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Complementary feeding to nourish and support the developing infant

A thesis presented in partial fulfilment of the requirements of the degree of

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Jacqueline Nicole Tonkie

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Abstract

During complementary feeding, the gut microbiota undergoes substantial changes in taxonomic composition, functional capacity, and metabolite production. Although diet is recognised as an important modulator of microbiota development, evidence remains limited regarding whether specific complementary foods can influence these trajectories during early infancy. This thesis investigated whether kūmara (New Zealand sweet potato) alone and combined with green banana extract as whole-food prebiotic interventions could influence the developing infant gut microbiota.

Using an integrated experimental approach, controlled *in vitro* fermentation experiments characterised infant gut microbiota responses to nine substrate mixtures spanning varying combinations of kūmara and green banana extract, which spanned resistant starch contents of 1.2–30.5%. This established intrinsic metabolic capacity under static conditions. The SUN Study, a three-arm randomised controlled trial (n=279), then evaluated kūmara alone, kūmara plus green banana extract (85:15 ratio), and standard complementary feeding from 6 to 10 months of age. Shotgun metagenomics characterised taxonomic composition and functional metabolic pathways, while targeted metabolomics quantified faecal organic acids. Dietary intake assessment confirmed resistant starch delivery and quantified total dietary exposure.

Three principal findings emerged. First, taxonomic composition remained stable across interventions in both *in vitro* and *in vivo* settings, despite clear differences in substrate exposure. Second, functional metabolic capacity was responsive to dietary substrates but was dominated by age-driven developmental trajectories *in vivo*. Functional pathway abundance exhibited a biphasic maturation pattern, with 55% of pathways increasing between 6–8 months and 67% decreasing between 8–10 months, reflecting expansion followed by consolidation characteristic of gut maturation. Intervention effects on pathways were detectable but modest relative to developmental change. Third, faecal organic acid profiles matured predictably with age, characterised by increasing dominance of acetate, propionate, and butyrate (rising from 59% to 92% of total organic acids), but did not differ between

intervention groups. Integration of metagenomic and metabolomic data revealed that functional pathway abundance explained three-fold more variation in organic acid profiles than taxonomic composition (12.1% versus 4%), with 94% of taxa-metabolite associations mediated through metabolic pathways.

These findings demonstrate that gut microbiota development during early complementary feeding follows robust, developmentally programmed trajectories that are relatively resistant to dietary variation when introduced alongside continued breastfeeding and other complementary foods. Functional pathway profiling provides substantially greater insight into metabolic outputs than taxonomic composition alone, with important implications for microbiome research design. These findings provide a foundation for designing targeted nutritional strategies that align with developmental timing and dietary context to support healthy microbiota maturation during infancy.

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Abbreviations

BCFA	- Branched-chain fatty acids
C	- Control group
CF1	- Complementary feeding baseline (6 months of age)
CF2	- Complementary feeding timepoint 2 (8 months of age)
CF4	- Complementary feeding timepoint 4 (10 months of age)
CPM	- Counts per million
FDR	- False discovery rate
GBE	- Green banana extract
GC-MS	- Gas chromatography-mass spectrometry
HMO	- Human milk oligosaccharides
K	- Kūmara group
K+	- Kūmara plus green banana extract group
KO	- KEGG Orthology
MaAsLin	- Microbiome Multivariable Associations with Linear Models
MetaPhlAn	- Metagenomic Phylogenetic Analysis
PERMANOVA	- Permutational multivariate analysis of variance
PLS	- Partial least squares
PS	- Potato starch
RCT	- Randomised controlled trial
RS	- Resistant starch
SCFA	- Short-chain fatty acids
SUN Study	- Seeding throUgh feediNg Study
VIP	- Variable importance in projection
WHO	- World Health Organisation

List of Supplementary Materials

Supplementary File 1. Full MaAsLin3 differential abundance results for species-level responses to kūmara: green banana extract mixture ratios.

Supplementary File 2. Significant MaAsLin3 differential abundance results for functional pathway responses to kumara: green banana extract mixture ratios.

Supplementary File 3. Pathway-level and KEGG Ortholog-level concordance analysis.

Supplementary File 4. MaAsLin3 differential abundance results for significant genera tested across timepoints (CF1, CF2, CF4).

Supplementary File 5. MaAsLin3 differential abundance results for significant genera tested across intervention groups (K = Kūmara, K+ = kumara + green banana extract, C = Control).

Supplementary File 6. MaAsLin3 differential abundance results for significant MetaCyc functional pathways across timepoints (CF1, CF2, CF4).

Supplementary File 7. MaAsLin3 differential abundance results for significant MetaCyc functional pathways across intervention groups (K = Kūmara, K+ = kumara + green banana extract, C = Control).

Chapter 1

General Introduction

During complementary feeding, which typically begins around 6 months of age, the gut microbiota undergoes rapid and dynamic development. The transition from a low-diversity, milk-adapted microbial community toward a more complex and functionally diverse ecosystem continues to mature throughout the first two years of life (Bäckhed et al., 2015; Homann et al., 2021; Koenig et al., 2011; Pannaraj et al., 2017; C. J. Stewart et al., 2018). During this transition, the gut microbiota is reshaped by dietary diversification, physiological maturation of the gastrointestinal tract, and ongoing host–microbe interactions (Cox et al., 2014; Tamburini et al., 2016). Understanding the factors that influence microbiota development during complementary feeding is critical, as early microbial trajectories have been associated with immune maturation, metabolic programming, and longer-term health outcomes.

Diet is widely recognised as one of the most influential and modifiable determinants of gut microbiota composition and function. There is growing interest in identifying complementary foods that may support healthy gut microbiota development during infancy. Dietary fibre, particularly resistant starch, a fermentable carbohydrate that escapes digestion in the small intestine and is metabolised by colonic bacteria, has emerged as a potent prebiotic substrate in adult populations (Martínez et al., 2010; Upadhyaya et al., 2016; Venkataraman et al., 2016; Vital et al., 2018). It consistently enriches starch-degrading taxa and increases short-chain fatty acid production, particularly butyrate. However, despite evidence in adults, comparatively little is known about how dietary fibre or resistant starch functions as a prebiotic during infancy, when the gut microbiota is still developmentally immature and strongly shaped by milk-derived substrates (Noles et al., 2025).

Kūmara (*Ipomoea batatas*; New Zealand sweet potato) provides a relevant and culturally appropriate whole-food model for investigating these prebiotic effects during complementary feeding. Kūmara is commonly consumed by infants in Aotearoa New Zealand and provides complex carbohydrates and

dietary fibre within a natural food matrix. When cooked and cooled, starch retrogradation increases resistant starch content, generating additional fermentable substrate available to the colonic microbiota. A pilot feasibility study (Nourish to Flourish) demonstrated that daily consumption of kūmara powder was acceptable, safe, and well-tolerated by infants (McKeen et al., 2022), providing a foundation for evaluating microbiota effects in a larger randomised controlled trial. Green banana extract, another natural source of dietary fibre and resistant starch, is another promising candidate for the formulation of prebiotic complementary foods (Munir et al., 2024).

In vitro fermentation models are widely used to investigate microbiota responses to dietary substrates under controlled conditions; however, they cannot fully replicate the complexity of the infant gut environment, including host–microbe interactions, immune regulation, intestinal maturation, nutrient absorption, and background dietary exposure. In contrast, longitudinal *in vivo* randomised intervention studies capture these processes. Understanding how microbial responses observed *in vitro* relate to those expressed *in vivo* is therefore essential for interpreting dietary intervention outcomes during infancy.

Much of the existing infant microbiota literature has prioritised taxonomic composition over microbial function. Integrating metagenomic functional profiling with targeted metabolite measurements is critical for understanding how dietary interventions influence microbial metabolism during infancy, yet few studies have combined these approaches at scale during the complementary feeding period (Bikel et al., 2015).

Together, these gaps highlight the need for well-powered studies that examine whole-food–based dietary interventions during complementary feeding, integrate *in vitro* and *in vivo* approaches, and move beyond taxonomic descriptions to functional and metabolic outcomes. Addressing these gaps is essential for determining whether physiologically realistic dietary interventions can meaningfully influence gut microbiota development during this critical developmental window and for informing evidence-based infant feeding guidance.

This PhD research builds on the foundations of the Nourish to Flourish dietary pilot study, in which the feasibility of introducing a kūmara-based complementary food to infants aged 6-12 months in the Auckland metro area was successfully assessed (Lovell et al., 2022). The pilot study refined sampling and processing protocols for research in infants transitioning to complementary foods and characterised the temporal changes in the gut microbiota composition and activity using faecal sample analysis of infants aged 4 to 12 months (McKeen, 2022; McKeen et al., 2019).

The study demonstrated that kūmara is a feasible prebiotic complementary food with positive effects on the gut microbiota composition and metabolic activity in infants. The study identified the period between 4 and 9 months as a critical window for microbial and metabolic maturation, underscoring the importance of diet during this stage of development (McKeen et al., 2022). These insights have guided the design of this thesis.

1.1 Research Aim and Objectives

This thesis aims to investigate whether kūmara alone or kūmara in combination with green banana extract (whole-food-based prebiotic interventions) can influence the developing infant gut microbiota. The research integrates controlled *in vitro* fermentation experiments with a large randomised controlled trial called the SUN Study (Seeding throUgh feediNg: Nourishing the Infant Microbiome to Support Immune Health). The addition of green banana extract is expected to enhance the prebiotic dietary fibre content, particularly resistant starch, which may exert more pronounced effects on gut microbiota development.

The primary objectives of this PhD research are as follows:

Objective 1: To characterise the metabolic responses of infant gut microbiota to kūmara, green banana extract, and combinations of the two in varying ratios under controlled *in vitro* conditions.

Using infant-adapted digestion followed by 24-hour batch fermentation, this objective establishes the intrinsic metabolic capacity of infant gut microbiota to respond to these substrates across a range of substrate combination. The *in vitro* system provides mechanistic insight into potential prebiotic effects under controlled conditions, examining taxonomic composition, functional pathway profiles, and metabolite production in response to substrate composition (Chapter 3).

Objective 2: To evaluate the effects of kūmara alone and kūmara combined with green banana extract on taxonomic composition and functional metabolic capacity during the complementary feeding period.

This objective relies on data from the SUN Study, a three-arm randomised controlled trial involving infants in the urban and central Auckland areas of New Zealand (Wall et al., 2024). The objective is to examine whether whole-food prebiotic interventions alter gut microbiota development trajectories. The trial compared kūmara alone, kūmara plus green banana extract (85:15 ratio), and standard complementary feeding from 6 to 10 months of age. Shotgun metagenomics is used to characterise microbiota maturation during this period and evaluate intervention effects on both taxonomic structure and functional metabolic pathways (Chapter 4).

Objective 3: To assess the impact of the dietary interventions on faecal organic acid profiles and examine how microbial taxonomic composition, functional metabolic capacity, and dietary intake influence these metabolic outputs.

This objective examines organic acid production and dietary intake patterns of the SUN Study cohort across the complementary feeding period and evaluates intervention effects. The analysis then integrates three complementary datasets from the SUN Study: dietary intake assessment, shotgun

metagenomics (taxonomic and functional profiling), and organic acid production, to determine the relative contribution of each in explaining metabolite production (Chapter 5).

In summary, this PhD research combines an infant dietary intervention study with *in vitro* modelling of infant colonic fermentation to examine the interaction between dietary interventions and the developing gut microbiota. By focusing on kūmara and green banana extract, the study aims to uncover how these interventions, with their varying types and quantities of dietary fibre, contribute to nourishing and supporting the composition and functionality of the infant gut microbiota during the crucial early months of complementary feeding.

The following chapters address these objectives: Chapter 2 reviews current literature on infant microbiota development and dietary influences during complementary feeding, establishing the knowledge base and gaps motivating this research. Chapters 3-5 then address Objectives 1-4 respectively, as indicated above. Chapter 6 synthesises findings across the *in vitro* and *in vivo* investigations and discusses implications for infant feeding practice and future research.

Chapter 2

Literature Review

2.1 Introduction

The first two years of an infant's life are pivotal for the development and maturation of the gut microbiome, which refers to the community of microbes, their genomes, and the environmental conditions within the large intestine (Lederberg & McCray, 2001). The gut microbiome consists of trillions of microbes, including bacteria, fungi, viruses, and archaea, forming a complex microbial community known as the gut microbiota (Marchesi & Ravel, 2015). The composition and activity of the gut microbiota evolve significantly during early postnatal life through dynamic and non-random processes, with distinct microbial interactions at different stages (Bäckhed et al., 2015; Hill et al., 2017; Koenig et al., 2011).

Nutrition is considered one of the primary factors influencing the colonisation and succession patterns of the gut microbiota. Infants usually consume a milk-based diet (breastmilk and/or formula) for the first six months of life, after which complementary foods are introduced to meet the growing nutritional demands (Krebs & Hambidge, 1986; Noles et al., 2025; Parkin et al., 2024; Sen et al., 2017; Wang et al., 2024; Young & Krebs, 2013). The new and varying dietary components introduced during the complementary feeding period drive changes in the composition and activity of the gut microbiota (Bäckhed et al., 2015; Bergstrom et al., 2014; Galazzo et al., 2020; Hill et al., 2017; Koenig et al., 2011; Laursen et al., 2017; Leong et al., 2020; Qasem et al., 2017).

Complementary foods containing non-digestible dietary fibre can have a prebiotic effect on the gut microbiota. Prebiotics are “a non-digestible compound that, through its metabolism by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host.” (Bindels et al., 2015). Thus, complementary foods with prebiotic properties can be strategically introduced to promote the beneficial development

of the gastrointestinal system, distal organs and the immune system, all of which co-mature with the gut microbiota (McKeen et al., 2022; Mishra et al., 2021; Praveen et al., 2015; Clarke et al., 2014).

This literature review summarises the current body of knowledge on the establishment and development of gut microbiota during infancy. It examines the effects of nutrition, including breastmilk, formula, complementary feeding, and prebiotic dietary fibre, on the gut microbiota and discusses the methods for studying its composition and activity.

2.2 Establishment of the Early Life Gut Microbiota

2.2.1 Bacterial colonisation of the postnatal gut

Although some studies suggest that bacterial colonisation begins *in utero* (Aagaard et al., 2014; Collado et al., 2016; DiGiulio et al., 2008; Dong et al., 2015; Hansen et al., 2015; Jiménez et al., 2005, 2008; Jones et al., 2009; Nagpal et al., 2016; Parnell et al., 2017; Rehbinder et al., 2018; Stinson et al., 2019; Zhu et al., 2018), concerns over contamination and inadequate controls cast doubt on these findings (de Goffau et al., 2019, 2021; Gschwind et al., 2020; Kuperman et al., 2020; Lauder et al., 2016; Laurence et al., 2014; Leiby et al., 2018; Lim et al., 2018; Olomu et al., 2020; Theis et al., 2019). It is, therefore, generally accepted that the first major colonisation by bacteria occurs at birth (Bäckhed et al., 2015; Ferretti et al., 2018; Penders et al., 2006).

Bacteria are the most extensively studied component of the gut microbiota due to their abundance, with densities in the colon estimated at 10^{11} per gram (Sender et al., 2016). Bacterial composition and establishment in the infant gut microbiota are influenced by several factors, including birth mode, gestational age, and genetics (Figure 2.1).

The transfer of bacteria from mother to baby during birth plays a crucial role in shaping the postnatal microbial community. Vaginally delivered infants acquire bacteria from the mother's vaginal and faecal microbiota, leading to higher microbial diversity dominated by *Bacteroides*, *Parabacteroides*,

Escherichia and *Bifidobacterium* spp. (Bäckhed et al., 2015; Hansen et al., 2015; Hill et al., 2017; Mitchell et al., 2020; Podlesny & Fricke, 2021; Wampach et al., 2018; Yassour et al., 2018).

In contrast, infants born by caesarean section are exposed to maternal skin and external environmental microbes, resulting in lower microbial diversity and delayed colonisation by *Bifidobacterium* and *Bacteroides* spp. (Jakobsson et al., 2014; Martin et al., 2016; Mitchell et al., 2020; Penders et al., 2006; Shao et al., 2019). Instead, these infants harbour more skin-related bacteria such as *Staphylococcus* and *Clostridium* spp. (Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Penders et al., 2006), which may not persist in the gut but could influence microbial succession. Caesarean birth is also associated with an increased risk of colonisation by pathogenic bacteria like *Klebsiella* and *Veillonella* spp. (Dogra et al., 2015; Shao et al., 2019; Stockholm et al., 2016).

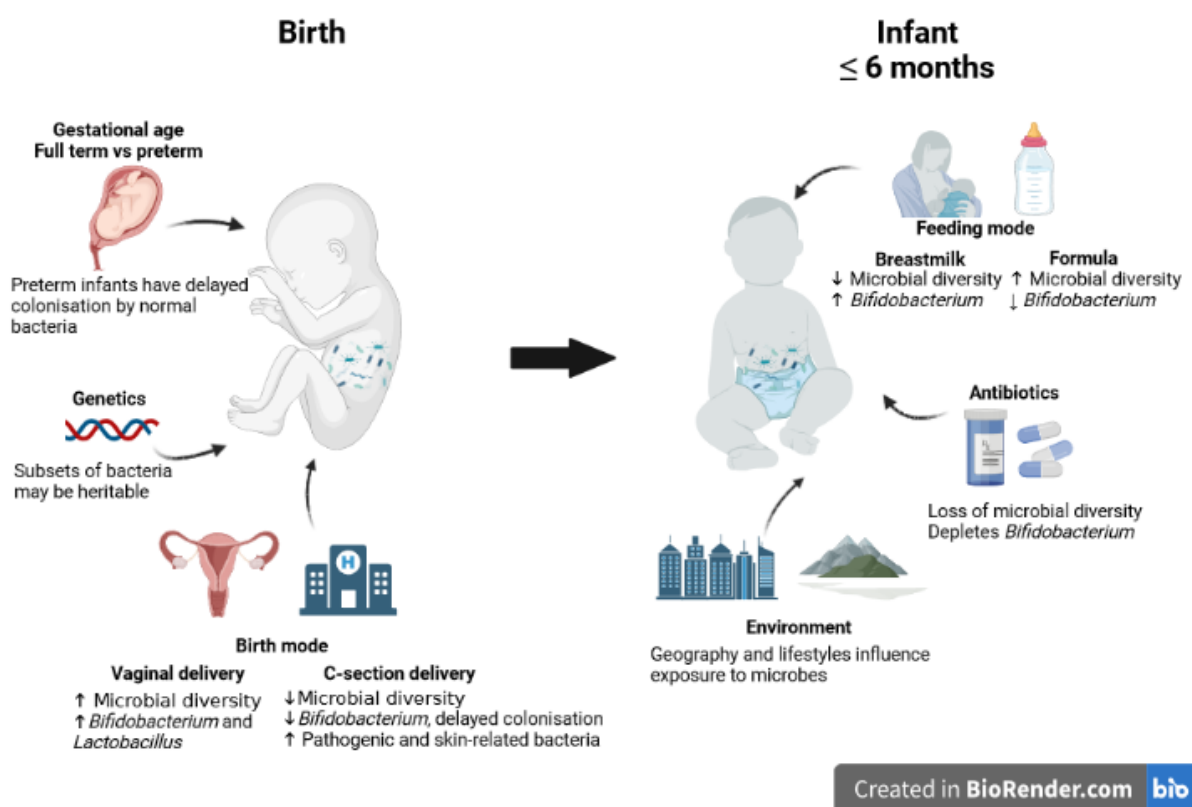


Figure 2.1. Factors influencing the initial colonisation and development of the infant bacterial gut microbiota.

Some studies indicate persistent compositional and functional differences between caesarean- and vaginally-born infants (Busi et al., 2021; Chu et al., 2017; Korpela et al., 2017; Neu & Rushing, 2011; Salminen et al., 2004; Tita et al., 2009), while others suggest microbial convergence over time (Bäckhed et al., 2015; Liu et al., 2019; Stewart et al., 2018).

Gestational age at birth also affects microbial colonisation. Infants born preterm (before 37 full weeks of gestation) often experience serious health challenges and may have some degree of immaturity in their digestive, respiratory, neurological and immune systems. Preterm infants are frequently hospitalised for prolonged periods and are exposed to various treatments, including antibiotics. In addition, preterm infants are often born by caesarean section. These factors alter the initial colonisation of their gut microbiota (Jacquot et al., 2011; Korpela et al., 2018; Penders et al., 2006; Rougé et al., 2010; Stewart et al., 2016).

Compared to full-term infants, premature infants typically have reduced microbial diversity and a lower abundance of *Bifidobacterium* and *Bacteroides* spp. Their microbiota is dominated by the *Enterobacteriaceae* family and *Enterococcus* and *Staphylococcus* genera (Arbolea et al., 2012, 2015; Barrett et al., 2013; Hill et al., 2017; Korpela et al., 2018; Rougé et al., 2010), with delayed colonisation by normal commensal anaerobic microbes (Hill et al., 2017; Milani et al., 2017), increasing susceptibility to pathogenic microbes (Jacquot et al., 2011; Korpela et al., 2018; Magne et al., 2006).

Emerging evidence suggests that host genetics plays a role in the acquisition and development of the infant's gut microbiota. Studies examining the gut microbiota of twins and triplets have shown a subset of bacteria that is heritable and affected by host genetics (Blekhman et al., 2015; Goodrich et al., 2014; Koo et al., 2019; Turpin et al., 2016). However, further research is needed to clarify the extent of genetic influence on early postnatal microbiota development.

2.2.2 Non-bacterial colonisation of the postnatal gut

While bacteria dominate the gut microbiota, viral, fungal, and archaeal communities also contribute to microbial establishment. Advancements in molecular technologies are helping to shed light on these components and their role in the early development of the gut microbiota.

2.2.3 Viral colonisation of the postnatal gut

Few studies have examined the gut virome (viral community) in healthy infants during early life. Evidence suggests viral colonisation at birth, with no detectable viral community detected in meconium and amniotic fluid (Breitbart et al., 2008; Lim et al., 2018). Viral colonisation proceeds quickly, with Virus Like Particles (VLPs) detected within a week after birth (Breitbart et al., 2008; Gregory et al., 2020; Liang et al., 2020; Lim et al., 2015; Maqsood et al., 2019).

Bacteriophages dominate the infant gut virome (Breitbart et al., 2008; Liang et al., 2020; Minot et al., 2013), suggesting initial viral colonisation is driven by prophage integration in pioneer bacteria. Bacteriophage abundance is highest in early life and decreases with age (Breitbart et al., 2008; Gregory et al., 2020; Lim et al., 2015; Sharon et al., 2013; Walters et al., 2023), with *Siphoviridae*, *Podoviridae* and *Myoviridae* families shifting to a predominantly *Microviridae* composition by 2 years of age.

Eukaryotic viruses, primarily *Anelloviruses*, are present in low abundance (Breitbart et al., 2008; Kapusinszky et al., 2020; Lim et al., 2015; Maqsood et al., 2019; McCann et al., 2018; Minot et al., 2013; Pannaraj et al., 2018; Tan et al., 2020), but increase with age, suggesting that environmental exposure primarily drives eukaryotic colonisation (Lim et al., 2015; Zuo et al., 2020). Archaeal viruses are rarely detected in the gut.

Birth mode influences virome diversity, with vaginally born infants exhibiting higher diversity (McCann et al., 2018). Viral transmission from mother to infant has been observed, but identifying

specific sources remains challenging due to limited data (Maqsood et al., 2019; Walters et al., 2023; Zeng et al., 2024).

Clear conclusions about the colonisation and transmission of the infant gut viral community are challenging due to the low abundance of viruses in the infant gut, which makes detection difficult, and incomplete viral reference databases hinder analysis.

2.2.4 Fungal colonisation of the postnatal gut

Fungi constitute a minor fraction of the gut microbiota, representing approximately 0.1% of the total microbial population (Qin et al., 2010). However, given their larger volume compared to bacteria, fungi likely constitute a sizeable portion of the gut microbiota (Schei et al., 2017). Despite increasing research into the gut fungal community, its composition and development in early life have not been fully established.

Fungal-bacterial interactions may influence the early development of the gut microbiota (Rao et al., 2021), and birth mode-specific bacteria may affect fungal colonisation (Bliss et al., 2008; Lupetti et al., 2002; Schei et al., 2017). However, the exact mechanisms of these interactions and the specific contributions of fungi are not fully understood.

The fungal community in the infant's gut is relatively unstable, with significant variation between individuals and within the same individual over time. Fungal diversity is low in early life but gradually increases with age (Schei et al., 2017; Wampach et al., 2017; Ward et al., 2017). This high variability suggests that some fungal species colonise the gut only transiently due to incidental introduction through diet and environmental exposure (James et al., 2020; David et al., 2014; Hoffmann et al., 2013; Schei et al., 2017; Wampach et al., 2017; Ward et al., 2017). For example, *Debaryomyces hansenii* is dominant in the infant gut during breastfeeding but does not persist after weaning (James et al., 2020; Schei et al., 2017).

Persistent fungal colonisers belong to phyla Ascomycota and Basidiomycota, with *Saccharomyces*, *Candida*, and *Malassezia* being the most abundant genera (Nel Van Zyl et al., 2022; Turunen et al., 2023; Wampach et al., 2017; Ward et al., 2017).

2.2.5 Archaeal colonisation of the postnatal gut

Few studies have detected archaea in the infant gut (Borrel et al., 2020; Neumann et al., 2024; Palmer et al., 2007; Wampach et al., 2017). Those present appear in low abundance and are transient, with potential sources including breastmilk (Togo et al., 2019) and bovine milk (0.13% abundance) (Hoque et al., 2019).

The dominant archaea are methanogens belonging to the orders *Methanobacteriales* and *Methanomassilliicoccales* (Borrel et al., 2020; Koskinen et al., 2017; Nadia Gaci et al., 2014). These methanogens consume fermentation by-products, such as hydrogen, carbon dioxide, formate, acetate and methyl compounds, and produce methane (Borrel et al., 2020; Hoegenauer et al., 2022). By reducing hydrogen concentrations and maintaining syntrophic relationships with other gut microbes, methanogens ensure efficient fermentation. As a result, archaea influence both the composition and activity of the gut microbiota (Kumpitsch et al., 2021; Nadia Gaci et al., 2014; Nkamga et al., 2017).

Under-representation of archaea in gut microbiome studies may be attributed to current methodological issues, which hinder the accurate detection and characterisation of archaea in the gut (Mahnert et al., 2018).

2.2.6 Development of the infant gut microbiota

Following birth, the gut microbiota composition undergoes a rapid shift. Facultative anaerobes initially dominate, consuming oxygen, making the luminal environment increasingly anoxic. This promotes colonisation by obligate anaerobes, such as *Bifidobacterium*, *Clostridia* and *Bacteroides* spp. (Bäckhed et al., 2015; Koenig et al., 2011; Martin et al., 2016; Nagpal et al., 2017). The continued

development of the gut microbiota is influenced by age-related maturation, antibiotic usage, environmental and lifestyle factors, and nutrition (breastfeeding and/or formula feeding) (Figure 2.1).

2.2.6.1 Age-related development of the gut microbiota

Host physiological maturation plays a central role in driving microbial succession during early postnatal life. The digestive system undergoes profound anatomical changes: the small intestine nearly doubles in length by one year of age, and this growth continues into adulthood (Weaver et al., 1991). This increased surface area, along with the progressive maturation of the enteric nervous system and muscular layers, enhances the gut's capacity for digestion, absorption, and motility, thereby influencing the availability of nutrients that reach the colon for microbial fermentation by gut microbes.

The intestinal epithelium undergoes substantial structural and functional development, including progressive maturation of the mucus layer, which increases in thickness and complexity of glycosylation patterns (Rokhsfat et al., 2016). As the mucus layer matures, it serves both as a scaffold and a carbon source for gut microorganisms, while gut microbes, including mucin degraders, reciprocally influence mucin gene expression, glycosylation, and secretion. This bidirectional interaction means that the maturation of the mucus layer actively shapes which microbial taxa can colonise and persist in the gut, while the microbes themselves remodel their physical environment. Additionally, intestinal transit time and peristaltic activity also shift, influencing substrate availability and microbial niche structure (Indrio et al., 2022).

The maturation of digestive function is characterised by asynchronous development of enzymes and changes in gastric pH. Beyond two weeks of age, fasting gastric pH in infants reaches adult levels. However, the frequent feeding intervals mean the stomach is rarely empty, often maintaining a higher, more neutral pH (Yu et al., 2014). Enzyme activity also follows distinct developmental trajectories: gastric lipase is mature and functional from birth. In contrast, pepsin activity is initially low and increases markedly over the first six months of life. Pancreatic lipase and pepsin mature progressively

until approximately two years of age, and bile salt concentrations increase. The maturation of digestive enzymes determines which dietary components are broken down in the small intestine, thereby shaping the substrates available for microbial fermentation and the ecological niches available to different bacterial taxa.

Host defence mechanisms that limit pathogen colonisation also develop postnatally. Gastric acid provides a barrier against ingested pathogens; however, limited secretion in early infancy may make this barrier less effective (Neu, 2007). Additionally, the progressive maturation of secretory IgA production further shapes community composition through selective coating of bacteria, promoting tolerance of commensals while limiting pathogen colonisation (Dogra et al., 2021).

Concurrently, the development of eating skills follows a predictable sequence that mirrors neurological and anatomical maturation. Newborns possess a reflexive suck-swallow-breathe cycle that is functional from birth, which facilitates liquid feeding. Over the first six months, the infant's orofacial motor control matures, with the ability to coordinate jaw, tongue, and lip movements improving. This enables the transition from sucking to spoon-feeding pureed foods. The subsequent eruption of teeth and maturation of salivary glands support the transition to a family diet, with salivary amylase activity increasing significantly between three and six months of age. The transition from sucking to chewing alters food particle size and structure, influencing how substrates reach the colon for microbial fermentation.

Together, these physiological, anatomical, and functional changes create a temporally structured habitat that constrains and directs microbial succession. The infant's gut microbial composition increases in number and diversity with age, and by around three years of age, the gut microbiota will attain an adult-like composition. The coordination of GI tract development, digestive maturation, and microbial succession helps to explain why microbiota developmental trajectories remain broadly predictable even across diverse populations, dietary and environmental contexts (Bottino et al., 2024; Hickman et al., 2024).

2.2.6.2 Antibiotic exposure

Postnatal antibiotic exposure disrupts gut microbiota, inhibiting microbial growth, reducing diversity, and delaying the colonisation of commensal microbes (Bokulich et al., 2016; Eck et al., 2020; Fouhy et al., 2012; Korpela et al., 2020; Mangin et al., 2010; Reyman et al., 2022; Tanaka et al., 2009; Yassour et al., 2016; Zhu et al., 2017).

The effects and recovery of the gut microbiota after antibiotic treatment are dependent on the antibiotic spectrum (broad or narrow), dose and duration of exposure (Fouhy et al., 2012; Koenig et al., 2011; Korpela et al., 2020; Zwiittink et al., 2018). Antibiotics administered during critical developmental windows can cause long-term disruption of gut microbiota, including depletion of taxa such as the *Bifidobacterium* genus. Such disruptions can interfere with the normal succession of the gut microbiota, affecting the infant's overall developmental trajectory (Fouhy et al., 2012; Korpela et al., 2016; Uzan-Yulzari et al., 2021).

Additionally, some gut microbes may also develop antibiotic resistance and possibly transfer these resistant genes to pathogens through horizontal gene transfer, potentially increasing the infant's susceptibility to infection (Gibson et al., 2016; Greenwood et al., 2014; Tapiainen et al., 2019).

