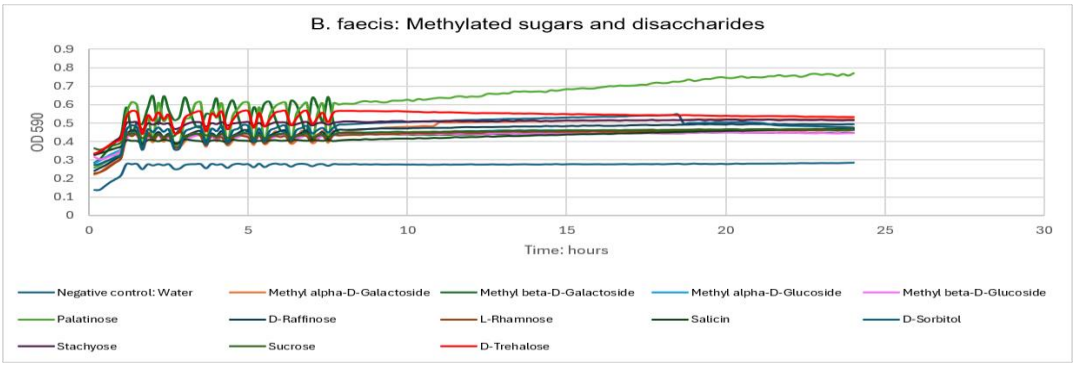
B



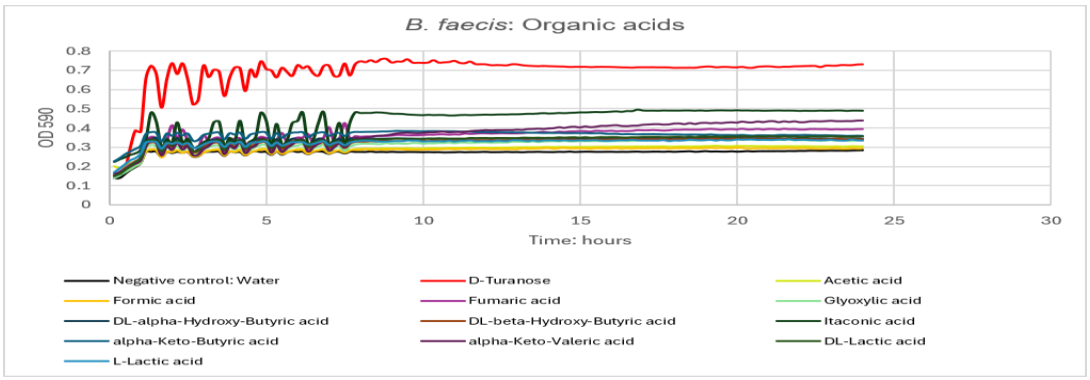
C



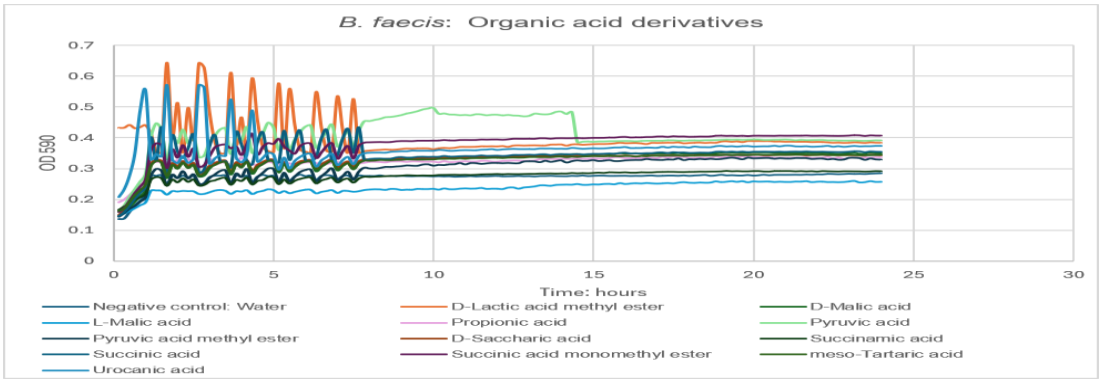
D


B. faecis: Methylated sugars and disaccharides
OD 590
Time: hours
Negative control: Water
Methyl alpha-D-Galactoside
Methyl beta-D-Galactoside
Methyl alpha-D-Glucoside
Methyl beta-D-Glucoside
Palatinose
D-Raffinose
L-Rhamnose
Salicin
D-Sorbitol
Stachyose
Sucrose
D-Trehalose

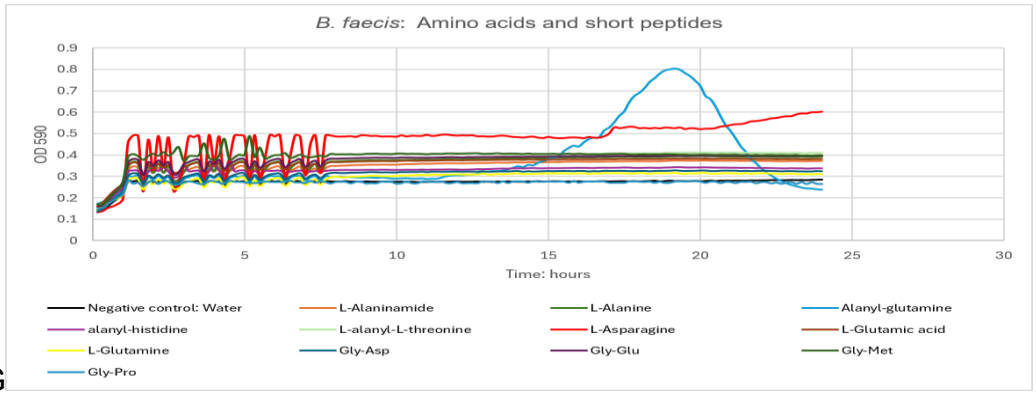
E

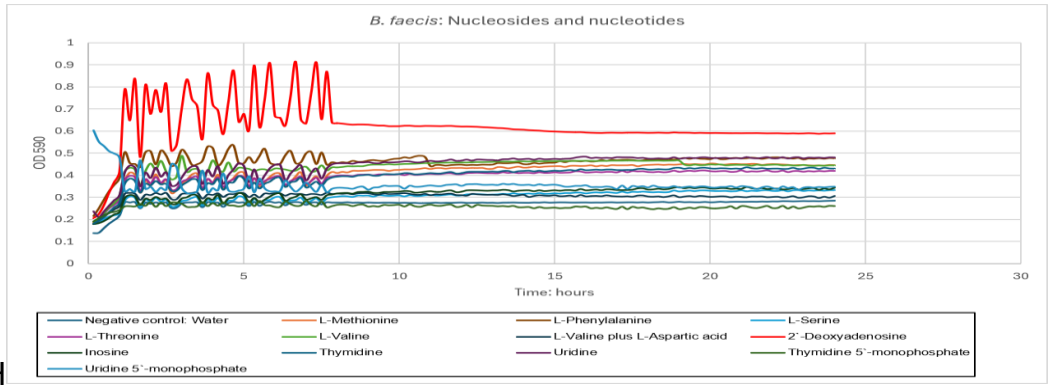

B. faecis: Organic acids
OD 590
Time: hours
Negative control: Water
D-Turanose
Acetic acid
Formic acid
Fumaric acid
Glyoxylic acid
DL-alpha-Hydroxy-Butyric acid
DL-beta-Hydroxy-Butyric acid
Itaconic acid
alpha-Keto-Butyric acid
alpha-Keto-Valeric acid
DL-Lactic acid
L-Lactic acid

F


B. faecis: Organic acid derivatives
OD 590
Time: hours
Negative control: Water
D-Lactic acid methyl ester
D-Malic acid
L-Malic acid
Propionic acid
Pyruvic acid
Pyruvic acid methyl ester
D-Saccharic acid
Succinamic acid
Succinic acid
Succinic acid monomethyl ester
meso-Tartaric acid
Urocanic acid

G


B. faecis: Amino acids and short peptides
OD 590
Time: hours
Negative control: Water
L-Alaninamide
L-Alanine
Alanyl-glutamine
alanyl-histidine
L-alanyl-L-threonine
L-Asparagine
L-Glutamic acid
L-Glutamine
Gly-Asp
Gly-Glu
Gly-Met
Gly-Pro



H

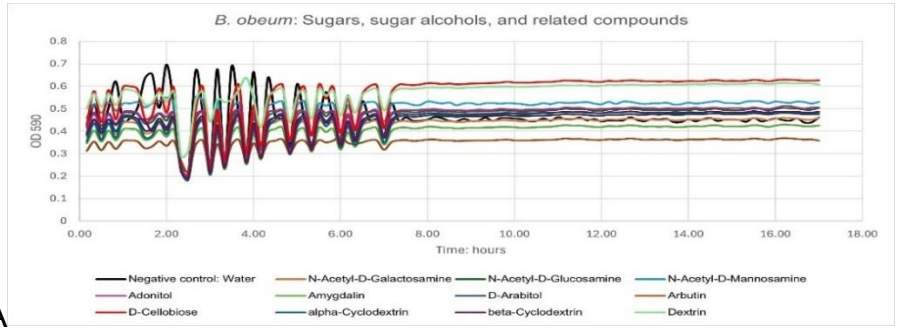
Figure 3.1.1 shows growth curves of B. faecis in sugar, sugar alcohols, and related compounds (A), sugar, sugar acids, and phosphorylated sugars (B), complex carbohydrates, sugar alcohol and derivatives (C), methylated sugars and disaccharides (D), organic acids (E), organic acid derivatives (F), amino acids and peptides (G), and nucleosides and nucleotides (H).

In figures A to H, the highest growth rate in *B. faecis* was observed in 2-Deoxyadenosine and D-turanose with OD reaching ≈0.9 and 0.8 respectively. On the other hand, L-Malic acid had OD readings lower than water (negative control). Complex carbohydrates had lower OD readings, whilst moderate growth was observed on sugars, sugar acids and alcohols of ≈ 0.5. Growth fluctuations were observed in the first 10-hour growth period of all substrates. In this lag phase, bacteria cells are metabolically active but do not yet start dividing. *B. faecis* showed modest growth rates on methylated sugars and organic acids compared to other carbohydrate sources. Overall, *B. faecis* showed moderate to high growth in methylated sugars, organic acid derivatives, nucleotides and nucleosides. The low level of bacterial growth on control (water) could be due to carryover from the previous culture.

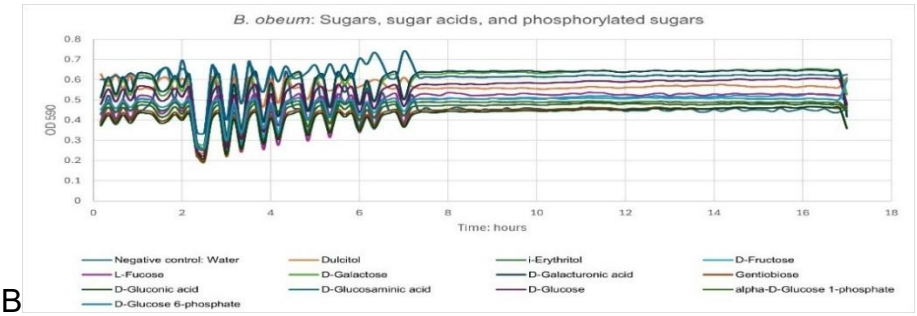
It should be noted that throughout the Biolog ODIN VIII tests, the water negative control yielded non-negligible OD readings of ≈ 0.3 to 0.4. This background signal puts a limit on the observed substrate growths. The transfer of leftover substrate and cells from the inoculum is a possible contributing factor that has not been independently confirmed. Since the Biolog growth curves were not statistically evaluated, many of the variances among substrates are equivalent to this background and the inter-curve noise. Therefore, the species-level substrate preference observations need to be verified by repeatable, baseline-corrected growth tests.