2.2.6.3 Environment and lifestyle

Daily contact between infants and their family members facilitates the postnatal transmission of microbes from the oral cavity and skin (Valles-Colomer et al., 2022). Infants with siblings generally exhibit higher levels of *Bifidobacterium* spp. and greater gut microbial diversity compared to those without siblings (Azad et al., 2013; Laursen et al., 2015; Martin et al., 2016; Penders et al., 2006). However, the specific influence of family size, structure, and birth order on gut microbiota remains inconclusive (Fouhy, et al., 2012).

Exposure to different environmental conditions also shapes the gut microbiota. Differences in colonisation patterns are observed between infants raised in industrial versus developing countries

(De Filippo et al., 2010; Fallani et al., 2010; Grześkowiak et al., 2012; Lin et al., 2013; Vatanen et al., 2016; Yatsunen et al., 2012) and between infants with different lifestyles (farms versus urban living, pets versus no pets) (Azad et al., 2013; Martin et al., 2016; Normand et al., 2011). These differences are mainly attributed to hygiene standards, so infants from rural areas and those with pets are more exposed to a wide array of environmental microbes.

2.2.6.4 Feeding mode

Feeding mode plays a key role in shaping the infant gut microbiota, with distinct differences between breastfed and formula-fed infants.

Breastmilk provides a complex bacterial community that can be vertically transferred from mother to infant (Asnicar et al., 2017; Biagi et al., 2017; Fehr et al., 2020; Lackey et al., 2019; Murphy et al., 2017; Pannaraj et al., 2017), along with components that support the growth of particular bacterial species, such as *Bifidobacterium* and *Bacteroides* spp. As a result, breastfed infants typically have a microbiota dominated by the *Bifidobacterium* genus, along with higher abundances of *Enterobacteriaceae*, *Bacteroidaceae* and *Clostridiaceae* families (Azad et al., 2013; Bäckhed et al., 2015; Bezirtzoglou et al., 2011; Fan et al., 2013; Lee et al., 2015; Liu et al., 2016; Lok et al., 2024; Ma et al., 2020; Penders et al., 2006; Stewart et al., 2018).

In contrast, formula-fed infants tend to have greater microbial diversity but lower *Bifidobacterium* abundance. Instead, their gut microbiota often includes taxa from the *Proteobacteria* phylum, the *Clostridiales* order, and the *Bacteroides* genus (Boudry et al., 2021; Hascoët et al., 2011; Ma et al., 2020).

Breastmilk and/or a formula-based diet is gradually supplemented with complementary foods while the infant is weaned from around 6 months until two years of age. This is an important milestone in the infant gut microbiota development, as major changes in the composition and diversity of the gut microbiota are induced (Homann et al., 2021; Laursen et al., 2016; Raspini et al., 2021; Thompson et

al., 2015; Vallès et al., 2014a). Early colonisers are progressively replaced by other microbial genera that can metabolise the new dietary components introduced during complementary feeding. After introducing complementary foods, the bacterial succession continues gradually, diversifying, and a stable state of adult-like gut microbiota is established at approximately three years of age.

Understanding the impact of early postnatal nutrition on gut microbiota development is crucial for optimising infant health and wellbeing.

2.3 Nutrition and Infant Microbiota Development

2.3.1 Breastmilk composition

Human breastmilk provides optimal nutrition as it has a unique composition of macronutrients (carbohydrates, proteins, and lipids), micronutrients (vitamins and minerals), digestive enzymes, hormones, and immunological components. As a result, the World Health Organisation (WHO) recommends exclusive breastfeeding for the first six months of life and sustained breastfeeding for up to two years of age to promote optimal growth, development, and health (World Health Organisation, 2023).

Breastmilk composition is dynamic, varying throughout each feeding session, across lactation stages, and from mother to mother (Akanalçı & Bilici, 2024; Bilston-John et al., 2021; Khan et al., 2013; Paulaviciene et al., 2020; Pu et al., 2023; Saarela et al., 2005; Soyyılmaz et al., 2021; Takumi et al., 2022; Thum et al., 2021, 2022; Vinjamuri et al., 2022). Colostrum, the first milk produced by mothers, is secreted in small quantities during the first two to four days postpartum and is rich in proteins and immunological components (secretory IgA, lactoferrin, leukocytes and growth factors). Transitional milk, produced from five days to two weeks postpartum, is similar in composition to colostrum but is produced in greater quantities. From two weeks postpartum, human milk is considered fully mature with increased production as well as higher carbohydrate and lipid content (Andreas et al., 2015;

Ballard & Morrow, 2013; Butte et al., 2002; Casey et al., 1986; Castellote et al., 2011; Moltó-Puigmartí et al., 2011; Sever et al., 2015; Yang et al., 2018).

Mature breastmilk typically consists of 87% water, 7% carbohydrates, 3.8% fat, 1% protein and 0.2% mineral constituents, providing around 65-70 kcal per 100 mL of energy (Guo, 2014, 2021; John et al., 2019; Kunz et al., 1999; Martin et al., 2016; Picciano, 2001). The detailed composition of mature breastmilk can be found in Table 2.1.

Lactose is the dominant carbohydrate in breastmilk and the most abundant macronutrient, contributing approximately 40% of its total energy content (Butte et al., 1984; Guo, 2014, 2021; Lönnerdal et al., 1976; Neville, 1999; Nommsen et al., 1991; Saarela et al., 2005). Unlike adults, who primarily metabolise glucose, infants rely on lactose due to their immature digestive systems. Lactose concentrations remain relatively stable throughout lactation, increasing from an average of 56 g/L in colostrum to an average of 69 g/L in mature milk, whereas other macronutrients fluctuate more significantly (Coppa et al., 1993; Kim & Yi, 2020; Wojcik et al., 2009).

Lipids are the second most abundant macronutrient in breastmilk, providing 40-55% of the total energy (Grote et al., 2016; Koletzko et al., 2001; Wan et al., 2010). Their concentration increases from 15-20 g/L in colostrum to 35-40 g/L in mature milk (Ballard & Morrow, 2013; Grote et al., 2016; Mosca & Gianni, 2017), and fluctuates throughout the day and within feedings. Overall concentrations decline as the day progresses (Italianer et al., 2020; Prentic et al., 1981; Selvalatchmanan et al., 2021), and hindmilk contains two to three times higher lipid concentration than foremilk (Daly et al., 1993; Khan et al., 2013; Saarela et al., 2005; Valentine et al., 1994).

Table 2.1. Composition of mature breastmilk (adapted from Guo (2021) and (Kim & Yi, 2020)), and the composition of bovine infant formula (adapted from the Codex Alimentarius Standard for Infant Formula CXS 72-1981 www.fao.org).

	Mature Breastmilk	Infant Formula
Energy	65-70 kcal/100mL	60-70 kcal/100mL
Macronutrients (g/L)		
Carbohydrates	60 - 70	54 - 98
Lipids	35 - 40	26.4 - 42
Protein	8 - 10	10.8 - 21
Micronutrients (mg/L)		
Vitamin B8	300	24 - 28
Vitamin C	47	60 - 70
Vitamin E	5.4	3 - 3.5
Vitamin B5	2.1	2.4 - 2.8
Niacin	1.7	1.8 - 2.1
Vitamin A	0.53	0.36 - 1.26
Vitamin B2	0.37	0.48 - 0.56
Carotene	0.24	-
Vitamin B1	0.15	0.36 - 0.42
Vitamin B6	0.1	0.21 - 0.245
Folate	0.043	0.06 - 0.07
Vitamin K	0.015	0.024 - 0.028
Vitamin B7	0.007	0.009 - 0.0105
Vitamin D	0.001	0.006 - 0.0175
Vitamin B12	0.0003	0.0006 - 0.0007
Minerals (mg/L)		
Potassium	400 - 550	360 - 1260
Chloride	400 - 450	300 - 1120
Calcium	200 - 250	300 - 350
Sodium	150 - 250	120 - 420
Phosphorus	120 - 140	150 - 1750
Magnesium	30 - 35	30 - 35
Iron	0.3 - 0.7	2.7 - 3.15
Iodine	0.14 - 0.15	0.06 - 0.07
Selenium	0.01 - 0.025	0.006 - 0.007
Manganese	0.003 - 0.004	0.006 - 0.007
Zinc	0.001 - 0.003	3 - 3.5
Copper	0.0001 - 0.0003	0.21 - 0.245

Protein concentration declines from 14-16 g/L in colostrum to 8-10 g/L in mature milk (Guo, 2014; Saarela et al., 2005) There are three major classes of proteins in breastmilk: whey, casein and mucin. The whey-to-casein ratio changes depending on the stage of milk: whey proteins decrease from 80% in colostrum to 60% in mature milk, while caseins increase from 20% to 40% (Forsum, 1974; Haschke et al., 2016; Lönnerdal, 2003; Lönnerdal et al., 2016). The main whey proteins include α -lactalbumin,

lactoferrin, lysozyme, and secretory immunoglobulin A (SIgA) (Ballard & Morrow, 2013), with α -lactalbumin constituting 20-25% of the total protein (Lönnerdal, 2003). The casein fraction includes α -, β - and κ -casein (Forsum, 1974; Lönnerdal et al., 2016).

Breastmilk provides most essential micronutrients for the first six months, except for vitamin D and vitamin K, which are present in low concentrations (0.001 mg/L and 0.015 mg/L, respectively) (Cancela et al., 1986; Chebet et al., 2022; Clarke et al., 2022; Kries et al., 1987; Shearer & Clarke, 2021; við Streym et al., 2016). Since infants have limited vitamin stores acquired during pregnancy that deplete quickly, breastmilk alone cannot meet their vitamin D and K requirements. While vitamin K is supplemented shortly after birth, vitamin D is not routinely given to infants in New Zealand.

Minerals in breastmilk play a crucial role in numerous physiological processes, serving as essential components of enzymes and contributing to the structure and function of various biological molecules. In breastmilk, the concentration of most minerals decreases as lactation progresses. This decline is notable for zinc, which decreases from 6.4 mg/L in colostrum to 1.98 mg/L in mature milk (Rios-Leyvraz & Yao, 2023), and iron, which decreases from 1 mg/L to 0.33 mg/L (Guo, 2021; Trinta et al., 2020). Despite this reduction, the bioavailability of these minerals in breastmilk remains high, allowing infants to absorb and use them efficiently during early development.

Beyond its role as a source of nutrition, breastmilk contains a diverse array of non-nutritive components that support infant development, immunity, and gut health. These include HMOs, immune factors, growth factors, and hormones.

HMOs are the third most abundant component in breastmilk, comprising about 1% of its total composition (Andreas et al., 2015; Guo, 2021). Although indigestible by infants, HMOs play a key role in shaping the infant gut microbiota, selectively promoting the growth of beneficial bacteria, inhibiting pathogenic bacteria, and maintaining gut barrier integrity (Bode, 2015; De Leoz et al., 2015; Garrido et al., 2013, 2015; Marcobal et al., 2010; Ruiz-Moyano et al., 2013; Thomson et al., 2018; Walsh et al., 2023). Breastmilk contains up to 200 structurally distinct HMOs, all derived from

a lactose core and five monosaccharides (glucose, galactose, N-acetylglucosamine, fucose and sialic acid derivative N-acetyl-neuraminic acid) which are joined by glycosidic linkages. Their concentration declines with lactation, from 20-25 g/L in colostrum to 5-15 g/L in mature milk (Elwakiel et al., 2018; Menzel et al., 2021; Sprenger et al., 2017; Thum et al., 2021; Thurl et al., 2010).

The composition and concentration of HMOs in breastmilk vary based on maternal genetics, particularly secretor statuses and Lewis blood groups (Kunz et al., 2017; Lefebvre et al., 2020; Tonon et al., 2019; Stahl et al., 2001; Thurl et al., 2010). These genetic factors determine whether a mother can express the enzymes α -1-2-fucosyltransferase FUT2 (secretor gene) and/or α -1-3/4-fucosyltransferase FUT3 (Lewis gene), which influence the presence and diversity of fucosylated HMOs (Tonon et al., 2019; Musumeci et al., 2006; Thurl et al., 2010). Secretor-positive and Lewis-positive mothers (those expressing both FUT2 and FUT3) produce breastmilk with a higher concentration and more diverse HMOs than the other milk phenotypes. Mothers who are secretor-negative or Lewis-negative produce fewer fucosylated HMOs. Other factors, such as geographic location, ethnicity, and seasonal variations, also contribute to variations in HMO composition (Azad et al., 2018; McGuire et al., 2017).

Immunological components in breastmilk provide immediate and long-term immunity and protect against infections and inflammation. These include immune cells (macrophages, T-cells, stem cells, and lymphocytes) (Järvinen & Suomalainen, 2002; Patki et al., 2010; Yagi et al., 2010), immunoglobulins (IgA, IgG, IgM, IgE, and IgD) (Bridgman et al., 2016; Corthésy, 2009; Gao et al., 2012), and non-specific immune components (lactoferrin, lactadherin, bile salt-stimulating lipase, mucin, cytokines, and chemokines) (Brenmoehl et al., 2018; Garofalo, 2010; Landberg et al., 2000; Lönnerdal, 2003; Munblit et al., 2016; Newburg et al., 1998; Schirmbeck et al., 2022; Yolken et al., 1992).

Growth factors are not directly part of the immune system, but they play a critical role in the maturation and repair of the gut mucosa (Chang & Chao, 2002; Hirai et al., 2002; Kobata et al., 2008). Hormones in breastmilk (leptin, adiponectin, insulin, cortisol) interact with the immune system to help regulate the infant's metabolism, appetite, and growth (Casabiell et al., 1997; Fields & Demerath, 2012; Savino et al., 2009).

Colostrum is particularly rich in secretory IgA (sIgA) and epidermal growth factor (EGF) (Munblit et al., 2017, 2018), ensuring early gut and immune protection for neonates, who cannot produce adequate immune responses for the first 30 days.

Together, these non-nutritive components contribute to microbial colonisation, immune development, and metabolic regulation, reinforcing breastmilk's role beyond basic nutrition.

2.3.1.1 Breastmilk and the gut microbiota

Breastmilk is an important medium for transferring microbes from mother to infant. An infant consuming approximately 800 mL of breastmilk ingests approximately 1×10^5 and 1×10^7 microbes daily (Jeurink et al., 2013). The most commonly found bacteria in breastmilk include species from the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Bifidobacterium* (Cabrera-Rubio et al., 2012; Ding et al., 2022; Jost et al., 2014, 2015; Kim & Yi, 2020; Pannaraj et al., 2017; Treven et al., 2019).

The exact origins of microbes are debated. While some microbes may originate from retrograde flow during breastfeeding (Biagi et al., 2017; Laursen, Pekmez, et al., 2021; Williams et al., 2019), some specific microbes appear to inhabit breast tissue, potentially colonising milk ducts (Urbaniak et al., 2014). Additionally, microbes from the maternal gut may translocate to the mammary glands via the entero-mammary pathway, further contributing to the microbial community in breastmilk (Jost et al., 2014; Kordy et al., 2020).

In addition to microbial transfer, HMOs further facilitate colonisation and persistence of specific bacteria in the infant gut. Resistant to digestion, HMOs reach the large intestine largely intact (Chaturvedi et al., 2001; Coppa et al., 2001; Engfer et al., 2000), where they serve as energy for some bacterial species. The *Bifidobacterium* genus is highly specialised for utilising HMOs. *Bifidobacterium longum* subsp. *infantis* is especially adept at using HMOs, possessing all five HMO-degrading gene clusters, making it a “superuser” of HMOs (Sela et al., 2008; Underwood et al., 2015). Other *Bifidobacterium* spp. include *B. breve*, *B. bifidum*, *B. longum*, and *B. catenulatum* subsp. *kashiwanohense* and *B. catenulatum* can also degrade HMOs, though their capacities vary due to genetic differences (Gotoh et al., 2018; LoCascio et al., 2007; Turrone et al., 2010).

By fermenting HMOs, *Bifidobacterium* spp. produce organic acids and short-chain fatty acids (SCFA), which support the development of beneficial microbiota and create energy sources for other bacteria that are non-HMO degrading species, creating a symbiotic microbial environment (Devika & Raman, 2019; Smith et al., 2019; Turrone et al., 2018).

Bifidobacteria are not the only species capable of utilising HMOs. Taxa from the *Lactobacillus* genus, *Akkermansia muciniphila*, certain *Staphylococcus* species, and certain *Bacteroides* species (*B. fragilis*, *B. vulgatus*) have some capacity to degrade HMOs (James et al., 2019; Kostopoulos et al., 2020; Marcobal et al., 2010; Rodríguez-Díaz et al., 2011; Salli et al., 2021; Thongaram et al., 2017).

These bacteria help inhibit pathogenic microbes by competing for nutrients and attachment sites and producing antimicrobial compounds (Lawson et al., 2020; Marcobal et al., 2010; Sakanaka et al., 2020; Salli et al., 2021; Yu et al., 2013). Additionally, some HMOs directly prevent pathogen adhesion by mimicking host cell receptors, blocking their ability to colonise the infant gut (Hill et al., 2021; Moore et al., 2021; Morozov et al., 2018).

Breastmilk’s impact extends beyond shaping microbiota composition and activity. Breastfeeding is associated with an enrichment of microbial genes related to carbohydrate and lipid metabolism,

including fatty acid biosynthesis, oxidative phosphorylation, and vitamin B synthesis (Bäckhed et al., 2015; Stewart et al., 2018).

Scientific evidence indicates that breastmilk is critical in establishing a healthy gut microbiota through microbial transmission, HMO degradation by various species, and the enrichment of microbial genes involved in carbohydrate metabolism, immune modulation, and gut barrier function.

2.3.2 Infant Formula Composition

Infant formula is intended as a suitable substitute or supplement for breastmilk when breastfeeding is not possible. The composition of infant formula is strictly regulated by global guidelines established by the Codex Alimentarius Commission and the World Health Organisation to ensure adequate nutrient concentrations (Koletzko et al., 2005). Despite significant advances in formula development, fully replicating its complex nutritional, immunological, and functional benefits remains challenging. Another major limitation of infant formula is its lack of the dynamic, evolving composition of breast milk, which changes throughout daily feedings to meet the developmental needs of infants.

Most infant formulas are derived from bovine milk. The differences in the composition of breastmilk and a standard bovine milk formula are summarised in Table 2.1. Bovine milk has a different protein, lipid and mineral content than human breastmilk (Guo, 2021; Park & Haenlein., 2013; Pietrzak-Fiećko & Kamelska-Sadowska, 2020; Roy et al., 2020). Standard bovine formula tends to have a higher protein content, which has been linked to rapid weight gain and an increased risk of obesity later in life (Günther et al., 2007; Rolland-Cachera et al., 1995). The caseins in bovine milk form firm curds, which are harder for infants to digest. Additionally, bovine milk contains β -lactoglobulin, a dominant whey protein absent in breastmilk. Both casein and β -lactoglobulin can act as allergens and contribute to bovine milk allergies in susceptible infants (Docena et al., 1996; Villa et al., 2018).

Bovine milk also has higher levels of some fatty acids not abundant in human breastmilk, and minerals, particularly calcium, phosphorus, sodium, potassium, chlorine and magnesium, but lower

levels of iron and copper. While both human and bovine milk contain relatively similar levels of vitamins, the chemical form of many vitamins can differ, affecting their absorption and bioactivity.

Furthermore, essential bioactive compounds, such as lactoferrin, immunoglobulin A (IgA) and lysozyme, are only in small quantities in bovine milk. While bovine milk contains some oligosaccharides, their concentration and diversity are significantly lower than in human breastmilk (Aldredge et al., 2013; Chauhan et al., 2023; Gopal & Gill, 2000; Shi et al., 2021).

To compensate for the oligosaccharide deficiency in bovine milk, prebiotics and probiotics may be added to the formula to simulate the beneficial effects of oligosaccharides and bacteria found in human breastmilk. However, further clinical studies are needed to confirm their impact on the gut microbiota (Alliet et al., 2016; Bertelsen et al., 2016; Li et al., 2021; Vandenplas et al., 2014). These prebiotics include galactooligosaccharides (GOS), polydextrose (PDX), lactulose (LOS), inulin, and fructooligosaccharides (FOS), along with synthesised HMOs (Bode et al., 2016; Pérez-Escalante et al., 2022; Vandenplas et al., 2015, 2022).

Lactose-free formulas, often used for lactose-intolerant infants, replace lactose with refined sugars such as glucose, dextrose, or maltodextrin. Although these formulas alleviate symptoms of lactose intolerance, refined sugars lack the prebiotic benefits of lactose and may affect the glycaemic load of the formula, potentially impacting the infant's gut health (Romero-Velarde et al., 2019; Slupsky et al., 2017).

In addition, specialised formulas are available for infants with specific dietary needs and severe allergies to bovine milk, including hydrolysed protein formulas and non-bovine milk-based formulas (Berg et al., 2017; Cabana, 2017; Knol et al., 2019).

Non-bovine milk-based formulas, particularly goat milk formulas, have gained popularity due to their closer composition resemblance to human milk fat composition and their lower allergenic potential (Gallier et al., 2020). Goat milk contains higher levels of short-chain fatty acids (SCFAs), medium-

chain fatty acids (MCFAs), and sn-2 palmitic acid, which are more easily digested. Studies have shown that goat milk forms a softer curd during digestion, making it gentler on the infant's digestive system (Hodgkinson et al., 2019; Maathuis et al., 2017). While goat milk formulas are considered safe, other non-bovine milk sources, such as sheep, donkey, horse, and camel milk, require further study to ensure their safety and efficacy in infant nutrition (Kumar et al., 2016; Li et al., 2020; Souroullas et al., 2018).

Plant-based formulas are also an option for infants who cannot tolerate bovine milk or for families preferring a vegan diet. Soy-based formula use is limited due to concerns about the oestrogenic effects of isoflavones found in soy, despite some studies showing normal growth in infants fed soy-based formulas (Gilchrist et al., 2010; Rozman et al., 2006; Vandenplas, 2017). Rice protein-based formulas offer alternatives but have their concerns, such as the potential presence of arsenic and an unpleasant taste that may not be well tolerated by infants (Vandenplas et al., 2014).

2.3.2.1 Infant formula and the gut microbiota

As discussed in section 1.2.3.3, there are notable differences between the gut microbiota of breastfed and formula-fed infants. These differences are influenced by formula composition, including milk source, protein type, and the presence or absence of non-nutritive components.

Studies suggest that modifying formula to reduce certain proteins and minerals, particularly by increasing the proportion of whey proteins, can result in a gut microbiota more similar to that of breastfed infants (Hascoët et al., 2011; Rochat et al., 2007; Ruiz-Ojeda et al., 2023). Additionally, Tannock and colleagues compared infants fed whole caprine milk formula, whey-adjusted bovine milk formula, and breastmilk, finding that infants fed caprine milk formula more closely resembled breastfed infants in microbial diversity (Tannock et al., 2013). Similar results were reported in another study (Gallier et al., 2020), suggesting that caprine milk's unique protein and fat composition foster a more favourable microbiota profile.

The type of prebiotic included in formula influences the growth of gut microbes in different ways. Formulas containing GOS, FOS, GOS/FOS mixtures, and oligofructose (OF) have been effective in promoting beneficial bacteria like those from *Bifidobacterium* and *Lactobacillus* genera (Béghin et al., 2021; Civardi et al., 2017; Holscher et al., 2012; Lee et al., 2015; Matsuki et al., 2016; Nomayo et al., 2020; Paineau et al., 2014; Salminen et al., 2016; Salvini et al., 2011; Sierra et al., 2015; Veereman-Wauters et al., 2011; Wernimont et al., 2015; Yao et al., 2014). However, a few studies indicate no difference in the abundance of *Bifidobacterium* spp. between these infant formula-supplemented groups and their controls (Giovannini et al., 2014; Vivatvakin et al., 2010; Xia et al., 2012). Other prebiotics, like inulin, maltodextrin, and polydextrose (PDX), are associated with poorer growth of these bacteria (Watson et al., 2013). Some studies have demonstrated that prebiotic-supplemented formula not only stimulates the growth of *Bifidobacterium* and *Lactobacillus* spp., but also reduces the prevalence or colonisation of pathogenic *Clostridia difficile* (Holscher et al., 2012; Huet et al., 2016; Sierra et al., 2015). Among the studies that assessed faecal SCFA, propionic, butyric, and isovaleric acid concentrations were lower, while acetic acid concentrations were higher in infants fed with formula supplemented with prebiotics (Béghin et al., 2021; Holscher et al., 2012; Huet et al., 2016; Sierra et al., 2015).

Recent developments in HMO-enriched formulas have shown promise in promoting a gut microbiota composition similar to breastfed infants. Formulas containing 2'-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnt) have been linked with increased abundance of *Bifidobacterium* spp. and reduced abundance of pathogenic bacteria (Alliet et al., 2016; Berger et al., 2020; Borewicz & Brück, 2024; Gold et al., 2022; Reverri et al., 2018). Emerging research suggests that formulas containing multiple HMOs may more effectively replicate the complex oligosaccharide profile of human milk (Bosheva et al., 2022).

The effects of probiotic supplementation in formula on the gut microbiota are inconsistent. While some studies suggest that probiotics, particularly *Bifidobacterium* and *Lactobacillus* species, may aid early colonisation, their effects are often short-lived. The correlation between *Bifidobacterium*

supplementation and faecal *Bifidobacterium* levels is generally weak (Gil-Campos et al., 2012; Maldonado et al., 2019; Papagaroufalis et al., 2014; Wu et al., 2016). In contrast, infants consistently exhibit an increased abundance of *Lactobacillus* spp. in faecal samples, regardless of the probiotic strain supplemented (Garcia Rodenas et al., 2016; Gil-Campos et al., 2012; Maldonado et al., 2019; Papagaroufalis et al., 2014). However, probiotic supplementation does not significantly impact faecal SCFA concentrations, as probiotics pass through the gut without sustained colonisation due to factors such as insufficient nutrients for growth and persistence, competition with native microbes, and host-mediated factors such as adhesion (Z. Chen et al., 2025; Monteagudo-Mera et al., 2019).

Formulas containing symbiotics (a combination of prebiotics and probiotics) show potential in enhancing the abundance of *Bifidobacterium* spp. (Abrahamse-Berkeveld et al., 2016; Cooper et al., 2016; Meli et al., 2014; Phavichitr et al., 2021; Radke et al., 2018; Simeoni et al., 2016). However, the impact on other gut bacterial genera, including *Lactobacillus*, *Clostridium*, *Staphylococcus*, and SCFAs, has been less consistent.

Overall, the effects of supplemented formulas are variable, due to differences in formulations, dosages, and individual responses.

2.3.3 Complementary feeding

By six months of age, breastmilk alone is insufficient to meet the full energy and nutrient requirements of infants (Butte et al., 2002; Prell & Koletzko, 2016). Consequently, infants need additional foods to support their increasing energy and nutrient requirements. This process is known as complementary feeding (Dewey, 2003).

Figure 2.2 outlines how breastmilk meets nutrient requirements from birth to 6 months and subsequently decreases from 7 to 12 months, with nutrient gaps emerging most significantly for iron and zinc (Figure 2.2) (McKeen et al., 2019). This is due to the depletion of iron stores (Friel et al.,

2018) and a decline in zinc levels throughout lactation (Ackland & Michalczyk, 2016; Krebs & Hambidge, 1986).

The timing and composition of complementary feeding is crucial. WHO recommends beginning complementary feeding at six months (WHO, 2023). This shift is supported by anatomical, physiological, and neurodevelopmental milestones for handling solid foods (Le Révérend et al., 2014), and the maturation of digestive enzymes – such as salivary α -amylase (Ben-Aryeh et al., 1984, 1990; Rossiter et al., 1974), gastric lipase and pepsin (Agunod et al., 1969; Lebenthal et al., 1983) and pancreatic amylase and lipase (Challacombe et al., 1975; Cleghorn et al., 1988; Otsuki et al., 1977) – necessary for starch, protein, and fat digestion.

The consistency, frequency and diversity of complementary foods are also essential for proper nutrition. Energy density and nutrient content determine how often and in what proportions these foods should be given. WHO recommends starting with pureed, mashed, or semi-solid foods 2-3 times daily at six months, gradually increasing the frequency, portion size, and consistencies after eight months (C. West, 2017; WHO, 2023). A balanced and varied diet that includes meat, dairy, eggs, legumes, fruits, vegetables and grains is crucial to meet the infant's nutritional needs (Lutter et al., 2021; WHO, 2005, 2023). Greater dietary diversity enhances nutrient absorption and promotes food acceptance in later childhood by exposing infants to various tastes and textures (Birch et al., 1998).

Complementary feeding is highly influenced by cultural, individual, and socioeconomic factors, which shape feeding practices and types of foods introduced (Akinsola et al., 2017; Caroli et al., 2012; Farella et al., 2020; Fein et al., 2008; Fu et al., 2018; Gatica-Domínguez et al., 2021; Jones, 2016; Komninou et al., 2019; Simeone et al., 2022; Sirkka et al., 2022).

Globally, starchy foods such as infant cereals, potatoes, and bananas are the most commonly consumed complementary foods, with 79% of children aged 6-23 months consuming on them. While starchy foods are convenient and frequently used during complementary feeding, they are often

relatively low in nutrient density and may be insufficient to meet infants' full nutrient requirements, particularly for key micronutrients like iron, zinc, and vitamin B12 (WHO, 2023).

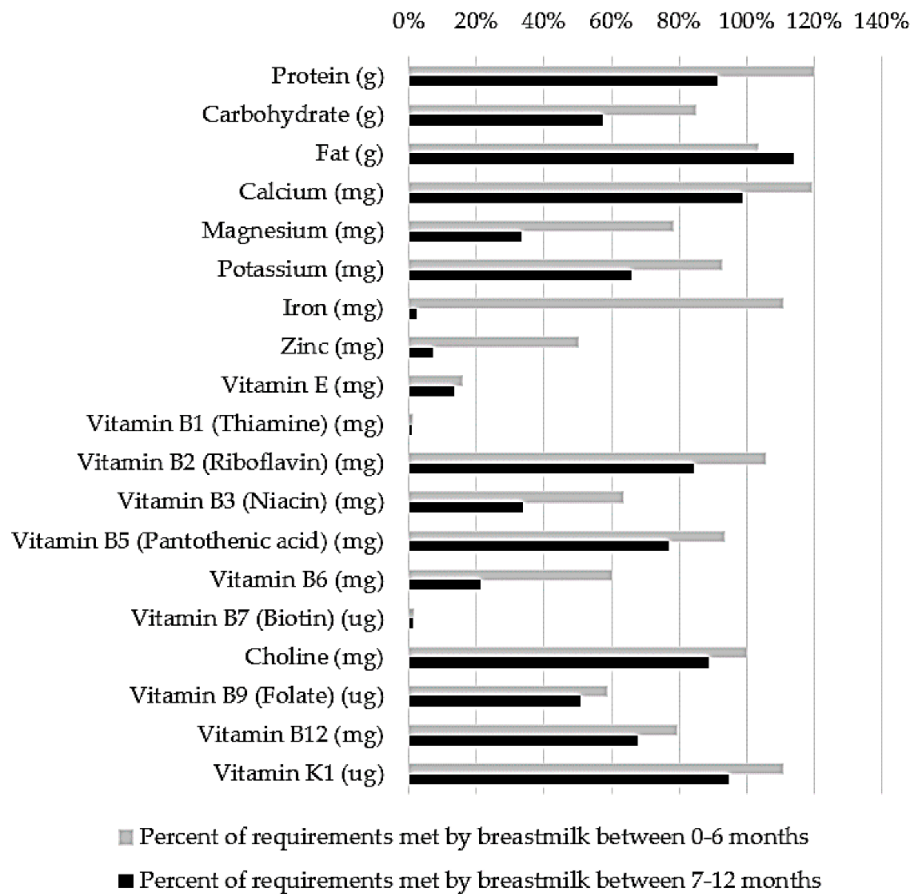


Figure 2.2. The recommended daily intakes that are met via average daily breastmilk consumption (750 mL from 0 to 6 months and 800 mL from 7 to 12 months) (Reproduced from McKeen et al. (2019), under a Creative Commons Attribution license (CC BY 4.0).