B. obeum substrate utilization:

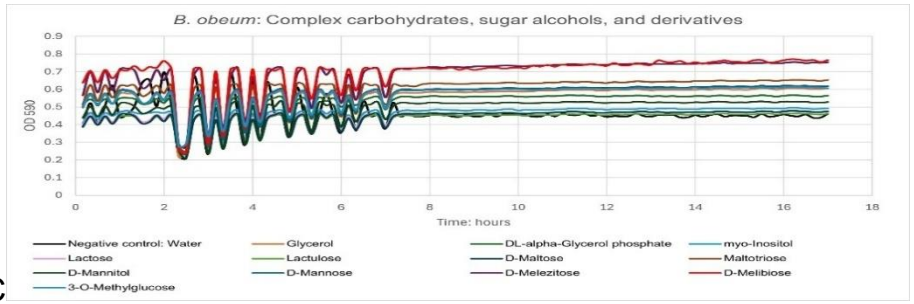
A



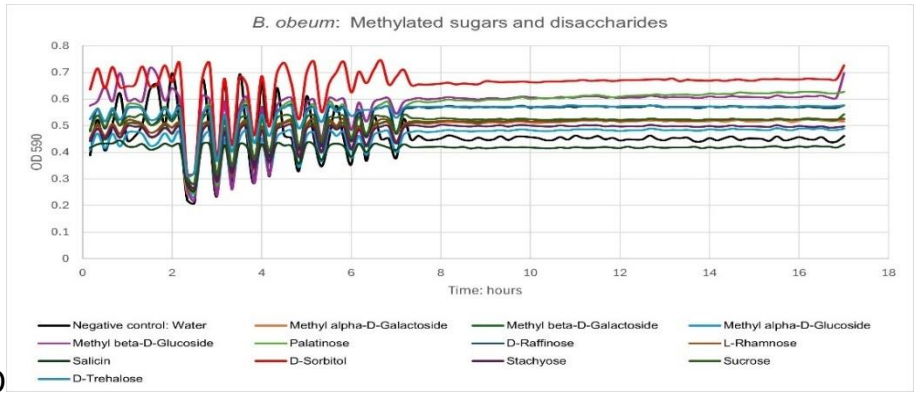
B



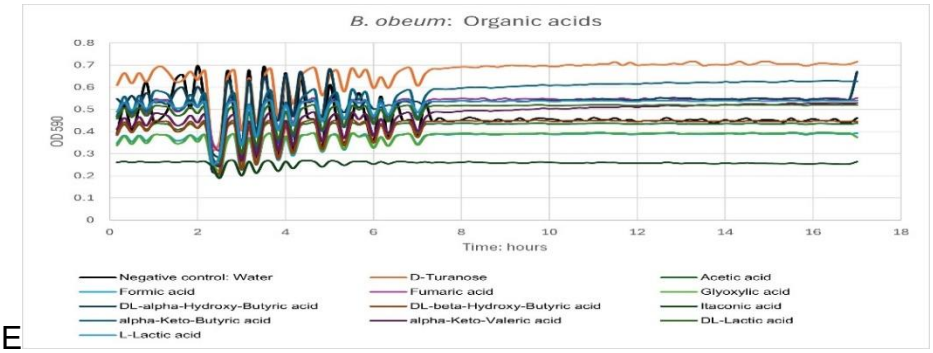
C



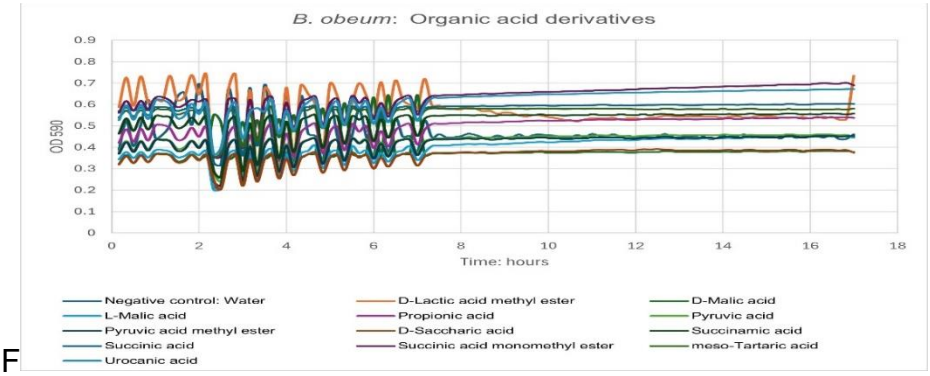
D



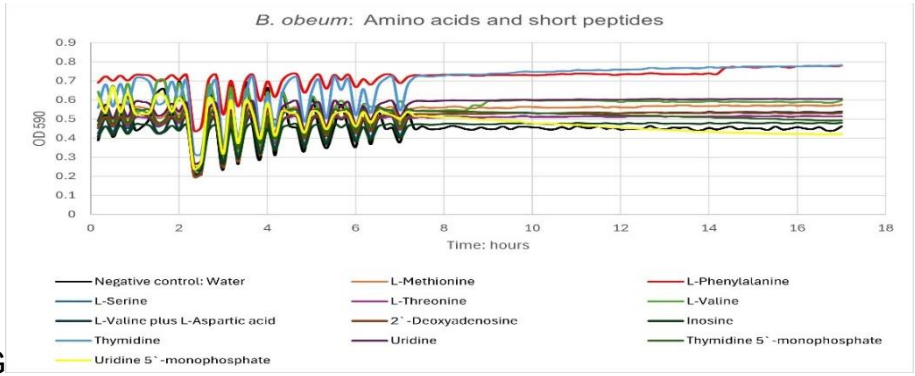
E



F



G



H

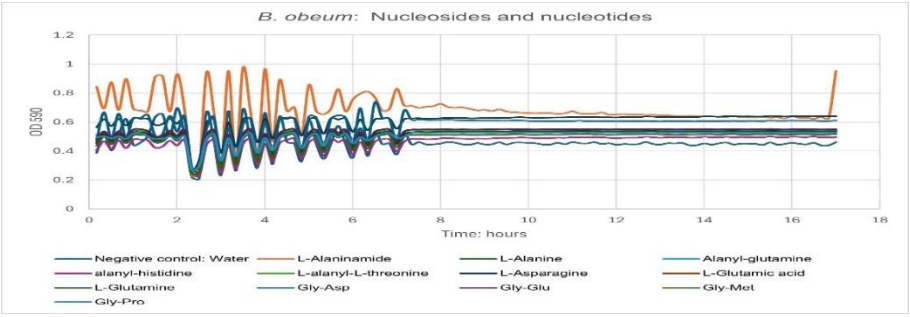


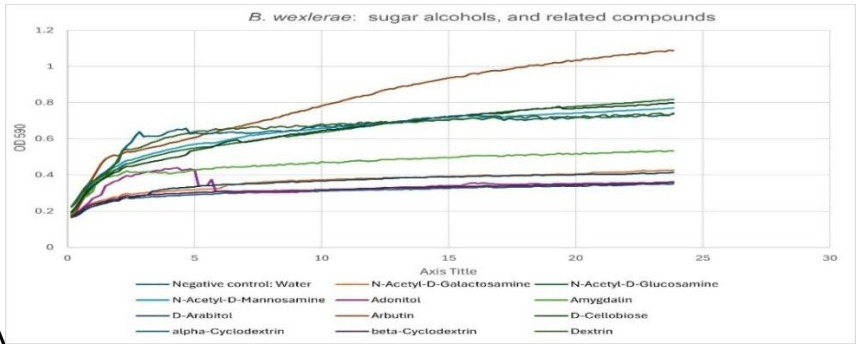
Figure 3.1.2 A to H shows B. obeum growth on sugars, sugar alcohols (A), sugars, sugar acids (B), complex carbohydrates (C), methylated sugars and disaccharides (D), organic acids (E), organic acid derivatives (F), amino acids and short peptides (G), and nucleosides and nucleotides (H).

In figures 3.1.2 A to D, the highest OD readings and *B. obeum* growth was in D-Glucosaminic acid, with OD readings going up to 0.740299 at 6.7 hours. *B. obeum* showed similar growth patterns between methylated sugars and complex

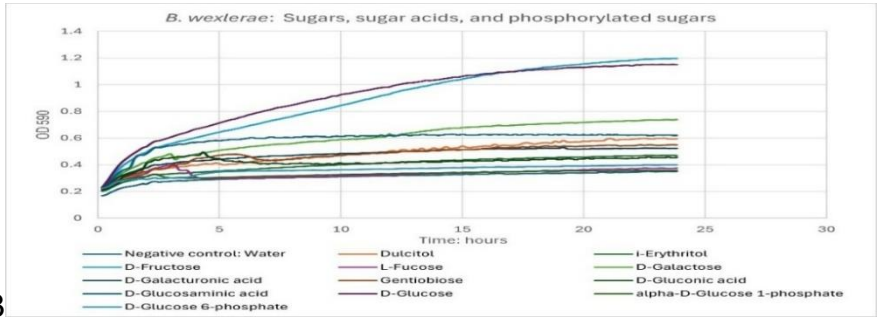
carbohydrates. The control (water) OD readings remained at ≈0.3 to 0.4. The highest OD readings from E to H were L-alaninamide, alanyl-histidine, D-turanose, and D-lactic acid methyl acid. *B. obeum* showed diverse carbohydrate utilization. Most substrates had similar growth patterns with some variations. *B. obeum* overall shows a broader substrate utilization pattern compared to *B. faecis.*

B. wexlerae substrate utilization:

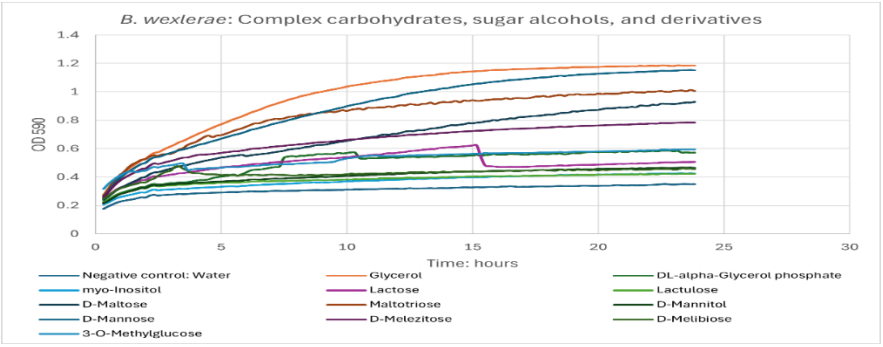
A



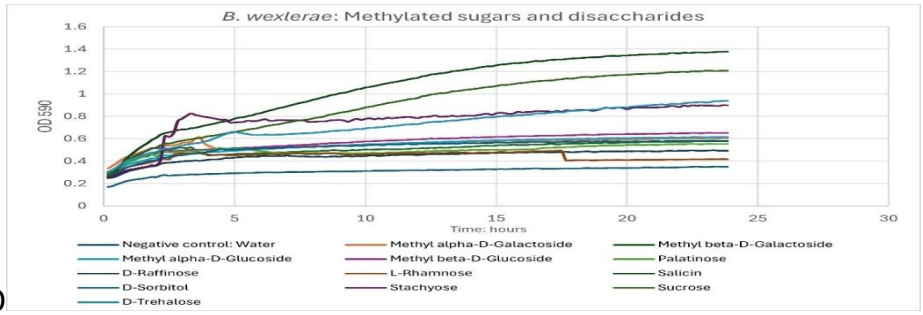
B



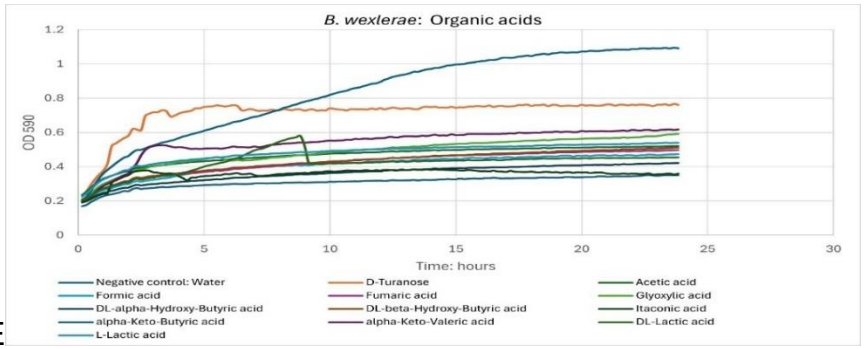
C



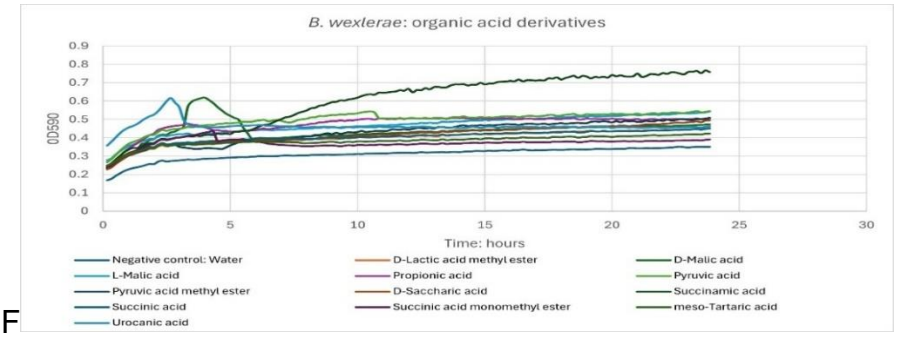
D


B. wexlerae: Methylated sugars and disaccharides
OD 590
Time: hours
Negative control: Water
Methyl alpha-D-Galactoside
Methyl beta-D-Galactoside
Methyl alpha-D-Glucoside
Methyl beta-D-Glucoside
Palatinose
D-Raffinose
L-Rhamnose
Salicin
D-Sorbitol
Stachyose
Sucrose
D-Trehalose

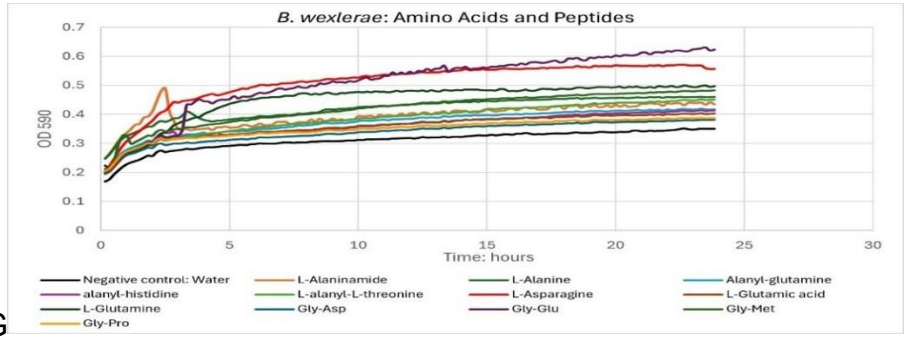
E

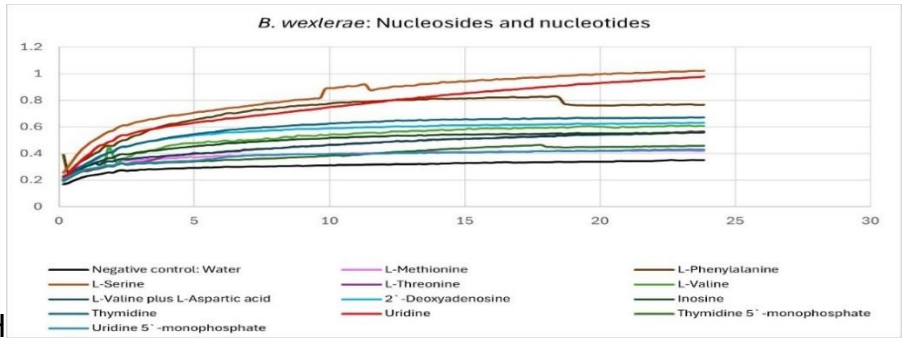

B. wexlerae: Organic acids
OD 590
Time: hours
Negative control: Water
D-Turanose
Acetic acid
Formic acid
Fumaric acid
Glyoxylic acid
DL-alpha-Hydroxy-Butyric acid
DL-beta-Hydroxy-Butyric acid
Itaconic acid
alpha-Keto-Butyric acid
alpha-Keto-Valeric acid
DL-Lactic acid
L-Lactic acid

F


B. wexlerae: organic acid derivatives
OD590
Time: hours
Negative control: Water
D-Lactic acid methyl ester
D-Malic acid
L-Malic acid
Propionic acid
Pyruvic acid
Pyruvic acid methyl ester
D-Saccharic acid
Succinamic acid
Succinic acid
Succinic acid monomethyl ester
meso-Tartaric acid
Urocanic acid

G


B. wexlerae: Amino Acids and Peptides
OD 590
Time: hours
Negative control: Water
L-Alaninamide
L-Alanine
Alanyl-glutamine
alanyl-histidine
L-alanyl-L-threonine
L-Asparagine
L-Glutamic acid
L-Glutamine
Gly-Asp
Gly-Glu
Gly-Met
Gly-Pro

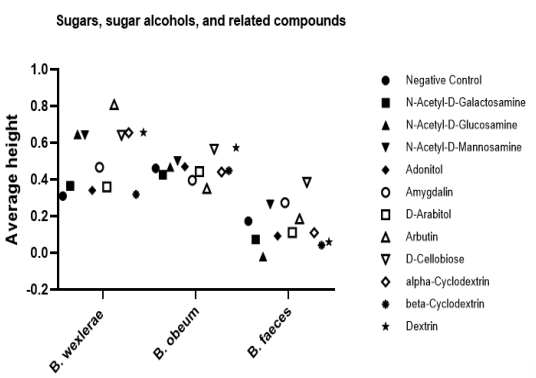


H

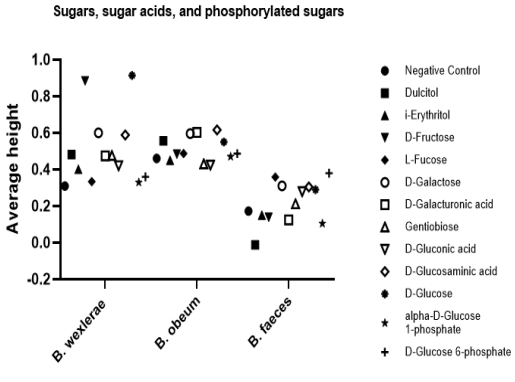
Figure 3.1.3 A to H shows B. wexlerae growth on sugar alcohols (A), sugar, sugar acids (B), complex carbohydrates(C), and methylated sugars and disaccharides (D), organic acids (E), organic acid derivatives (F), amino acids (G), and peptides, nucleosides and nucleotides (H).

B. wexlerae's growth in the above substrates shows methyl beta-D-galactose had the highest OD readings of 1.377, and D-fructose and glycerol sucrose with the highest OD readings of ≈1.2. *B. wexlerae* generally had higher OD readings suggesting better growth compared to *B. faecis* and *B. obeum*. Overall, *B. wexlerae*, showed a more consistent growth pattern compared to *B. obeum* and *B. faecis. B. wexlerae* showed a broader utilization profile with observes high OD readings of up to ≈1.4. However, it showed less growth on amino acids at OD ≈ 0.62 in Gly-Glu. Negative control shows minimal growth of *B. wexlerae*.

An alternative way to represent the metabolic activity of *B. wexlerae, B. obeum,* and *B. faecis* across different substrates was to perform kinetic analysis (OD 590) for all substrates and time points, which is represented below as "average height". This analysis clearly shows species-specific nutrient preferences.



A



B

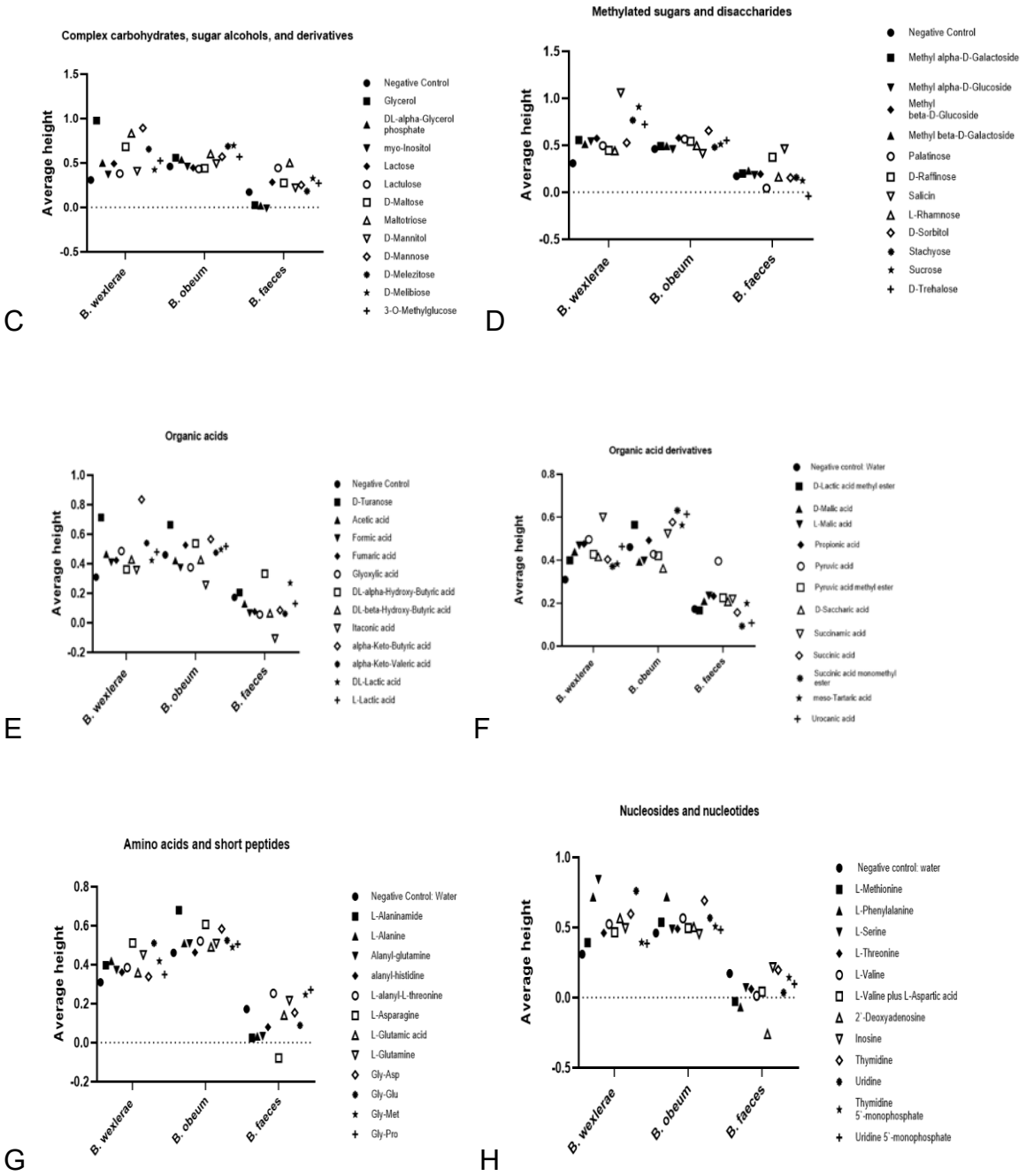


Figure 3.1.4 (A) to (H) Represents metabolic activity of different substrates of Blautia strains on sugar, sugar alcohols (A), sugar, sugar acids, phosphorylated sugars (B), complex carbohydrates (C), methylated sugars and disaccharides (D), organic acids (E), organic acid derivatives (F), amino acids and short peptides (G), and nucleosides and nucleotides (H).

The lowest metabolic activity was seen for *B. faecis*, with some substrates showing no metabolic activity such as dulcitol, myo-inositol, and methylated sugar D-Trehalose. Amongst all substrates shown above, *B. wexlerae* had the highest metabolic activity, whilst *B. obeum* was more versatile in substrate utilisation but shows similar metabolic activity to *B. wexlerae* with complex carbohydrates and methylated sugar

fermentations. *Blautia* strains show diverse substrate utilization capabilities with species specific metabolic patterns.

Amino acids and organic acid derivatives metabolism also showed species-level differences, with *B. obeum* displaying higher metabolic activity with average height values of 0.679 and 0.631 for L-alaninamide and succinic acid monomethyl ester, respectively. *B. wexlerae* had the highest recorded average height of organic acids and nucleosides and nucleosides with average height of 0.835 and 0.842 for alpha-keto-butyric acid and L-serine, respectively. Overall, *B. faecis* had the lowest substrate utilization profile, with average height values for L-methionine -0.028, L-phenylalanine -0.0673, and L-asparagine -0.0784. *B. obeum* is more substrate diverse compared to the other two *Blautia* species, with strong metabolic activity across peptides and carbohydrate classes, suggesting *B. obeum* is the most versatile strain tested. Despite low growth, *B. faecis* still showed a robust, inducible metabolism on amino sugars, dipeptides, disaccharides, phosphorylated sugars, and nucleosides.

Bayes Factor Inference on Pairwise Correlations[a]

		B. wexlerae	B. obeum	B. faecis
B. wexlerae	Pearson Correlation	1	.194	.081
	Bayes Factor		2.064	9.114
	N	96	96	96
B. obeum	Pearson Correlation	.194	1	-.024
	Bayes Factor	2.064		12.071
	N	96	96	96
B. faecis	Pearson Correlation	.081	-.024	1
	Bayes Factor	9.114	12.071	
	N	96	96	96

a. Bayes factor: Null versus alternative hypothesis.

Figure 3.1.5. Bayesian and Pearson correlation analysis of metabolic activity among Blautia species. Pairwise Pearson correlation coefficients and corresponding Bayes factors are shown for B. wexlerae, B. obeum, and B. faecis across (N = 96 substrates).

Bayesian correlation analysis found weak relationship amongst three *Blautia* species, with moderate to strong evidence supported by species-specific growth and metabolic data. Pearson correlation between *B. wexlerae* and *B. faecis* shows a weak but positive correlation with r= 0.081. However, *B. obeum* and *B. faecis* show a negative correlation at r= -0.024.

The Bayes factors measure the quality of evidence rather than its statistical significance. The typically weak pairwise Pearson coefficients show that the three *Blautia* species do not share a similar substrate-utilization profile. The three *Blautia* species exhibit functional divergence, as evidenced by the weak positive association between *B. wexlerae* and *B. faecis* and the weak negative association between *B. obeum* and *B. faecis* exhibits a distinct and more limited metabolic profile. This pattern suggests that the three species are most likely occupying complementary metabolic niches within the community and supports the substrate-preference variations seen in the individual growth trials.

Quantom TX

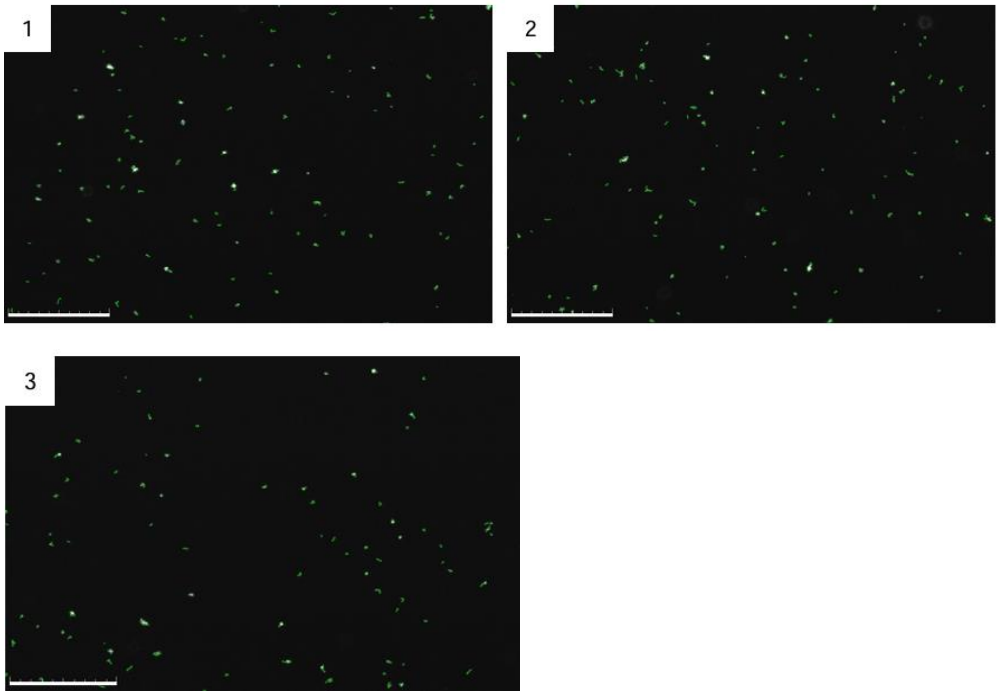


Figure 3.1.6. (1) B. wexlerae 3.30 x 10^6 cells/mL with average fluorescent object size 3.7µm. (2). B. faecis with an average fluorescent object size of 4.2 µm. (3) B. obeum with average fluorescent object size of 3.9 µm.

Representative microscopic images from Quantom TX analysis of *Blautia strains* following growth in complete media. These illustrate good growth of all species examined in complete media.

3.2 Peanut substrate utilization

I next determined if *B. wexlerae*, *B. obeum*, and *B. faecis* could grow and metabolize peanut substrates. To compare growth under control conditions, strains were grown in limited YCFA broth (no glucose), as well as YCFA broth supplemented with raw, digested, or defatted peanuts at 1% 3%, and 5% v/v. Optical density (OD600) and pH measurements were taken at predetermined intervals over 24 hours.

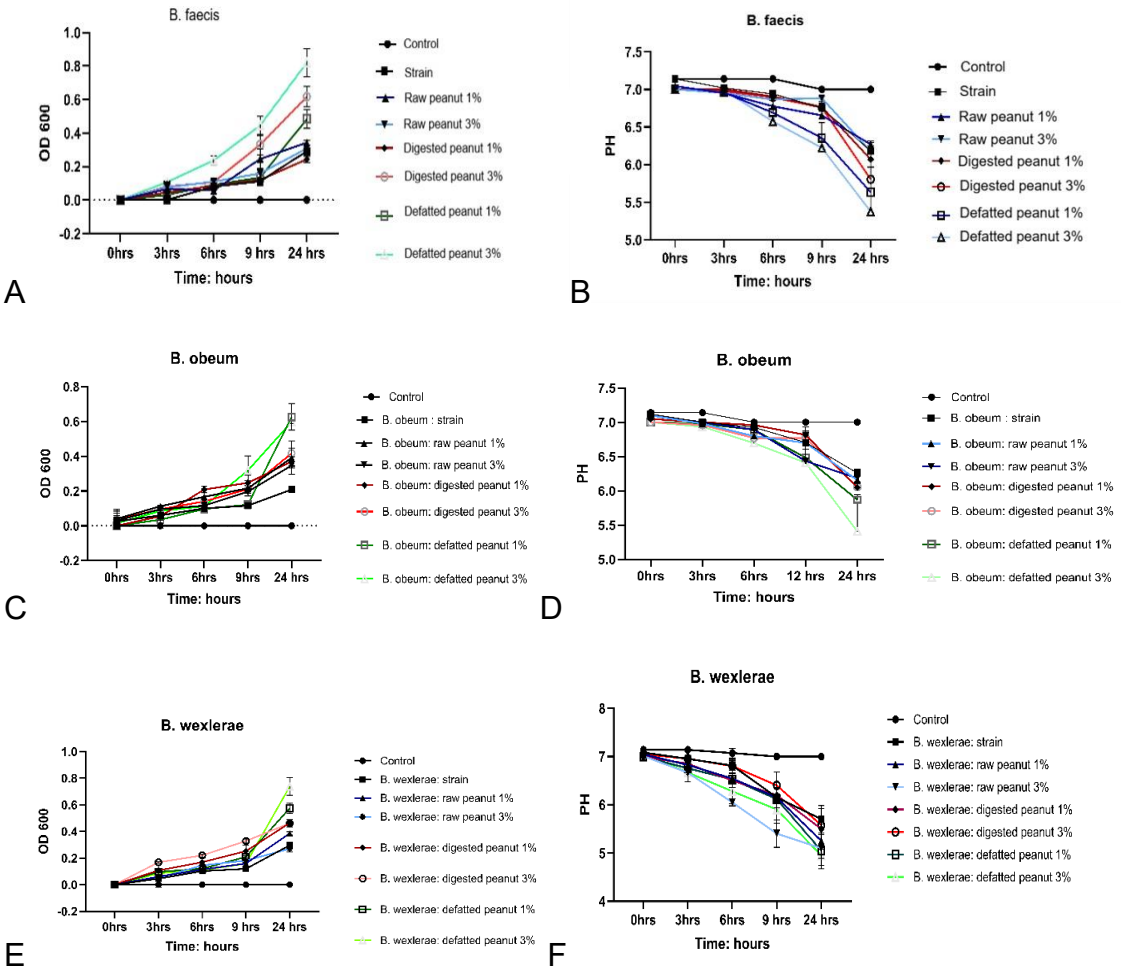


Figure 3.2.1 (A) to (F) Peanut substrate utilization of B. faecis, B. wexlerae, and B. obeum in 1% and 3% (w/v) peanut supplementation. Growth curves of B. faecis (OD600) (A) and pH (B). Growth curves of B. obeum (OD600) (C) and pH (D). Growth curves of B. wexlerae (OD600) (E) and pH (F). The control was YCFA broth (no supplementation).

All strains showed increased OD readings with defatted peanut, coupled with a decrease in pH over 24 hours. No growth was observed in the control (YCFA broth alone), demonstrating media sterility. Digested peanut showed moderate growth patterns across all strains, with raw peanut having the lowest OD and pH readings across all strains.

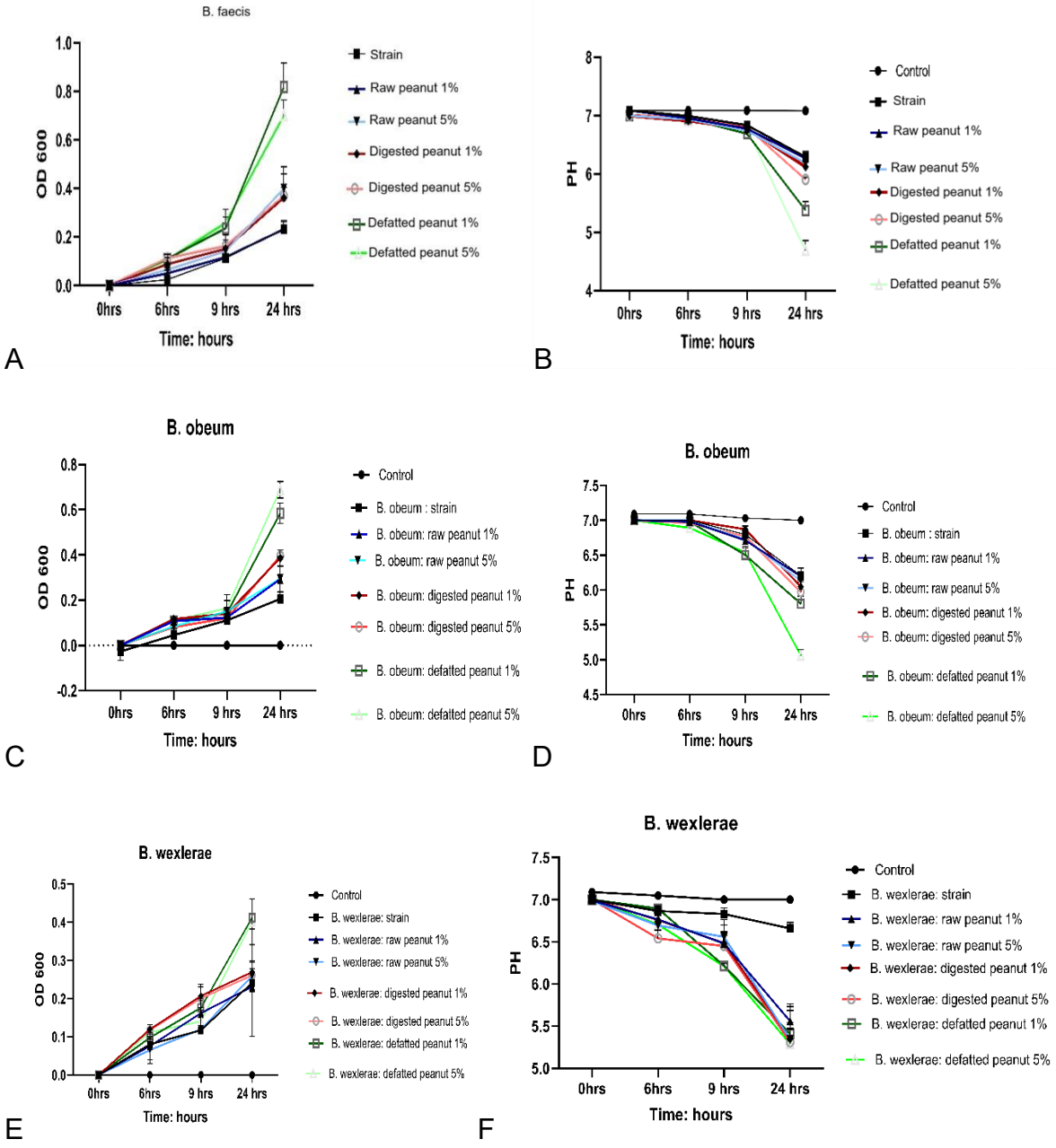


Figure 3.2.2 (A) to (F) Peanut substrate utilization of B. wexlerae, B. obeum, and B. faecis, at 1% and 5% (w/v) peanut supplementation. Growth curves of B. faecis (OD600) (A) and pH (B). Growth curves of B. obeum (OD600) (C) and pH (D). Growth curves of B. wexlerae (OD600) (E) and pH (F). The control was YCFA broth (no supplementation).