There is also an increased consumption of unhealthy, ultra-processed foods and beverages, including commercially prepared infant foods that are high in free sugars, salt, saturated and trans fats (Cogswell et al., 2015; Foterek et al., 2015; Garcia et al., 2016; Haszard et al., 2024; Hinnouho et al., 2023; Katiforis et al., 2021; Mesch et al., 2014; Mir-Marqués et al., 2015; Pries et al., 2019; Swanepoel et al., 2019). Early exposure to these unhealthy, sweet, and salty foods reinforces a preference for less nutritious options (Chambers, 2016; Foterek et al., 2015; Grimm et al., 2014), and

displaces nutrient-rich foods, contributing to poor health outcomes (Brown et al., 2024; Costa et al., 2018; Emmett & Jones, 2015; Rauber et al., 2015; Rousham et al., 2022). Early and repeated exposure to a varied diet, particularly nutrient-dense fruits and vegetables, can enhance acceptance of these flavours, promoting greater dietary diversity and healthier eating habits later in life (Coulthard et al., 2010; Hetherington et al., 2015; Maier et al., 2007; Maier et al., 2008; Remy et al., 2013).

Breastmilk remains an important source of nutrients throughout the complementary feeding period, particularly for energy, protein, vitamin A, calcium and riboflavin. Thus, WHO recommends continued breastfeeding for up to two years (WHO, 2024). However, in cases where breastfeeding stops before then, alternative milk beverages, including infant formula or fortified dairy products, can help provide essential nutrients (Csölle et al., 2021).

Conclusions about the optimal type and quantity of complementary foods are challenging to draw due to significant inconsistencies in study designs and the wide variety of complementary foods examined (English et al., 2019). Additionally, studies differ in the age of infants, timing of dietary assessment and duration of feeding interventions.

2.3.3.1 Complementary feeding and the gut microbiota

As discussed in Section 2.2.6.4, the gut microbiota follows a predictable, age-dependent developmental trajectory that is largely conserved across populations. The introduction of complementary foods acts as a major driver that accelerates this intrinsic maturation process. Although extensive research has focused on the effects of breastmilk and formula feeding on the infant gut microbiota, comparatively little attention has been given to the impacts of complementary feeding and the cessation of breastmilk which occurs in conjunction with complementary feeding.

As the diet expands, infants acquire a diverse microbial community capable of producing the necessary enzymes and metabolic pathways required to efficiently break down these complex dietary components (Anthamatten et al., 2023; McKeen et al., 2022; Ouyang et al., 2023). In terms of the

substrates available to the colonic microbiota, the primary carbohydrates shift from lactose and HMOs in breastmilk to non-digestible complex plant-derived polysaccharides found in fruit, vegetables, and grains (Bernal et al., 2013; Jovanovic-Malinoversuska et al., 2014). The composition and chemical structures of these polysaccharides and dietary fibres are complex and highly diverse, requiring specific bacteria and enzymes for their digestion (Parkar et al., 2021; Payling et al., 2020). Since the human gut has limited genes encoding for these enzymes (El Kaoutari et al., 2013), most complex carbohydrate digestion depends on enzymes from the gut microbiota, which harbour tens of thousands of carbohydrate-active enzymes (Flint et al., 2012; Koenig et al., 2011; Vaishampayan et al., 2010; Xu et al., 2023).

Microbes specialised in breaking down complex carbohydrates, including members of the *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroidaceae* families, increase in abundance with complementary feeding (Bäckhed et al., 2015; Bergström et al., 2014; Borowitz, 2021; Laursen et al., 2016, 2017; Roswall et al., 2021; Tannock, 2021). As breastmilk and/or formula intake decreases, so does the abundance of HMO-degrading *Bifidobacterium* species. However, certain *Bifidobacterium* species, such as *B. longum*, *B. adolescentis*, and *B. catenilatum*, continue to persist in the gut microbiota during and beyond complementary feeding as they are metabolically flexible and adapt to the changing diet (Milani et al., 2015; Pokusaeva et al., 2011; Turrone, Milani, Duranti, Ferrario, et al., 2018; Turrone, Milani, Duranti, Mahony, et al., 2018). Figure 2.3 illustrates the changes in the relative abundance of different microbial families throughout the exclusive breastfeeding and complementary feeding period.

Proteins from meat, dairy and legumes contribute to the gut microbiota development by providing nitrogen essential for microbial growth. Different protein sources exert distinct effects on the gut microbiota. For instance, meat-derived proteins have been linked to increased abundance of the *Lachnospiraceae* family, the *Ruminococcus* genus, the *Roseburia* genus, and *Clostridium* group XIVa clade, as well as an increase in diversity, compared to dairy or plant proteins (Tang et al., 2019; Tang, Ma, et al., 2022; Tang, Matz, et al., 2022). High doses of sugars in commercially manufactured infant

food can disrupt microbial diversity, increasing the Firmicutes/Bacteroidota ratio and promoting the abundance of taxa from the *Proteobacteria* phylum and *Escherichia-Shigella* (Burr et al., 2023; Plaza-Diaz et al., 2021).

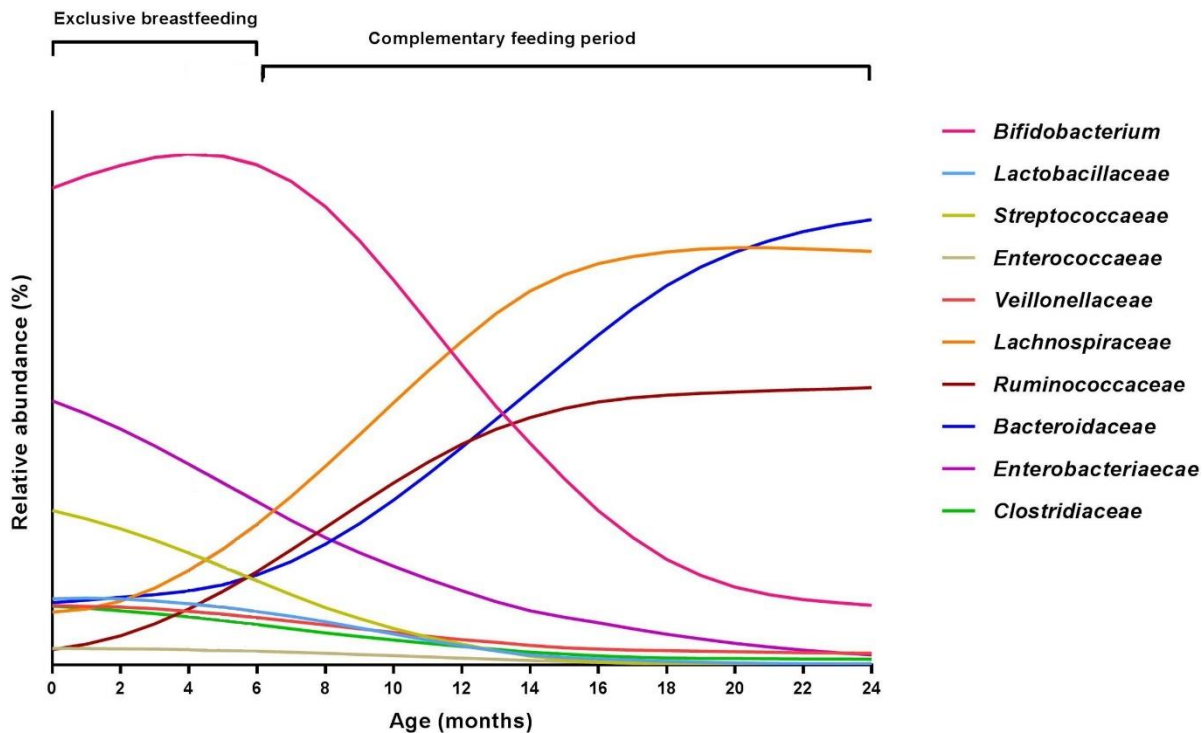


Figure 2.3. Changes in bacterial gut microbial taxa during the first 1000 days of postnatal life (adapted from Laursen et al. (2017), under a Creative Commons Attribution license (CC BY 4.0)).

Beyond compositional changes, complementary feeding enhances the gut's fermentative capacity, leading to changes in metabolite production, including SCFAs. Infants initially produce lower concentrations of propionate and butyrate due to the immaturity of their gut microbiota. However, as complementary foods are introduced, microbial communities adapt to utilise all the new dietary components efficiently (Milani, Duranti, et al., 2017).

The introduction of complex carbohydrates increases the production of SCFAs, particularly acetate, propionate, and butyrate (Shi et al., 2024; Tsukuda et al., 2021). Meanwhile, protein fermentation contributes to both the production of SCFAs and branched-chain fatty acids (BCFAs), reflecting increased proteolytic activity (Hellmuth et al., 2016; Kirchberg et al., 2015; Smith & Macfarlane,

1998; Tsukuda et al., 2021). While SCFAs generally have beneficial effects on gut health, excessive protein fermentation and the resultant BCFAs are considered detrimental to the gut microbiota and metabolic health in adults (Russell et al., 2011).

In vitro studies and an *in silico* study have investigated the fermentative capacity of various complementary foods and ingredients and their impact on the infant gut microbiota (da Silva et al., 2024; Flickinger et al., 2002; Parkar et al., 2021). Research has primarily focused on infant cereal, legumes, meat, dairy, and certain fruits and vegetables (Bierut et al., 2023; Krebs et al., 2013; Ordiz et al., 2020; Plaza-Diaz et al., 2021; Qasem et al., 2017; Tang, Ma, et al., 2022; Tang, Matz, et al., 2022; Zambrana et al., 2019). These studies demonstrated that different foods or combinations influence fermentation rates and microbial metabolic production in distinct ways. However, consensus on the specific effects of various foods, ingredients, and food components is hindered by a limited number of studies, particularly randomised controlled trials.

2.4 Complementary Feeding and Dietary Fibre

Dietary fibre is defined as “carbohydrate polymers with ten or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine”. This encompasses naturally occurring fibre polymers, as well as those from raw food or synthetically produced, provided the latter two demonstrate a proven physiological benefit to health (Codex Alimentarius Commission, 2009; Zielinski et al., 2013). Dietary fibre plays a significant role in human nutrition due to its resistance to digestive enzymes in the small intestine (Hamaker & Tuncil, 2014; Holscher, 2017; Makki et al., 2018; Wong et al., 2017). This resistance arises not only from the limited repertoire of human glycosidases but also from the fibres’ complex chemical structure, physical properties, and interactions with other food components, which collectively hinder enzymatic breakdown (Cantarel et al., 2012). Consequently, most dietary fibre enters the colon intact, where it undergoes microbial fermentation and is metabolised by microbial enzymes released during the process (Rakoff-Nahoum et al., 2016).

Research on the effects of dietary fibre in infants below the age of one year is limited, and there are currently no standardised guidelines for fibre intake for infants in this age group. For infants above 1-3 years, existing recommendations vary by country from 10 to 19 grams per day (Edwards et al., 2015; Hojsak et al., 2022; Stephen et al., 2017; Stewart & Schroeder, 2013). These recommendations, however, do not account for the heterogeneous nature of dietary fibres or their diverse physiological effects.

2.4.1 Dietary fibre types

The structural characteristics of dietary fibres are defined by the number, type, sequence, and bonding of the monosaccharides. These vary significantly depending on their source (plant cell walls, intracellular components, outer layer of seeds, etc.) and origin (fruits, vegetables, legumes, grains). Traditionally, dietary fibres have been classified based on their solubility and fermentability (Holscher, 2017; McRorie & McKeown, 2017). Soluble fibres dissolve in water, forming gels that slow digestion and are readily fermented by gut microbes. Insoluble fibres do not dissolve and mainly function as bulking agents, aiding intestinal motility (Mudgil, 2017). However, this binary system is increasingly recognised as oversimplified, as it fails to capture the structural complexity and functional diversity of dietary fibres.

A more comprehensive framework for classifying dietary fibres considers the structural complexity and functional properties, including chain length (degree of polymerisation), backbone structure, structural charge, water-holding capacity (WHC), fibre matrix, and fermentation rate (Opperman et al., 2025). Figure 2.4 presents a classification of dietary fibres based on this framework.

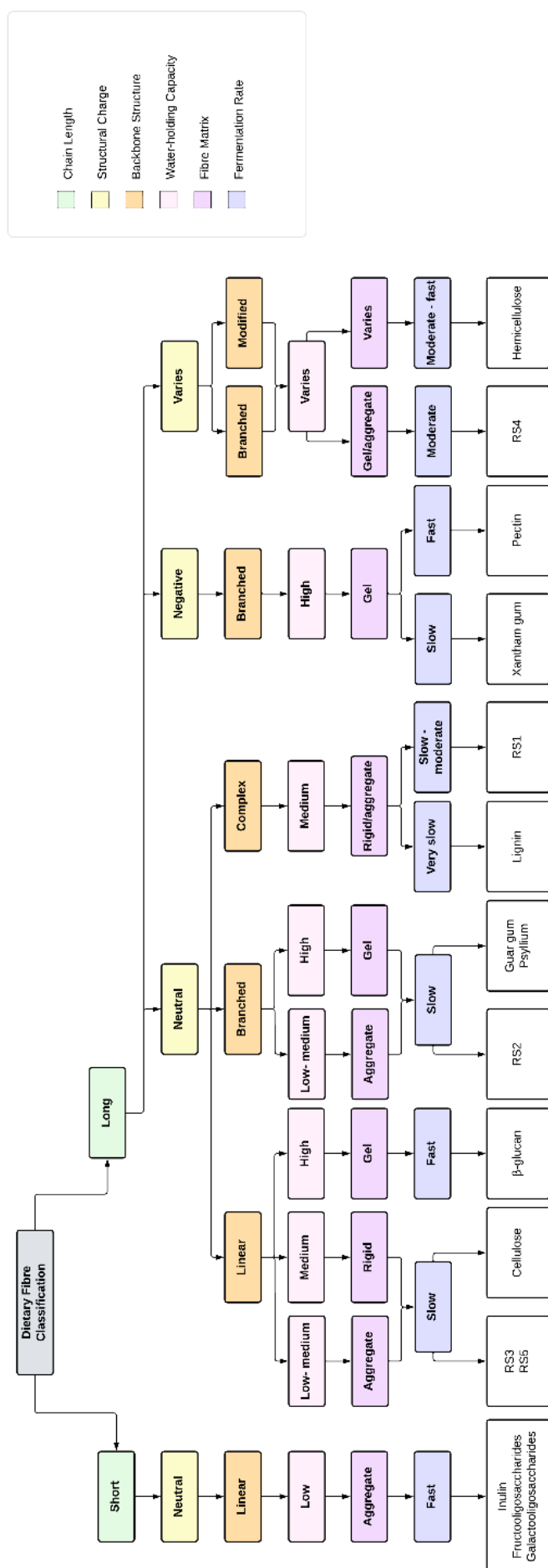


Figure 2.4. Classification of dietary fibre types and their physiochemical (chain length, structural charge, backbone structure, water-holding capacity, fibre matrix) and fermentation properties. The properties for each fibre type were compiled from established literature, and synthesised within the framework of Opperman et al. (2025).

Short-chain fibres (DP < 10), such as FOS and GOS, are rapidly fermented in the proximal colon (Li et al., 2015), while longer-chain fibres, such as resistant starch, reach the distal colon before being metabolised (Plongbunjong et al., 2017). Backbone structure, which can be linear, branched, complex or modified, influences solubility, charge and enzymatic breakdown. The structural charge of fibre influences its interaction with other molecules; negatively charged fibres such as pectin can bind minerals and bile acids, while neutral fibres such as cellulose do not exhibit this capacity (Dhingra et al., 2012; Mudgil & Barak, 2013). Water-holding capacity ranges from low to very high; fibres with high WHC, such as β -glucan and psyllium, form viscous gels that slow gastric emptying and lower cholesterol, whereas fibres with low WHC, such as cellulose and resistant starch, provide more bulking and laxative effects (McBurney et al., 1985; Mudgil & Barak, 2013). The fibre matrix, whether rigid (e.g. cellulose and lignin), gel (e.g. pectin and β -glucan), or aggregate (e.g. resistant starch types), determines accessibility to digestive enzymes and gut microbiota. Fermentation rate, which ranges from very low (lignin) to fast (inulin and pectin), determines the rate of organic acid production and prebiotic effects (Bourquin et al., 1993; Opperman et al., 2025).

The distribution of dietary fibre across food groups varies (Dhingra et al., 2012). Fruits and vegetables are rich in pectins and insoluble fibres, starchy foods such as legumes, tubers, and green bananas provide resistant starch and non-starch polysaccharides, while cereals are excellent sources of β -glucans, lignin and cellulose (Lovegrove et al., 2015; Stephen et al., 2017).

The speed and extent of fermentation vary considerably, influenced not only by the source of fibre but also the context in which it is consumed. Factors such as food combinations, preparation methods (raw versus cooked), and stored state (fresh versus dried) can have markedly different fermentation dynamics. The presence or absence of outer layers, such as the skin of fruits and vegetables, further modulates fermentation by altering fibre accessibility to gut microbes (Cao et al., 2022; Carmody et al., 2019; Garcia-Amezquita et al., 2018; Lamothe et al., 2021; Smith et al., 2020; Tuncil et al., 2018). Additionally, chemical and mechanical modifications of fibres, such as those introduced through processing methods like milling, extrusion, or chemical treatments, can alter their physiological

properties (Payling et al., 2020). These modifications may enhance or reduce microbial accessibility and fermentation potential.

2.4.2 Digestion and fermentation of dietary fibre

In infants, the transition from sucking to chewing during complementary feeding extends the contact of semi-solid foods with the oral cavity, enhancing the enzymatic exposure of food (Sarkar et al., 2017). Salivary enzymes initiate digestion, with enzyme activity reaching adult levels by six months of age (Ben-Aryeh et al., 1984, 1990; Lebenthal et al., 1981; Sevenhuysen et al., 1984). After swallowing, the food bolus passes through the oesophagus to the stomach, where mechanical churning, acidic gastric juice, and enzymatic activity further degrade food (Bornhorst & Singh, 2014). The oral and gastric phases have minimal impact on fibre digestion, but structural disruption to cell wall components occurs, and starch digestion begins (Goodman, 2010; Hoebler et al., 1998).

The small intestine is the primary site of pancreatic enzymatic digestion and nutrient absorption (Borgstrom et al., 1957). In adults, enzymes efficiently digest simple carbohydrates and oligosaccharides (Boland et al., 2014). However, infants have lower enzyme concentrations during the early stages of complementary feeding (Cleghorn et al., 1988; Otsuki et al., 1977), leading to reduced hydrolysis of most carbohydrates and oligosaccharides.

Certain fibre types, particularly soluble and structurally disrupted fibres, undergo hydration, swelling, and partial structural modification during passage through the small intestine. These processes can increase apparent solubility and release low-molecular-weight fractions from the fibre matrix, thereby influencing subsequent fermentation dynamics (Hardacre et al., 2015; Ulmius et al., 2012). The small intestine also harbours a microbial community capable of degrading a minor fraction of dietary fibre (Hillman et al., 2017; Kastl et al., 2020).

The colon is the primary site for fibre fermentation. Depending on the structural and chemical changes that occur during the earlier phases, dietary fibre arrives in various states of solubility and chain

lengths, influencing the extent of fermentation. Fermentation efficiency is highest in the proximal colon, where rapidly fermentable dietary fibres are metabolised, leaving insoluble and less fermentable fibres for the distal colon (Flint et al., 2008). Only metabolites, especially SCFAs and water, are absorbed while non-fermentable and degradation remnants are expelled as waste.

2.4.3 Dietary fibre and gut microbiota

Colonic fermentation plays a critical role in gut microbiota development. Some evidence suggests that an infant's gut microbiota may lack the capacity to ferment dietary fibre until several months (up to 4 months) after the introduction of complementary foods (Parrett et al., 1997). However, other studies indicate that at 7–10 months of age, the infant gut microbiota can ferment complex carbohydrates (Schwab, 2022).

Dietary fibre promotes the growth of particular microbial taxa equipped with carbohydrate-active enzymes (CAZymes), which include glycosyl hydrolase and polysaccharide lyase, which are necessary for fibre degradation (Figure 2.5) (El Kaoutari et al., 2013; Kurokawa et al., 2007; Laursen, 2021; Laursen et al., 2017; Scott et al., 2013; Walker et al., 2011; Yatsunencko et al., 2012). Different microbial populations express distinct pools of CAZymes, leading to variation in fibre fermentation across microbes or groups of microbes (Hamaker & Tuncil, 2014; Koropatkin et al., 2012; Makki et al., 2018; Martens et al., 2011). This enzymatic flexibility allows the microbiota to adapt depending on the fibre availability (Flint et al. 2012).

Bacterial species from the Firmicutes and Actinomycetota phyla are among the first to degrade dietary fibre (Deehan et al., 2017). Specific species thrive on different fibre types, *Bifidobacterium adolescentis*, *Ruminococcus bromii*, *Eubacterium rectale*, and *Parabacteroides distasonis*, specialise in resistant starch, while inulin and GOS primarily promote *Bifidobacterium* species (Deehan et al., 2017, 2020; Martínez et al., 2010; Walker et al., 2011). Fibre-rich diets in infants promote beneficial microbial taxa, including *Bacteroides* and several specialised starch-degrading species from the genera *Prevotella* and *Xylanibacter* (De Filippo et al., 2010). Fibre fermentation supports cooperative

microbial networks, where the by-products of primary degraders serve as substrates for secondary degraders that are less suited to degrading fibre (Flint et al., 2012; Peterson et al., 2022; Rogowski et al., 2015). Thus, combining different fibres that stimulate various microbial genera may be important to maintain microbial diversity and richness, as well as SCFAs generated from the fermentation of dietary fibre (Koh et al., 2016; Louis & Flint, 2017).

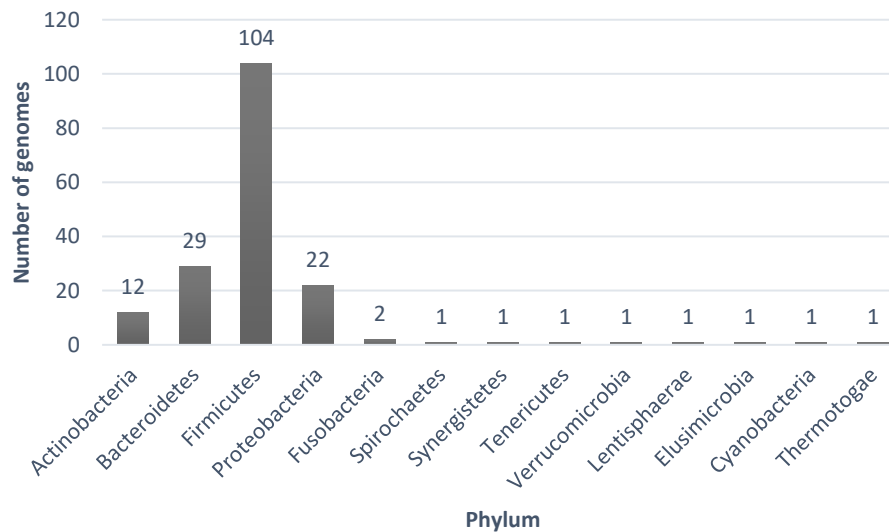


Figure 2.5. The abundance and variety of carbohydrate-active enzymes (CAZymes) in the gut microbiota (adapted from El Kaoutari et al. (2013)).

2.4.3.1 Resistant Starch and Gut Microbiota

Resistant starch (RS) is a unique and accessible form of dietary fibre naturally found in starchy food such as potatoes, grains and green bananas. Unlike rapidly and slowly digestible starch, which are broken down entirely in the small intestine, resistant starch is defined as the fraction of starch that is resistant to digestion by starch-degrading enzymes in the small intestine and passes to the colon and undergoes fermentation by the gut microbiota (Englyst et al., 1992). Resistant starch is categorised into five distinct types (RS1, RS2, RS3, RS4 and RS5) based on the physical and chemical basis for its resistance to enzymatic breakdown, including source, structure, and processing (Bird et al., 2010; Warren et al., 2018) (Table 2.2). RS1 refers to starch trapped in food matrices which are insoluble and undergo slow, partial digestion and are fermentable in the colon (Meenu & Xu, 2019). RS2

includes native ungelatinised starch, which resists digestion due to its low solubility and highly crystalline structure, resulting in moderate fermentation in the colon (Moslemi et al., 2014; N. Yao et al., 2009). RS3, known as retrograded starch, is formed when gelatinised starches are cooked and cooled, resulting in starch that is less digestible and exhibits high fermentability (Kapelko-Żeberska et al., 2016). RS4 is produced through chemical or physical modification, with varying digestibility and fermentability depending on the degree of modification (Remya et al., 2017). RS5 is formed when high-amylose starches interact with lipids under high temperatures, creating a complex that resists digestion but has lower fermentability than other RS types (Gutiérrez & Tovar, 2021). A detailed description of each RS type and its formation has recently been reviewed by [Bello-Perez et al. \(2020\)](#) and [Wen et al. \(2022\)](#).

The relative abundance of species from the families Bifidobacteriaceae, Ruminococcaceae, Eubacteriaceae, Lachnospiraceae and Bacteroidiaceae increases in response to resistant starch (Maier et al., 2017; Martínez et al., 2010; Upadhyaya et al., 2016; Venkataraman et al., 2016). Primary starch-degrading bacteria like *Ruminococcus bromii* and *Bifidobacterium adolescentis*, possess specialised enzymes, such as carbohydrate-binding modules (CBMs), to hydrolyse insoluble resistant starch particles (Duranti et al., 2014; Photenhauer et al., 2022; Ze et al., 2012). The by-products of resistant starch degradation, including oligosaccharides, are fermented by secondary degraders such as *Eubacterium rectale*, *Roseburia* spp., *Butyrivibrio* spp., and *Bacteroides thetaiotaomicron* creating a cooperative microbial network (Dobranowski & Stintzi, 2021). The impacts of resistant starch on enzyme pathways, species, and metabolic outputs are depicted in Figure 2.6 (Maier et al., 2017).

The microbial response varies with RS type and source due to differences in structure and physiochemical properties (Gu et al., 2020; Tuncil, Thakkar, Arioglu-Tuncil, et al., 2018). RS2 from potato starch selectively enriches *B. faecale*, *B. adolescentis*, and *B. stercoris*, while maize-derived RS2 predominantly increases *R. bromii* (Baxter Nielson T. et al., n.d.). RS3 increases the abundance of *R. bromii* and *Roseburia* spp. RS4, derived from chemical modification, has been shown to increase the abundance of *Parabacteroides distasonis* and *B. adolescentis* while reducing that of Firmicutes

populations (Jiang et al., 2020; Martínez et al., 2010). RS5 (amylose-lipid complexes) fosters the growth of the *Bacteroides* genus and *B. adolescentis*, which can efficiently utilise this modified starch to enhance saccharolytic fermentation (Jiang et al., 2020; Klosterman, 2021).

Table 2.2. Categories of resistant starch (RS) and their structure, source, digestibility and fermentability properties. Adapted from (Raigond et al., 2015).

RS Type	Description	Food source	Solubility	Digestibility	Fermentability
RS1	Physically inaccessible	Whole/partially milled grains and seeds, legumes, raw fruit & vegetables	Insoluble	Slow, partial digestion	Slowly fermented
RS2	Native starch granules (uncooked starch)	Green banana, raw potato, cereals, raw legumes, fruit and vegetables	Low	Very slow digestion	Moderately fermentable
RS3	Retrograded starch	Cooked and cooled potatoes, bread, cornflakes, and foods exposed to repeated heating and cooling treatments	Low	Slow rate of partial digestion	Most fermentable
RS4	Chemically and/or physically modified starch	High-fibre drinks, bread and cakes with modified starches	Low to high	May resist digestion	Variable (high to low)
RS5	Amylose-lipid complexes	Foods with high amylose	Low	Resists digestion	Least fermentable

Consumption of a diet enriched in resistant starch (RS) has been shown to increase SCFA concentrations, particularly butyrate. Adult studies have used varying RS amounts depending on the source and study design. *In vitro*, Arcila & Rose (2015) used a diet with 8.07% RS to model adult gut fermentation. Adult human intervention studies reviewed by Shen et al. (2017), reported RS supplementation ranging from 22 g/day to 45 g/day, with an average intake of 33 g/day. Venkataraman et al. (2016) investigated RS from potato starch (50% RS), with participants consuming increasing doses of 12 g, 24 g, and 48 g per day. Similarly, Yang et al. (2013) used a high-amylose corn starch diet containing 70% RS. In animal models, Haenen et al. (2013) supplemented pig diets with 34% RS.

Across these studies, RS consistently enhanced SCFA production, particularly butyrate, though the extent of its effects depends on the host microbiota composition and RS type. RS3 specifically increases butyrate levels by enriching key butyrate-producing bacteria such as *R. bromii* and *Roseburia* spp. Comparisons of different RS4 sources (maize, potato, and tapioca) revealed distinct microbial responses: maize-derived RS4 significantly increased butyrate production, while tapioca RS4 enhanced propionate levels (Deehan et al., 2017).

In infants, the gut microbiota is immature, and introducing RS during complementary feeding may support microbiota maturation and dynamics (Lin, 2018; Lin & Nichols, 2017). For example, *in vitro* studies have demonstrated that high-amylose maize starch (HAMS) increases microbial diversity and the abundance of taxa from the genera *Bacteroides* and *Bifidobacterium* (Gopalsamy et al., 2019). A study involving Malawian infants also highlighted that resistant starch fermentation enhances microbial diversity, particularly within the *Bifidobacterium* genus, among other starch-degrading species (Wang et al., 2019). Specific strains such as *Bifidobacterium longum* (including subspecies *infantis* and *longum*) are essential during this transitional period, showing nutrient-specific adaptations that enhance starch utilisation (Kujawska et al., 2020; Millar et al., 2024; Vatanen et al., 2022). However, while key resistant starch degraders like those from the *Bifidobacterium* genus are well-documented, the molecular mechanisms underlying RS utilisation remain understudied.

The consumption of resistant starch must be carefully managed to minimise gastrointestinal discomfort, such as bloating and flatulence, particularly in individuals unaccustomed to high fibre intake or with an underdeveloped gut microbiota, such as infants (H.-K. Ma et al., 2025; Salvatore et al., 2023). During complementary feeding, incorporating RS-rich foods such as green bananas (RS2), cooled starchy foods like potatoes and rice (RS3), and high-amylose cereals (RS5) supports the gradual development of a diverse and functional gut microbiota.