B. wexlerae and *B. faecis* showed highest OD readings and lowest pH values when grown with 1% v/v defatted peanut. *B. obeum* however had higher growth in 5% defatted peanut. Overall, *B. faecis* had the highest growth curves recoded with defatted peanut containing media.

3.3 Faecal bioreactor fermentation

The gut microbiome's response to peanut supplementation was studied under controlled conditions using the MicroMatrix™ bioreactor. SCFA production was quantified with GC-FID following 24-hour fermentation to assess metabolic changes. Four conditions were studied: the control (faecal slurry only), raw peanut, digested peanut, and defatted peanut.

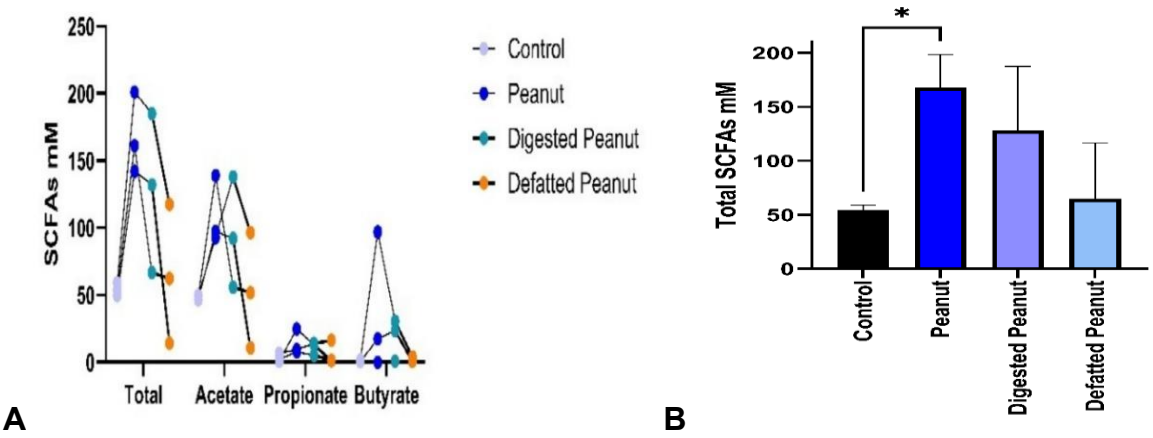


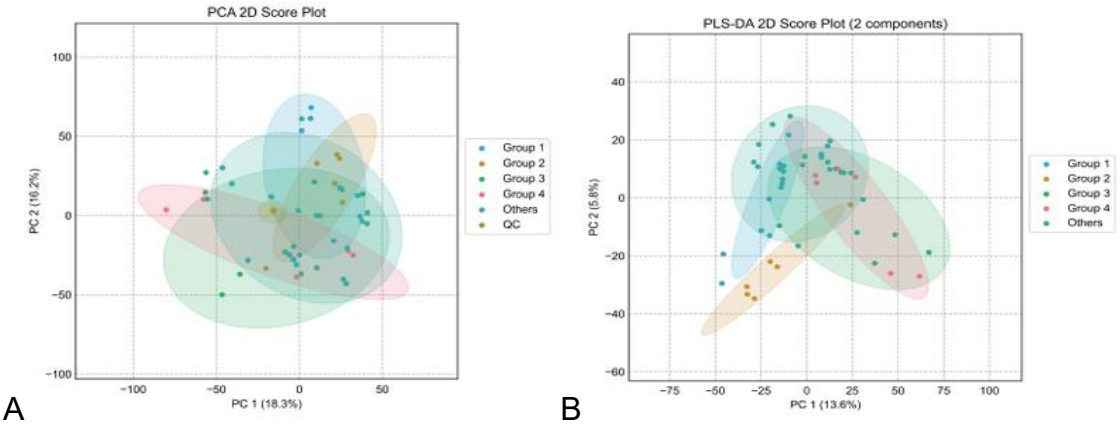
Figure 3.3.1. Short-chain fatty acid (SCFA) production following a triplicate run 24-hour faecal slurry fermentation of peanut substrates using the MicroMatrix™. (A) Line graph showing SCFA concentrations of acetate, propionate, butyrate, and total SCFAs (mM) in three experiments across four conditions: Control (faecal slurry), peanut, digested peanut, and defatted peanut. (B) Bar graph of averaged SCFA concentrations (mM) across three experiments; asterisk (*) indicates statistically significant increase versus control ($p < 0.05$) (n=3 independent experiments).

Table 3.3.1 Average SCFA concentrations across all conditions (mM)

	Acetate	Propionate	Butyrate	Total SCFA
FS+ Peanut	109.8056	14.03519	38.17263	162.0134
FS+ Digested peanut	95.33742	10.66111	18.32	124.3185
FS+ Defatted peanut	52.97926	6.457728	1.74037	61.17736
Control (FS)	54.2401	3.215556	0.75037	58.20603

Faecal microbiota fermentation of raw peanut generated the highest levels of SCFAs (162.013 mM), with high acetate and butyrate production. Digested peanut fermentation generated the second highest levels of total SCFAs, with lowest levels observed for defatted peanut fermentation. Of the SCFAs quantified, the highest produced by peanut fermentation by the faecal microbiota was acetate, followed by butyrate, and propionate was the lowest generated. This data suggests that the defatting process removes fermentable substrates from peanut that would otherwise contribute to the generation of SCFAs.

Untargeted metabolomic analysis was performed on faecal fermentates of different peanut preparations and control to futher explore the wider metabolic consequences of peanut fermentation by the gut microbiota. Untargetted metabolomic analysis was performed with LC-MS, with each channel's data set included 55 samples. After data processing, two channels of LC-MS data from the same sample were pooled. After filtering, 4609 ± 225 peak pairs per sample were recovered. The highest level of confidence is represented by Tier 1 metabolites, which are identified with high confidence. Conversely, tier 2 metabolites represent high-confidence putative identifications of metabolites. Peak pairs were compared in tier 1 using precise mass and retention time to a labelled metabolite library (CIL Library). There are about 1,500 experimental entries in the CIL Library. In tier 1, 541 peak pairings were conclusively identified. The remaining peak pairs were found in Tier 2 using the linked identity library (LI Library). The LI Library's over 9,000 pathway-related metabolites provide high-confidence presumptive identification results based on accurate mass and expected retention time correlations. There were 697 peak pairings in tier 2, and 541 metabolites positively identified in tier 1. Multivariate analyses were firstly performed to uncover metabolite patterns amongst different peanut groups. Differences in metabolite production between different peanut fermentations can be clearly observed in the Figure 3.3.2 PLS-DA plots.



A B

Figure 3.3.2 (A). Principal component analysis (PCA) score plot visualises the global metabolic variation across four experimental groups. Group 1(control: faecal slurry only), peanut (group 2), digested peanut (group 3), and defatted peanut (group 4). Quality control (QC) samples and reference samples are also shown. (B) Partial least square discriminant analysis (PLS-DA) across 4 groups without quality control.

Principal component 1 (PC1) shows 18.3% of the variation between groups, while PC2 accounts for 16.2%. Both PCA analyses account for 34.5% of data variability between

groups. Peanut (Group 2) and digested peanut (Group 3) form distinct clusters, appearing separated from the control and defatted peanut groups. This indicates substantial metabolic shifts between raw peanut, control, and defatted peanut substrates. Defatted peanut (Group 4) cluster closely with the Control (Group 1), suggesting similar metabolic profiles. Quality control samples are densely clustered, indicating high reproducibility of experiments. Partial least square discriminant analysis (PLS-DA) shows visual differences between treatment groups. PLS-DA shows distinct differences, especially between raw peanut and defatted peanut. Visual group separation is still visible in the PLS-DA plot, particularly for the raw and digested peanuts as opposed to the control and defatted peanuts.

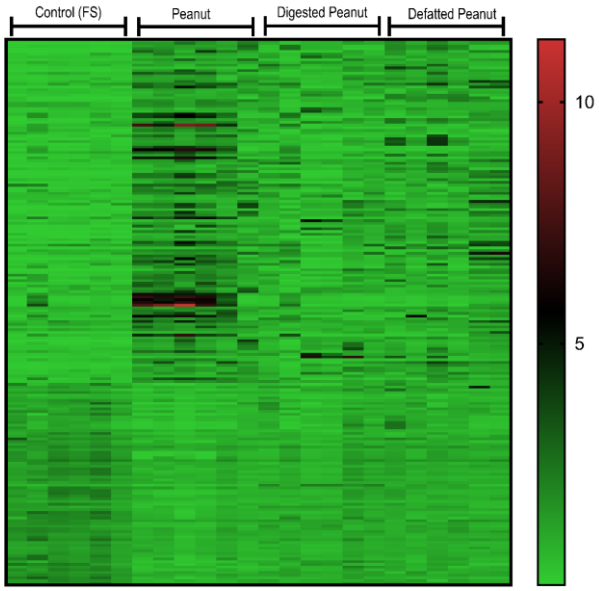


Figure 3.3.3. Clustered heatmap representing the relative abundance of the top 200 metabolites across four experimental groups with replicates of 6 per group. The heatmap uses a colour gradient from green (low) to red (high) to show metabolite intensity values. A metabolite is represented by each row, and a sample replicate is represented by each column, where n=55.

Raw peanut fermentation resulted in the greatest number of metabolite changes, as can be observed in the top 200 metabolite changes in Figure 3.3.3. Digested peanut fermentation by the gut microbiota showed moderate changes compared to the control fermentation, however, not as robust as fermentation with raw peanut. Defatted peanut fermentation showed far fewer changes with a metabolite profile remaining similar to control fermentation. Overall, metabolite clustering shows that raw and digested peanut fermentation result in a much broader metabolic pattern compared to defatted peanuts and control fermentations.

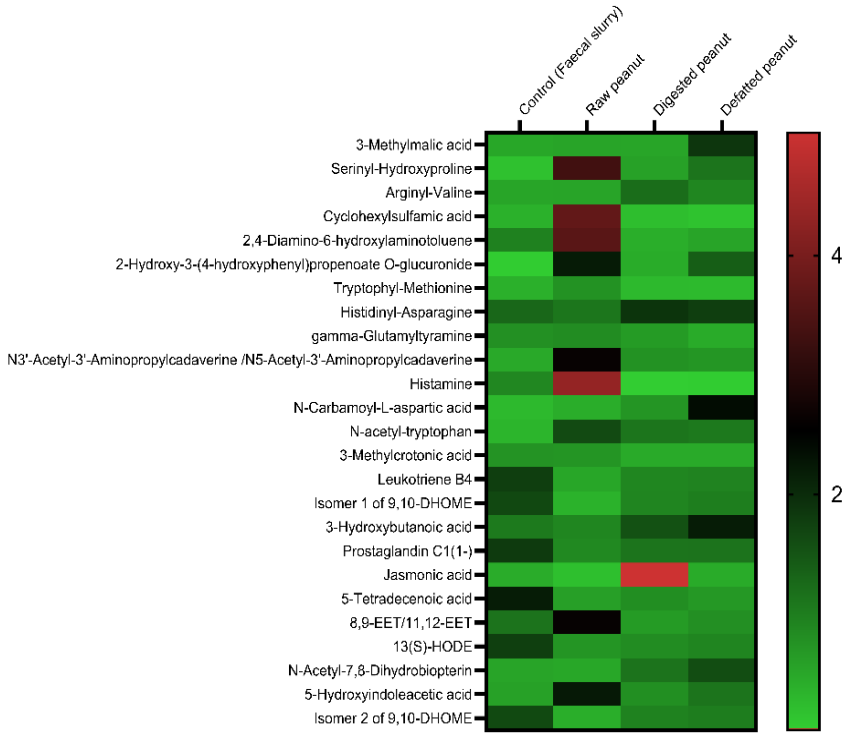


Figure 2.3.4. Clustered heatmap of averaged (replicates of 6) top 25 Tier 1 and Tier 2 identified metabolites ranked by p-value.

When we focus on the top 25 metabolite changes (ranked by p-value), both metabolite increases and metabolite decreases can be seen with peanut fermentation compared to control fermentation (Figure 3.3.4). For example, 5-tetradecenoic acid levels were highest in the control, while levels were significantly less following fermentation with all peanut preparations. This suggests that 5-tetradecenoic acid might be utilized by microbes metabolizing peanut substrates, or that 5-tetradecenoic acid might only be produced in the control fermentation and the alternate metabolic network driven by peanut substrates did not support 5-tetradecenoic production. Raw peanut fermentation resulted in increased levels of many metabolites such as serinyl-hydroxyproline, N-acetyl-tryptophan and 5-hydroxyindoleacetic acid, which was not observed for fermentation with the other peanut preparations. In contrast, digested and defatted peanut fermentation resulted in the highest levels of histidinyl-asparagine, while only defatted peanut fermentation increased levels of 3-methylmalic acid. Overall, peanut preparation specific differences were clearly observed, suggesting that substrate availability is critically important in driving the metabolic changes seen with gut microbiota fermentation.

A volcano plot was created by comparing the $\log_2$ fold change (FC) to the $-\log_{10}$ p-value for each metabolite. Fold change was determined as Mean (Group 1) / Mean (Group 2), with values >1 indicating greater abundance in Group 1. Metabolites significantly higher in the control fermentation are represented by red dots on the volcano plot, while the metabolites significantly higher in the peanut fermentation are represented by blue dots.

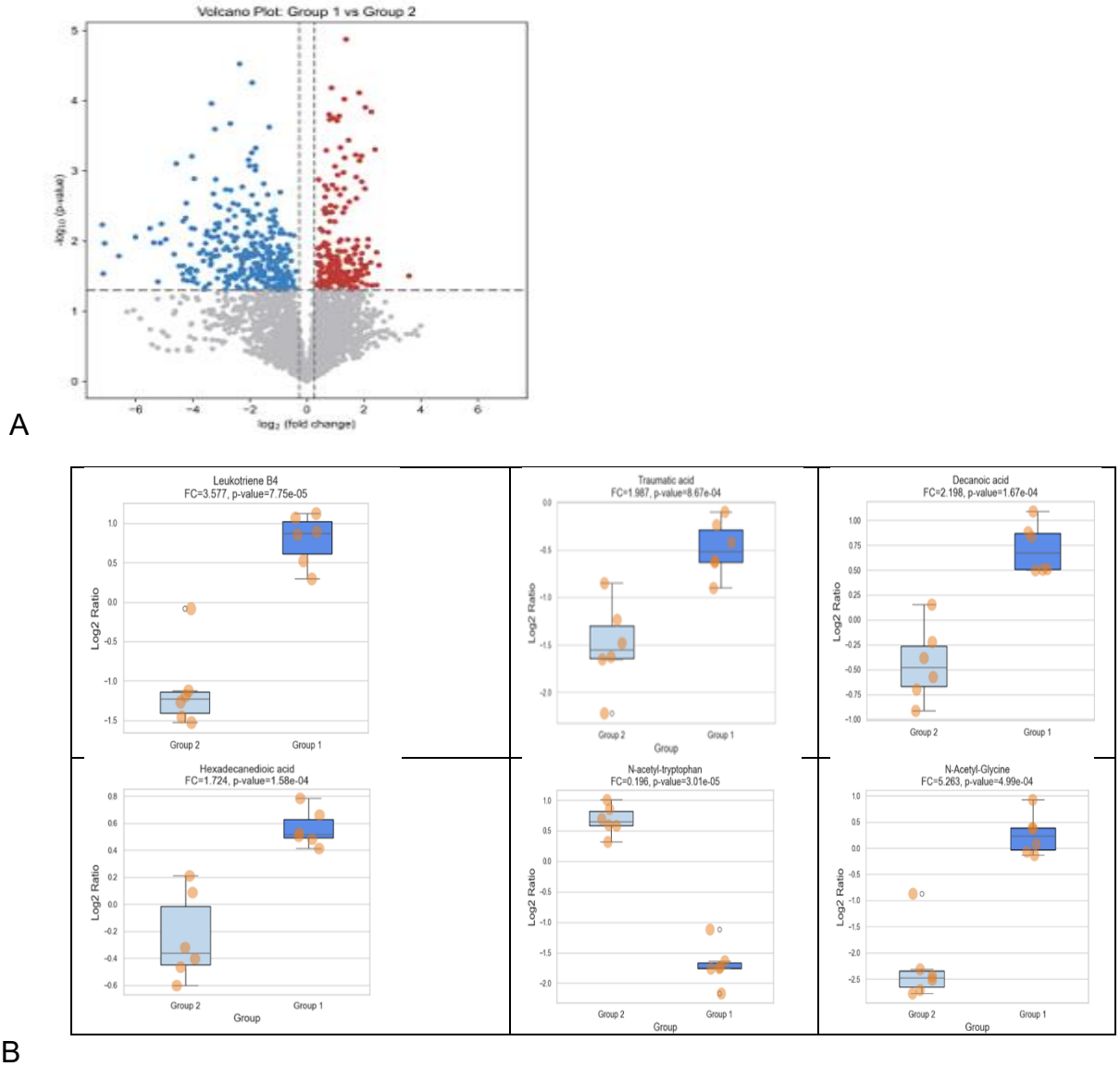
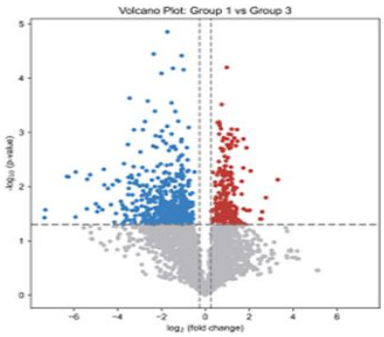


Figure 3.3.5. (A) A Volcano plot showing significant metabolite changes between group 1 (control) and group 2 (raw peanut). (B) Examples of specific metabolite differences between group 1 (control) and group 2 (raw peanut). Log_2 fold-change distributions are displayed in boxplots for top metabolite changes between two groups.

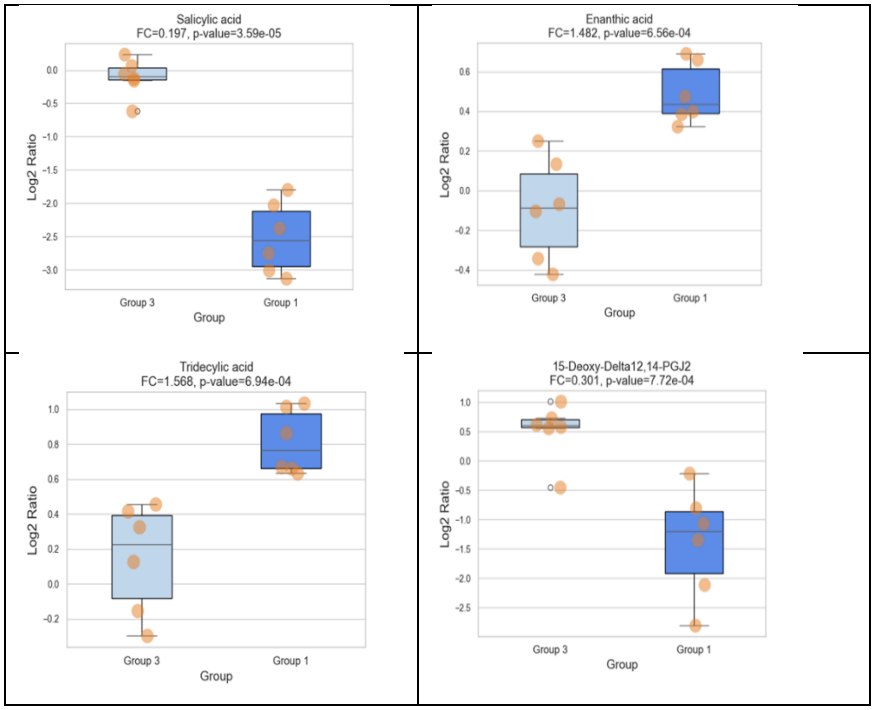
Leukotriene B4, traumatic acid, decanoic acid, hexadecanedioic acid, and N-acetyl-glycine were higher in the control fermentation, while N-acetyl-tryptophan was

significantly elevated following fermentation with raw peanut (fold change of 0.196 and p-value=3.01e-05).

The effect of digested peanut supplementation (group 3) on faecal microbiota fermentation (group 3) compared to control fermentation (group 1) can be observed in Figure 3.3.6 below. The $\log_2$ (fold change) versus -$\log_{10}$ (p-value) was plotted with the fold change calculated as mean (group 1) / mean (group 3).



A



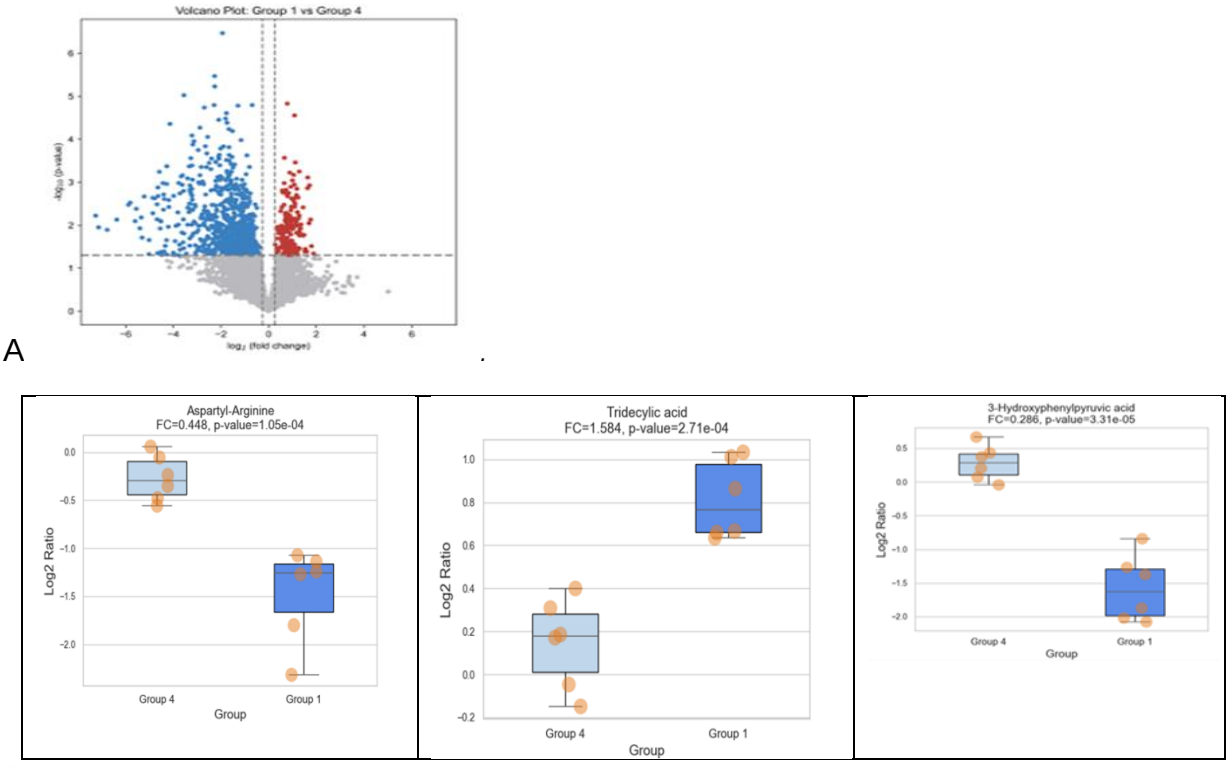
B

Figure 3.3.6. (A) Volcano plot showing significant changes in metabolites between group 1 (control) and group 3 (digested peanut). (B) Box plots showing significant differences in metabolite abundance between faecal fermentation (group 1) and digested peanut substrate (group 3).

Digested peanut (group 3) had higher levels of salicylic acid and 15-Deoxy-Δ12,14-PGJ2 compared to group 1, with fold changes of 0.197 (p-value 3.59e-05) and 0.301 (p-value 7.72e-04) respectively. On the other hand, enanthic acid and tridecylic acid

were higher in group 1 with fold changes of 1.482 (p-value 6.56e-04) and 1.568 (p-value 6.94e-04) respectively.

The volcano plot (Figure 3.3.7 below) comparing control fermentation (group 1) versus fermentation with defatted peanut (group 4) was created by graphing $\log_2$ (fold change) against -$\log_{10}$ (p-value). Fold change was computed as mean (Group 1)/mean (Group 4).



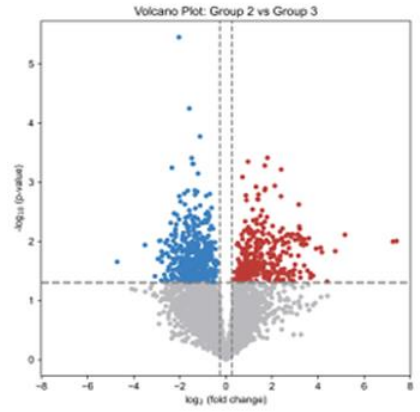
A

B

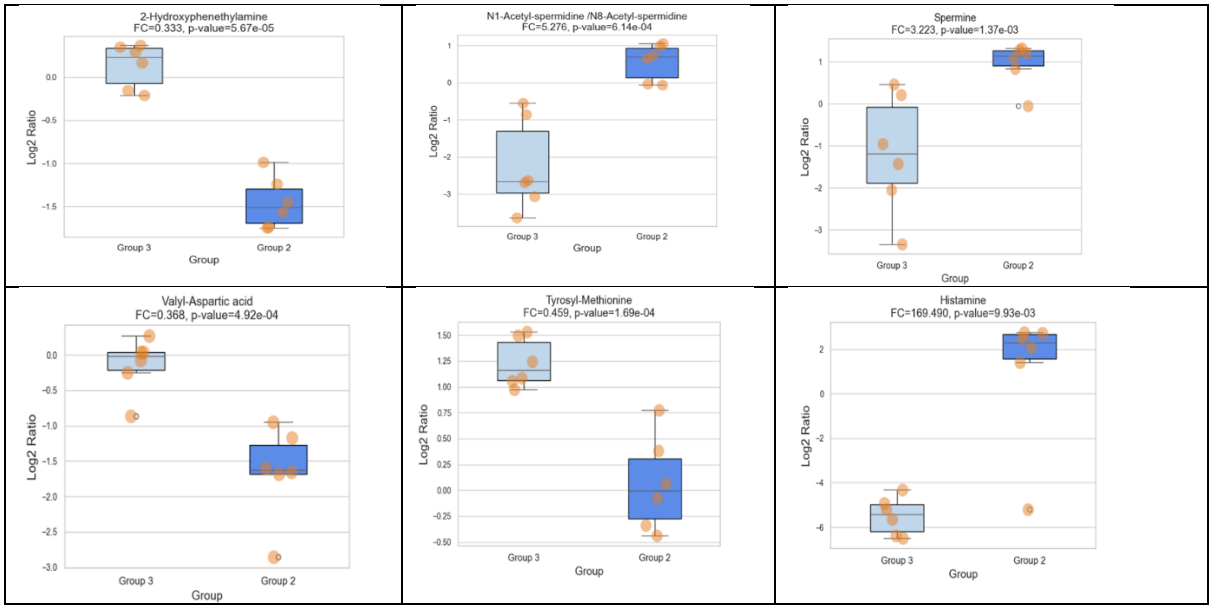
Figure 3.3.7. (A) A Volcano plot of significant changes in metabolites between group 1 (control and group 4 (defatted peanut). (B) Box plots showing examples of significant differences in metabolite abundance between control faecal fermentation (group 1) and defatted peanut fermentation (group 4).

Defatted peanut (group 4) showed increased levels of aspartyl-arginine and 3-hydroxyphenylpyruvic acid, with fold changes of 0.448 (p-value 1.05e-04) and 0.286 (p-value 3.31e-05) respectively. On the other hand, group 1 had a 1.584-fold higher levels of tridecylic acid (p-value 2.71e-04).

To better identify the differences in metabolite levels between raw peanut and digested peanut fermentation, we calculated the fold change as Mean (Group 2) / Mean (Group 3), and this is illustrated in the volcano plot in Figure 3.3.8 below.



A

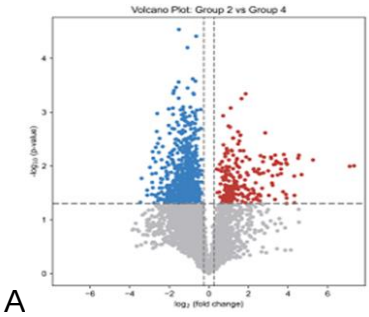


B

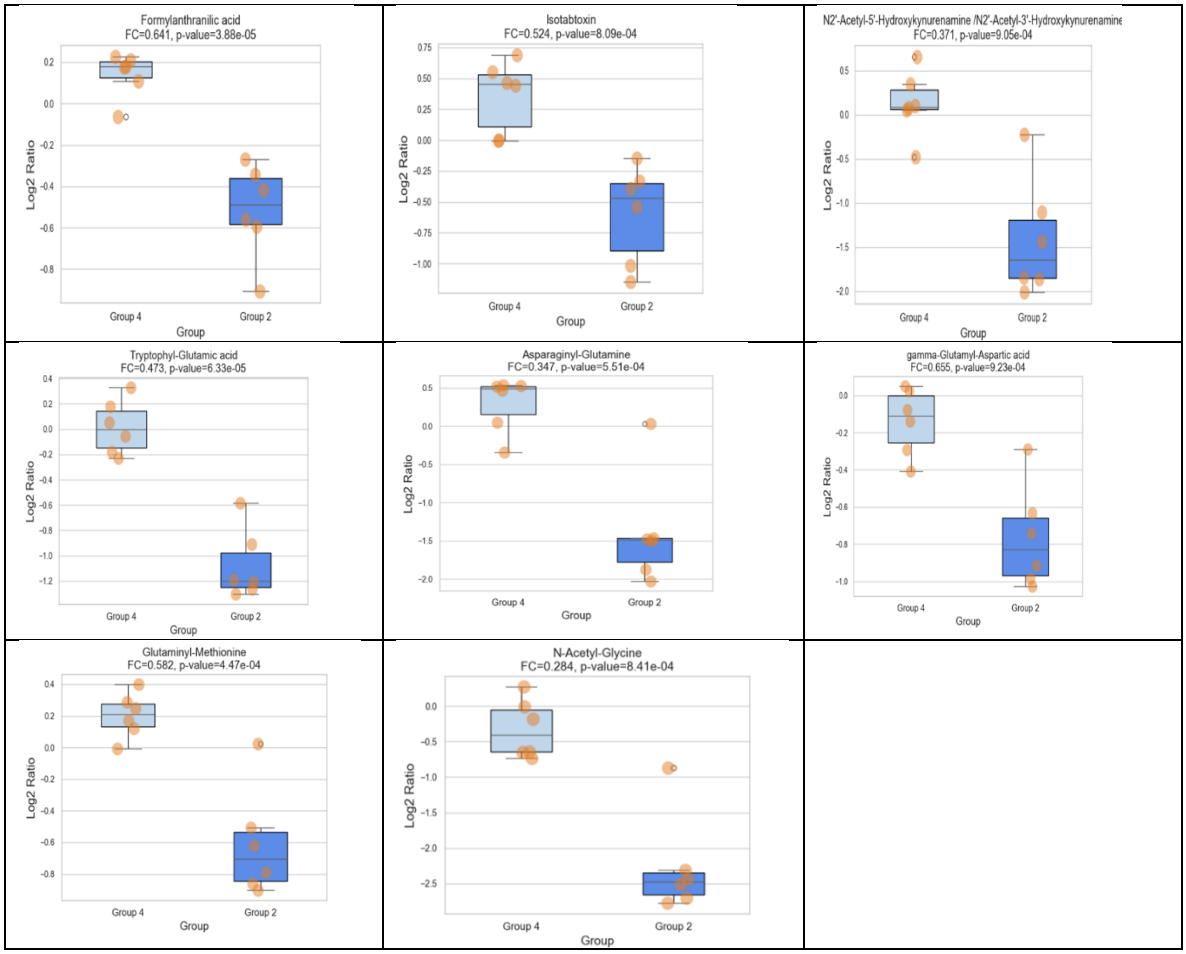
Figure 3.3.8. (A) A volcano plot of significant metabolite changes between group 2 (raw peanut) and group 3 (digested peanut). (B) Box plots showing examples of significant differences in metabolite abundance between raw peanut fermentation (group 2) and digested peanut fermentation (group 3).