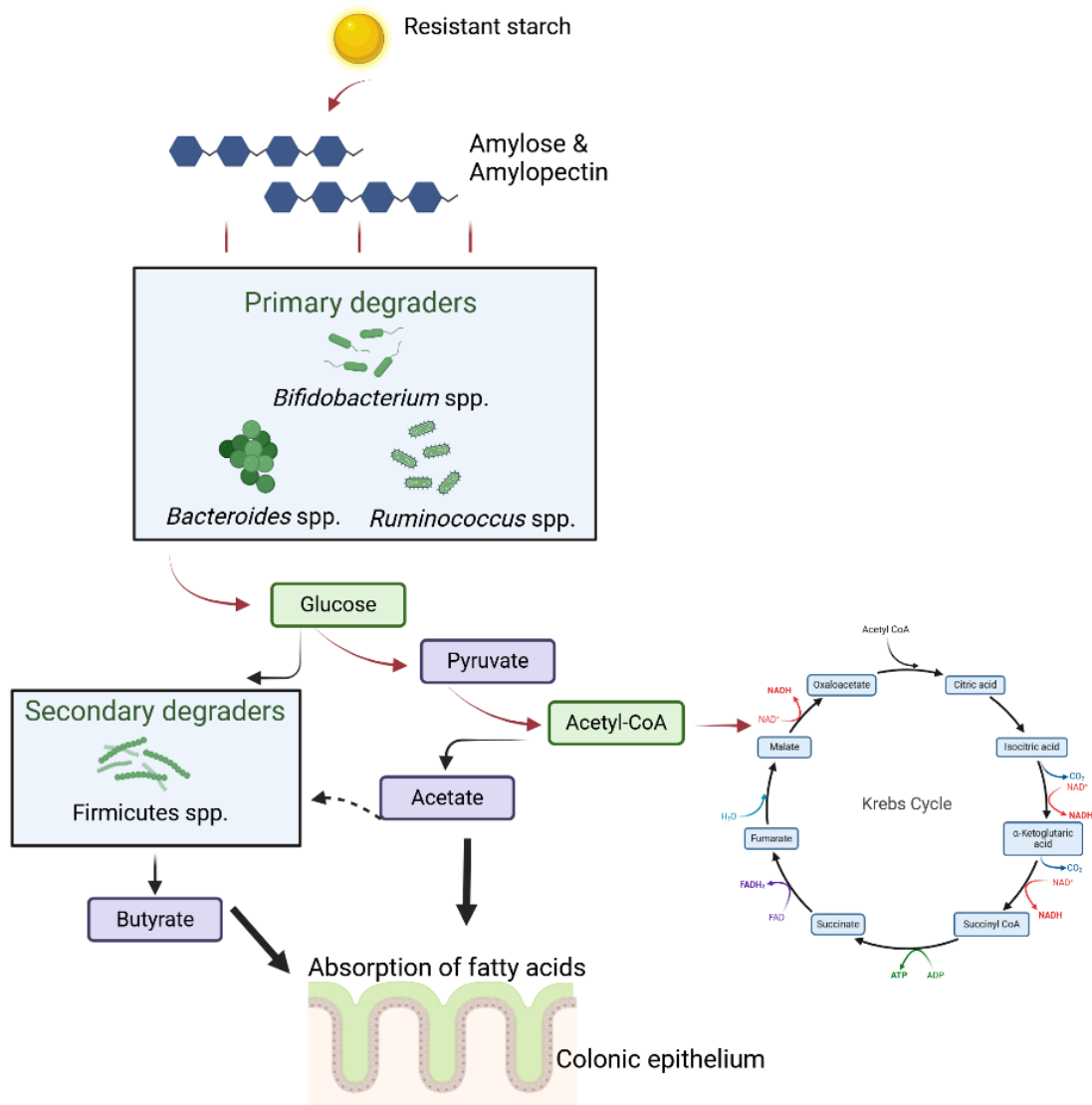


Figure 2.6. Key bacterial taxa and enzymatic pathways involved in RS fermentation and SCFA production (adapted from Maier et al. (2017)). Resistant starch, composed of amylose and amylopectin, reaches the colon where it undergoes fermentation. RS is broken down by primary degraders into glucose. Glucose undergoes glycolysis, generating pyruvate. Pyruvate is converted into acetyl-CoA, which is metabolised into acetate or can enter the Krebs cycle for energy production. Secondary degraders further metabolise fermentation products to butyrate. Short-chain fatty acids, including acetate and butyrate, are absorbed by the colonic epithelium, where they serve as an energy source for colonocytes.

2.5 Dietary Fibre, Gut Microbiota and Infant Health

Early postnatal life, particularly the complementary feeding period, represents a critical window in which the gut microbiota supports the development of an infant's immune system (Ganal-Vonarburg

et al., 2020; Hornef & Torow, 2020). Colonisation by beneficial microbes ensures the developing immune system accurately interprets microbiota-derived signals, promoting coordinated maturation of both innate and adaptive immune responses (Al Nabhani & Eberl, 2020; Hornef & Torow, 2020).

During early postnatal life, infants rely on maternal antibodies and immune factors transferred via the placenta and breastmilk for protection against pathogens (Brambell, 1966; Le Doare et al., 2018; Stelzer et al., 2021). However, the transition to complementary feeding exposes infants to dietary antigens and a diverse microbial community, which facilitates the maturation of innate immune cells, including monocytes, macrophages, dendritic cells, neutrophils, and natural killer (NK) cells. These cells detect dietary and microbial antigens through pattern recognition receptors (PRPs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain (NOD)-like receptors, triggering signalling pathways essential for immune defence and coordination (Fulde et al., 2018; J. C. Yu et al., 2018).

Gut-associated lymphoid tissue (GALT), also develops substantially during this period, promoting the endogenous production of secretory immunoglobulin A (SIgA). SIgA coats mucosal surfaces, reinforcing mucosal barrier integrity, limiting pathogen adhesion, and supporting tolerance to commensal microbes and dietary antigens (Dogra et al., 2021).

Adaptive immunity simultaneously matures, driven by antigen presentation by dendritic cells to naïve T and B cells. This interaction promotes the differentiation of T cells into regulatory (Tregs), helper (Th), and cytotoxic subsets. Regulatory T cells are especially critical, as they establish immune tolerance towards dietary and microbial antigens, reducing the risk of autoimmune or allergic responses (Kawamoto et al., 2014; Lathrop et al., 2011). Additionally, diversified antigen exposure promotes B cell activation and high-affinity antibody production, particularly IgA, which stabilises the gut microbiota, reinforcing homeostasis and the maturation of adaptive immune responses (Dogra et al., 2021).

Dietary fibres, particularly prebiotic fibres, play a pivotal role in shaping immune development by several key mechanisms, as illustrated in Figure 2.7 (McKeen et al., 2019). Prebiotic fibres directly interact with innate immune cells and epithelial cells through PRPs, promoting a tolerogenic environment and anti-inflammatory responses (Bermudez-Brito et al., 2015; Kong et al., 2019; Lehmann et al., 2015; Majima et al., 2017).

They can also indirectly modulate immune function by shaping the gut microbiota and increasing the production of SCFAs. SCFAs (acetate, propionate, and butyrate) influence both innate and adaptive immunity. Butyrate, for example, nourishes colonocytes, enhances gut barrier integrity, and modulates inflammatory responses, while acetate drives Treg differentiation and supports mucosal immunity. The expanding microbial community provides constant signals to the immune system, ensuring a balanced response to dietary and microbial antigens while maintaining tolerance to commensals (Sanidad & Zeng, 2020; Mendez et al., 2021).

Specific prebiotic fibres, such as RS, have demonstrated immunomodulatory effects through targeted enhancement of beneficial microbes, such as *Bifidobacterium* and *Lactobacillus* genera. These fibres promote IgA production, strengthen mucosal immunity, and support immune homeostasis (Abokor et al., 2021; Mantis et al., 2011). RS is particularly notable for its ability to produce high levels of butyrate during fermentation (Baxter et al., 2018; Jiang et al., 2020; Morrison & Preston, 2016; Nugent, 2005), a SCFA which modulates immune cell activity, cytokine release, and inflammatory balance (Donohoe et al., 2012).

The relationship between prebiotic dietary fibres and the immune system highlights the intricate connection between diet, the gut microbiota, and immune function. Research examining the effects of different prebiotic dietary fibres on the infant microbiota and immune system remains limited. Consequently, much of the evidence supporting the benefits of prebiotic dietary fibres on microbiota and immune health is based on adult studies and extrapolated assumptions rather than direct studies in infants.

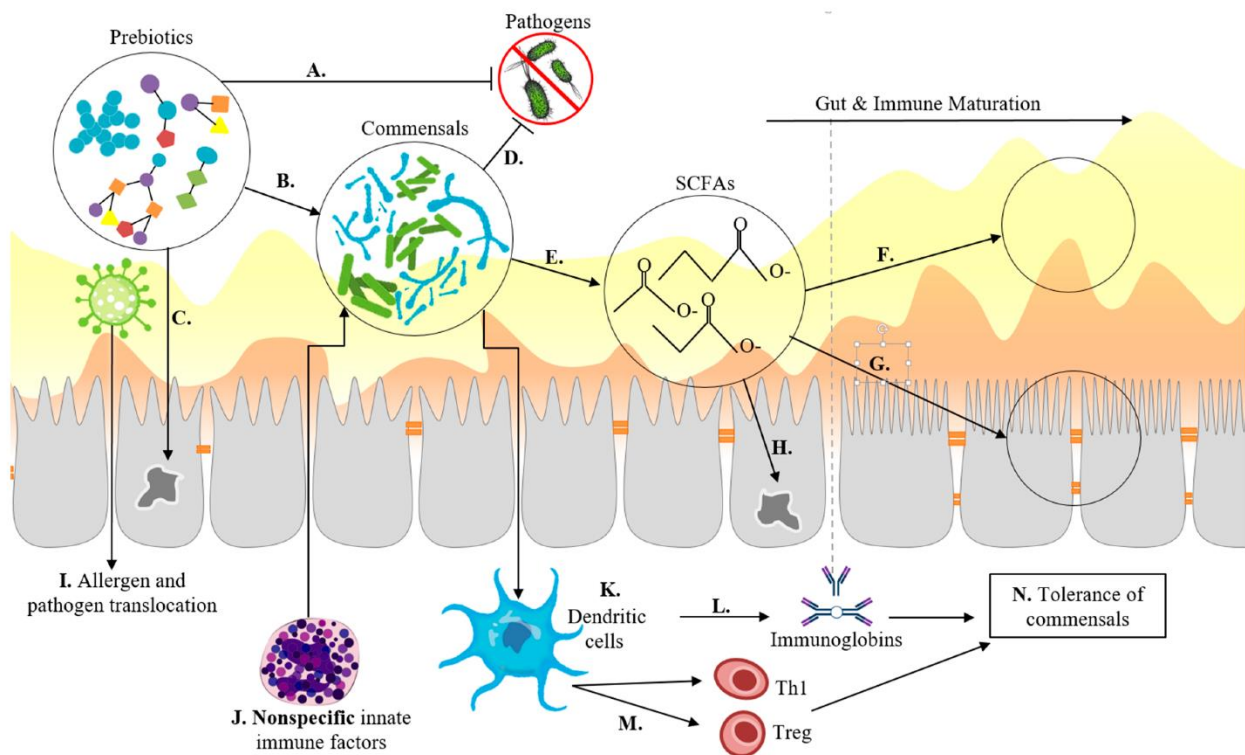


Figure 2.7. A schematic of multiple mechanisms by which prebiotics modulate immune and gut development (reproduced from McKeen et al. (2019), Creative Commons Attribution license (CC BY 4.0).). (A) Prebiotics bind to pathogens as receptor analogues, preventing adhesion to the epithelial surface and subsequent infection. (B) Prebiotics promote populations of commensal microbes, which outcompete pathogens for resources (D), reducing infections. (C) Prebiotics act directly upon the epithelium, promoting the mRNA transcription of proteins involved in barrier integrity. (E) Commensal microbes produce metabolites, such as short-chain fatty acids (SCFAs), which decrease the lumen pH and increase mucus F, increase tight junction proteins and crypt and villi development (G), and serve as an energy source for enterocytes that form the epithelium (H). The immature gut is susceptible to allergy and pathogen translocation (I) in infants through a leaky gut barrier. (J). Non-specific immune factors, such as macrophages and neutrophils, attack commensals and pathogens in poorly regulated inflammatory responses. During immune development, dendritic cells (K) sample commensal microbes through Toll-Like Receptor (TLR) recognition, allowing for antigen-specific immunoglobulin production (L) and promoting the differentiation of T and B cells (M), resulting in improved tolerance to commensals and targeted response to pathogens (N).

2.6 Fibre-rich complementary foods

A study by Parkar and colleagues (2021) assessed 32 different infant food ingredients in an *in vitro* setting and observed that the easily metabolised dietary fibre in kūmara, sweetcorn, pea, pumpkin and oat powder generated high amounts of lactate and acetate, attributed to the promotion of taxa from the *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* genera. Conversely, ingredients such as carrots, kiwifruit, apples, blackcurrants and other berries, which contain pectin-rich cell-wall polysaccharides, produced lactate and acetate, which facilitate a more diverse microbiota (Larsen et al., 2019; Liu et al., 2020; Parkar et al., 2021). Spinach and tomato, mainly composed of cellulose, hemicellulose and lignin, were slowly fermented (Dziedzic et al., 2019; Fry, 1982), resulting in lower lactate and acetate and higher propionate and butyrate, as some members utilise the easily accessible sugars while others further metabolise the primary end products.

Michelini et al. (2018) employed an *in silico* reverse metabolic technique to identify complementary foods that support gut and immune health. This study identified sweet potato (kūmara) as a suitable candidate. A recent *in silico* and *in vitro* study by da Silva and colleagues (2024) further supports the potential of kūmara alongside pumpkin, raspberries, blackcurrants, and soybeans, demonstrating their capacity to promote SCFA production.

Sweet potato offers significant nutritional and prebiotic potential due to its rich composition of vitamins, amino acids, minerals and dietary fibre (Cartier et al., 2017; Dako et al., 2016; Fitri et al., 2023; Kim et al., 2011; Mu & Singh, 2019; Olatunde et al., 2020; Wang et al., 2016). The dietary fibre content in sweet potato residues (after starch extraction) of 10 different varieties is, on average, 75.2% (Mei et al., 2010). Cellulose (31.2%), lignin (16.9%), pectin (15.7%), and hemicellulose (11.3%) were the main contributors to the dietary fibre fraction of kūmara. Another study found dietary fibre contents in the range of 1.85 to 2.35% in freeze-dried sweet potato roots of different varieties (Ji et al., 2015). Starch is found in the highest amounts in sweet potato roots, with the content varying between varieties from 50 to 80 % (Brabet et al., 1999). The resistant starch content in sweet

potato, a critical prebiotic component, varies between 3 to 68 % (Cui & Zhu, 2019; Kwon et al., 2019; Lv et al., 2018; Remya et al., 2018; Song et al., 2014). Another study found that the resistant starch fraction in flours from the roots of five different sweet potato varieties ranges from 13.2% to 17.2% (Senanayake et al., 2013). In contrast, some reports show that resistant starch content does not vary (24-25%) between different varieties (Trung et al., 2017). Similarly, the starch structure of New Zealand kūmara (sweet potato) exhibits a C-type crystalline pattern with moderate amylose content (19.8–25.3%) and strong granule integrity, features that confer thermal stability and moderate resistance to enzymatic digestion, suggesting a comparable prebiotic potential (F. Zhu & Xie, 2018).

Several processing techniques (physical modification, enzymatic modification, and chemical modification) may be used to further increase the resistant starch in kūmara (Dupuis et al., 2014; Fuentes-Zaragoza et al., 2010; Jiang et al., 2020). Physical modification is the preferred method for use in the food industry, as it is safe, environmentally friendly and economical. Physical modification includes heat-moisture treatment, annealing, autoclaving and retrogradation cycles. These methods have been effective in increasing the resistant starch content in some foods (Chung et al., 2009; Dunder & Gocmen, 2013; Xie et al., 2018).

Studies related to the health-promoting effects of different kūmara varieties can be found in de Albuquerque et al. (2019). The macronutrients and bioactives found in kūmara, such as carbohydrates (polysaccharides, dietary fibre, and resistant starch), phenolics, peptides and carotenoids, can potentially have antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, antidiabetic and antiobesity effects on health.

Similarly, banana, one of the first complementary foods introduced to infants, offers prebiotic potential, particularly unripe/green bananas due to their high resistant starch content (up to 77%) (Jiang et al., 2015). While unripe/green bananas are not naturally sweet and don't align with infants' innate sweet preference (Liem & Mennella, 2002), processing green banana into powder may improve palatability.

Green bananas are also rich in essential minerals, vitamins, polyphenols, and antioxidants (Juarez-Garcia et al., 2006). Green banana flour has been developed as a functional ingredient to increase the prebiotic dietary fibre content in food products (Almanza-Benitez et al., 2015; Ovando-Martinez et al., 2009). Heating and cooling treatments can enhance the resistant starch levels in green banana powder (Aparicio-Saguilán et al., 2005).

Whole foods or ingredients, such as kūmara and green banana, are promising candidates for the formulation of complementary foods with high nutritional value and prebiotic potential, which position them as valuable components in infant diets, supporting gut microbiota maturation and immune development during the critical early life stages.

2.7 Methods to Study the Infant Gut Microbiota during Complementary Feeding

2.7.1 *In vitro* digestion and fermentation methods

In vitro models are indispensable tools for exploring the intricate interactions between diet and the gut microbiota (Ashaolu et al., 2021). These models replicate the physiological and biochemical conditions of the human digestive system, encompassing temperature, pH, enzymatic activity, and anaerobic environments, to simulate digestion from the mouth to the large intestine (Minekus et al., 2014). Analyses of samples from *in vitro* fermentation models provide insights into the composition of the gut microbiota, microbial responses to dietary inputs, and the niches made accessible for microbial colonisation during food digestion and fermentation.

Static batch cultures, as simplified systems, are commonly used for controlled studies of microbial metabolism under specific conditions. These models are highly accessible, relatively inexpensive, and straightforward to set up, making them ideal for initial hypothesis testing and preliminary studies. Static systems are particularly useful for short-term studies investigating the metabolism of specific substrates or for generating mechanistic insights into microbial activity (Pham & Mohajeri, 2018). Despite these advantages, static models are limited in their ability to simulate dynamic digestive

processes, nutrient absorption, and microbial shifts over time due to substrate depletion and accumulation of metabolic end-products.

Dynamic models offer a significant advancement by incorporating features that replicate the physiological changes occurring during digestion. These models often simulate peristaltic mixing, gradual nutrient absorption, and the progressive movement of digesta through different compartments, closely resembling the natural digestive process. They can also account for shifts in pH, enzyme activity, and microbial growth that occur over time. For example, systems like the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and TNO Gastro-Intestinal Model (TIM) mimic the transit of food through the gastrointestinal tract and provide environments representative of the proximal and distal colon (Venema, 2015; Minekus et al., 2014). Dynamic models enable more accurate assessments of microbial interactions and functional responses to dietary substrates, offering a better approximation of *in vivo* conditions compared to static systems. However, dynamic models come with several limitations. Their complexity makes them resource-intensive and challenging to operate, requiring specialised equipment, expertise, and maintenance (Verhoeckx et al., 2015). Additionally, the high cost of these systems may limit their accessibility to many research facilities. Dynamic models are also less reproducible compared to static systems, as their inherent variability in conditions (e.g., peristalsis and nutrient absorption rates) can introduce inconsistencies in results.

A major limitation of all *in vitro* fermentation models lies in their reliance on faecal samples as inoculum, which introduces inherent biases. Faecal homogenisation obscures both radial spatial variability (differences between microbial communities in the lumen, mucosa, and digesta) and axial spatial heterogeneity (differences between proximal and distal colon), limiting the ability to capture site-specific microbial activities (Donaldson, Lee, & Mazmanian, 2016). Furthermore, while standardised protocols like the INFOGEST digestion method exist for adults (Brodtkorb et al., 2019; Minekus et al., 2014), there is a lack of equivalent guidelines tailored to infant-specific conditions. Recent evaluations of infant *in vitro* models underscore the need for refined methods to better capture

the unique gut microbiota characteristics during this critical developmental phase (Bourlieu et al., 2014; Fournier et al., 2021).

Despite these limitations, *in vitro* methods remain valuable for rapidly and safely evaluating the prebiotic potential of ingredients in complementary infant foods. Static models, with their simplicity and cost-effectiveness, are particularly advantageous for exploratory studies, while dynamic and continuous systems provide greater physiological relevance for investigating complex microbial dynamics. Together, these methods offer complementary insights into how dietary substrates shape microbial composition and function, supporting the development of optimised dietary interventions (Gamage et al., 2017).

2.7.2 Human intervention studies

Human intervention studies offer numerous advantages over *in vitro* models, particularly in understanding diet-microbiota interactions and their health implications. These studies capture the complexity of the gut ecosystem, integrating microbial, host, and environmental factors that cannot be replicated *in vitro*. For instance, human studies reflect dynamic interactions between the gut microbiota, gut physiology, immune responses, and systemic factors such as hormonal signalling and nutrient absorption. They also provide insights into the long-term effects of dietary interventions on gut microbiota composition and function, which are challenging to assess in short-term *in vitro* experiments (Johnson et al., 2020).

In the case of infants, intervention studies are uniquely suited to investigate the acquisition and maturation of the gut microbiota, capturing developmental changes influenced by diet, such as the introduction of complementary foods or prebiotic supplementation (Yatsunenکو et al., 2012). These studies are essential for understanding the temporal and individual-specific factors that shape the infant gut microbiota, offering real-world evidence of how dietary interventions affect health outcomes during this critical developmental period. Additionally, human intervention studies have higher clinical relevance and translational value than *in vitro* studies, as they directly assess dietary

effects under natural physiological conditions, accounting for individual variability in factors like genetics and lifestyle (Penders et al., 2006). Such studies complement mechanistic findings from *in vitro* models, providing a holistic understanding of diet-gut microbiota-host interactions and their potential for improving health.

Longitudinal sampling plays a vital role in intervention studies, as it captures the dynamic nature of the gut microbiota over time, which is particularly important in infants as the microbiota changes rapidly over the first 1000 days of life. Unlike cross-sectional studies, which provide a single snapshot, longitudinal studies assess intra-individual variations and microbial shifts, offering a detailed understanding of gut microbiota maturation and responses to dietary interventions (Wu et al., 2019). Frequent and consistent faecal sample collection is essential to monitor these changes accurately. However, such studies face challenges in sample collection, transportation, and storage, as improper handling—such as temperature fluctuations or freeze-thaw cycles—can alter microbial composition. Standardised protocols, including stabilising solutions and appropriate storage methods, are crucial for preserving the integrity of microbial communities in faecal samples (McDonald et al., 2018; International Human Microbiome Standards, 2015). Even with rigorous protocols, longitudinal faecal samples cannot overcome the inherent spatial limitation of the sample type, a limitation shared with *in vitro* models that rely on faecal inocula.

In human dietary studies, the reliance on self-reported dietary intake (food frequency questionnaires, food records, dietary recalls) introduces substantial inaccuracies, with as much as 50% of food records showing underreporting (Burrows et al., 2019). This is particularly problematic for carbohydrates and other nutrients affected by processing and preparation methods. To improve accuracy, guidelines recommend collecting multiple days of dietary data prior to faecal sampling and using three to five consecutive faecal samples to account for intra-individual variation (Barabási et al., 2020; Johnson et al., 2020). However, logistical constraints often limit the implementation of these protocols.

2.7.3 Assessment of the gut microbiota

Culture-based methods involve isolating and growing microbes under controlled laboratory conditions to identify specific taxa. While valuable for characterising culturable species, these methods are limited by their inability to capture the vast diversity of the gut microbiota, as many species are not amenable to standard culturing techniques (Lau et al., 2016). Advances in culturing, such as using specialised media and anaerobic conditions, have expanded the range of detectable organisms, but these methods remain insufficient for comprehensive microbiota profiling.

The advent of molecular techniques has revolutionised microbiota research by enabling culture-independent analysis. Next-Generation Sequencing (NGS) methods, such as 16S rRNA sequencing, target the conserved 16S ribosomal RNA gene to identify bacterial taxa. This widely used, cost-effective, and efficient approach provides insights into community composition and relative abundance. However, 16S rRNA sequencing has limitations, including amplification bias during PCR, limited resolution for distinguishing closely related taxa, and the inability to detect non-bacterial components such as archaea, viruses, and fungi (Campanaro et al., 2018; Johnson et al., 2019).

On the other hand, shotgun metagenomic sequencing sequences all genetic material in a sample, offering a more comprehensive approach. This method provides species-level resolution and functional insights, identifying genes and pathways involved in microbial metabolism. Shotgun sequencing can also detect archaea, viruses, and eukaryotes, making it a more versatile tool than 16S sequencing. However, it is more resource-intensive, requiring greater computational power, higher costs, and specialised bioinformatics expertise (Laudadio et al., 2018; Durazzi et al., 2021). Therefore, the choice between these methods should be guided by specific research objectives, budget considerations, and the desired depth of taxonomic and functional insights.

Functional profiling methods extend beyond taxonomic analysis to explore the activity and metabolic potential of the microbiota. Metatranscriptomics focuses on RNA transcripts to provide insights into active gene expression, offering a dynamic view of microbial function. However, challenges such as

RNA instability and limited reference databases can complicate data interpretation (Jovel et al., 2016). Metaproteomics analyses the proteins expressed in a microbial community, including both microbial and host-derived proteins, offering insights into microbial functions, host-microbe interactions, and post-transcriptional regulation. This approach is particularly useful for understanding functional activity beyond genomic potential. However, it is technically demanding, requiring efficient protein extraction, high sample purity, and advanced mass spectrometry techniques (Petriz & Franco, 2017).

Metabolomics and SCFA quantification are complementary, yet distinct approaches used to study gut microbiota functionality. Metabolomics is a comprehensive method that analyses all detectable small molecules (metabolites) in a biological sample, including SCFAs, bile acids, amino acids, lipids, sugars, and phenolic compounds (Chen et al., 2019; Turnbaugh & Gordon, 2008; Wikoff et al., 2009). In contrast, SCFA analysis is a targeted approach within metabolomics, focusing specifically on microbial fermentation products like acetate, propionate, and butyrate. While SCFA analysis offers detailed insights into the production and abundance of these specific metabolites, metabolomics captures the broader metabolic context, enabling the study of diverse pathways and potential biomarkers for disease (Gall et al., 2018). Both approaches commonly employ techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (Emwas et al., 2019), but metabolomics requires more extensive data analysis due to the diversity of metabolites studied. Together, metabolomics and SCFA quantification provide a complementary understanding of microbiota functionality, with SCFA analysis offering depth on specific microbial outputs and metabolomics delivering a broader, systems-level perspective.

Each method for assessing the gut microbiota has its positives and limitations; therefore, it often adopts a multi-omic approach, integrating several methods to achieve a holistic understanding of the gut microbiota, its functions and complex diet-microbiota-host interactions (albeit with some limitations). These advanced methods are particularly valuable for studying the gut microbiota during infancy, a period of rapid microbial and functional development, to provide insights on how early

postnatal life nutrition shapes the gut microbiota and its functional role in health and disease. The advantages and limitations of each method are outlined in Table 2.3.

Table 2.3. Advantages and limitations of multi-omic methods used to study the gut microbiota (adapted from Schwartz (2016) and Simpson & Campbell (2015)).

Method	Advantages	Limitations
16S rRNA gene sequencing	Cheaper than other sequencing methods. Faster, less computational power required for analysis. Well-suited for broad taxonomic profiling.	Amplification bias due to PCR. Comparison of results requires amplification of the same region. Only describes the bacterial fraction of the microbial community. Does not distinguish between active and inactive/dead cells.
Shotgun metagenomics	High coverage, deep sequencing of total genetic material present. No amplification bias. Identifies novel genes. Simultaneous profiling of functional capabilities and taxonomic composition of microbial community. Detects archaea, viruses, fungi, and bacteriophages.	Comparatively more expensive. Bioinformatic analyses are complex and time consuming. Does not distinguish between active and inactive/dead cells. Requires high computational power.
Metabolomics	Provides comprehensive metabolic profiles. Identifies microbiota and host metabolic pathways and responses.	Differentiates poorly between host- and microbial-derived metabolites. Reference databases are less advanced. Data analysis is complex and computationally intense.
Short-chain fatty acid (SCFA)	Focused on physiologically relevant microbial outputs. Easily linked to diet and gut microbiota functionality. Relatively targeted and cost-effective compared to metabolomics.	Limited scope; only analyses SCFAs. Misses' broader metabolic context. Requires optimisation for low-abundance metabolites. Less comprehensive than full metabolomics.

Method	Advantages	Limitations
Metatranscriptomics	Captures RNA-based gene expression. Provides data on active microbial processes. Highlights functional pathways like SCFA production.	RNA instability requires careful handling. Technically challenging; multiple purification steps needed. Limited reference databases. Computationally demanding. Provides a snapshot of gene activity without linking directly to protein output.
Metaproteomics	Directly measure active proteins. Provides functional insights into microbial processes. Detects post-transcriptional regulation.	Technology demanding, requiring efficient protein extraction. Complex bioinformatics for analysing vast protein diversity. Requires high-quality samples with minimal contamination. Computationally and resource intensive.

2.8 Knowledge Gaps and Future Perspectives

Dietary modulation of the gut microbiota and subsequent effects on host health are well established, particularly in adults. However, significant knowledge gaps persist regarding the impact of diet on the gut microbiota during infancy, especially during the complementary feeding period - a critical window for establishing a healthy gut microbiota. While studies have focused on the role of breastmilk and infant formula in shaping the infant gut microbiota, fewer have investigated the profound dietary transition from a milk-based diet to solid foods, which occurs during the first 1000 days of life. This transition represents the most significant dietary shift in the human lifespan and coincides with rapid changes in both the gut microbiota and host-microbial interactions.

Existing studies on complementary feeding in infants typically examine different stages between 6 months and 2 years. However, these timeframes often do not align or provide a continuous perspective, making it difficult to capture the full progression of gut microbiota changes from the

onset of complementary feeding. Moreover, limited research has explored how different types of complementary foods, the sequence of their introduction, and their specific dietary components influence gut microbiota development and functional maturation during this period. Mechanistic insights into how diet modulates immune-beneficial microbes and supports immune system maturation during complementary feeding are particularly lacking.

Dietary interventions with prebiotics, such as resistant oligosaccharides (e.g., inulin, FOS, and GOS), have shown promise in promoting beneficial microbial communities and improving immune health outcomes. However, little is known about the prebiotic functionality of dietary fibre components during complementary feeding, including their impact on microbiota composition, diversity, and metabolic outputs such as SCFAs. This knowledge gap presents an opportunity to investigate complementary foods rich in prebiotic dietary fibres, which can fulfil infants' nutritional needs while promoting microbial populations beneficial for gut and immune system development.

Kūmara (sweet potato) and banana, two commonly introduced foods during complementary feeding, are rich in prebiotic dietary fibre and resistant starch and are widely used in infant food products (Garcia et al., 2016; Moding et al., 2018; Padarath et al., 2020). While kūmara has been studied for its potential to modulate the infant gut microbiota, there is a lack of published evidence from *in vitro*, animal, or human studies on the effects of ripe or unripe (green) bananas on microbiota modulation, particularly during complementary feeding. Given their high nutritional value and potential prebiotic effects, further research is needed to evaluate their impact on gut microbiota composition, functional activity, and host health outcomes during this critical developmental phase.

Studies utilising *in vitro* colonic fermentation models with infant faecal inoculum offer a controlled approach to investigating how dietary fibres influence microbial composition and metabolic activity. However, most existing research has focused on non-starch polysaccharides (e.g., inulin, hemicellulose, guar gum, and beta-glucan) and resistant oligosaccharides (e.g., inulin, FOS, and GOS). Research on resistant starch and other dietary fibre types or combinations remains limited,

despite their potential to influence the gut microbiota's composition and functional outputs, such as SCFA production. Combining different dietary fibres may provide synergistic effects, enhancing the diversity and activity of beneficial microbes.