2-Hydroxyphenethylamine, Valyl-Aspartic acid, and Tyrosyl-Methionine were higher for digested peanut fermentation with fold changes of 0.333 (p-value 5.67e-05), 0.368 (p-value 4.92e-04), and 0.459 (p-value 1.69e-04) respectively. N1-Acetyl-spermine/N8-Acetyl-spermidine, spermine, and histamine had the highest levels for raw peanut fermentation with fold changes of 5.276 (6.14e-04), 3.223 (p-value 1.37e-03), and 169.49 (p-value 9.93e-03) respectively.

The effects of raw and defatted peanut supplementation on faecal microbiota fermentation were compared in Figure 3.3.9 by plotting $\log_2$ (fold change) against -$\log_{10}$ (p-value), with fold change computed as Mean (Group 2) / Mean (Group 4).



A

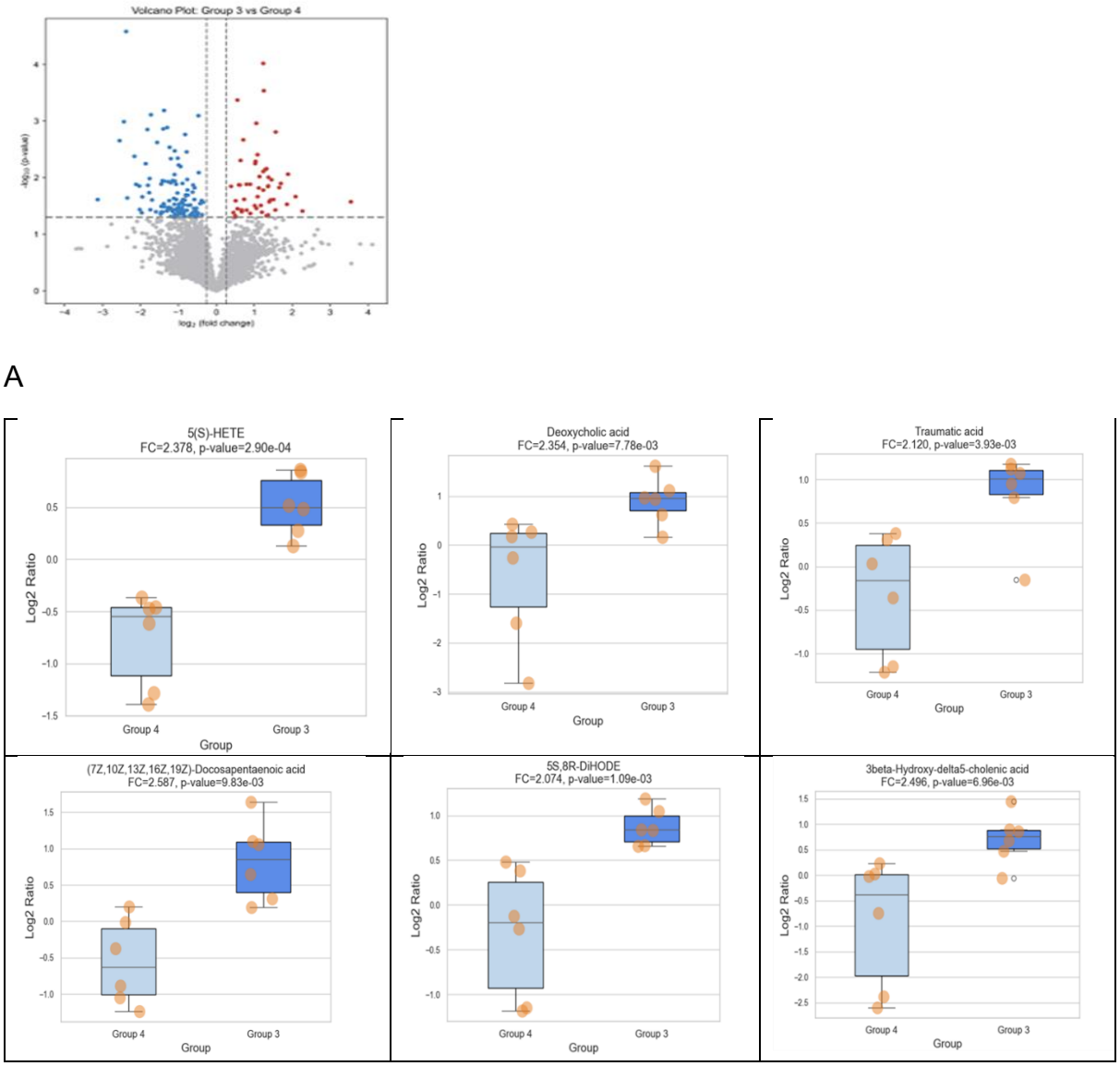


B

Figure 3.3.9.(A) A Volcano plot of significant metabolite changes between group 2 (raw peanut) and group 4 (defatted peanut). (B) Box plots showing examples of significant differences in metabolite abundance between raw peanut fermentation (group 2) and defatted peanut fermentation (group 4).

The 9 examples illustrated were all higher in defatted peanut fermentations compared to raw peanut fermentation, with the highest fold changes in gamma-glutamyl-aspartic acid and formylanthranilic acid (fold changes of 0.655 (p-value 9.23e-04) and 0.641 (p-value 3.88e-05)).

The effects of digested versus defatted peanut fermentation on faecal microbiota fermentation were compared in Figure 3.3.10, by plotting $\log_2$ (fold change) versus -$\log_{10}$ (p-value), with fold change determined as Mean (Group 3) / Mean (Group 4).



A

B

Figure 3.3.10. (A) Volcano plot of significant metabolite changes between group 3 (digested peanut) and group 4 (defatted peanut). (B) Box plots showing examples of significant differences in metabolite abundance between digested peanut fermentation (group 3) and defatted peanut fermentation (group 4).

All 6 significant metabolites examples shown were higher in group 3, with the highest being Docosapentaenoic acid and 3beta-hydroxy-delta5-cholenic acid (fold changes of 2.587 (p-value 9.83e-03) and 2.496 (p-value 6.96e-03) respectively).

Pathway enrichments for each peanut fermentation compared to control fermentations were identified using MetaboAnalyst, which provides a pathway-level interpretation of microbial metabolism.

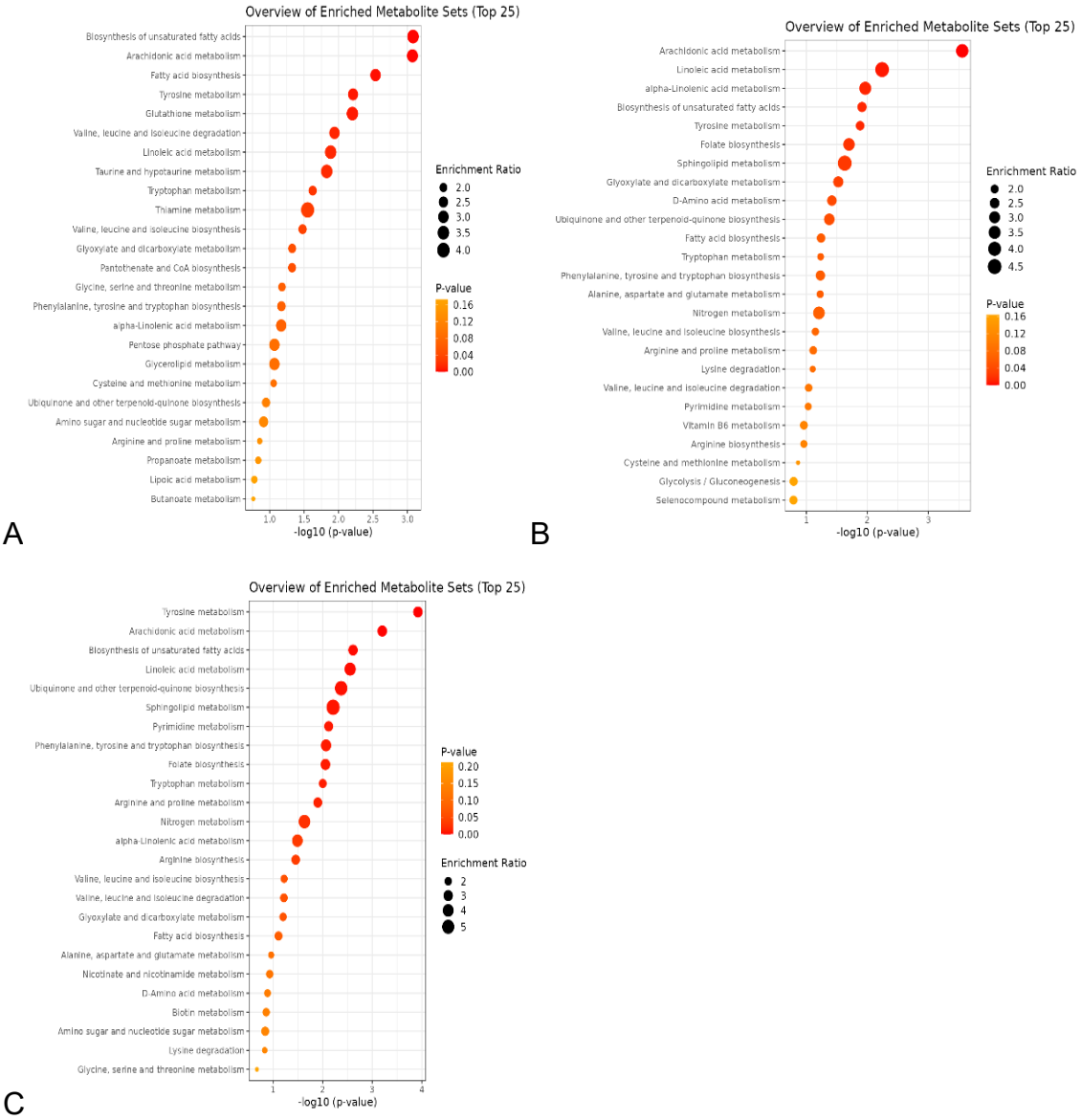


Figure 3.3.11. A Bubble plot showing pathway enrichments for (A) raw peanut metabolism, (B) digested peanut metabolism, (C) defatted peanut metabolism, each compared to control fermentation.

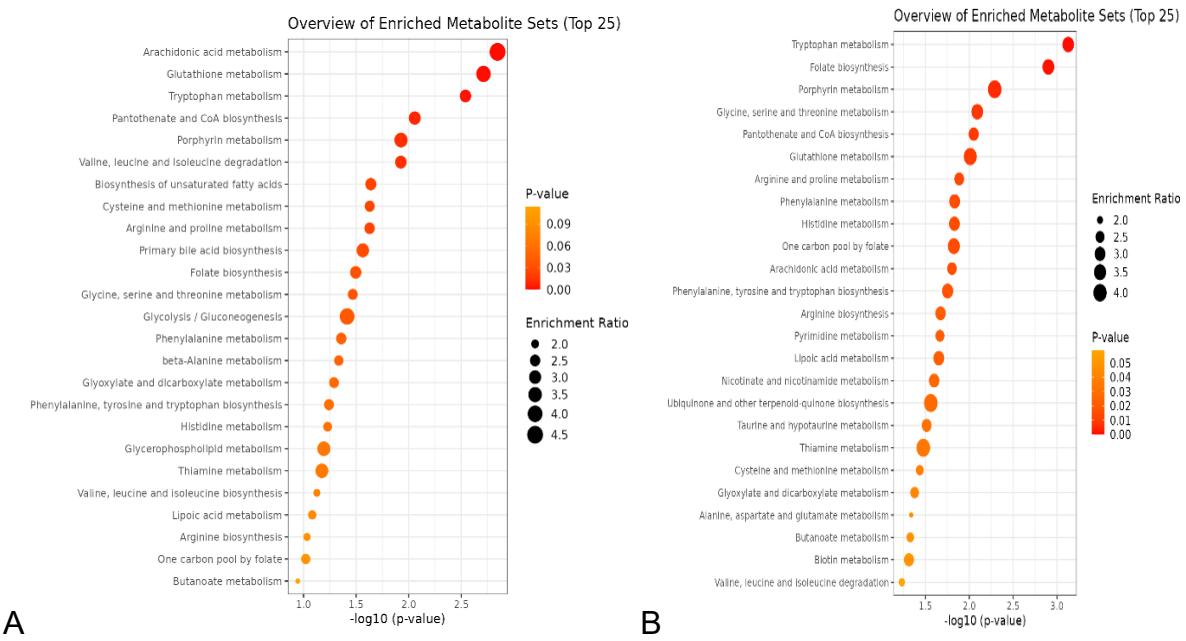


Figure 3.3.12. Bubble plot of pathway enrichment analysis. (A) group 2 (raw peanut) vs group 3 (digested peanut) and (B) Group 2 (raw peanut) vs group 4 (defatted peanut).

Pathway enrichment analysis revealed that raw peanut fermentation was predominantly associated with changes in lipid and fatty acid metabolism. Defatted peanut fermentation was characterized by arachidonic acid metabolism and linoleic acid metabolism, while defatted peanut fermentation was mostly characterized by tyrosine and arachidonic acid metabolism.

Pathway analysis comparisons between raw and digested peanut fermentation suggested enrichment in arachidonic acid metabolism, glutathione, and tryptophan metabolism for raw peanut. When comparing raw versus defatted peanut fermentation, tryptophan metabolism remained enriched for raw peanut fermentation, with addition pathways including folate biosynthesis and porphyrin metabolism being enriched for raw peanuts.

3.4 16s rRNA analysis

Microbial community profiling was carried out to determine if different peanut preparations affect gut bacterial population composition during faecal microbiota fermentation. Firstly, taxa changes normalised for changes observed in control faecal

fermentation after 24 hours were evaluated for raw peanut (group 2), digested peanut (group 3), and defatted peanut (group 4).