Furthermore, as technology advances and the demand for a more comprehensive understanding of microbiota's taxonomic and functional profiles grows, studies integrating multi-omics approaches are essential for establishing links between microbiota and host health outcomes. More longitudinal studies with frequent faecal sampling are essential to capture the dynamic nature of the infant microbiota during complementary feeding and to investigate the specific mechanisms by which dietary fibres influence gut microbial communities and host responses over time.

Future research should focus on dietary clinical interventions designed to modulate the gut microbiota and immune system maturation during the early stages of complementary feeding. Such studies should evaluate the effects of individual and combined dietary fibres, particularly resistant starch, on microbial composition, metabolic activity, and functional outputs. This knowledge will be critical for designing complementary foods that support the development of healthy gut microbiota and optimise health outcomes in infancy and beyond.

Chapter 3

***In vitro* digestion and fermentation of kūmara and green banana extract combinations by infant gut microbiota**

3.1 Abstract

The introduction of complementary foods is crucial in shaping the developing infant gut microbiota. This study used infant-adapted *in vitro* models to investigate how varying mixtures of kūmara (New Zealand sweet potato) and green banana extract (GBE) influence gut microbial composition, metabolic pathways, and fermentation outputs. We examined nine mixtures with varying kūmara: green banana extract ratios, plus a FreshAs mixture and a potato starch control. Resistant starch (RS) content increased systematically from 1.2% (100% kūmara) to 30.5% (100% green banana extract), while simple sugars decreased correspondingly from 54.2% to 1.5%. Microbial composition (shotgun metagenomics), functional potential (MetaCyc metabolic pathways), and fermentation metabolites (gases and organic acids) were assessed following 24-hour fermentation with pooled infant faecal inocula (n=3 replicates per mixture).

Fermentation validation confirmed substantial microbial activity: paired PERMANOVA detected significant community restructuring from baseline ($R^2=0.999$, $p=0.012$), Shannon diversity decreased from 1.80 to 1.59, and total organic acid production increased 6-fold. However, taxonomic composition after fermentation showed remarkable stability across substrate mixtures, with zero species reaching significance among mixtures (0 of 85 species, $q<0.05$, Maaslin3). Despite this taxonomic stability, functional profiling revealed substantial metabolic reorganisation: 25 MetaCyc pathways showed significant mixture associations ($q<0.05$), with 16 nucleotide biosynthesis pathways (64% of significant pathways) coordinately suppressed in green banana extract-rich formulations. Gene-level validation demonstrated exceptional concordance (mean 94.8%; nucleotide pathways 99.3%), confirming genuine metabolic reorganisation rather than statistical artefacts.

Metabolite production showed substrate-dependent patterns without significant categorical mixture effects: acetate relative abundance correlated positively with kūmara content (Spearman $\rho=0.42$), while organic acid production did not differ significantly among mixtures (Maaslin3, $q<0.05$). Multi-omics integration revealed that 94% of species-metabolite associations were mediated through pathway intermediates, with only 6% representing direct relationships.

The findings demonstrate that infant gut microbiota exhibit functional plasticity without taxonomic turnover: metabolic pathway activity reorganises substantially in response to substrate composition while community membership remains stable. These results provide mechanistic insight into how kūmara and green banana extract substrates differentially influence infant gut microbial metabolism, informing interpretation of the SUN clinical trial and highlighting age-specific microbial adaptation strategies during complementary feeding.

3.2 Introduction

The complementary feeding period, which lasts roughly from 6 to 24 months of age, is a vital developmental phase during which the gut microbiota undergoes significant compositional and functional development, with potential effects on both immediate and long-term health outcomes. Recent evidence shows that dietary fibres, including resistant starch (RS), act as prebiotics that support intestinal barrier integrity, modulate immune development, and influence microbial succession during this important developmental window (Lalli et al., 2025).

The infant faecal microbiota demonstrates RS fermentation capacity even before weaning, with this ability increasing substantially following complementary food introduction, resulting in elevated short-chain fatty acid (SCFA) production and enhanced microbial diversity (Gopalsamy et al., 2019).

Despite the recognised importance of complementary feeding for microbiota development, conclusive evidence linking specific complementary foods to gut microbiota outcomes in infants remains limited, with studies producing conflicting results regarding individual dietary components

(da Silva et al., 2025; Noles et al., 2025). This knowledge gap is particularly relevant for starchy staple foods that constitute primary weaning foods globally. Kūmara is a promising complementary food, widely used in infant feeding practices and suitable for introduction during weaning. Recent *in vitro* fermentation studies using infant faecal inocula demonstrate that kūmara effectively promotes lactate production by stimulating lactic acid bacteria from the genera *Enterococcus* and *Lactobacillus* (Silva et al., 2025). Similarly, green banana represents a concentrated source of Type 2 resistant starch with documented prebiotic properties in adult populations (Munir et al., 2024), though its effects in infants remain underexplored.

Kūmara and green banana also differ markedly in their carbohydrate structures: kūmara contains predominantly soluble pectic polysaccharides with low RS content (C. Liu et al., 2025; Zhao et al., 2024), whereas green banana flour typically contains 20–40% RS2 depending on cultivar and processing (Agama-Acevedo et al., 2015; Cordoba et al., 2018). These contrasting fibre profiles are expected to generate distinct fermentation kinetics and microbial responses, providing a mechanistic basis for comparing the substrates across a controlled gradient.

The Seeding throUgh feediNg (SUN) randomised controlled trial (ACTRN12620000026921), detailed in the published trial protocol (Wall et al., 2024), is designed to evaluate whether kūmara alone or a mixture of kūmara (85%) and green banana extract (15%) can beneficially modulate infant gut microbiota during the first year of life. Because the SUN Study interventions differ in their intended characteristics, with the kūmara and green banana extract mixture targeting a higher resistant starch content, a key step in interpreting clinical outcomes is understanding how these substrates interact with the infant gut microbiota.

In vitro fermentation models provide controlled experimental platforms to mechanistically investigate how substrate composition influences microbial metabolism without the confounding variable inherent to clinical trials. By systematically testing multiple ratios of kūmara and green banana extract, we can characterise how incremental changes in substrate properties influence not only

microbial community composition but also metabolic pathway activity and fermentation outputs. Critically, combining compositional profiling (shotgun metagenomics) with functional profiling (metabolic pathway abundance) and metabolite quantification (SCFAs, organic acids, gases) enables assessment of whether substrate effects manifest primarily at the taxonomic level, the functional level, or both.

3.3 Aims

This study aims to characterise the digestion, fermentation, and microbiota-modulating properties of kūmara and green banana extract (GBE) mixtures as a mechanistic foundation for interpreting outcomes from the SUN randomised controlled trial. To achieve this, we investigated how substrate composition across a controlled kūmara–GBE gradient influences nutrient profiles, microbial fermentation dynamics, and downstream metabolic function.

Specific objectives were to:

1. Characterise the nutritional composition of kūmara–GBE mixtures across defined ratios.
2. Assess colonic fermentation responses to these substrates using infant faecal inoculum in a 24 h batch fermentation model, including microbial community composition, functional pathway profiles, and metabolite production.
3. Identify mechanistic signatures—taxonomic, functional, and metabolite-based—that can inform interpretation of substrate effects observed in the SUN clinical trial.

3.4 Materials and Methods

3.4.1 Preparation of Substrates and Chemical Characterisation

Powdered kūmara was produced by FreshAs (Auckland, New Zealand) through repeated cooking and cooling of whole kūmara (a variety of New Zealand sweet potato, including the skin), followed by lyophilisation. Powdered green banana extract (Green Banana Resistant Starch) was purchased from Natural Evolution (Queensland, Australia).

The mixtures outlined in Table 3.1 were prepared by mechanically blending powdered kūmara and powdered green banana extract in varying ratios. Milling was conducted using a mechanical blender to ensure uniform particle size across all mixtures. Mixture 4 was provided premixed by FreshAs, which combined their kūmara powder with green banana extract sourced from Natural Evolution, QLD, Australia; the mixture was also milled to ensure consistency.

In addition to the experimental mixtures, potato starch (PS) was included as a positive fermentation control to confirm that the *in vitro* fermentation system was functioning correctly, and as an inter-batch control to assess reproducibility across experimental runs.

Table 3.1. Composition of powdered kūmara and green banana extract mixtures.

	Ratio	
Mixture	Kūmara (%)	Green Banana Extract (%)
100/0	100	0
92.5/7.5	92.5	7.5
85/15	85	15
FreshAs	85	15
70/30	70	30
50/50	50	50
30/70	30	70
15/85	15	85
0/100	0	100

The chemical composition of the mixtures was analysed by the Nutrition Laboratory at Massey University. Dry matter content was determined using AOAC methods 925.10 and 930.15. Ash content was measured according to AOAC method 942.05, with incineration at 550°C. Fat content was determined using the Mojonnier method (AOAC 922.06). Crude protein content was quantified using the Dumas method (AOAC 968.06), applying a nitrogen-to-protein conversion factor of 6.25. In addition, non-protein nitrogen (NPN) was assessed through protein precipitation followed by Kjeldahl analysis.

Carbohydrate content was calculated by difference, subtracting the sum of crude protein, fat, and ash from the dry matter content. Starch concentration was measured using the Megazyme α -amylase/amyloglucosidase kit following the AOAC method 996.11. Resistant starch (RS) content was determined using the Megazyme Resistant Starch Assay Kit (K-RSTAR), according to the AOAC method 2002.02. β -Glucan concentration was analysed using the Megazyme assay based on the AOAC method 995.16.

Total dietary fibre (TDF) was determined using the Megazyme enzymatic-gravimetric method in accordance with the AOAC method 991.43. Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF) were quantified using the Fibertec system, following AOAC methods 2002.04 and 973.18, respectively.

Energy content was calculated using the Atwater general factors by multiplying the gram amounts of carbohydrates and protein by 16.7 kJ/g and fat by 37.7 kJ/g, then summing the values to express total energy in kJ per 100 g. The sugar profile was analysed by gas-liquid chromatography (GLC), using a subcontracted analytical service.

The non-starch polysaccharide composition of kūmara was analysed by the Ferrier Research Institute (Victoria University of Wellington, New Zealand). The Ferrier team employed a stepwise fractionation protocol to isolate polysaccharide-rich fractions, with each extraction stage designed to target specific structural components of the plant cell wall. Glycosyl linkage analysis was

subsequently performed on each fraction to determine the abundance and structural characteristics of the constituent polysaccharides. The interpretation of linkage profiles was guided by established frameworks (Pettolino et al., 2012).

3.4.2 *In vitro* Digestion

An *in vitro* static digestion model simulating infant gastrointestinal conditions at the onset of complementary feeding (6–7 months old) was used to assess the digestibility of the powdered mixtures. The simulated *in-vitro* digestion conditions used in this study are summarised in Figure 3.1.

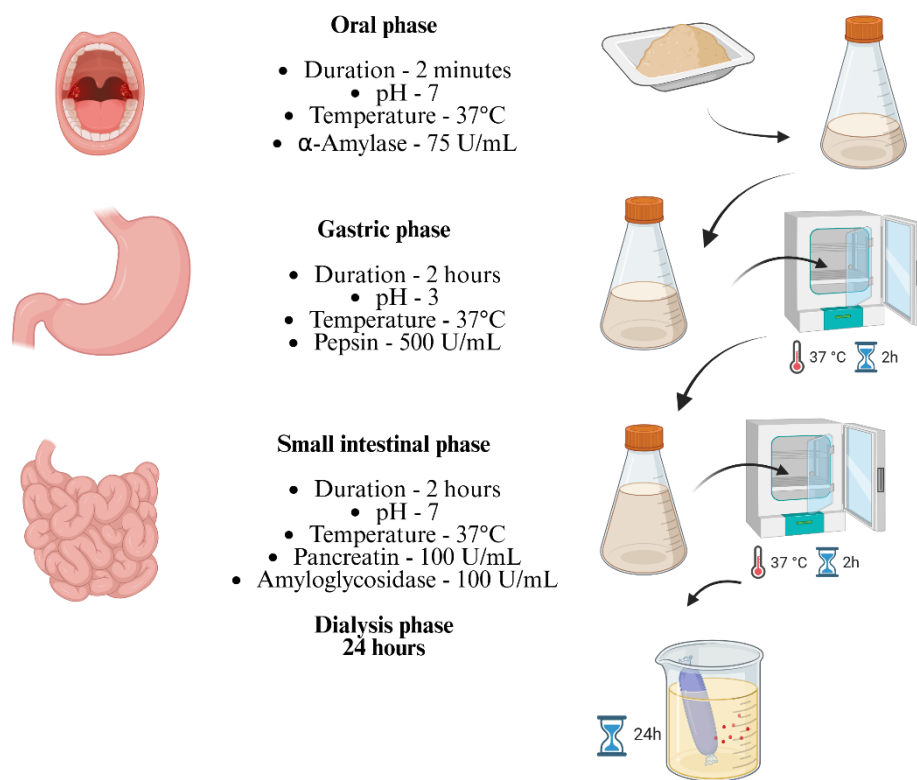


Figure 3.1. Composition of powdered kūmara and green banana extract mixtures. Created with BioRender.com.

3.4.2.1 Simulated Digestive Fluid Preparation

Electrolytes for the simulated fluids for the different phases—namely, electrolyte simulated salivary fluid (eSSF), electrolyte simulated gastric fluid (eSGF), and electrolyte simulated intestinal fluid (eSIF)—were prepared by mixing the appropriate electrolyte stock solutions, following the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014).

The electrolyte simulated digestion fluids were prepared at 1.25× concentration and stored at 4°C for no longer than one week. The addition of enzymes, CaCl₂ solution, and sterile H₂O to these stock solutions yielded final simulated digestive fluids at the correct 1× concentration, specifically: simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF).

The preparation of simulated digestive fluids and enzymes used in this experiment is provided in Appendix A. All enzymes were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

3.4.2.2 Oral Phase

The nine mixtures were digested in triplicate. A total of 7–8 digests were run simultaneously across four separate batches. The mixtures were randomised across the batches to account for batch effects. Potato starch, used as a control, was included in each batch. In total, 31 digests were conducted.

The SSF solution was prepared a day in advance by combining 7 mL of the eSSF solution, 1.95 mL sterile H₂O, and 0.05 mL CaCl₂ (1.5 M) solution. α -Amylase (A4551-100mg, Sigma) was not added to the pre-prepared SSF solution to allow better control over the short duration of the oral phase. The SSF solution was stored at 4°C overnight.

To reconstitute each mixture, 5 g of powdered mixture was added to pre-weighed containers, followed by 10 mL of sterile H₂O pre-warmed to 37°C. The SSF solution (also pre-warmed to 37°C) was added to the reconstituted mixture. Then, 1 mL of α -amylase solution (75 U/mL) was added to initiate the oral phase. Digests were incubated for 2 minutes.

The composition of the oral phase was: 50% 'food mixture' + 35% eSSF + 9.75% H₂O + 0.25% CaCl₂ solution + 5% α -amylase solution.

After 2 minutes, 0.32 mL of HCl (6M) was added to the digests to stop the oral phase reaction.

3.4.2.3 Gastric Phase

The SGF solution was prepared by mixing 12 mL eSGF solution with 1.112 mL H₂O, 0.008 mL CaCl₂ (1.5 M) solution, and 2.56 mL pepsin solution (500 U/mL, P7125-250U/mg, Sigma). This solution was stored at 4°C overnight.

The SGF solution was pre-warmed to 37°C and added to the 16 mL of oral phase digest. The pH was adjusted to 3, if required, using HCl (6 M) and NaOH (1 M). The digest was then incubated at 37°C in a shaking incubator at 150 rpm for 2 hours.

The composition of the gastric phase was: 50% oral digest + 1% HCl solution + 37.5% eSGF solution + 3.475% H₂O + 0.025% CaCl₂ solution + 8% pepsin solution.

After 2 hours, 0.18 mL of NaOH (1M) was added to stop the gastric phase reaction.

3.4.2.4 Intestinal Phase

The SIF solution was prepared using 13.2 mL eSIF, 1.572 mL H₂O, 0.048 mL CaCl₂ (1.5M) solution and 6 mL pancreatin (from porcine pancreas, P7545-100G, Sigma) and amyloglucosidase (from *Aspergillus niger*, 10113-1G/5G, Sigma) solution (100 U/mL concentration for each enzyme). This solution was stored at 4°C overnight. The bile salt solution (B8756, Sigma) is not included in the SIF solution but prepared on the same day of digestion to prevent contamination and maintain enzyme stability and activity.

Both the SIF solution and 3 mL of bile solution (0.01M) were pre-warmed to 37°C and added to the remaining 24 mL from the gastric phase. The pH was adjusted to pH 7 using HCl (6M) and NaOH (1M). The digest was then incubated at 37°C in a shaking incubator at 150 rpm for 2 hours.

The composition of the intestinal phase was: 50% gastric digest + 0.375% NaOH solution + 27.5% eSIF solution + 3.275% H₂O + 6.25% bile solution + 0.1% CaCl₂ solution + 12% Pancreatin & Amyloglucosidase solution.

After 2 hours, the digests were transferred to a water bath set to 95°C and incubated for 3 minutes to stop enzyme activity.

3.4.2.5 Dialysis Phase

Dialysis tubing (Spectra/Por 131060 Biotech-Grade CE Dialysis Tubing, 100-500 MWCO) was cut to 22-25 cm lengths (equivalent to 3.1 mL per cm of tubing). The tubing was washed inside and outside, then soaked in reverse osmosis (RO) water for 5 minutes.

The total volume of each digest after the intestinal phase was measured and recorded. Digests were transferred into the prepared dialysis tubing and sealed with dialysis clips. Each tube was placed in a 15 L bucket containing RO water (approximately 100x the digest volume). Dialysis was carried out over 24 h with the RO water changed three times.

After 24 h, the retentate was removed from the dialysis tubing, and the volume was measured and recorded.

3.4.3 *In vitro* Colonic Fermentation

Following simulated *in vitro* digestion, the digests were subjected to *in vitro* colonic fermentation. Faecal samples from healthy infant donors were used as microbial inocula to represent the infant gut microbiota, necessitating ethical approval and participant recruitment.

3.4.3.1 Ethics Approval and Participant Recruitment

The study was reviewed and approved by the Massey University Human Ethics Committee (Southern A, Application SOA 21/69, 30/11/2021).

Participant recruitment took place from September 2022 to January 2023. A recruitment email was distributed via the staff and student mailing lists at Massey University. In addition, recruitment posters were shared on Facebook groups and community pages.

Potential participants were first sent a study information sheet, followed by a screening questionnaire designed to assess eligibility. Screening was conducted based on the inclusion and exclusion criteria outlined below.

3.4.3.1.1 Inclusion Criteria

- Are healthy and free of illness for the past week.
- Aged between 5-7 months.
- Born at or after 32 weeks' gestation.
- Have not yet, or have only recently, been introduced to their first complementary foods.
- Have parents or legal guardians who can provide written informed consent.

3.4.3.1.2 Exclusion Criteria

- Born before 32 weeks' gestation.
- Small for gestational age (due to potential special dietary requirements).
- Have an illness likely to influence their nutritional status (e.g. chronic illness known to cause malabsorption, any digestive or metabolic disorders).
- Have health conditions affecting feeding.
- Currently receiving or have received antibiotic treatment in the last 3 weeks.
- Currently receiving supplements containing prebiotics and/or probiotics.

Of the 14 participants screened, five were ineligible, and two could not be contacted to collect samples after screening. Faecal samples were successfully collected from seven participants. Written informed consent was obtained from parents or guardians at the time of enrolment. Each participant provided a faecal sample, which was subsequently used to inoculate an *in vitro* model of colonic fermentation.

3.4.3.2 Faecal Sample Collection

Parents or guardians were provided with a faecal sample collection kit containing the following items:

- A chilly bin/cool box
- A freezer pack
- Gloves
- Antibacterial wipes
- Standard bamboo nappy liners
- Zip-lock bags labelled with the participant's Study ID
- Sample collection instructions

Parents/guardians were instructed to cleanse the infant's skin of powders or lotions using the antibacterial wipes and to place a bamboo nappy liner inside the infant's nappy. Once the infant defecated (without urine contamination), the soiled liner was transferred into the labelled zip-lock bag, and the time and date of collection were recorded. The sealed bag could then be stored either in a refrigerator or inside the cool box with the frozen freezer pack. Samples were collected or delivered to the Riddet Institute research facility within a few hours of collection.

At the laboratory, the faecal samples were immediately diluted in sterile 50 mM potassium phosphate buffer (pH 7.2-7.4), homogenised by palpitating, and strained through a filter bag. The amount of buffer added was recorded. The filtered faecal samples were aliquoted into 2 mL tubes and stored at -80°C until use in batch fermentation inoculum preparation.

3.4.3.3 Batch fermentation

Sterilised Hungate tubes were labelled for each retentate: one for the 0-hour timepoint and one for the 24 h timepoint. The fermentation media was prepared by mixing 2 mL of sterile 150 mM potassium phosphate buffer (pH 7.4) with 6 mL of the digestion retentate. Each tube was flushed with nitrogen, the headspace was filled with CO₂, and the tubes were sealed securely with stoppers.

Using an 18-gauge needle and a sterile 1 mL syringe, 100 μ L of 3% L-cysteine solution (filter sterilised and stored at -4°C for no longer than a week) was added to each tube. The tubes were left to stand for 5 minutes to allow the L-cysteine to consume any residual oxygen.

Additional Hungate tubes for gas pressure controls were prepared using 10 mL of 50 mM potassium phosphate buffer (pH 7.2-7.4) and 100 μ L of 3% L-cysteine solution. They were equalised at the same time as the experimental tubes and measured after the 48 h incubation.

The fermentation inoculum was prepared by defrosting the filtered faecal samples at -20°C for 48 h, followed by 1 hour at room temperature prior to use. Equal volumes of faecal material from each participant were pooled to create a composite inoculum. This was prepared under sterile conditions in a Class II Biosafety Hood, using a sterile glass beaker with a magnetic stirrer.

To each tube, 2 mL of inoculum (at 32% w/v) was added using a 20 mL syringe fitted with an 18-gauge needle to pierce the stopper. A second syringe was simultaneously used to equalise the internal pressure. An atmospheric pressure reading was taken at the time of inoculations.

Immediately after inoculation (0-hours), one Hungate tube per retentate was placed on ice for at least 20 minutes to slow microbial activity (Feng et al., 2018; Warren et al., 2018). The pH was measured and recorded. The contents were then vortexed, and a 1 mL aliquot (in duplicate) of fermentation fluid was collected for faecal organic acid and DNA analysis. This aliquot was centrifuged at 10 000 rpm for 1 minute, and the supernatant was transferred to a fresh tube, leaving the pellet in the original tube.

After 24 h of incubation in a shaking incubator at 37°C at 50 rpm, gas pressure was measured by connecting a needle to the pressure sensor and piercing the tube stopper. Headspace gas (2 mL) was extracted from the tube using a syringe and transferred into a 5.9 mL vial (Exetainer, Labco, Lampeter, UK) for hydrogen and methane analysis. The tubes were then placed on ice for 20 minutes to slow microbial activity. The pH was measured and recorded. A 1 mL aliquot (in duplicate) was removed

and processed as described for the 0-hour timepoint for faecal organic acids and DNA analysis. An additional 0.1 mL aliquot (in duplicate) was removed and mixed with 1 mL of a 2% zinc acetate solution for sulphide determination.

All subsamples were stored at -80°C until analysis.

3.4.4 Analyses

3.4.4.1 Total Gas Production and pH

A calibration curve was established to convert gas pressure readings (kPa) into gas volume (mL) (Appendix B). Calibration was conducted under experimental conditions by injecting known volumes of nitrogen gas (0-10 mL) into Hungate tubes and measuring the resulting pressure with a pressure probe. The measured atmospheric pressure was 101.72 kPa. A linear regression was fitted to the data ($R^2 > 0.99$), resulting in the equation:

$$\text{Gas volume (mL)} = (\text{measured pressure} - 106.19) / 13.439$$

This equation was used to calculate gas production during fermentation from pressure measurements.

During collection of fermentation supernatants at 0 and 48 h, pH was measured with a pH meter (Thermo Fisher Scientific, Waltham, MA, USA).

3.4.4.2 Headspace Gas (hydrogen and methane)

Hydrogen and methane concentrations in the headspace gas were analysed using a gas chromatograph (GC-2030 BID, Shimadzu, Kyoto, Japan) fitted with a Restek Msieve 5A PLOT column (30 m × 0.53 mm ID × 50 µM) and a helium photoinisation pulsed discharge detector (VIVI, Valco Instruments, Houston, USA), following the method described by Muetzel et al. (2018). A PAL Combi-xt autosampler (CTC Analytics AG, Zwingen, Switzerland) was used with a 32-well tray, 10 µL gas-tight syringe (Hamilton Ltd., Reno, USA).

Gas samples (10 μL) were injected using a splitless mode at an injector temperature of 250°C. Helium was used as the carrier gas under linear velocity mode with the following GC parameters: column flow 8.00 mL/min, total flow 11.0 mL/min, linear velocity 60.9 cm/sec, and purge flow 3.0 mL/min. The inlet pressure was set to 70.3 kPa. The column temperature was held isothermally at 80°C, and the BID detector was maintained at 300°C. The sampling rate was 40 ms, and the run time was set to 3 minutes.

Hydrogen and methane concentrations were quantified using calibration curves (Appendix C) generated from standard gas mixtures containing 0–10% hydrogen and 0–20% methane in nitrogen (BOC, Auckland, New Zealand). An air sample was included as a blank control. Peak areas ($\text{V} \cdot \text{min}$) were plotted against known concentrations, and linear regression equations were derived and used to calculate gas concentrations in fermentation samples. The hydrogen calibration curve used the equation:

$$\text{Hydrogen (\% v/v)} = (\text{peak area} \times 2.00 \times 10^{-7}) - 0.00475,$$

and the methane calibration used:

$$\text{Methane (\% v/v)} = (\text{peak area} \times 5.00 \times 10^{-9}) - 0.01.$$

3.4.4.3 Sulphide Determination

Hydrogen sulphide was quantified as described in Payling et al. (2021). Sulphide standards were prepared by handling sodium sulphide nonahydrate ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) crystals under a continuous stream of nitrogen gas to prevent oxidation. Crystals were washed with degassed water, gently blotted dry, and immediately transferred to a stoppered weighing bottle containing 5–10 mL of degassed reagent water. This process was repeated until the required mass of sodium sulphide was prepared. A 1 mg/mL stock solution (equivalent to 31 mmol/L) was prepared by dissolving 7.5 g of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in 1 L of degassed distilled water. Two working standards ranging from 0.02 to 0.16 $\mu\text{g/mL}$ were prepared from the stock solution and analysed to generate a standard curve (Appendix D).

Frozen fermentation aliquots preserved in zinc acetate were thawed at 4°C, and the pH was measured. The 0.1 mL aliquots were diluted 11-fold with distilled water and mixed with 0.1 mL “diamine solution” (0.2% 4-amino-N, -N-dimethylaniline HCl₂ w/v in 20% H₂SO₄ v/v) and 0.1 mL “Fe solution” (0.5% FeNH₄(SO₄)₂ w/v in 2% H₂SO₄ v/v). Samples were incubated at room temperature (21-27°C) for ten minutes to allow colour development. The absorbance of both standards and samples was measured at 670 nm using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Hydrogen sulphide (H₂S) production was estimated by first correcting the measured sulphide concentrations for pH-dependent speciation, using the known pK_a of H₂S (6.84). The proportion of undissociated H₂S in solution was calculated and used to estimate the gas-phase concentration based on the liquid–gas partition coefficient and fermentation volume. Final H₂S gas volumes were then derived using the ideal gas law and expressed as the change in H₂S volume ($\Delta[\text{H}_2\text{S}]$) over time, normalised to the dry weight of the fermentation substrate (L/kg DM).

3.4.4.4 Shotgun Metagenomics

Faecal DNA was extracted from fermentation samples collected at 0h (baseline, 3 randomly selected samples) and at 24 h (all 31 samples). Frozen faecal pellet fractions were thawed at 4°C prior to DNA extraction using the Macherey-Nagel NucleoSpin Soil Kit, following the manufacturer's instructions with the addition of a bead beating step (4 minutes, Mini-Beadbeater 96, BioSpec, Bartlesville, USA) as detailed by Young et al. (2020).

DNA quality was initially assessed using the Qiaxcel DNA High Resolution Kit on the QiAxcel Connect (Qiagen, Hilden, Germany), following the manufacturer's instructions. Results were visualised and analysed using ScreenGel 1.6 software (Qiagen, Germantown, MD, USA). DNA concentration was quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Subsequently, DNA samples underwent further quality control testing by Custom Science Ltd (Auckland, New Zealand), including integrity and potential contamination assessment on 1% agarose gels. DNA purity, determined by optical density (OD) at 260/280 and 280/230, was evaluated using a NanoPhotometer (IMPLEN, CA, USA), and DNA concentrations were measured with a Qubit dsDNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). Only samples with OD values between 1.8 and 2.0 and the total DNA content above 1 µg were selected for library preparation.

For library preparation, 500 ng of DNA per sample was used as input material. Sequencing libraries were constructed using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's recommendations. Briefly, DNA was fragmented by sonication to an average size of 300 bp, followed by end polishing, A-tailing, and ligation with full-length adaptors suitable for Illumina sequencing. Amplified libraries were purified using the AMPure XP system, library size distribution was confirmed using an Agilent 2100 Bioanalyzer, and library concentration was quantified via real-time PCR.

Indexed libraries were pooled and sequenced on an Illumina NovaSeq X Plus (Illumina, USA) platform to generate 150-bp paired-end reads. Sequencing services were provided by Annoroad Gene Technology Corporation (Beijing, China).

Sequencing data were supplied as raw and filtered reads. Subsequent processing of data was performed by Jane Mullaney at AgResearch. Initial sequence quality was assessed using FastQC (version 0.12.1), with results summarised across samples using MultiQC (version 1.25.1). Biobakery tools kneaddata v0.12.2 was used with Trimmomatic (version 0.33 automatically installed as part of kneaddata) for removal of adapters, low quality (quality score < Q20), and short (< 36 bp) sequencing reads. Host contamination reads were removed using Bowtie2 version 2.5.4 with default settings, mapping to the reference human genome database (Homo_sapiens_hg39_T2T). Paired reads and

surviving unpaired forward and reverse reads were concatenated and compiled into a final “clean” read fastq sample file for downstream taxonomy assignment and functional analyses.

Taxonomic profiling was performed using Metagenomic Phylogenetic Analysis (MetaPhlAn version 4.0.6 (1 Mar 2023)) (Blanco-Míguez et al., 2023), with accompanying CHOCOPhlan database (mpa_vOct22_CHOCOPhlanSGB_202403). The analysis was run with default parameters, enabling strain-level microbial profiling and resolution down to the species level where possible.

Following taxonomic profiling, we performed functional analysis using *Human Microbiome Project Unified Metabolic Analysis Network* (HUMAN 3 v4.0.0. alpha.1) (Beghini et al., 2021). In this pipeline, the taxonomic profile was used to perform species-specific pangenome mapping. Remaining unmapped reads were then aligned directly to the UniRef90 protein database. This approach allowed for comprehensive quantification of gene families and MetaCyc metabolic pathways, producing outputs with both community-wide and taxon-stratified abundances.

3.4.4.5 Organic Acid Production

Fermentation supernatants (900 µL) were thawed on ice and mixed with 100 µL of 50 mM 2-ethyl butyric acid solution, resulting in a final standard concentration of 5 mM. An aliquot of 500 µL from this solution was transferred to a tube containing 250 µL of concentrated HCL (37% v/v) and 1250 µL of diethyl ether. The mixture was shaken vigorously to extract organic acids into the ether phase. Following phase separation of the solvent and liquid phases, 200 µL of ether layer was transferred to a 2 mL glass gas chromatography (GC) vial with 40 µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) derivatisation agent. Vials were sealed with 11 mm crimp top caps fitted with PTFE/rubber septa and mixed thoroughly. Derivatisation proceeded through a two-step process: initial incubation at 80°C for 20 minutes, followed by 48 h at room temperature to ensure complete derivatisation of all organic acids.

Derivatised samples were analysed on a Shimadzu Nexus GC-2010 (Kyoto, Japan) equipped with a flame ionisation detector and an Agilent HP-1 capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness) (Santa Clara, CA, USA). The autosampler (AOC-20i) injected 1 µL of sample using a split injection mode with a split ratio of 10:1. The syringe was rinsed six times with solvent before and after injection, twice with sample, and underwent an 8 µL washing cycle using diethyl ether (solvents A and C) and methanol (solvent B) to prevent carryover.

The injection port was maintained at 260°C. High purity helium served as the carrier gas with a constant linear velocity of 37.5 cm/s, resulting in a column flow rate of 1.66 mL/min and an inlet pressure of 131.2 kPa. The purge flow was set to 3.0 mL/min. The oven temperature began at 70°C with a 0.4 minute hold, then ramped at 6°C/min to 115°C, followed by a second ramp at 60°C/min to 300°C, where it was held for 3 minutes. The total run time was 13.58 minutes.

The flame ionisation detector operated at 310°C with hydrogen flow at 40 mL/min, and nitrogen makeup gas at 30 mL/min. Data was acquired at 120 ms intervals over a 14-minute detection window. Chromatographic data were processed using LabSolutions software (Version 5.98, Shimadzu). The column inlet liner and septum were replaced before each analytical batch, and the system was conditioned with 5-10 consecutive injections of a representative sample before analysing experimental samples.

A multipoint calibration curve was generated for eleven organic acids in order of retention time: formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, hexanoate, heptanoate, lactate and succinate. Calibration solutions were at 0.15, 0.25, 0.5, 1, 2, 5, 10, 15 and 20 mM concentrations. Due to the high yields of acetate and lactate observed in preliminary fermentations, the calibration range for these two acids was extended to 40 and 80 mM, respectively (Appendix E).

Individual organic acid concentrations were determined by integrating the peak area for each analyte, normalising this to the peak area of the internal standard (2-ethylbutric acid) and comparing the resulting ratio to the appropriate calibration curve. Net organic acid production during fermentation

was calculated as the difference in concentrations between paired 0 h and 24 h samples. Organic acid concentrations are reported in millimolar (mM) on a fresh weight basis, representing the concentration in the fermentation supernatant without correction for dry matter content.

To enable comparison across different substrates, organic acid production was normalised to the theoretical dry matter content of each fermentation. This value was calculated by determining the concentration of food material in the post-dialysis digest (retentate), accounting for all dilution factors introduced during digestion and dialysis, and back-calculating the equivalent mass that originated from the oral phase. This normalisation provides a standardised metric of millimoles of organic acid produced or consumed per gram of dry matter over 24 h fermentation period, allowing direct comparison of fermentative capacity across diverse food matrices.

3.4.4.6 Data Analyses

All statistical analyses were performed in R version 4.4.3 (R Core Team, 2023). Key packages included: phyloseq for data management, vegan (v 2.6.10) for diversity indices and PERMANOVA, MaAsLin3 (v 1.1.2) for differential abundance testing, mixOmics (v 6.30.0) for multi-omics integration, and ggplot2 (v 4.0.0) for visualisation.

Nutrient composition data (proximate measures, starch fractions, sugars and fibre components) were summarised descriptively for each mixture, and trends across the kūmara–GBE gradient. Sugar profiles were expressed as a percentage of total carbohydrate, and kūmara polysaccharide composition was summarised as the proportion of each polysaccharide class relative to total destarched cell wall material.

Gas production parameters (total gas pressure, hydrogen concentration and hydrogen sulphide) were compared among mixtures using Kruskal–Wallis tests where assumptions of normality or equal variance were not met, and one-way ANOVA where assumptions were satisfied. Dunn tests (Kruskal–

Wallis) or Tukey's HSD (ANOVA) were used for post-hoc contrasts. Methane was below detection limits in all samples and therefore not statistically analysed.

Fermentation validity was confirmed by comparing three randomly selected baseline (0 h) samples with their paired post-fermentation (24 h) samples. Alpha diversity metrics (Shannon index and observed species) were calculated, and differences were tested using Wilcoxon signed-rank tests. Beta diversity was assessed using Bray-Curtis dissimilarity, with paired PERMANOVA (999 permutations) evaluating compositional shifts between baseline and post-fermentation samples.

Potato starch (PS) was included as a positive control to assess technical reproducibility across fermentation batches. PS was evaluated alongside the kūmara-GBE mixtures for fermentation validation using diversity-based quality control metrics. Alpha diversity (Shannon diversity and observed richness) was compared across batches to assess consistency. Beta-diversity patterns were examined descriptively using Bray-Curtis distances; however, due to the presence of a single PS sample per batch, formal inferential testing of batch effects using PERMANOVA and dispersion analyses was not statistically evaluable.

For downstream taxonomic analysis of diversity, species were retained if they had a minimum total abundance of ≥ 20 reads across all samples and were present in more than 10% of samples. This removed low-abundance and highly sparse data, resulting in 18 of 117 species being retained.

Alpha diversity (Shannon and observed features) was compared among mixtures using Kruskal-Wallis tests with Wilcoxon rank-sum post hoc tests. Associations between Shannon diversity and kūmara concentration were assessed using Spearman correlations. Beta diversity was evaluated using Bray-Curtis dissimilarity on the same filtered dataset. PERMANOVA (999 permutations) tested for community differences among mixtures, and homogeneity of dispersion was assessed prior to interpretation. Principal coordinates analysis (PCoA) was used for visualisation.

Differential abundance analysis was performed using MaAsLin3 (Nickols et al., 2024), which accounts for compositional data while controlling for multiple covariates. MaAsLin3 handles zero-inflation (a common challenge in sequencing data) by automatically filtering taxa with low prevalence and incorporates zero-inflation into its modelling framework by using a zero-inflated Gaussian model. The MaAsLin3 pipeline retained 5 of 6 phyla, 54 of 62 genera, and 101 of 117 species.

Two complementary models were fitted:

1. a categorical mixture model with 100% kūmara (100/0 mixture) as the reference group, and
2. a continuous kūmara gradient model with kūmara proportion as a numeric predictor.

Batch was included as a fixed effect in both models. PS and FreshAs were omitted from the gradient model. PS does not contain kūmara and FreshAs, although matching the 85/15 kūmara-GBE mixture ratio, was a prepared product, mixed independently of the other mixtures, and the GBE used came from a different batch. Including it would therefore introduce uncontrolled technical variation into the continuous gradient model, hence its exclusion. All analyses used total sum scaling (TSS) normalisation, log transformation, and Benjamini-Hochberg FDR correction, with significance defined at $q < 0.05$.

Functional pathway abundance was performed on MetaCyc pathway-level abundance data using the same MaAsLin3 framework. Pathways present in $\geq 20\%$ of samples (427 of 6349) were retained for analysis. For significant pathways, taxonomic contributors were identified using species-stratified pathway abundances to link functional shifts to specific microbial taxa. Concordance between pathway-level and gene-level (KEGG Orthology, KO) differential abundance was evaluated. Constituent KO families were examined to determine the percentage showing concordant directional effects.

Organic acid concentrations were calculated as Δ = concentration at 24 h minus concentration at 0 h. Differential organic acid production among mixtures was assessed using MaAsLin3. Each organic acid was modelled separately as a continuous outcome, using mixture as a categorical predictor

(100% kūmara mixture reference) and, in a second model, kūmara proportion as a continuous predictor (PS and FreshAs mixtures excluded). Benjamini–Hochberg FDR correction was applied across organic acids tested. Relative abundances of organic acids (each organic acid expressed as a percentage of total acids at 24 h) were analysed using the same models.

Multi-omics integration combined four data blocks: (1) nutritional composition (fat, carbohydrates, starch, sugars, resistant starch, total dietary fibre, neutral detergent fibre, acid detergent fibre, β -glucan, non-protein nitrogen, crude protein), microbial taxonomy, functional pathways, and fermentation outputs (organic acids FW concentration: acetate, propionate, butyrate, lactate, succinate, formate, isobutyrate, valerate, isovalerate, hexanoate, heptanoate; gas pressure, hydrogen, and hydrogen sulphide concentrations). Multi-block principal component analysis (mbPCA) was performed using the *mixOmics* package (Rohart et al., 2017) to identify coordinated patterns of variation across and dominant sources of variance across the datasets. Partial least squares (PLS) regression models were then fitted to predict fermentation outputs from nutritional, taxonomic, and functional pathway features, with Variable Importance in Projection (VIP) scores (Mehmood et al., 2021) identifying key contributors.

3.5 Results

3.5.1 Nutrient composition

The nutritional composition of the different kūmara and GBE mixtures is presented in Table 3.2. Dry matter (DM) content ranged from 98.25% in the 85/15 mixture to 95.20% in the 100% banana mixture. Ash content remained relatively consistent across mixtures, ranging from 2.68% to 2.88%.

Carbohydrates were the major component across all mixtures, ranging from 79% to 82.5%. Protein and fat contents were relatively consistent across mixtures, with only minor variations.

Starch and resistant starch (RS) contents differed markedly between mixtures (Table 3.2). Starch content increased with greater inclusion of GBE, from 46.4% (100% kūmara) to 73.2% (100% GBE).

This trend corresponded with a substantial increase in RS content, increasing from 1.18% to over 30%.

Table 3.2. Proximate and nutritional composition of the different kūmara and green banana extract mixtures. All components are given as a percentage of the sample weight.

Nutrients (%)	Mixture								
	100/0	92.5/7.5	85/15	FreshAs	70/30	50/50	30/70	15/85	0/100
DM	98	98.2	98.3	96.8	97.8	97	96.7	96.1	95.2
Ash	2.9	2.8	2.8	2.7	2.7	2.7	2.7	2.6	2.6
Fat	0.6	0.8	0.7	0.8	0.9	1	0.8	0.8	0.8
Carb	82.5	82.5	82.4	81.3	82	79	80.2	79.5	78.4
Starch	46.4	44.1	48.1	46.2	52.3	57.9	63.5	69.6	73.2
Sugars	54.2	38.9	41.1	37.7	37.6	24.6	23.4	12.4	1.5
RS	1.18	3.53	4.01	5.51	9.43	16.52	20.42	25.80	30.52
TDF	7	7	7	7.2	7.1	9.2	7.7	7.9	8.1
NDF	4.1	4.7	5.8	6.4	6.1	4.5	8	6	6.4
ADF	2.2	2.2	2.4	1.8	2.7	2.1	2.8	2.2	2.9
B-glucan	<0.1	0.12	0.08	<0.1	0.09	<0.1	<0.1	<0.1	<0.1
NPN	0.18	0.14	0.19	0.17	0.2	0.16	0.23	0.23	0.26
Crude protein	5	5	5.3	4.8	5.1	5.1	5.2	5.2	5.3

Abbreviations: DM, dry matter; Carb, carbohydrate; RS, resistant starch; β -Glucan, beta-glucan; TDF, total dietary fibre; NDF, neutral detergent fibre; ADF, acid detergent fibre; NPN, non-protein nitrogen.

Conversely, total sugar content decreased markedly from 54.2% (100% kūmara) to 1.5% (100% GBE)(Appendix F). Maltose and sucrose were the dominant sugars present in the kūmara-rich mixtures, with maltose concentration decreasing from 36.8% to undetectable concentrations (< 0.1%), and sucrose declining from 14.9% to 0.9% across the mixtures (Figure 3.2). The high maltose concentration in kūmara likely reflects the impact of cooking, as raw kūmara typically contains lower concentrations.

Total dietary fibre (TDF) increased slightly with higher proportions of GBE, (7.0% to 8.1%), while β -glucan content remained low across all mixtures (<0.1%). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) varied between mixtures (NDF: 4.1–8.0%; ADF: 2.0–2.9%). Notably, the 50/50

mixture exhibited the highest TDF (9.2%) but lower NDF and ADF than other banana-rich mixtures, suggesting a greater proportion of soluble fibre components.

The FreshAs blended mixture (85:15 ratio) exhibited differences from the self-prepared 85/15 mixture (despite having the same ingredient ratio: lower DM (96.8% versus 98.3%), higher RS (5.5% versus 4.0%), higher NDF (6.4% versus 5.8%), lower ADF (1.8% versus 2.4%), and lower crude protein (4.8% versus 5.3%)).

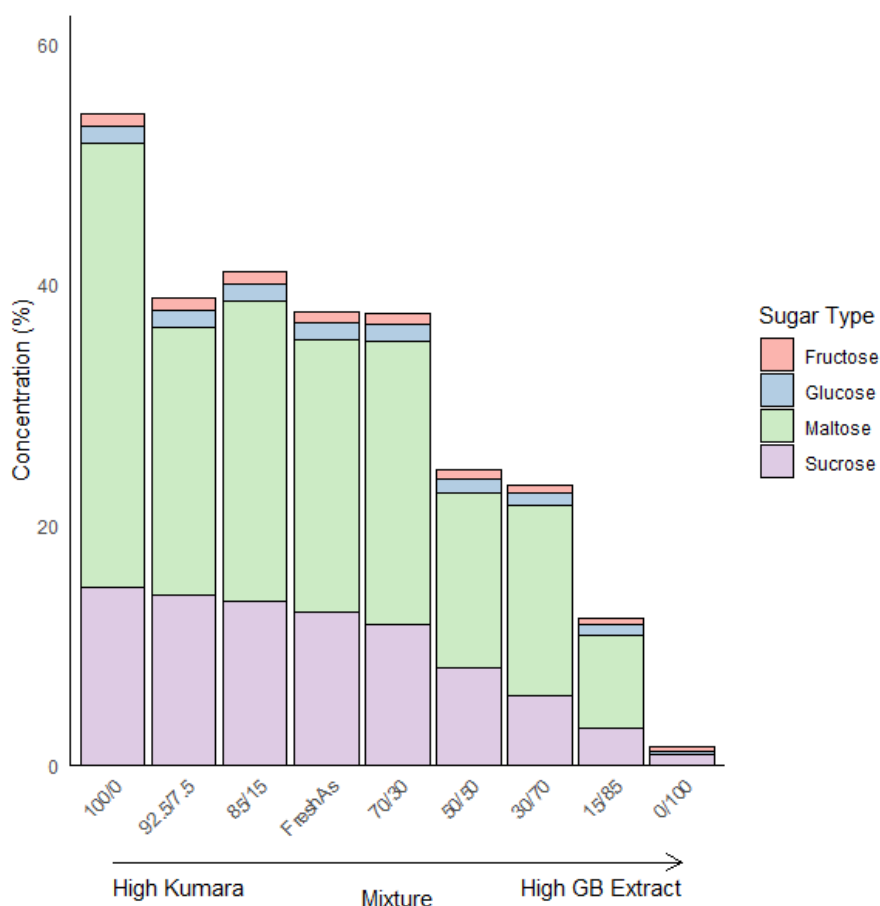


Figure 3.2. Sugar composition of kumara and green banana extract mixtures.

3.5.2 Polysaccharide composition of kumara

A total of nine major polysaccharide types were identified across the extracts (Figure 3.3), with pectins as the dominant fibre class. Homogalacturonan (HG), a galacturonic acid-rich pectin backbone, was the most abundant (40.48% of total destarched cell wall polysaccharides). Additional

pectic polysaccharides included rhamnogalacturonan-I (RG-I) backbone (6.07%), arabinan (2.45%), arabinogalactan I (AG-I, 4.33%), and arabinogalactan II (AG-II, 6.63%). Collectively, pectin-type polysaccharides represented approximately 60% of total polysaccharide composition. These represent the major non-cellulosic components of the kūmara powder.

Hemicelluloses were present in lower amounts: xyloglucan (9.5%), heteroxylan (4.04%), and heteromannan (0.73%), primarily extracted in alkaline fractions. The final residue was interpreted to consist primarily of cellulose (approximately 90% based on 1→4-linked glucose linkage profile), with minor contributions from tightly bound hemicellulosic components.

These results confirm that kūmara cell wall fibre is predominantly composed of soluble pectic polysaccharides, with a notable cellulose fraction and comparatively low hemicellulose content.

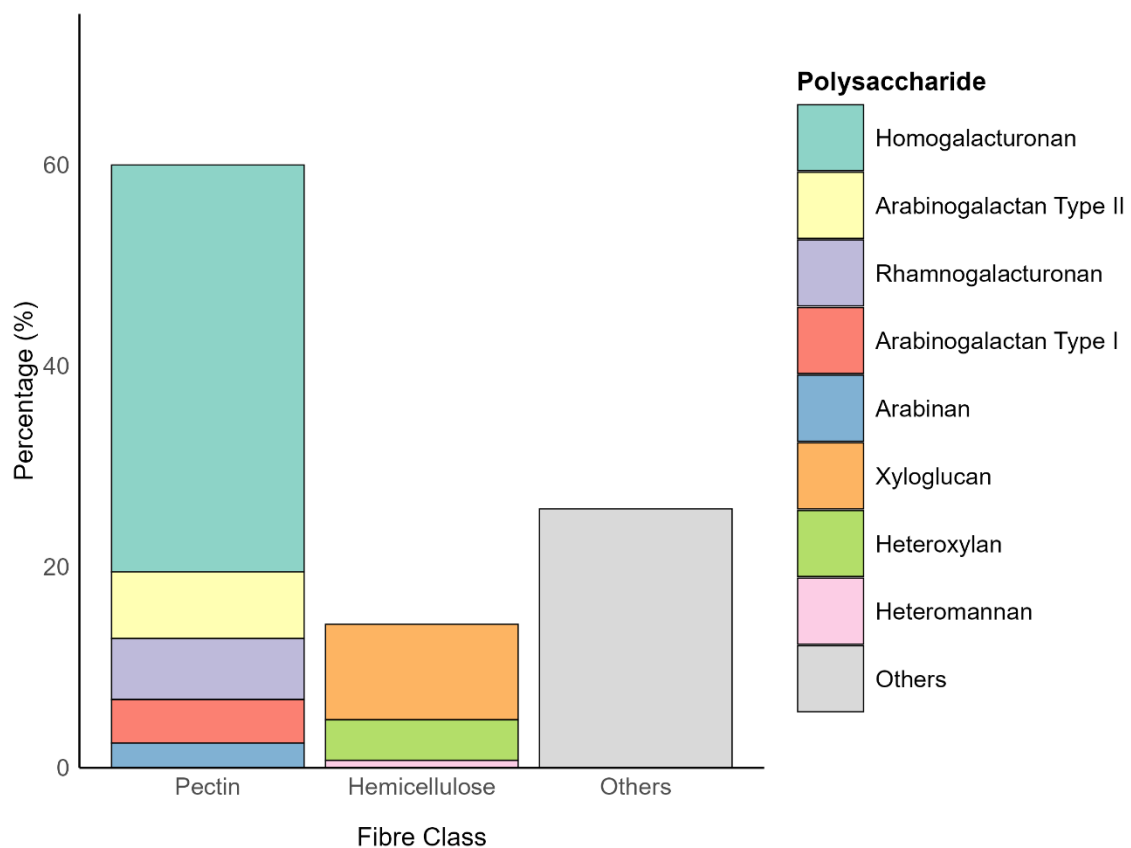


Figure 3.3. Polysaccharide composition of 100% kūmara mixture by fibre class. Stacked bars sum to 100% of the total polysaccharides

3.5.3 Gas production and composition

Gas production parameters after 24 h fermentation are presented in Table 3.3. No significant differences were observed among mixtures for total gas pressure (Kruskal–Wallis: $\chi^2 = 4.86$, $p = 0.77$), hydrogen concentration (Kruskal–Wallis: $\chi^2 = 12.72$, $p = 0.18$), or hydrogen sulphide production (Kruskal–Wallis: $\chi^2 = 5.25$, $p = 0.73$). Methane production was negligible (<0.1%) across all mixtures. Collectively, these results indicate comparable overall fermentation activity across substrate formulations.

Table 3.3. Summary of gas pressure, hydrogen concentration, and hydrogen sulphide per ferment across mixtures.

Mixture	Gas pressure (mL)	Hydrogen (%)	H ₂ S per ferment (L/kg DM)
100/0	1870 ± 29.3	2.74 ± 2.11	0.004 ± 0.004
92.5/7.5	1853 ± 29.4	2.63 ± 1.13	0.015 ± 0.018
85/15	1848 ± 54.5	5.06 ± 1.25	0.001 ± 0.015
FreshAs	1842 ± 12.8	3.02 ± 2.42	−0.003 ± 0.013
70/30	1839 ± 30.2	5.05 ± 1.08	0.071 ± 0.121
50/50	1882 ± 71.3	4.53 ± 1.13	0.019 ± 0.049
30/70	1812 ± 41.0	4.85 ± 1.56	−0.009 ± 0.020
15/85	1855 ± 63.2	3.81 ± 0.65	0.009 ± 0.012
0/100	1893 ± 85.6	5.19 ± 0.58	−0.004 ± 0.007

3.5.4 Validation of Fermentation and Inter-Batch Performance Using the Potato Starch (PS) Control

Three randomly selected baseline samples (0 h) paired with their corresponding 24 h fermentations were included to confirm that microbial fermentation occurred during incubation. Alpha diversity decreased consistently across all three baseline–fermentation pairs (mean Shannon: 2.50 at 0 h versus 2.08 at 24 h; mean richness: 18 versus 16.7). Paired Wilcoxon tests did not reach statistical significance (Shannon $p = 0.25$; richness $p = 0.17$), reflecting the very limited power with $n = 3$;

however, the uniform direction and magnitude of change across all pairs are consistent with selective enrichment during active fermentation.

Beta diversity provided clear evidence of fermentation-driven community restructuring. PERMANOVA identified a strong effect of timepoint on microbial community composition when baseline and 24 h samples were considered collectively ($R^2 = 0.86$, $p = 0.001$), with no corresponding difference in dispersion (PERMDISP $p = 0.82$). Pairwise comparisons between baseline and individual mixtures did not reach statistical significance, likely reflecting limited permutation resolution at small sample sizes, but 24 h communities consistently separated from baseline in ordination space and showed no evidence of mixture-specific divergence.

Potato starch (PS) was included as a positive control to assess technical consistency across fermentation batches. Alpha diversity of PS samples was highly consistent between batches, with no significant differences in Shannon diversity (Kruskal–Wallis $\chi^2 = 3$, $p = 0.39$), and richness was invariant across all samples (17 taxa). Due to the presence of only one PS sample per batch, formal beta-diversity tests for batch effects were not statistically evaluable. Nevertheless, PS samples clustered within the same overall compositional space as the kūmara–GBE mixtures, supporting the interpretation that observed variation among mixtures reflects biological differences rather than batch-related artefacts.

3.5.5 Microbial Diversity

Following quality filtering, 18 core microbial taxa by 8 taxonomic ranks were retained for analysis. A Kruskal-Wallis test comparing Shannon diversity across mixtures (excluding PS) indicated that differences between mixture groups approached, but did not reach, statistical significance ($\chi^2 = 15.35$, $p = 0.053$). Subsequent pairwise Wilcoxon tests did not identify any significant pairwise contrasts after multiple-testing correction (all BH-adjusted $p > 0.30$). This pattern suggests that the observed variability was weak and distributed across multiple mixtures with no individual mixture producing a distinct alpha-diversity response (Figure 3.4). Spearman rank correlation between kūmara mixture

percentage and Shannon diversity indicated no evidence of a monotonic relationship between kūmara content and alpha diversity; coefficient was very close to zero ($\rho = -0.043$) with a high p-value ($p = 0.833$).

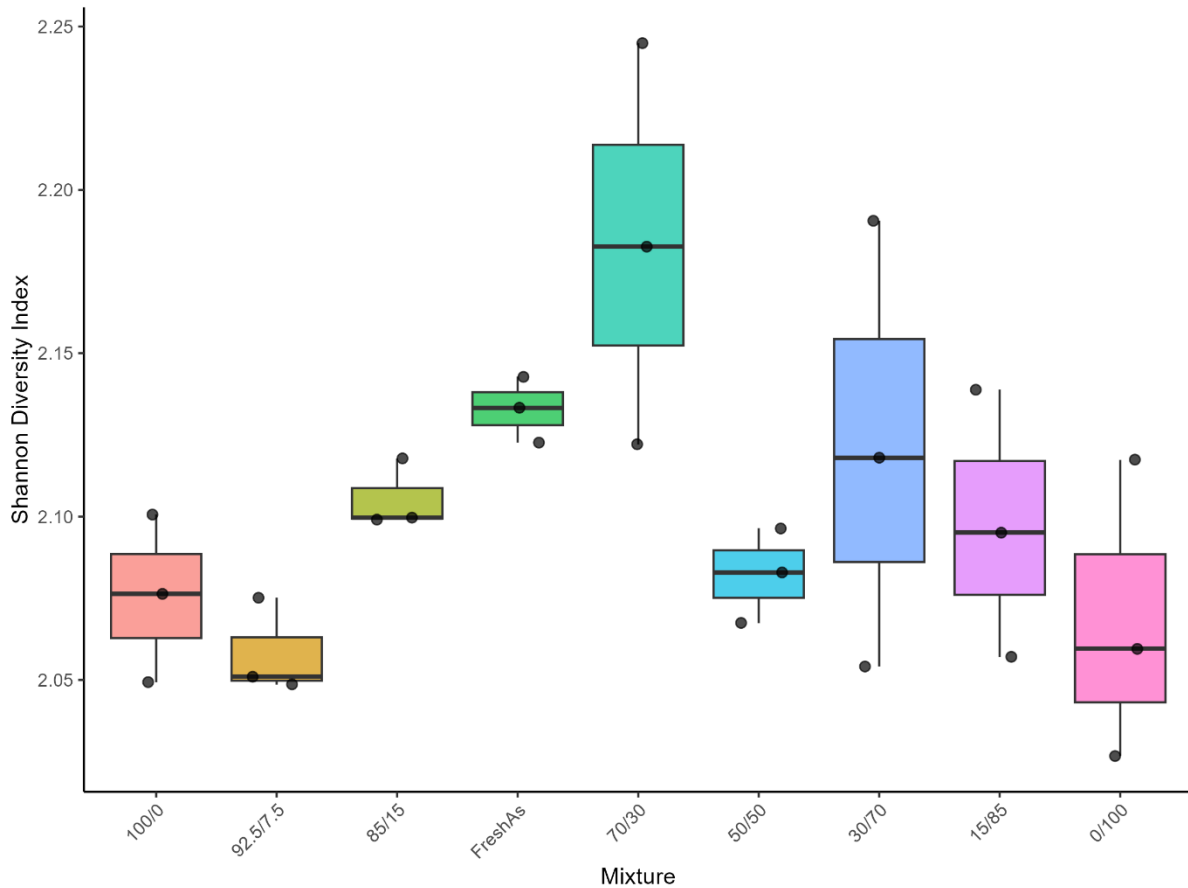


Figure 3.4. Alpha diversity (Shannon) across mixtures, ordered from highest to lowest kūmara content.

No significant mixture-driven differences in beta diversity were detected. Bray-Curtis PERMANOVA showed that neither mixture ($R^2 = 0.346$, $p = 0.27$) nor kūmara content ($R^2 = 0.077$, $p = 0.078$) contributed significantly to variation in microbial community composition. Pairwise comparisons supported this finding with no mixture combination exhibiting significant differences after multiple-testing adjustment. Community dispersion was also homogeneous across groups ($p = 0.889$). This suggests that the fermenting community has responded similarly to all mixture formulations and that differences in mixture composition were insufficient to drive detectable shifts in global microbial community structure.

When included alongside the kūmara-GBE mixtures, potato starch showed alpha-diversity values within the same overall range, and no significant pairwise differences were detected between PS and any other mixture. Including PS in the beta-diversity analysis did not alter the results (R^2 and p-value were unchanged), indicating that it fosters a similar microbial composition and does not form a distinct cluster compared to the mixtures.

3.5.6 Taxonomic Composition

Across all fermentations, the microbial communities were consistently dominated by Actinomycetota, which comprised 65-70% of total relative abundance across all mixtures, including PS. This phylum signal was driven primarily by *Bifidobacterium* species, which was the most abundant genus in every mixture. The most abundant representatives were *Bifidobacterium longum*, *B. breve*, and *B. pseudocatenulatum*. Secondary contributions were derived from Firmicutes, including *Ruminococcus gnavus*, *Erysipelatoclostridium ramosum*, and enterococci, as well as low-abundance Proteobacteria and Bacteroidota taxa. No mixture produced unique dominant taxa, and PS community closely mirrored the mixtures. This compositional stability is visualised in Figure 3.5.

MaAsLin3 was applied to species-level abundance data using 100/0 (100% kūmara) as the reference and batch included as a covariate. Among 85 species tested, none were statistically significant after FDR correction ($q < 0.1$).

Despite the absence of statistically significant associations in the mixture-based MaAsLin models (Supplementary File 1), several taxa exhibited consistent directional trends across multiple kūmara-GBE formulations. *Clostridium butyricum* showed a tendency toward increased abundance in mixtures containing higher proportions of green banana extract, corresponding to approximately two-fold higher abundance in the highest-GBE formulations. *Bacteroides ovatus* displayed a similar pattern, with higher relative abundance observed across several mixtures enriched in green banana extract compared with kūmara-dominant formulations. These trends were consistent across multiple

mixtures but did not reach statistical significance after false-discovery rate correction, indicating weak and distributed effects rather than a strong response driven by any single formulation.

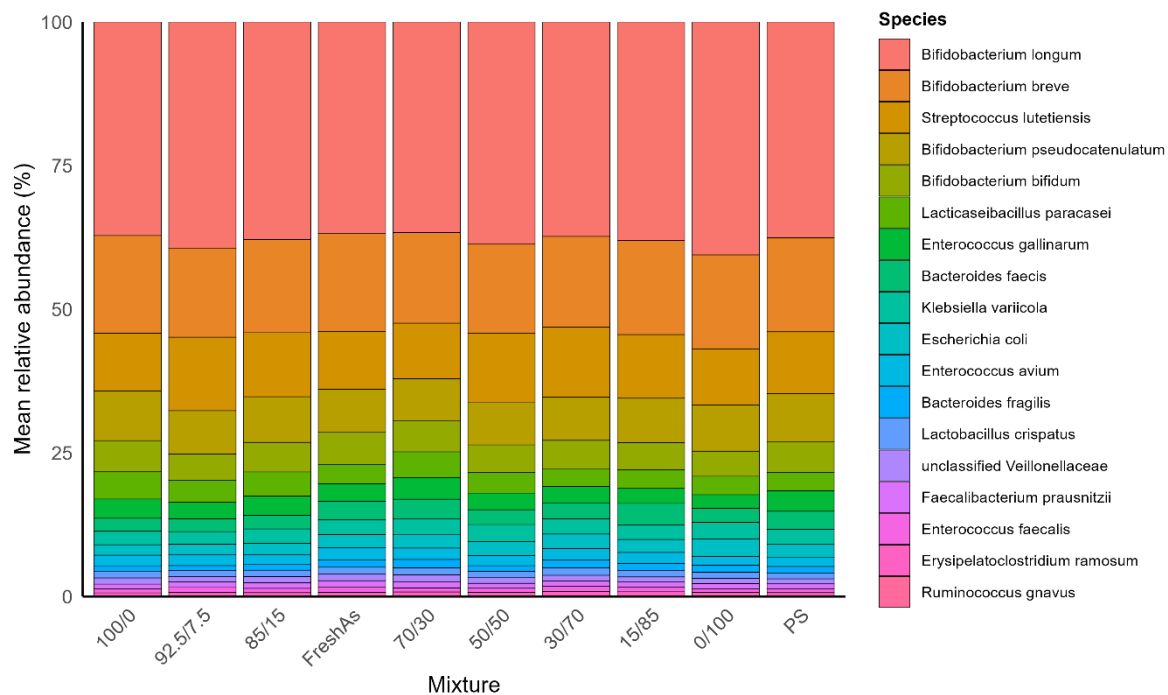


Figure 3.5. Mean species-level relative abundance (%) across the kūmara-green banana extract mixtures and potato starch (PS) following 24 h fermentation.