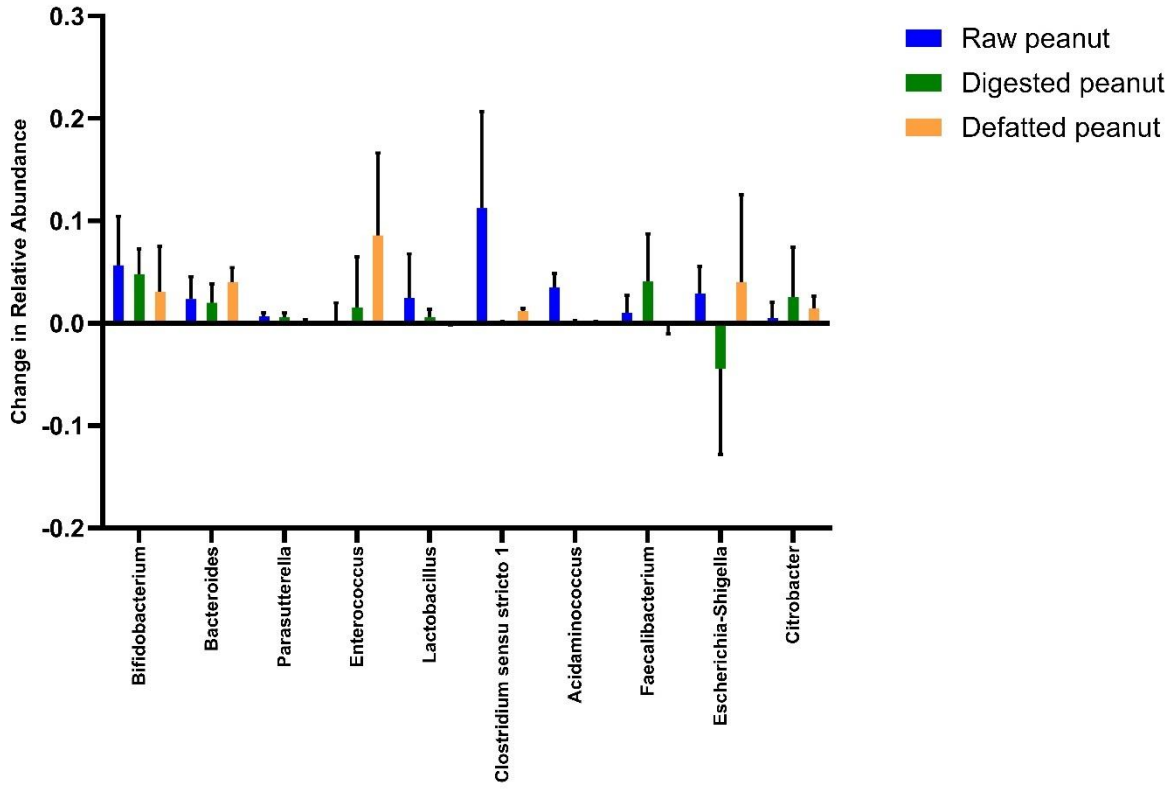


Figure 3.4.1. The bar graph shows the mean (+/- SD) changes in relative abundance of specific taxa following faecal microbiota fermentation of raw peanut (blue), digested peanut (green), and defatted peanut (orange).

Raw peanut fermentation resulted in substantial changes in relative abundance of *Megasphaera, Clostridium sensu stricto 1, Bifidobacterium*, and *Acidaminococcus* with mean change of 0.109, 0.099, 0.0566, and 0.035, respectively. *Bacteroidetes* had the greatest change in relative abundance for defatted peanut fermentation. Digested peanut had the lowest change in relative abundance of *Megasphera, Clostridium sensu stricto 1, Acidaminococcus* and *Bacteroidetes* with mean values of 0.00385, -0.01045, 0.00116, and 0.00936, respectively. An increase of *Bifidobacteria* and *Bacteroidetes* was observed across all peanut fermentations, with raw peanut showing the highest change in relative abundance of *Bifidobacteria*.

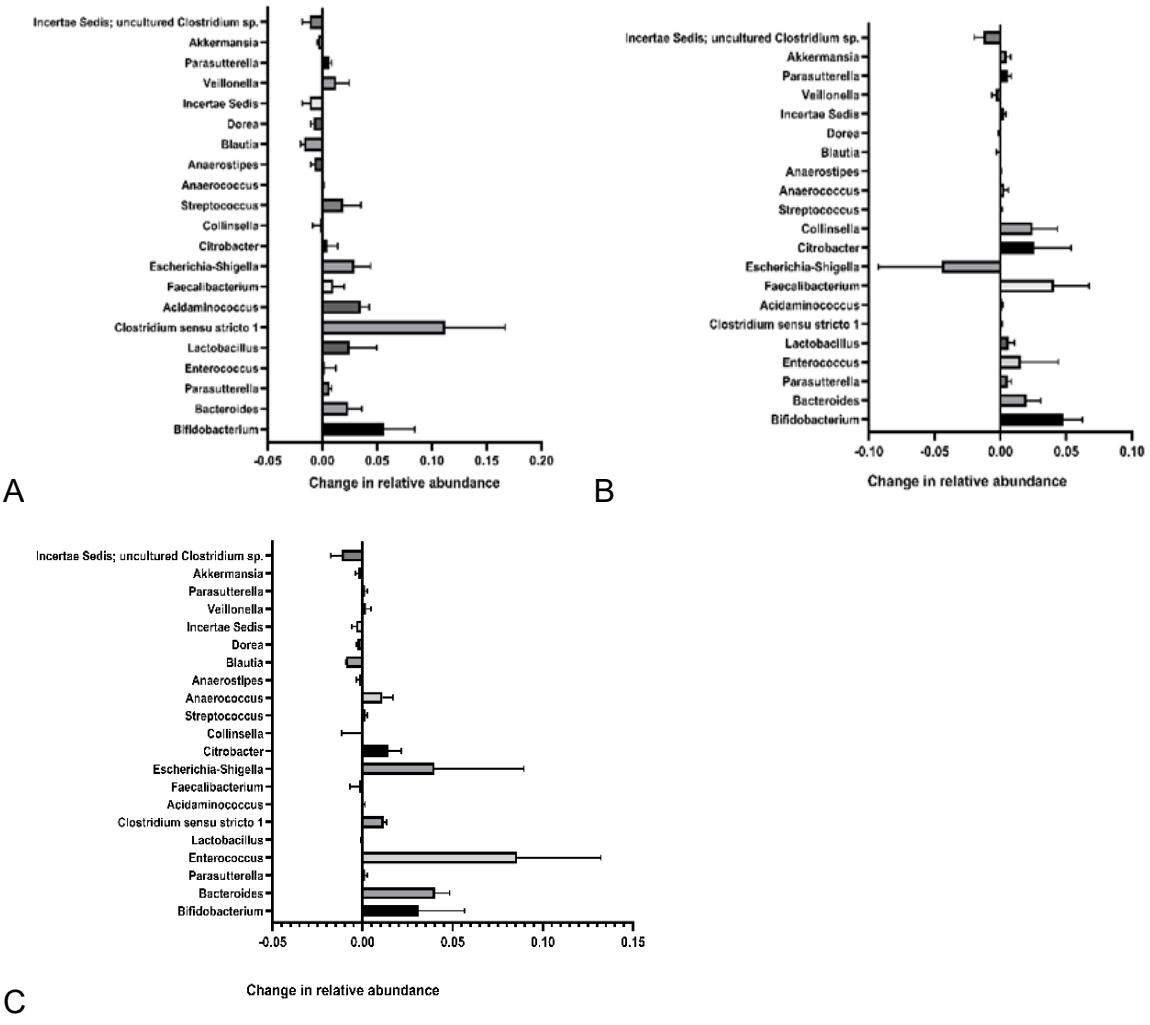


Figure 3.4.2 Positive and negative changes in relative abundance of bacterial taxa for (A) raw peanut, (B) digested peanut and (C) defatted peanut fermentation compared to control fermentation. The bars indicate positive (rightward) or negative (leftward) movements relative to the baseline (error bars represent standard deviation).

In addition to comparisons to control fermentations at 24 hours, we also evaluated the changes in taxa from baseline time 0 hours without fermentation. *Clostridium sensu stricto 1, Acidaminococcus, Escherichia-Shigella, Bifidobacterium*, and *Lactobacillus* all increased in relative abundance with raw peanut fermentation, whilst *Blautia, Insertae Serdis, Dorea* and *Anaerostipes* were decreased, and *Faecalibacterium*, *Veillonella,* and *Streptococcus* was moderately increased. In digested peanut fermentations, *Bifidobacterium*, *Faecalibacterium*, Bacteroidetes, and Citrobacter are increased, while *Escherichia-Shigella* was significantly reduced. In defatted peanut fermentation*, Enterococcus, Escherichia-Shigella, Bacteroidetes,* and *Bifidobacterium* were increased, while *Blautia, Dorea,* and an uncultured *Clostridium* species were decreased compared to baseline.

3.5 AhR activation by *Blautia* and faecal microbiota fermentates

The aryl hydrocarbon receptor (AhR) is an important signal transduction pathway that integrates metabolite production by microbes with generation of beneficial host responses. Using the HT-29 AhR reporter epithelial cell line, we investigated if *Blautia* culture on peanuts, or faecal fermentation of peanuts, could generate ligands that activate the AhR.

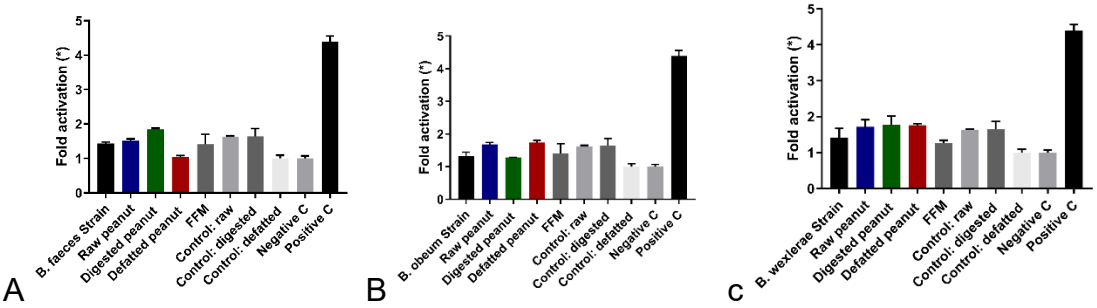


Figure 3.5.1. AhR activation assay of B. faecis, B obeum, and B. wexlerae strains following in vitro culture with raw, digested or defatted peanuts. Positive control was L-kynurenine, and negative control was HT-29 cells alone. 50,000 HT-29 cells were assessed per well and bacterial supernatants were added at 5% (v/v).

L-kynurenine, as a positive control, induced strong AhR activation. Culture supernatants from all *Blautia* strains grown without peanut substrates showed a slight activation of the AhR (5% (v/v) (*Figure 3.5.1*)). Culture of *Blautia* strains with peanuts preparations however did not significantly change AhR activation compared to culture without peanuts.

In addition to assessing *Blautia* culture supernatant effects on AhR activation, we evaluated if faecal microbiota fermentation with different peanut preparations might generate AhR ligands.

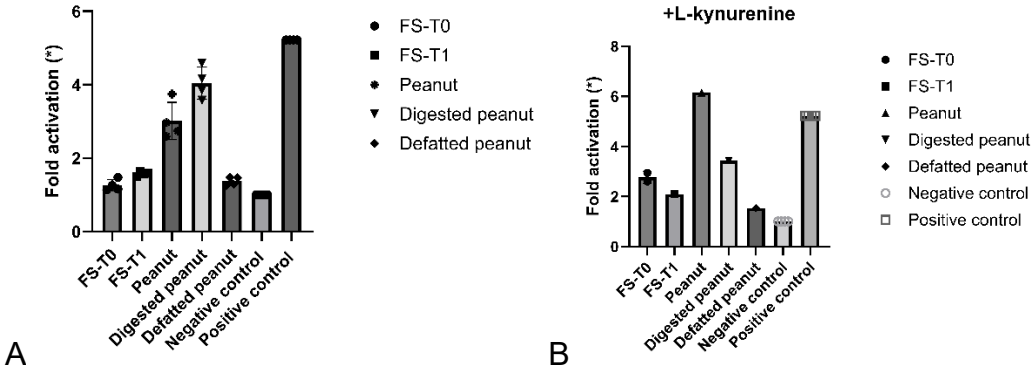


Figure 3.5.2. (A) AhR activation by faecal microbiota fermented peanut substrates. (B) AhR activation when in L-kynurenine cultures with supernatants from faecal fermentations.

Control faecal fermentation supernatants induced low levels of AhR activation, while supernatants from faecal microbiota fermentation with raw peanut or digested peanut, but not defatted peanut, strongly activated AhR signalling (Figure 3.5.2A). In HT-29 cells stimulated by L-kynurenine, control fermentation, digested and defatted peanut fermentation culture supernatants showed inhibitory effects on AhR activation (Figure 3.5.2B). Supernatants from raw peanut fermentation did not reduce L-kynurenine induced AhR activation but in fact slightly increased AhR reporter activity, suggesting that raw peanut fermentation by the gut microbiota results in secretion of metabolites that activate, rather than suppress the AhR. While we do not know which specific metabolites are critical for AhR activation in this experiment, perhaps this might correlate with the higher level of tryptophan metabolism shown in the pathway enrichment analysis shown above as multiple microbial-derived tryptophan metabolites have been previously shown to activate the AhR.

4. Discussion and Conclusions

This thesis explored the metabolic effect of peanuts by fermentation using pre-selected Blautia strains and faecal microbiota cultures. Blautia was chosen due to earlier discoveries of its abundance and SCFA producing capacities in peanut-consuming children. The resultant metabolites were then tested for immune-modulatory properties, namely their ability to serve as aryl hydrocarbon receptor (AhR) ligands.

Firstly, growth and metabolic activities of *B. wexlerae, B. obeum,* and *B. faecis* were studied across many standard growth substrates. Broad substrate utilization was observed across all species, but species-specific effects were noted. This result is in line with previous observations by Rui et al. (2024). Microbial growth and metabolic analysis using the Biolog ODIN VIII system showed a wider substrate utilization by *B. wexlerae*, followed by *B. obeum* and lower growth and metabolic activity with *B. faecis*. All tested *Blautia* species can metabolize complex carbohydrates, but significant differences were observed when grown on different peanut preparations, suggesting distinct enzymatic capabilities associated with each species.

Perhaps unrelated to peanut fermentation, a noteworthy finding was the high growth of *B. wexlerae* and *B. obeum* on glycerol. A gut microbiome study by Degnan et al. (2014) revealed that approximately 11% of gut microbes have the vitamin B12-dependent glycerol dehydratase (GDH) gene. Based on the high growth rates in glycerol, it can be suggested that *B. wexlerae* and *B. obeum* possess these genes but *B. faecis* does not. This gene converts glycerol to 3-hydroxypropionaldehyde (3-HPA). 3-HPA is a reuterin intermediate metabolite and has been shown to have antimicrobial properties (El-Ziney, 1998; Degnan, Taga and Goodman, 2014b). Metagenomic analysis of adult faecal samples identified GDH-encoding genes in various *Bacillota (formerly Firmicutes)*, including *Ruminococcus obeum*, which was reclassified as *B. obeum* (Engels *et al.*, 2016). By utilizing glycerol, this suggests that *B. obeum* and *B. wexlerae* could produce compounds that support host health. The growth and metabolic activity of *B. faecis* showed the lowest activity across all substrates. This result is consistent with earlier research by Sorbara et al. (2020), which showed *B.*

wexlerae to have higher acidification and production of SCFAs when compared to *B. faecis* and *Blautia schinkii* (Sorbara *et al.*, 2020). Of note, the highest OD reading for *B. faecis* was on D-Turanose. *B. faecis* can tolerate and partially metabolise short-chain fatty acids but prefers sugar-based substrates. Even though *B. faecis* growth across all substrates was low, this still shows active glycolytic and peptidase mechanisms. *B. faecis'* preferred substrates were glucose 6-phosphate, trehalose and N-Acetyl-D-Glucosamine. In summary, these growth and metabolic differences show functional differences among the three *Blautia* species examined.