Akkermansia muciniphila exhibited comparatively large effect sizes but substantial variability across mixtures. While positive associations were observed in both high-GBE mixtures and the potato starch control, detection was limited to a small subset of samples and variance was high, preventing these associations from reaching statistical significance. As potato starch was included solely as a fermentation validation control, interpretation here focuses on the kūmara–GBE mixtures, with PS shown only for contextual comparison in the figure.

Overall, these findings suggest that while individual taxa may exhibit directional responses across the mixture range, the magnitude of these effects was insufficient to produce robust, mixture-specific taxonomic shifts under the conditions tested.

3.5.7 Microbial Pathway Abundance

Pathway-level differential abundance was assessed using MaAsLin3 with mixture as the main effect (100/0 kūmara as the reference level) and batch included as a covariate to control for technical variation. Using the joint FDR threshold ($q < 0.05$) and focusing on the abundance model, 148 significant pathway-mixture associations were identified, corresponding to 94 distinct MetaCyc pathways across the non-reference mixtures (Supplementary File 2).

The vast majority (142 of 148, 96%) of significant associations showed negative coefficients, indicating that these pathways were less abundant in mixtures with higher GBE content than in the 100% kūmara reference mixture. Six pathways showed positive coefficients, all in the 100% GBE mixture only, representing enrichment of catabolic pathways including allantoin degradation (URDEGR-PWY, PWY-5692, PWY-5705) and aromatic compound catabolism (HCAMHPDEG-PWY, PWY-6690, PWY-6318).

The 100% GBE mixture (0/100) showed the largest number of significant differences (93 pathways), followed by FreshAs (22 pathways), PS (9 pathways), 30/70 (8 pathways), 50/50 (6 pathways), 15/85 (5 pathways), 70/30 (3 pathways), and 92.5/7.5 (2 pathways). Notably, the 85/15 kūmara:GBE mixture showed no significant pathway differences from the 100% kūmara reference, indicating that GBE content at or below 15% had minimal impact on overall functional potential. This threshold effect is directly relevant to the SUN Study intervention, which used an 85:15 kūmara:GBE ratio.

The most prominent functional pattern among suppressed pathways was the coordinate reduction of nucleotide metabolism. Nineteen pathways related to purine and pyrimidine biosynthesis, salvage, and phosphorylation, representing 22% of all significantly suppressed pathways, were markedly reduced in GBE-containing mixtures relative to 100% kūmara. These nucleotide metabolism pathways showed the most extensive mixture effects, with pyrimidine deoxyribonucleotides de novo biosynthesis IV (PWY-7185) and pyrimidine deoxyribonucleotides de novo biosynthesis I (PWY-7210) each significant across seven different mixture comparisons.

The glycolysis III (from glucose) pathway (ANAGLYCOLYSIS-PWY) was significantly reduced in seven non-reference mixtures (0/100, 15/85, 30/70, 50/50, 70/30, FreshAs, and PS), showing the most widespread suppression across substrate types. However, the magnitude of suppression was modest, ranging from 0.93-fold in the PS control (7% decrease) to 0.98-fold in the 70/30 mixture (2% decrease).

Beyond nucleotide metabolism, GBE-containing mixtures showed coordinate suppression of pathways involved in core biosynthetic processes. Amino acid biosynthesis was broadly affected, with eight pathways showing mixture-dependent reductions. These included pathways for both L-arginine biosynthesis routes (suppressed in five to six mixtures), branched-chain amino acids (four mixtures), and pathways for L-tryptophan, L-threonine, L-isoleucine, L-histidine (each suppressed in four to five mixtures), as well as L-lysine and L-valine biosynthesis (suppressed in 100% GBE and two mixtures, respectively).

Cell wall biosynthesis pathways showed similarly coordinated suppression. Peptidoglycan biosynthesis was reduced in eight mixtures, while pathways producing its precursors—UDP-N-acetyl-D-glucosamine and dTDP-L-rhamnose—were suppressed in six and four mixtures respectively. The tRNA charging pathway, essential for protein synthesis, was also reduced in the 100% GBE and FreshAs mixtures.

The distribution of pathway-level effects across mixtures showed a clear dose-dependent pattern. The 100% GBE mixture exhibited the greatest metabolic divergence from the reference, with 93 pathways showing altered abundance (87 suppressed, 6 enriched). The FreshAs blend displayed a suppression profile (22 pathways) intermediate between the 70/30 and 30/70 mixtures, suggesting its resistant starch content was comparable to these ratios. The 85/15 and 92.5/7.5 mixtures showed minimal (2 pathways) or no significant differences from the 100% kūmara reference, indicating a threshold effect where GBE content below approximately 15% did not detectably alter functional potential.

To validate that pathway-level shifts reflected underlying gene-level patterns, we examined concordance between pathway-level and KO-level (gene family) differential abundance using MaAsLin3 with batch adjustment for both analyses (Supplementary File 3). Across the 94 significant pathways, 91 pathways had detectable KO coverage. Gene-level concordance was exceptionally high, with a mean of 97.5% of constituent KOs changing in the same direction as the pathway-level effect (median: 100%). Sixty-three percent of pathway-mixture combinations showed perfect concordance with all detected KOs matching the pathway direction, and 62% of pathways showed 100% concordance in at least one mixture. Pathway coefficients were strongly correlated with mean KO-level coefficients ($r = 0.91$), demonstrating robust agreement in effect size magnitude between pathway and gene-level analyses.

The nucleotide metabolism pathways showed particularly strong concordance (mean 98.0%; median 100%), with 90% of pathway-mixture combinations showing $\geq 95\%$ of constituent genes changing in the pathway direction. Eighteen of 20 nucleotide pathways (90%) demonstrated $\geq 95\%$ concordance across mixtures, and 13 pathways showed perfect 100% concordance across all tested mixtures. This strong gene-level agreement demonstrates that the observed nucleotide pathway suppression reflects coordinated reductions in underlying functional gene potential rather than artefacts of pathway aggregation, supporting genuine metabolic reorganisation in response to GBE content.

Taxonomic annotation of the significant pathways provided insight into the microbial contributors underpinning the observed functional shifts (Figure 3.6). The pathways reduced in GBE-rich formulations were overwhelmingly annotated to members of the Enterobacteriaceae (*Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp.), which contributed to 88 of the 94 significant pathways (94%). These taxa contributed substantially to nucleotide biosynthesis pathways (18 of 18 nucleotide pathways), amino acid biosynthesis pathways (21 pathways), and cell wall biosynthesis (4 pathways).

Broader taxonomic representation was observed across functional categories, with pathways also annotated to Gram-positive taxa including *Bifidobacterium* spp., *Clostridioides* spp., *Clostridium*

spp., *Enterococcus* spp., and *Lactobacillus* spp. (collectively represented in 71 pathways). The six pathways showing increased abundance in 100% GBE (allantoin and aromatic compound degradation) were exclusively annotated to Enterobacteriaceae, suggesting enhanced catabolic activity of these taxa in response to high GBE content. This suggests that pathway differences predominantly reflect changes in the metabolic activity of metabolically versatile Enterobacteriaceae rather than wholesale taxonomic shifts.

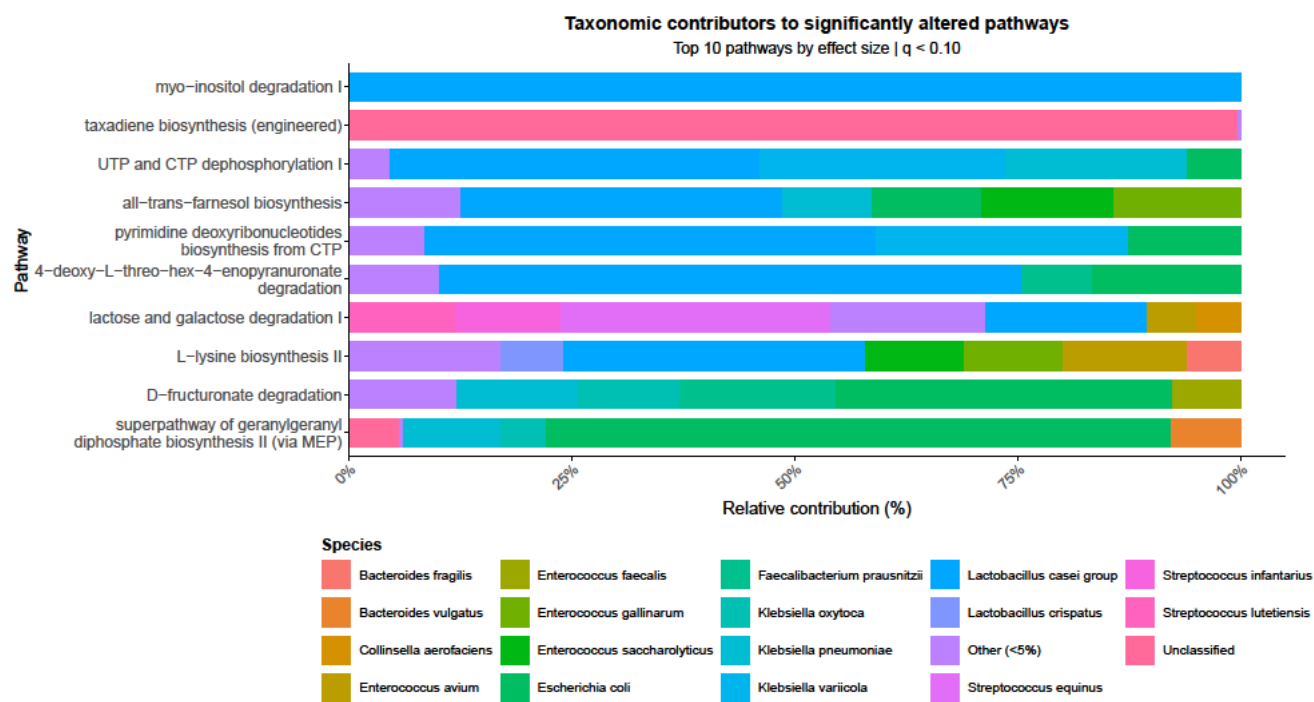


Figure 3.6. Taxonomic contributors to significantly altered pathways. Stacked bars illustrate the relative contributions of species to the top 10 MetaCyc pathways with the largest mixture effects across all mixtures. Taxa contributing < 5% to a given pathway are grouped as “Others (< 5%)”.

3.5.8 Organic acid production

Organic acid production over 24 h fermentation (reported on a fresh weight basis for concentrations) was strongly dominated by acetate and lactate, which together accounted for more than 90% of total measured acids across all mixtures. Net acetate production ($\Delta = 24 \text{ h} - 0 \text{ h}$) averaged 31.26 ± 7.54 mM (range 15.56–50.72 mM) across the kūmara–GBE gradient and PS control mixtures, while lactate

averaged 20.23 ± 5.81 mM (range 9.75–35.90 mM). Formate and succinate were produced in much smaller quantities (0.79 ± 0.48 mM and 0.70 ± 0.20 mM, respectively). Other acids, including propionate, butyrate, isobutyrate, isovalerate, valerate, hexanoate and heptanoate, were detected only at trace levels (mean ≤ 0.03 mM), with mean net changes close to zero for several of these (Figure 3.7). Small negative mean values for some minor acids (e.g., butyrate -0.005 mM, valerate -0.006 mM) reflect minimal net change around baseline after subtraction of 0 h concentrations rather than biologically meaningful consumption.

Analysis of absolute production values ($\Delta = 24 \text{ h} - 0 \text{ h}$), modelled using MaAsLin3 with 100% kūmara as the reference mixture, revealed no mixture-organic acid associations, indicating that total organic acid output remained remarkably stable across the full kūmara-GBE mixture gradient.

Relative abundance (each acid as a percentage of total organic acids at 24 h) showed that the composition of the organic acid pool was also highly conserved across mixtures. Acetate comprised 57-60% of total organic acids, while lactate contributed ~34-38%, with only moderate differences between the 100% kūmara, 100% GBE and the intermediate mixtures. Minor acids collectively accounted for the remaining ~5% on average, with formate and succinate each contributing ~2% and all other acids each contributing <1%. The FreshAs and PS controls fell within the same compositional range as the gradient mixtures (Figure 3.8).

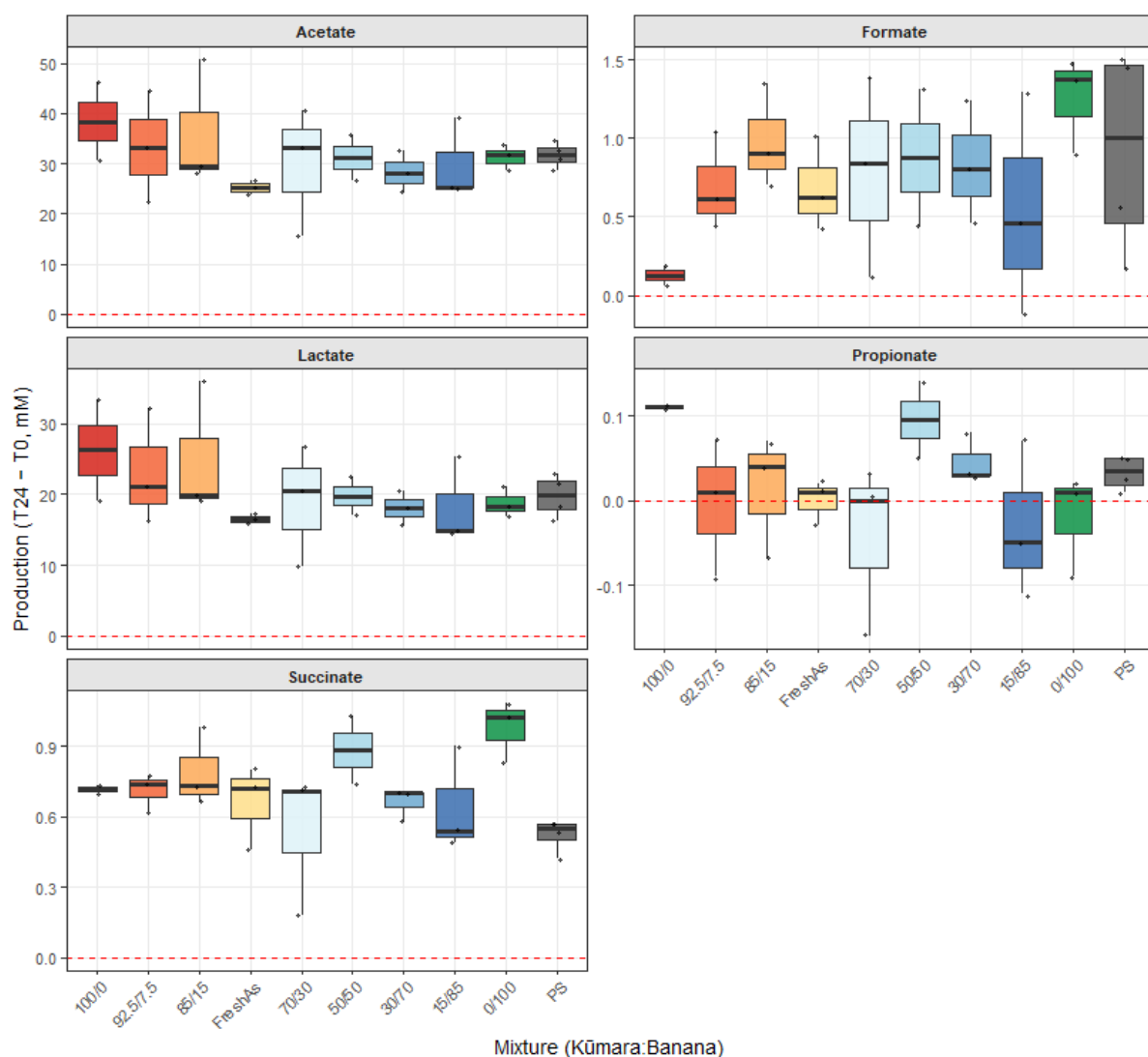


Figure 3.7. Net production ($\Delta = 24 \text{ h} - 0 \text{ h}$, mM) of individual organic acids across mixtures and PS control. Bars represent mean $\pm$ SD; only quantitatively measurable acids are shown.

Taken together, these results show that infant faecal microbiota consistently produced an acetate-lactate-dominated profile across all mixtures and the control. Neither kūmara proportion, GBE inclusion, nor PS control substantially altered the magnitude or relative composition of organic acid production. The near absence of butyrate and propionate does not indicate an inability to ferment resistant starch. The infant microbiota clearly metabolised the substrates as evidenced by gas

production, pH reduction, and robust organic acid output. Rather, this acetate-lactate dominance reflects the developmental stage of the infant gut microbiota.

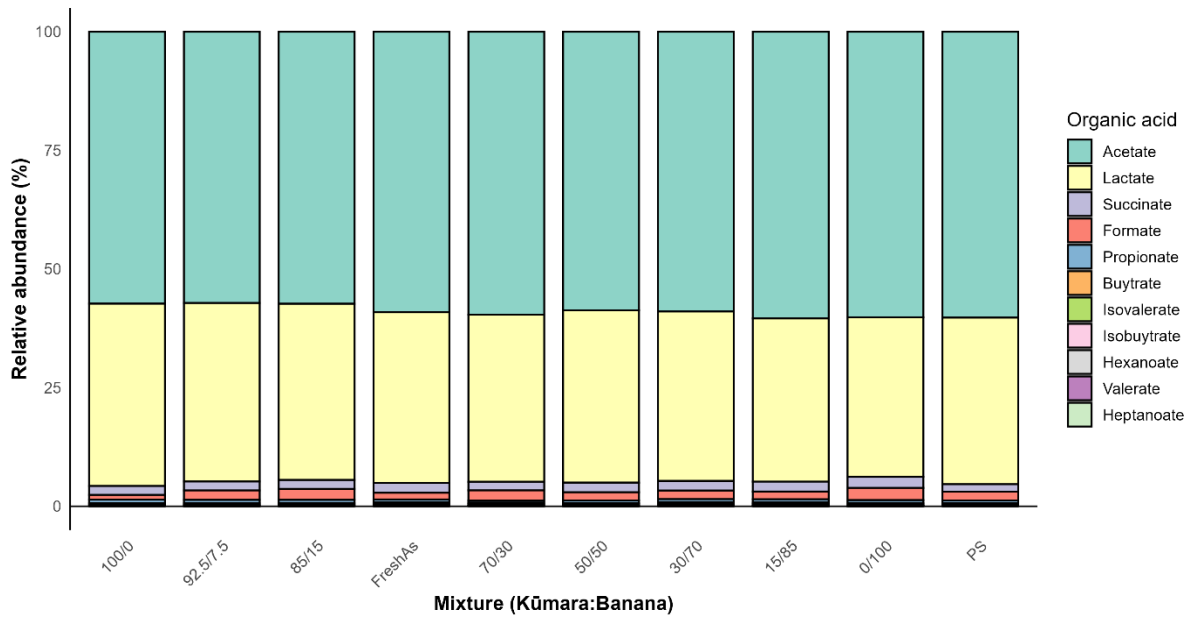


Figure 3.8. Mean relative abundance (% of total organic acids) of individual organic acids following 24 h *in vitro* fermentation of mixtures.

3.5.9 Integrated multivariate analysis

To comprehensively characterise relationships between substrate composition, gut microbiome structure and function, and fermentation outputs, an integrated multivariate analysis was performed incorporating nutritional composition (11 variables), microbial taxonomy (32 species), functional metagenomics (435 pathways), and fermentation metabolites (13 outputs) across nine substrate formulations at 24 h post-inoculation.

Multi-block PCA identified major sources of variation across microbial species, metabolic pathways, and nutritional composition. The first two principal components collectively explained 60.7% of total variance, indicating substantial coordinated variation (Figure 3.). Component 1 primarily distinguished kūmara-dominant formulations from green banana extract-dominant formulations. Component 2 captured additional variation in microbial composition and pathway abundance.

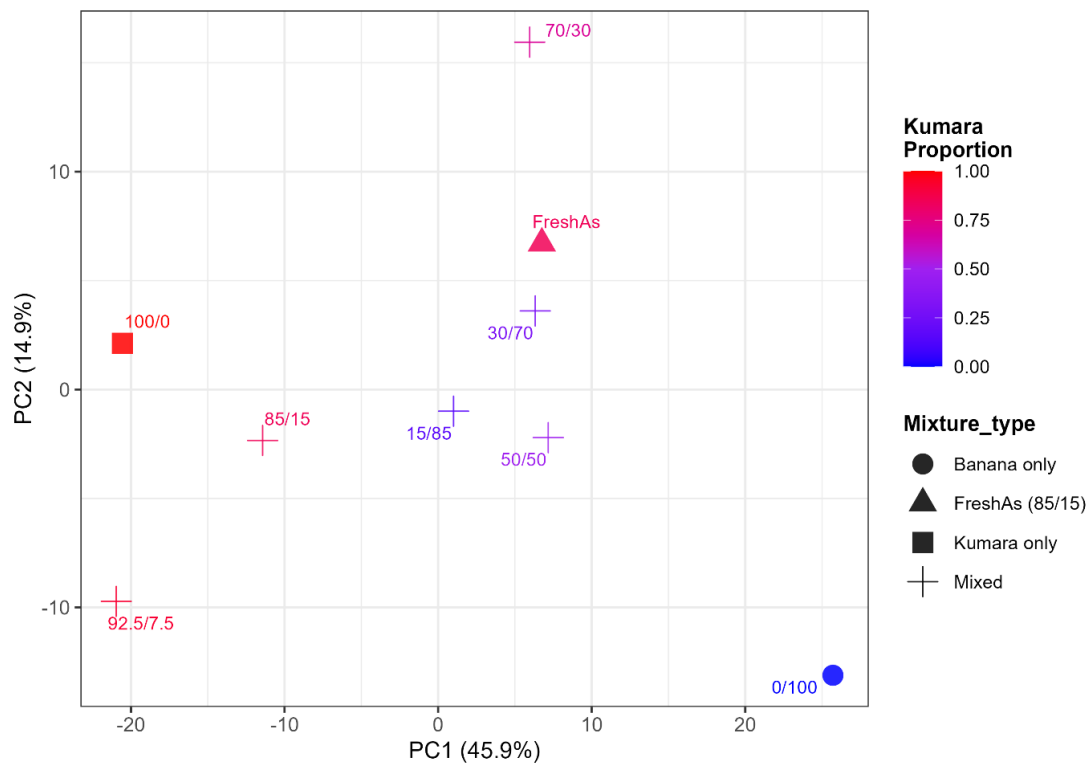


Figure 3.16. Multi-block principal component analysis of integrated data showing mixture distribution across PC1 and PC2.

Six PLS regression models were fitted to predict metabolic outputs from different predictor combinations. Variable Importance in Projection (VIP) scores identified the most influential predictors (VIP > 1.0 threshold), revealing consistent patterns across nutritional, taxonomic, and functional dimensions (Table 3.4).

Fermentable carbohydrate composition emerged as the primary nutritional driver, with total sugars, total carbohydrate, and resistant starch showing highest importance (45.5% of nutritional variables exceeded the VIP threshold). *Bacteroides* species dominated influential microbial predictors (31.3% of species), consistent with their role as versatile carbohydrate degraders in the infant gut microbiome. Metabolic pathways showed extensive linkages to fermentation outputs, with over half (51.9%) exceeding the VIP threshold. Biosynthetic pathways, particularly membrane lipid and cofactor production, predominated amongst top predictors, suggesting active microbial growth under fermentation conditions.

Importantly, high VIP scores reflect overall importance in multivariate prediction but do not necessarily indicate positive correlations with individual metabolites. For example, *Bacteroides fragilis* exhibited the highest species VIP yet was negatively correlated with acetate ($r = -0.867$), demonstrating that influential predictors modulate outcomes through complex, pathway-mediated effects rather than simple unidirectional relationships.

A correlation network analysis of strong correlations ($|r| > 0.7$) identified 2,653 edges connecting variables across all four data categories. The majority of strong correlations occurred between species and pathways (1,466 edges; 55.2%), followed by nutrition–pathway (659 edges; 24.8%) and pathway–output (461 edges; 17.4%) relationships. Direct species–metabolite correlations were sparse (26 edges; 1.0%), indicating that associations between taxonomic composition and metabolite production were largely mediated by microbial metabolic pathways rather than species presence alone.

Table 3.4. Partial Least Squares Regression: top predictors of fermentation organic acids. Variable Importance in Projection (VIP) scores for nutritional composition, bacterial species, and metabolic pathways predicting fermentation outputs. Only variables with VIP > 1.0 shown for nutritional and species predictors; top 5 of 226 pathways with VIP > 1.0 shown for metabolic pathways.

A. Nutritional Predictors		
Variable	VIP score	
Total sugars	1.252	
Total carbohydrate	1.216	
Resistant Starch	1.156	
Fat	1.134	
Sucrose	1.096	
B. Microbial Species Predictors		
Species	VIP score	
<i>Bacteroides fragilis</i>	1.759	
<i>Bacteroides ovatus</i>	1.754	
<i>Bifidobacterium pseudocatenulatum</i>	1.655	
<i>Erysipelatoclostridium ramosum</i>	1.533	
<i>Bacteroides faecis</i>	1.461	
C. Metabolic Pathway Predictors		
Pathway	VIP score	Category
Phosphatidylglycerol biosynthesis II	1.516	Lipid biosynthesis
Phosphatidylcholine biosynthesis I	1.515	Lipid biosynthesis
Arginine biosynthesis II	1.506	Amino acid biosynthesis
Phospholipid biosynthesis I	1.487	Lipid biosynthesis
Ubiquinol-8 biosynthesis	1.453	Cofactor biosynthesis

3.6 Discussion

This study used infant-adapted *in vitro* digestion and fermentation models to characterise how varying mixtures of kūmara and green banana extract (GBE) influence substrate composition, microbial community composition, functional potential and fermentation end-products.

3.6.1 Nutritional composition and substrate gradient

The nutritional profiles of the kūmara–GBE mixtures aligned closely with characteristics of these substrates and created a controlled gradient in fermentable carbohydrate composition. As expected, resistant starch (RS) content increased proportionally with the proportion of green banana extract (from 1.18% in 100% kūmara to over 30% in 100% GBE), consistent with reports that green banana

flour typically contains 20–40% RS2 depending on cultivar and processing conditions (Agama-Acevedo et al., 2015; Cordoba et al., 2018). In contrast, kūmara contributed predominantly digestible starch and soluble pectic polysaccharides, with only minor RS fractions after cooking. This aligns with analyses of cooked sweet potato, which generally show low RS content and a non-starch polysaccharide profile dominated by homogalacturonan and rhamnogalacturonan-I pectins (C. Liu et al., 2025; Zhao et al., 2024).

The sugar profiles of the mixtures further reflect known thermal transformations of sweet potato carbohydrates. Raw sweet potato typically contains sucrose as the major sugar, but cooking induces starch hydrolysis, producing substantial maltose through β -amylase activity (Lai et al., 2013; Lebot, 2017; Wei et al., 2017). The high maltose content observed in the kūmara-rich mixtures is therefore consistent with thermal conversion during preparation. The subsequent decline of both maltose and sucrose across mixtures with increasing green banana extract (36.8% maltose in 100% kūmara to < 0.1% in 100% GBE, sucrose declined from 14.9% to 0.9%), reflects dilution by a low-sugar, RS-rich substrate, while the near absence of simple sugars in the 100% GBE treatment matches published compositional profiles of green banana flour (Cordoba et al., 2018; Liao & Hung, 2015).

Importantly, despite differences in carbohydrate type, the mixtures maintained a narrow total carbohydrate range (79–82.5%). This minimised confounding effects of total fermentable substrate load and ensured that observed fermentation and microbiota differences could be attributed primarily to shifts in carbohydrate types. Soluble pectic polysaccharides from kūmara are typically fermented rapidly in adults and support saccharolytic taxa capable of producing acetate, butyrate, and propionate early in fermentation (Cao et al., 2022b; Xiong et al., 2023; Zhang et al., 2023). In contrast, RS2 requires enzymatic debranching and granule disruption prior to microbial utilisation, favouring taxa with amylolytic capabilities and often resulting in slower but more sustained organic acid production profiles (Jung et al., 2020; Zheng et al., 2025). These distinctions provide a strong foundation for examining how carbohydrate type and structure, rather than carbohydrate quantity, modulate infant-

relevant microbial metabolism. However, the infant microbiota, which differs substantially from that of adults, may show different fermentation kinetics and SCFA profiles.

3.6.2 Fermentation capacity and validation

The substrates underwent infant-adapted digestion prior to fermentation, mimicking upper gastrointestinal transit. This pre-fermentation digestion removed rapidly digestible starch (RDS) and slowly digestible starch (SDS), ensuring slowly fermentable-driven effects reflected true colonic fermentation rather than preferential utilisation of accessible starch fractions. Recent work demonstrates that the presence of even small digestible starch fractions ($\geq 25\%$) during *in vitro* fermentation can substantially influence microbial responses and may override the evaluation of intrinsic RS fermentability (Klostermann et al., 2024).

Validation analyses confirmed robust fermentation activity. Significant community restructuring from baseline was detected, demonstrating substantial microbial community change during fermentation. Although alpha diversity changes were not statistically significant in the validation subset, the uniform direction of decrease with fermentation indicates a consistent transition from a mixed viable/non-viable state to an active fermenting community. These changes establish that active microbial metabolism occurred and that the inoculum retained fermentative capacity despite pooling and anaerobic incubation. It also confirms that infants at the onset of complementary feeding possess the capacity to degrade complex carbohydrates, consistent with other studies (Gopalsamy et al., 2019).

The potato starch control demonstrated excellent reproducibility across batches, with no significant difference in alpha and beta diversity. This technical consistency establishes confidence in between-mixture comparisons and confirms that variation observed among mixtures reflects true biological differences rather than batch-related artefacts.

The absence of significant differences in total gas production, hydrogen concentration, or hydrogen sulphide among mixtures likely reflects similar total fermentable carbohydrate content across mixtures. Methane production remained negligible (<0.1%) across all mixtures, consistent with the known immaturity of methanogenic communities in infants (Edwards & Parrett, 2003).

3.6.3 Taxonomic stability despite substrate variation

The infant faecal microbiota used in this study exhibited a composition dominated by Actinomycetota (65-70%), primarily *Bifidobacterium* species, consistent with the expected profile of healthy breastfed infants.

Despite the substantial gradient in resistant starch content across the nine kūmara-GBE mixtures (ranging from 1.18% to 30.52%) and variation in simple sugars (maltose ranging from <0.1% to 36.8%, sucrose ranging from 0.9% to 14.9%), no species-level taxonomic differences reached statistical significance (0 of 85 species tested, $q < 0.05$). While alpha diversity showed a trend toward significance (Shannon index $\chi^2 = 15.35$, $p = 0.053$) and beta diversity exhibited moderate separation ($R^2 = 0.346$, $p = 0.27$), these compositional shifts were modest compared to the magnitude of functional differences observed.