One type of microbial metabolites that are important are SCFAs. A previous study showed 400 to 600 mmol production of SCFAs daily (Høverstad, 1986). The data suggests that different peanut preparations may have distinct effects on their SCFA metabolism by microbes. Raw peanut had the highest SCFA production overall, followed closely by digested peanut. This result suggests that some peanut components were partially degraded during INFOGEST digestion, yielding less substrates for SCFA production. Defatted peanut had the lowest level of total SCFA production. N-hexane defatting reduces fat content by over 70%. Fat removal may have also removed other available nutrients. However, this was not evaluated in this study. The most prevalent SCFA across all peanut substrates was acetate. Acetate has been shown to modify the metabolism of fats and carbohydrates and activates GPR43 on host cells to control inflammatory reactions (Sankarganesh *et al.*, 2025).

Peanut's complex matrix was associated with microbial fermentation pathways that promoted the production of acetate and butyrate at higher concentrations for raw peanut fermentation compared to digested and defatted peanuts. The majority of SCFAs are readily absorbed by the host's epithelial cells through active transport mechanisms or passive diffusion (den Besten *et al.*, 2013). Most of the microbial butyrate in the human intestine comes from carbohydrate metabolism. It is promoted by the butyrate kinase and butyryl-CoA: acetate CoA-transferase pathways (Altemani *et al.*, 2021). Absorbed SCFAs are mostly used as energy sources by the host epithelial cells (Imoto and Namioka, 1978). Interestingly, while colon levels of acetate, propionate, and butyrate are in a molar ratio of 60:25:15 (Tazoe et al., 2008), we found

a relative enrichment of butyrate following raw peanut fermentation as the ratio was68:9:23. A study by Paparo et al. (2021) showed a decrease in allergic response in three rodent models supplemented with butyrate. This was due to Th2 cytokines, and the modulation of oxidative stress (Paparo *et al.*, 2021). Increased SCFA production and increased change in relative abundance of beneficial bacteria shows some level of allergy protection, with some allergy related factors.

Given the fermentation experiment's low statistical power, these changes were evaluated cautiously. The bioreactor fermentation was carried out in triplicate (three independent runs), with duplicate wells for each condition. The SCFA concentrations in Table 3.3.1 are shown as means. In this design, only the rise in total SCFA with raw peanut attained statistical significance relative to the control ($p < 0.05$; Figure 3.3.1B), whereas the ranking of digested peanut and defatted peanut variations between specific SCFA species did not. The ability to resolve inter-run variability and identify minor effects is limited by the small number of biological replicates. To assess the robustness of these comparisons, future research should include more independent donors and runs in addition to measurements of dispersion (standard deviation or standard error) alongside the means in Table 3.3.1.

Fermented peanut showed distinctive differences from the control. PLS-DA models showed distinct clusters of raw peanuts. This shows substantial metabolic shifts between the peanut preparations. Metabolomic analysis of peanut supplemented faecal microbiota fermentations showed higher concentrations of histamine, serinyl-hydroxyproline, cyclohexysulfuric acid, and 2,4-diamino-6-hydroxylaminotuluene. Their production suggests microbial metabolism of amino acids and aromatic chemicals. Histamine is a byproduct of histidine decarboxylation (Moriguchi and Takai, 2020). Histamine increase (a 169.49-fold), relative to digested peanut ($p=0.009$) is the most significant metabolite change in the entire dataset. This could be linked to the microbial decarboxylation of raw peanut-derived histidine by bacteria such as Enterobacteriaceae and Escherichia-Shigella. These bacteria possess the histidine decarboxylase (HDC) expression (Zhang et al., 2018). The observed increase in Escherichia-Shigella and Enterobacteriaceae during raw peanut fermentation is

consistent with microbial decarboxylation of peanut-derived histidine. This metabolite is immunologically relevant to the allergy alignment of the study. Histamine has immunoregulatory properties which depend on the binding to four subtypes of histamine receptors (HRs), which are called H1R through H4R in chronological order of discovery (O'Mahony, Akdis and Akdis, 2011). A gut microbiome study of asthma patients by Barcik et al. (2019) showed that bacteria secreted histamine can modulate host-immune responses at mucosal sites via H1-H4 receptor signalling. Histamine, leukotrienes, platelet-activating factor, and prostaglandins are some of the many inflammatory mediators released by mast cells when peanut allergens crosslink the IgE antibodies on mast cells and basophils (Chu et al., 2024; Shimamura et al., 2011).

The high concentration of histamine in raw peanut fermentates, compared to raw and digested peanut fermentates shows that the processed peanuts may influence their allergy potential. This statement, however, warrants qualification: net luminal histamine concentration reflects a dynamic equilibrium between histamine-producing taxa expression. This, however, needs qualification. Future research will need to home in on establishing the microbial origin of histamine, as well as the quantification of free histidine across substrates.

The most enriched pathways were observed in raw peanut fermentates including α-linolenic acid metabolism, fatty acid biosynthesis, glycerol-lipid metabolism, and linoleic acid metabolism. Peanuts are high in lipid precursors polyunsaturated and fatty acids. Incorporating them into the fermentation system supplies additional substrates and nutrients for microbial lipid transformation (Arya, Salve and Chauhan, 2024; Jardim, Domingues and Alves, 2024). The observed enrichment of tryptophan metabolism and high levels of N-acetyl-tryptophan indicates high aromatic amino acid synthesis. Tryptophan metabolism microbes are linked to the production of bioactive metabolites that alter host signalling pathways, related to immune control and epithelial homeostasis (Taleb, 2019).

Clostridium sensu stricto 1, Acidaminococcus, Escherichia-Shigella, Bifidobacterium, and *Lactobacillus* were increased in relative abundance in peanut fermented cultures. *Bifidobacterium* and *Lactobacillus* are essential SCFA producers, while *Clostridium sensu stricto* 1 are medium-chain fatty acid (MFA) producers (Yu *et al.*, 2023; Sankarganesh *et al.*, 2025). However, in this study, peanut fermentation was associated with a reduction in *Blautia*. This contradicts previous infant cohort observations (Fitzgerald *et al.*, 2021; Sapp *et al.*, 2022) and therefore emphasises the need for further investigation. There may be several reasons behind this. The in vitro fermentation system's inability to accurately mimic the intricacy of the gastrointestinal environment could be one reason. Peanut intake takes place in vivo in the context of host digestion, absorption, immunological interactions, gastrointestinal transit, and ongoing nutrient exposure, all of which may have an impact on the composition of microbial communities (Birla, Hyderabadwala and Das, 2019; He *et al.*, 2021; Arya, Salve and Chauhan, 2024). The batch fermentation model, on the other hand, only records microbial reactions over a brief period in a controlled laboratory setting. Additionally, prior research on elevated *Blautia* after peanut consumption mostly evaluated long-term dietary consumption in humans. All things considered, the discrepancy between the present in vitro findings and previous in vivo observations suggests that the relationship between *Blautia* abundance and peanut consumption is most likely context-dependent and influenced by factors not fully captured in the experimental model employed in this study.

The faecal fermentations of raw peanut, as well as the univariate analysis revealed changes in some important immunomodulatory metabolites, including decreased levels of N-acetyl-glycine and Leukotriene B4, while N-acetyl tryptophan was increased. Leukotriene B4 is a strong inducer of leukocyte activation and promotes leukocyte migration into tissues during inflammation (Tamás et al., 2023). This molecule was significantly reduced following peanut fermentation. N-Acetyl tryptophan is the N-acetyl derivative of L-tryptophan, which can be utilized by gut microbes to generate immunomodulatory indoles (Taleb, 2019).

The digestion of peanut resulted in a slight reduction in SCFA production when compared to raw peanut, with acetate and butyrate still being the predominant metabolites. Dietary fibres are the major end substrates for SCFAs production (Vinelli et al., 2022). Reduced SCFA production by digested peanut suggests altered substrate availability. A recent in vitro digestion study by Chinnadurai et al (2025) supports this finding. They found that a small quantity of peanut carbohydrates was released during gastrointestinal digestion (Chinnadurai *et al.*, 2025). Even though food analysis was not performed, based on the slight differences in SCFA production of raw peanut and digested peanut, it is suggested that their dietary fibre compositions were similar, but not identical. In both raw and digested peanut fermentations, there was a positive change in relative abundance of *Bifidobacterium*, *Faecalibacterium*, and *Citrobacter*, which are major SCFA producers. *Faecalibacterium* are major butyrate producers whilst *Bifidobacterium* are major acetate producers. A significant finding was a decrease in the change of relative abundance of *Escherichia-Shigella* in digested peanut fermentate. Members of the *Escherichia-Shigella* group are pathogens closely related to *Escherichia coli* and have been linked to gut inflammation and dysbiosis(Baltazar-Díaz *et al.*, 2022). Digested peanut selectively suppresses potentially pathogenic taxa while favouring beneficial bacteria that produce beneficial SCFAs.

Digested peanut showed a significant upregulation of jasmonic acid and histidinyl-asparagine. Jasmonic acid is a plant hormone that controls plant growth, development, and defence mechanisms. Jasmonic acid has anti-inflammatory and immunomodulatory benefits in humans through the modification of cytokine production (Jarocka-Karpowicz and Markowska, 2021b). The first studies on the anti-inflammatory benefits of jasmonates (derived from *Gracilaria verrucosa*) showed similar positive results to prostaglandin (Dang *et al.*, 2008; Jarocka-Karpowicz and Markowska, 2021a). Prostaglandins are hormone-like molecules generated in the body in locations of tissue infection or damage (Ricciotti and FitzGerald, 2011).

Digested peanut fermentation also showed an upregulation of 15-deoxy-Δ12,14-PGJ2. It is a lipid-derived molecule linked to resolution of inflammatory processes

(Surh *et al.*, 2011). They are known sources of lipid mediators that control inflammation. Enrichment analysis showed increased levels of arachidonic acid and unsaturated fatty acid pathways. The enrichment of these pathways, combined with elevated levels of 15-deoxy-Δ12,14-PGJ2 supports the hypothesis that digested peanut fermentation supports microbial metabolism of lipids. Overall, the reduction of *Escherichia-Shigella* and the up-regulation of jasmonic acid and histidinyl-asparagine suggests that fermentation of digested peanut by gut microbes produces compounds with immunomodulatory effects while also suppressing pathogenic and pro-inflammatory related pathogens.

Further univariate analysis of control and defatted peanut showed upregulation in aspartyl-arginine and 3-hydroxyphenylpyruvic acid. While no literature on the effects of aspartyl-arginine was found, a previous study did link the gastrointestinal tract's physiology with arginine. It has been shown that oral administration of arginine enhanced mucosal healing after intestinal damage (Pretorius, 2012). Group comparisons between raw peanut and defatted peanut fermentation showed higher levels of gamma-glutamyl-aspartic acid and formylanthranilic acid. Gamma-Glutamylaspartic acid is a dipeptide made up of gamma-glutamate and aspartic acid formed during the proteolysis of large proteins. Gamma-Glutamylaspartic acid is a partial breakdown product of protein digestion. Their elevated concentration is indicative of incomplete digestion of defatted peanut. This also correlates with the taxa changes described for defatted peanut faecal microbiota fermentations.

The lowest concentrations of SCFA were for defatted peanut, with a total concentration of 61.18 mM, like the control (faecal slurry) with 58.21 mM, which suggests poor utilization of defatted peanut for SCFA production. The metabolic similarities are supported by principal component analysis, which showed defatted peanut clustering closely with the control. Similar to control, metabolite profiling of defatted peanut showed higher levels of N-Acetyl-L-aspartic acid and Hydroxybutanoic acid. N-acetyl-L-aspartic acid (NAA) is an amino acid derivative that has primarily been associated with brain metabolism. The expression of NAA in the gut impacts proteins linked to inflammation, and transcription factors. It is responsible for abnormal gastrointestinal

function (Surendran, 2009). Unlike raw peanut, defatted peanut supplementation has higher relative abundance of nosocomial pathogens such as *Escherichia-Shigella* and *Enterococcus*, which correlates with a study by Baltazar-Díaz et al. (2022) where it was found that the dominance of *Escherichia* taxa was associated with low SCFA production and an increase in pathogenic metabolic pathways and the production of endotoxic chemicals (Baltazar-Díaz et al., 2022).

Pathway enrichment analysis of defatted peanut fermentates revealed increased lipid and aromatic metabolism. The most enhanced pathways were α-linolenic acid metabolism, unsaturated fatty acid biosynthesis, arachidonic acid metabolism, and linoleic. This suggests that lipid metabolism pathways by the gut microbiota are still impacted even after peanut defatting. The enrichment of aromatic amino acid pathways supports that defatted peanuts preferentially enhance amino acid and nitrogen metabolism. This sets defatted peanut apart metabolically from raw and digested peanut. Overall, defatting peanut alters their metabolic fate, directing microbial activity towards aromatic and peptide associated metabolic pathways but still links to lipid pathways. Defatted peanuts have higher levels of protein compared to raw peanuts (Wu et al., 2009; Asen et al., 2021). Microbial fermentation of defatted peanuts promoted proteolytic microbial metabolism, hence the increased abundance of peptide and amino acid derived metabolites (Diether and Willing, 2019; Wang *et al.*, 2019).

These findings give a mechanistic insight into not only how diet affects host–microbiome interactions, but how food processing can change their microbial metabolic effects. Food composition can impact gut microbial enrichment and metabolic pathway activation which can either have beneficial or negative effects. These findings contribute to the growing body of evidence linking functional outcomes in gut ecology and metabolic health. Whole foods elicit positive microbiome effects, that processed foods are incapable of reproducing. The structure of food plays a vital role in human health.

This study identified significant research findings and lays the groundwork for further studies on the relationships between host, diet, and gut microbiota. However, some gaps were identified. The lack of physicochemical characterisation of the three peanut preparations before fermentation is the more basic research gap. Because of the lack of quantitative data on the protein, lipid, fibre, and free amino acid content of the peanut substrates, the differential metabolic outcomes in the study can only be understood inferentially and cannot be verified without compositional data.

The use of in vitro faecal fermentation models is extremely regulated but cannot accurately mimic host-mediated processes such intestinal absorption and immunological signalling. Therefore, to close those gaps, future research should include multi-omics, in vivo models and, or human dietary intervention trials. Future research focusing on genetic factors that influence the unique substrate preferences of the three *Blautia* species need to be identified. Metatranscriptomics could help explain why *B. faecis* has a specialised metabolic profile compared to *B. wexlerae* and *B. obeum.* Integrating transcriptomics or proteomics into future fermentations could show how these species regulate carbohydrate and amino acid utilisation pathways. Mechanistic insights can reveal strain level features that impact host metabolic health. Additionally, mono-association Micromatrix^{TM} fermentation experiments could be conducted to identify which species produce anti-inflammatories, as well as which species supress pathogenic microbes. Due to the lack of composition analyses of peanut substrates, there is also a gap in the characterisation of the food matrix. To clearly connect nutrient availability to microbial metabolic results, future research should incorporate physicochemical analysis of peanut substrates before and after digestion. To translate microbial metabolic changes into health effects. It would also be worthwhile to investigate epithelial barrier integrity of peanut fermentates using human epithelial cells (HT-29). Lastly, conducting humanised mice models and or clinical research could be used to evaluate the positive metabolic changes from eating whole, defatted or digested peanuts result in gastrointestinal health or allergy alleviation. These methods will improve our knowledge of how food processing affects host health and microbial function.

This study gives molecular insights into how *Blautia* species and peanut substrates influence gut microbial metabolism. The three *Blautia* species studied have distinct metabolic profiles, with *B. wexlerae* and *B. obeum* showing similar profiles. However, *B. faecis* showed limited but functionally tailored substrate preferences. These differences emphasise the functional variety of the *Blautia* genus and their possible contributions to gut homeostasis.

Peanut fermentations demonstrated that whole and digested peanuts have the most beneficial impacts on human gut microbiota metabolism. Positive effects include increased anti-inflammatory metabolites such as jasmonic acid and the reduction of pro-inflammatory taxa such as *Escherichia-shigella*. Defatted peanut, on the other hand, produced less SCFAs, more proteolytic metabolites, and a higher concentration of opportunistic pathogens. This shows a significant shift towards a less favourable gut microbial metabolism.

In conclusion, my research study shows that gut microbes do ferment peanut, and peanut fermentation might contribute to the observed clinical effects of peanut consumption and peanut allergy prevention.

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