This finding contrasts with a previous infant fermentation study, where resistant starch increased both alpha diversity and abundances of specific taxa (Gopalsamy et al., 2019). However, these findings are highly consistent with those reported by da Silva et al. (2025) in their *in vitro* fermentation study of complementary foods. Their work also used infant faecal inocula to ferment plant-based substrates, including sweet potato, and similarly observed that alpha diversity decreased after 24 h, with no significant beta-diversity separation following fermentation. Their communities remained strongly dominated by *Bifidobacterium* throughout, despite differences in the carbohydrate composition of test foods.

The minimal taxonomic response observed here may reflect several factors. First, the 24-hour fermentation captures relatively short-term metabolic responses before substantial population shifts occur. Studies examining longer fermentation periods or *in vivo* interventions have detected more pronounced compositional changes as the microbiota adapts to the substrates (Gopalsamy et al., 2019; Y. Wang et al., 2019). Second, the inoculum source—pooled faecal samples from exclusively breastfed infants—represents a microbiota primarily adapted to the utilisation of human milk oligosaccharides rather than resistant starch. However, early-life gut communities are characterised by high functional plasticity and metabolic flexibility, which may permit adaptive responses to novel substrates at the level of pathway activity without necessitating marked changes in species composition (Milani, Mangifesta, et al., 2017; Saturio et al., 2021; Turrone et al., 2012; Walsh et al., 2022).

Although no species reached the threshold for statistical significance, some taxa showed directional trends that, while not statistically significant, are consistent with known metabolic preferences. For instance, *Clostridium butyricum* demonstrated increased abundance in mixtures rich in GBE (aligned with RS preference), while *Bacteroides* species showed a slight enrichment in kūmara-rich mixtures (aligned with pectin/simple sugar utilisation). These trends imply that substrate composition can exert selective pressure, but the effect size is too small to overcome inter-replicate variation and strict FDR correction. Larger sample sizes, longer incubation periods, or longitudinal sampling might be needed to observe these effects more clearly at the taxonomic level.

3.6.4 Functional pathway responses reveal coordinated metabolic reorganisation

In stark contrast to taxonomic stability, functional pathway analysis revealed extensive metabolic reorganisation in response to substrate composition. Using batch-adjusted MaAsLin3 models with stringent FDR correction ($q < 0.05$), 94 distinct pathways showed significant mixture effects, demonstrating that substrate composition profoundly modulates microbial metabolism without necessitating taxonomic shifts.

Ninety-six percent of pathway effects involved suppression of biosynthetic pathways in GBE-rich mixtures, overwhelmingly contributed by Enterobacteriaceae (94% of pathways). These suppressed pathways spanned nucleotide metabolism (18 pathways), amino acid biosynthesis (21 pathways), cell wall biosynthesis, and cofactor production—collectively indicating reduced anabolic activity under RS-dominant conditions. Conversely, six catabolic pathways showed 2.1–2.6-fold enrichment exclusively in 100% GBE, including allantoin degradation and aromatic compound catabolism. This bidirectional pattern (biosynthesis suppressed, catabolism enhanced) suggests a metabolic shift from growth-associated processes toward scavenging and maintenance metabolism when readily fermentable sugars are limited.

Gene-level validation confirmed these pathway-level patterns represent genuine coordinated regulation rather than aggregation artefacts. Concordance between pathway coefficients and constituent gene abundances was exceptional (mean 97.5%; $r = 0.91$), with nucleotide metabolism pathways showing particularly strong agreement (98.0% concordance). This validates functional profiling as capturing mechanistically relevant information that taxonomic profiling alone would miss.

Importantly, a threshold effect emerged at approximately 15% GBE content. The 85:15 and 92.5:7.5 kūmara:GBE mixtures showed minimal functional divergence from 100% kūmara, indicating that modest RS supplementation preserves the anabolic metabolic environment. This threshold is directly relevant to the SUN Study intervention design and suggests that the metabolic benefits of kūmara-based complementary foods can be maintained while incorporating prebiotic RS at physiologically achievable doses.

These findings demonstrate that the infant gut microbiota exhibits remarkable functional plasticity, maintaining stable taxonomic composition while substantially altering metabolic programs in response to substrate availability. Rather than selecting for different taxa, dietary substrates appear to

modulate the metabolic state of the existing community, creating environments more or less permissive for growth versus maintenance metabolism.

3.6.5 Organic acid production patterns

Despite clear functional pathway differences among the kūmara–GBE mixtures, fermentation end-products remained remarkably stable. Acetate dominated across all formulations, lactate accumulated consistently, and butyrate stayed at or below detection limits. No significant associations between mixture composition and individual organic acids were detected.

Three infant *in vitro* studies using plant-based complementary foods, including sweet potato, show striking similarity to our results. They observed robust acetate production, lactate accumulation, and little to no butyrate (Parkar et al., 2021; Parkar et al., 2021; da Silva et al., 2025).

Several mechanisms explain the stability of organic acid outputs despite substrate differences and despite the significant functional pathway restructuring. With Actinomycetota comprising ~65–70% of the community, the metabolic landscape is dominated by the “bifid shunt”, a unique pathway of hexose catabolism in *Bifidobacteria* that produces primarily acetate and lactate (Wolin et al., 1998). This metabolic dominance ensures acetate–lactate predominance regardless of substrate. Kūmara supplies fermentable carbohydrates, including starch and soluble pectic polysaccharides, which the gut microbiota may preferentially utilise first, producing acetate-dominant profiles. The positive correlation between acetate and kūmara percentage is consistent with fast carbohydrate metabolism by *bifidobacteria* and lactic acid bacteria. RS-dominated mixtures (higher in GBE) favoured lactate accumulation. The acetate-lactate profile observed here differs markedly from typical adult gut fermentation, where butyrate and propionate dominate alongside acetate. In adult gut microbiota, acetate and lactate are efficiently converted to butyrate by secondary fermenters, however, the limited availability of secondary fermenters in the infant gut likely restricts conversion of lactate to butyrate or propionate. Even though 94 MetaCyc pathways differed significantly between mixtures, multiple

metabolic routes may converge on identical end-products, allowing substantial pathway-level differences to coexist with near-identical organic acid outputs.

3.6.6 Integrated multivariate patterns

The multi-block PCA, which integrated nutritional, taxonomic, functional, and metabolic data, showed that the primary source of variation (60.7% variance) separated samples along the kūmara-green banana extract gradient. PLS regression confirmed fermentable carbohydrates as the primary drivers, with *Bacteroides* species being key microbial predictors.

PLS regression identified metabolic pathways as the strongest predictor of organic acid production (VIP scores > 2 for multiple nucleotide and carbohydrate pathways), outperforming species-level abundance as predictors. This validates that pathway-level functional profiling captures mechanistically relevant metabolic information that taxonomic profiling misses.

3.6.7 Implications for infant nutrition and microbiome development

The finding that dietary substrates modulate the metabolic state of the existing microbial community, rather than selecting for different taxa, has important implications for understanding early-life gut ecology. The bidirectional metabolic response demonstrates that substrate composition influences the balance between microbial growth and maintenance metabolism, with kūmara supporting anabolic environments characterised by active biosynthesis while RS-dominant conditions shift toward catabolic processes and nutrient scavenging. This metabolic plasticity, maintaining stable taxonomic composition while substantially altering functional profiles, may represent an important resilience mechanism during the transition to solid foods, allowing the community to accommodate diverse dietary inputs without destabilising compositional shifts.

The coordinate suppression of multiple biosynthetic pathways in GBE-rich mixtures, including nucleotide metabolism, amino acid biosynthesis, and cell wall synthesis, suggests that kūmara provides a more permissive environment for anabolic activity. While the significance of enhanced

nucleotide biosynthesis capacity specifically remains to be established, dietary nucleotides have been shown to enhance *Bifidobacterium* growth in minimal culture media and influence gut microbiota composition in formula-fed infants (Singhal et al., 2008). The capacity of the infant microbiota to modulate biosynthetic pathway expression in response to substrate availability may represent an adaptive mechanism, though whether this translates to functional advantages for the host requires further investigation.

The resistant starch provided by both kūmara and banana represents an important prebiotic component that resists digestion and reaches the colon for microbial fermentation. However, the non-linear responses observed, including threshold effects below 15% GBE content and the divergence between nominal and functional composition in the commercial FreshAs product, demonstrate that simple dose-response relationships do not fully capture the complexity of substrate-microbiota interactions. The capacity to ferment RS was present across all substrate formulations, indicating functional versatility of the infant gut community.

These findings highlight that not all resistant starch sources are functionally equivalent. While both kūmara and banana provide fermentable carbohydrates, the broader nutritional matrix, including the balance between simple sugars and complex carbohydrates, micronutrients, phytochemicals, and protein content collectively shapes whether the metabolic environment favours biosynthetic or catabolic activity. These foods differ substantially in bioactive compounds, vitamins, minerals, and fibre types beyond resistant starch content alone, and these factors appear to influence the metabolic programs expressed by the gut microbiota.

3.6.8 Strengths and Limitations

This study addresses a critical knowledge gap by providing a detailed, mechanistic characterisation of complementary food fermentation using infant-adapted *in vitro* models. The stringent FDR correction and exceptional gene-level validation (97.5% overall concordance, 98% for nucleotide pathways) ensure robust findings that reflect coordinated metabolic regulation rather than isolated

gene expression artefacts. Furthermore, by focusing on locally-relevant foods (kūmara and GBE) this work provides foundational data for ingredients underrepresented in infant nutrition models.

The systematic examination of nine mixture ratios with biological replicates enabled detection of both linear and threshold responses across the composition gradient, providing nuanced insights into substrate-microbiome interactions that would not emerge from simple binary comparisons. The identification of a functional threshold effect at ~15% GBE content is particularly relevant for intervention design, as it establishes that modest RS supplementation can be achieved without substantially disrupting the metabolic environment supported by kūmara.

The commercial FreshAs formulation merits particular attention for translation to real-world products. Although produced at a nominal kūmara:GBE ratio of 85:15, FreshAs consistently showed functional pathway profiles and fermentation metabolites more similar to the 70:30 laboratory-prepared mixtures than to its declared composition. This discrepancy likely reflects effects of commercial processing or variability in the green banana extract used. These findings highlight the challenges of standardising whole-food substrates and emphasise that microbiome responses depend on the specific product formulation rather than ingredient ratios alone.

Several important limitations must be considered when interpreting these findings. First, the *in vitro* batch fermentation model, while providing controlled conditions for mechanistic investigation, lacks *in vivo* dynamics, including continuous substrate flow and pH control. The static nature of the system may influence microbial activity differently than the dynamic infant gut environment, and the absence of pH control means that acid accumulation during fermentation could have altered pathway activity and metabolite production.

Second, the use of pooled faecal inocula, while reducing individual variation and enabling detection of substrate-driven effects, masks the considerable inter-individual variability known to exist in infant microbiomes. The extent to which the functional plasticity observed here is representative of individual infant microbiomes versus a property emerging from the pooled community remains

uncertain. Additionally, the experimental model simplified the complementary feeding environment by excluding breastmilk or formula, which would be present in real infant feeding contexts and could modulate fermentation through the provision of additional substrates.

Third, the literature available for contextualising these findings has important gaps. Limited studies have specifically examined sweet potato fermentation using infant faecal inocula at the onset of complementary feeding, requiring extrapolation from generalised or adult fermentation studies. Similarly, while green banana resistant starch has been studied extensively, most work has used adult or murine models.

Finally, the clinical relevance of the observed functional differences remains to be established. While the metabolic pathway changes documented here are statistically robust and biologically coherent, it requires validation through *in vivo* studies. The relationship between responses *in vitro* and responses *in vivo* is complex and mediated by many factors beyond those captured in the current experimental system.

Despite these constraints, which are typical for foundational *in vitro* work, this model successfully isolated the specific effects of kūmara and GBE on infant gut microbiota metabolism, providing a clear mechanistic baseline that will be invaluable for interpreting more complex *in vivo* data. Longitudinal sampling with multiple timepoints would capture the dynamic evolution of microbial communities and functions as infants adapt to new dietary substrates, revealing kinetic patterns obscured by *in vitro* analyses.

Chapter 4

Kūmara-Based Interventions During Complementary Feeding: Impacts on Gut Microbiota Composition and Functional Capacity in the SUN Study cohort

4.1 Abstract

The complementary feeding period represents a critical window in gut microbiota development, yet the functional maturation of the infant microbiota and its responsiveness to whole-food dietary fibres remain poorly defined. This randomised controlled trial ($n = 279$; 789 samples), with 89% of participants providing complete longitudinal data, evaluated the effects of daily kūmara or kūmara + green banana extract supplementation, foods naturally containing dietary fibre and modest amounts of resistant starch, on gut microbiota composition and functional capacity in healthy infants aged 6–10 months using shotgun metagenomic sequencing.

Across all intervention groups, temporal maturation overwhelmingly dominated microbiota development. Shannon diversity substantially increased by 65% (from a mean 1.74 to 2.88, $p < 0.001$), with marked taxonomic remodelling (PERMANOVA $R^2 = 0.04$, $p < 0.001$) and with extensive functional reorganisation (PERMANOVA $R^2 = 0.05$, $p < 0.001$). This reflects the transition from a *Bifidobacterium*-rich, milk-adapted community to a more diverse, fibre-responsive microbiota enriched in Firmicutes and Bacteroidota. Functional pathway profiling revealed a biphasic maturation pattern: the introductory complementary feeding period (CF1 to CF2, 6–8 months of age) was characterised by broad metabolic expansion, whereas the establishing complementary feeding period (CF2 to CF4, 8–10 months of age) involved pronounced metabolic consolidation, particularly within amino-acid, nucleotide, and lipid metabolism.

Although kūmara-based interventions ($\approx 1.5\%$ in the kūmara intervention; $\approx 7\%$ in the kūmara–green banana extract intervention) increased exposure to dietary fibre, particularly resistant starch, they did not significantly alter diversity trajectories or community composition relative to the control group. Group-level differences emerged only at the level of functional pathways, demonstrating that whole-

food dietary intervention can produce measurable metabolic effects without perturbing overarching maturational patterns.

This study provides a comprehensive characterisation of infant gut microbiota maturation during the early complementary feeding period, integrating diversity, taxonomic composition, and functional capacity to establish that while age-driven developmental programming is the primary force shaping the infant gut microbiota, kūmara-based interventions can modulate functional metabolic capacity.

4.2 Introduction

The complementary feeding period represents a critical window in gut microbiota development, during which the introduction of solid foods triggers rapid restructuring of the infant gut microbiota. (Bäckhed et al., 2015; Bergstrom et al., 2014; Koenig et al., 2011; Laursen, 2021; C. J. Stewart et al., 2018). This restructuring sees the gut microbiota shift from a milk-adapted community dominated by *Bifidobacterium* species towards a more diverse, adult-like configuration enriched in Bacteroidota and Firmicutes. Understanding the factors that influence this developmental trajectory is important for optimising infant health outcomes, as aberrant microbiota maturation during this sensitive period has been associated with increased impairment of metabolic and immune development (Cox et al., 2014; Tamburini et al., 2016). Identifying modifiable factors that support healthy microbiota development could provide practical intervention strategies, potentially reduce disease risk and support long-term metabolic and immune function.

Diet has been found to exert a profound influence on gut microbiota composition and function across populations and age groups. Geographic comparisons highlight the role of the effect of diet on the gut microbiota. Children consuming high-fibre diets in rural Africa show microbiotas enriched in fibre-degrading *Prevotella* and *Xylanibacter* species, harbouring genes involved in cellulose and xylan hydrolysis, compared with European children consuming low-fibre westernised diets (De Filippo et al., 2010). Collectively, these findings suggest that dietary substrate availability is a primary driver of microbiota assembly during infancy.

Despite this recognition, important questions remain about how specific complementary foods, particularly those with prebiotic potential, influence microbiota development. Most intervention studies examining prebiotic effects on the infant microbiota have focused on purified oligosaccharides (galacto-oligosaccharides, fructo-oligosaccharides) added to infant formula, with few examining potentially prebiotic whole foods as they are actually consumed during complementary feeding (Ferro et al., 2023). Translating prebiotic effects from purified compounds to whole foods is complicated by food structure, cooking methods, fibre-matrix interactions, and co-consumption of other foods, all of which modulate fermentation.

Among whole food fibre sources, resistant starch has emerged as a promising prebiotic candidate. Most work on the effects of resistant starch and other dietary fibre components on the gut microbiota has been on adults (R. Chen et al., 2023; Sobh et al., 2022). However, translating these findings to actual infant feeding contexts is challenging, and few studies have examined resistant starch or whole foods rich in resistant starch during complementary feeding, with inconsistent results due to differences in type, dose, delivery format, timing, and baseline microbiota composition.

Understanding the functional metabolic capacity of the microbiota, i.e., what the microbes are doing, is critical, as taxonomic profiling alone provides limited insight into functional maturation. Shotgun metagenomic sequencing enables assessment of gene content beyond phylogenetic markers, capturing metabolic potential and functional redundancy, whereby distinct species encode similar functions to maintain key community processes such as fibre fermentation, vitamin biosynthesis, and amino acid metabolism (Franzosa et al., 2015; Gevers et al., 2012).

This chapter presents the compositional and functional metagenomic findings from The Seeding Through Feeding (SUN) Study, which addresses these gaps through a three-arm randomised controlled trial examining the effects of a prebiotic kūmara and kūmara with green banana extract supplementation on gut microbiota development. Building on pilot feasibility work (McKeen et al., 2022), the study aimed to recruit 300 healthy infants and followed them from 4–6 months to 10

months of age. By integrating shotgun metagenomics with comprehensive taxonomic and functional analyses, including VIP-based prioritisation, the SUN Study provides a comprehensive characterisation of how complementary food composition influences both the structure and function of the developing infant gut microbiota.

4.3 Aims

We hypothesised that, while all infants would follow characteristic microbiota maturation patterns during complementary feeding, those receiving the interventions, particularly the kūmara plus green banana extract supplementation, would show enrichment of fibre-degrading bacterial taxa and fermentative metabolic pathways. The specific objectives of this chapter were:

1. To quantify temporal changes in gut microbiota diversity, taxonomic composition, and functional metabolic pathways across the complementary feeding period.
2. To determine whether supplementation with kūmara alone or in combination with green banana extract modulates microbiota maturation trajectories.

4.4 Materials and Methods

4.4.1 Study Design and Participants

The Seeding Through Feeding (SUN) Study is a three-arm, parallel-group, randomised controlled trial conducted in Auckland, Aotearoa, New Zealand, between January 2022 and January 2024. The full trial protocol, including study rationale, recruitment procedures, eligibility criteria, randomisation, intervention allocation, and planned outcomes, has been published (Wall et al., 2024). This thesis forms part of the SUN Study's secondary objectives.

Healthy infants aged 3–6 months who had not yet commenced complementary feeding were enrolled and randomly assigned (1:1:1) to receive: (i) standard kūmara powder (K), (ii) kūmara powder with added green-banana extract (K+), or (iii) no study product (control; C). The intervention freeze-dried

powders were administered as a 5g daily dose ($\pm$ 30g when reconstituted). Data and biological samples were collected at three timepoints: baseline (CF1: before complementary feeding), mid-intervention (CF2: approximately 2 months after starting complementary foods, infants ~8 months of age), and end-intervention (CF4: approximately 4 months after starting complementary foods, infants ~10 months of age).

A total of 282 participants were enrolled in the study. Three participants were allocated to an intervention arm but withdrew or were lost to follow up before commencing the study. Eighteen participants withdrew or were lost to follow up after baseline (CF1), and two were lost to follow up after CF2.

4.4.2 Intervention product composition

The nutrient composition and resistant starch (RS) content of the kūmara and kūmara + banana extract (GBE) products were determined by MU Lab. Table 4.1 summarises the nutrient composition per 100 g of kūmara powder, and the final kūmara + green banana extract (GBE) intervention product.

The selected doses were evaluated against the FSANZ inulin standard, which permits up to 0.8 g inulin per 100 g infant food or 0.3 g per 100 mL infant formula. RS levels in the study products remained well below these regulatory allowances, ensuring safety and compliance with infant feeding guidelines.

Table 4.1. Nutrient composition of kūmara (K) and kūmara + green banana extract (K+) intervention products per 100 g.

Component	K intervention	K+ intervention
Energy (kJ)	~1450	~1444.5
Protein (g)	~2	~2.2
Carbohydrate (g)	~30	31
Dietary fibre (g)	~8	8.6
Resistant starch (g)	1.5	7

4.4.3 Sample collection

Parents collected stool samples during the three days before clinic visits. At baseline (4-6 months of age), a nappy liner was provided to accommodate the liquid consistency of infant stools before introducing complementary foods. Samples collected at CF2 and CF4 were scooped into collection pottles. Parents were asked to immediately store all samples in their home freezer (-20°C). They were provided with Styrofoam boxes and ice packs for transport to the clinic. Upon arrival at the clinic, samples were transferred to a -80°C freezer, then transported on dry ice to the Riddet Institute/AgResearch in Palmerston North, New Zealand, where they were immediately stored at -80°C until processed for analysis.

4.4.4 DNA extraction and Metagenomic sequencing

Samples stored at -80 °C were transferred to -20 °C overnight before DNA extraction. For baseline samples collected in nappy liners, faecal material was obtained by scooping or squeezing from the liner. In cases where the material had a watery consistency and was embedded within the liner, a section of the liner containing approximately 100 mg of faecal content was cut and included in the initial extraction steps.

DNA extraction, quantification, and quality assessment were performed following the protocols detailed in Chapter 3 (Section 3.4.4.4). Briefly, DNA was extracted using the Macherey–Nagel NucleoSpin Soil Kit with an added bead-beating step, and DNA integrity, purity, and concentration were assessed using electrophoretic, spectrophotometric, and fluorometric methods. Only samples meeting the quality thresholds described in Chapter 3 were taken forward for library preparation.

Shotgun metagenomic sequencing was conducted by Annoroad Gene Technology Corporation (Beijing, China) on an Illumina NovaSeq X Plus (Illumina, USA). Sequencing data (raw and filtered reads) were processed by Jane Mullaney (AgResearch, New Zealand) following the same workflow described in Chapter 3 (Section 3.4.4.4), including initial quality assessment with FastQC/MultiQC, read trimming and decontamination using KneadData with Trimmomatic and Bowtie2, and generation of final clean read sets for downstream analyses.

Taxonomic and functional profiling were performed using MetaPhlAn (version 4.0.6 (1 Mar 2023)) and HUMAnN3 (v4.0.0. alpha 1), respectively. All samples were processed using identical parameters to those used in Chapter 3 to ensure methodological consistency across datasets. Samples were sequenced and processed in three batches.

4.4.5 Data Analysis

In total, 789 samples were available from 279 participants. To ensure balanced longitudinal comparisons, analyses were restricted to complete cases, comprising samples from 279 participants (747 samples).

4.4.5.1 Microbial diversity

Alpha diversity metrics were calculated from genus-level taxonomic classifications using the *vegan* package (version 2.6-4) in R (version 4.3.0). Three complementary metrics were computed: Shannon diversity index (accounting for both richness and evenness), Simpson diversity index (emphasising dominant taxa), and observed genus richness (total number of genera detected per sample). Linear mixed-effects models were fitted using the *lmerTest* package (v 3.1.3) to assess the effects of temporal changes (CF1, CF2, CF4), and intervention groups (K, K+, C), as well as their interaction. Timepoints were treated as a factor to reflect the longitudinal design. The sequencing batch was included as a fixed effect to account for technical variation. Participant ID was included as a random intercept to account for repeated measures, allowing each infant to contribute data for whichever

timepoints were available. This approach accommodates missing measurements without excluding participants entirely. Type III analysis of variance with Satterthwaite's approximation for degrees of freedom was used to test main effects and interactions.

Post-hoc pairwise comparisons were conducted using estimated marginal means with Bonferroni correction for multiple testing. Effect sizes were calculated as Cohen's *d* using the pooled standard deviation from the final model.

Beta diversity was assessed using the Aitchison distance calculated from centred log-ratio (CLR)–transformed genus-level relative abundance data. This approach was selected to appropriately account for the compositional nature of microbiome data. Permutational multivariate analysis of variance (PERMANOVA) was performed using the *adonis2* function (vegan v2.6-4) with 10,000 permutations. Permutations were restricted by participant ID to account for the repeated-measures design. The full model tested the effects of timepoint (CF1, CF2, CF4), intervention group (K, K+, C), and their interaction, with sequencing batch included as a covariate. Homogeneity of multivariate dispersion was assessed using PERMDISP to confirm that observed PERMANOVA effects reflected differences in community composition rather than within-group variability. Ordination of Aitchison distances was visualised using principal coordinates analysis (PCoA), including stratification by intervention group, timepoint, and sequencing batch.

4.4.5.2 Taxonomic composition analyses

Differential abundance analysis at the genus level was performed using MaAsLin3 (v1.1.2), applying linear mixed-effects models to account for the longitudinal structure of the dataset. Genus-level abundance tables were normalised using total sum scaling (TSS) and log-transformed prior to analysis. Genera were filtered to retain features with a minimum relative abundance of 0.1% and prevalence in at least 10% of samples.

For temporal analyses, linear mixed-effects models included Timepoint and Batch as fixed effects, with Participant ID specified as a random intercept to account for repeated measures within infants. CF1 was set as the reference level, enabling direct estimation of CF2 versus CF1 and CF4 versus CF1 contrasts. As MaAsLin3 does not natively provide a CF4 versus CF2 contrast when CF1 is used as the reference, timepoint was additionally re-levelled to CF2, allowing direct estimation of the CF2 versus CF4 comparison.

To evaluate intervention effects, two Group $\times$ Timepoint interaction analyses were conducted. The primary analysis included all three timepoints (CF1, CF2, and CF4), with the Control group at CF1 specified as the reference level. Linear mixed-effects models included Group, Batch, and the Group $\times$ Timepoint interaction as fixed effects, with Participant ID included as a random intercept. This model enabled estimation of group-specific changes at CF2 and CF4 relative to the Control group at CF1.

A secondary, focused analysis examined changes during the established complementary feeding period (CF2 to CF4 only). In this model, the Control group at CF2 was set as the reference level, with the same fixed and random effects structure applied, enabling direct comparison of intervention-specific trajectories during this complementary feeding period.

All p-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate procedure. Genes with q-values < 0.05 were considered significantly associated with temporal or intervention-related changes during complementary feeding.

MaAsLin3 reports natural log-transformed coefficients. Fold changes were calculated as $\exp(\text{coefficient})$, where coefficient represents effect size on the natural log scale. Positive coefficients indicate increases and negative coefficients indicate decreases relative to the reference group. All fold changes reported in the results represent these exponentially transformed coefficients.

4.4.5.3 Functional pathway analyses

For the functional pathway analysis, a total of 31,039 MetaCyc pathways were initially detected across all samples. After filtering for prevalence ($\geq 20\%$ of samples) and mean abundance (≥ 0.0001 counts per million [CPM]), 3,030 pathways were retained for downstream analysis. Specifically, 27,457 pathways were removed for low prevalence ($< 20\%$ samples), 187 for low mean abundance (< 0.0001 CPM), and 365 unintegrated or ungrouped pathways were discarded, leaving 3,030 pathways from the initial 31,039. The 20% prevalence threshold was chosen to focus on core metabolic functions present across the majority of the cohort while excluding rare pathways that may reflect transient colonisers or sequencing artefacts.

Differential abundance analyses were performed using MaAsLin3, controlling for batch effects. For temporal comparisons, two separate analyses were conducted: CF1 versus CF2 and CF1 versus CF4 (using CF1 as reference), and CF2 versus CF4 (using CF2 as reference). Each temporal model included timepoint and batch as fixed effects with participant as a random effect. For intervention analyses, Group $\times$ Timepoint interaction models were fitted with Control and CF1 as reference levels. Pathway abundances were analysed as counts per million (CPM); reported effect directions therefore reflect relative differences in CPM-scaled pathway abundance rather than absolute pathway copy number. Fold changes were calculated as $\exp(\text{coefficient})$ from the natural log-transformed model coefficients. Significantly changed pathways ($q < 0.05$, Benjamini-Hochberg correction) were manually categorised into 14 functional categories based on MetaCyc pathway annotations for easier interpretation.

To assess whether overall functional pathway composition differed across the complementary feeding period and between intervention groups, permutational multivariate analysis of variance (PERMANOVA) using Bray–Curtis dissimilarities was conducted with 10,000 permutations. For temporal models, timepoint was included as a fixed factor, with permutations constrained within participants to account for repeated measures. A parallel model was tested for global differences

between intervention groups at each timepoint. A Group x Timepoint interaction model was tested to assess whether intervention effects varied across developmental stages. Multivariate dispersion of homogeneity was assessed using PERMDISP (betadisper) with 10 000 permutations to validate PERMANOVA assumption.

Genus-level contributions to metabolic shifts were quantified by linking microbial genera to their associated pathways across functional categories. For each genus, the number of pathways contributed and the direction of change (based on calculated fold changes from MaAsLin3 models) were summarised. Proportional contributions to each functional category were calculated for genera contributing to multiple categories. Temporal trends were assessed across CF1, CF2, and CF4, and intervention effects were evaluated by comparing the number of contributing genera and their pathway distributions across groups (K, K+, C). This approach allowed characterisation of core metabolically active taxa and the distribution of functional activity among genera.

4.4.5.4 sPLS-DA and VIP analyses

Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed using the mixOmics R package (version 6.26.0) to identify microbial metabolic pathways with the highest discrimination power across timepoints (temporal VIP) and between intervention groups (intervention VIP). For temporal discrimination, pathway abundances (CPM-normalised) were used as predictor variables and timepoint (CF1, CF2, CF4) as the outcome categorical variable. The optimal number of components and features per component was determined via cross-validation. Variable Importance in Projection (VIP) scores were calculated for each pathway, with $VIP > 1$ indicating above-average contribution and $VIP > 3$ denoting highly discriminative pathways. Temporal VIP scores were validated by correlating them with differential abundance effect sizes from MaAsLin3 (absolute $\log_2$ fold-changes) using Spearman correlation.

For *intervention-specific discrimination*, separate sPLS-DA models were run at each timepoint (CF1, CF2, CF4) with the intervention group (K, K+, C) as the outcome. This approach allowed

identification of pathways driving differences between groups at distinct developmental stages and evaluation of changes in group discrimination over time. Intervention VIP scores were interpreted alongside pathway abundances to characterise diet-specific metabolic signatures.

4.5 Results

4.5.1 Microbial diversity

Sequencing batch explained a small but significant proportion of variation in alpha diversity and was therefore retained as a covariate in the final models ($\chi^2 = 9.54$, $p = 0.0085$). Shannon diversity was modestly lower in Batch 2 and Batch 3 compared with Batch 1 (Batch 2 = -0.18 , $p = 0.002$; Batch 3 = -0.15 , $p = 0.031$), indicating measurable technical variation across sequencing runs.

Alpha diversity increased substantially across the complementary feeding period at the genus level, with significant time effects for all metrics (Shannon: $F = 236.03$, $p < 0.001$; Simpson: $F = 124.20$, $p < 0.001$; Observed richness: $F = 138.62$, $p < 0.001$; Figure 4.1). Shannon diversity increased progressively from 1.75 ± 0.61 (mean $\pm$ SD) at baseline (CF1, 4-6 months of age) to 2.40 ± 0.62 at CF2 (8 months of age) and 2.90 ± 0.61 at CF4 (10 months of age). Post-hoc pairwise comparisons confirmed significant differences each pair of timepoints (all $p < 0.001$). Similar temporal patterns were observed for Simpson diversity and observed genus richness (Appendix G and H).

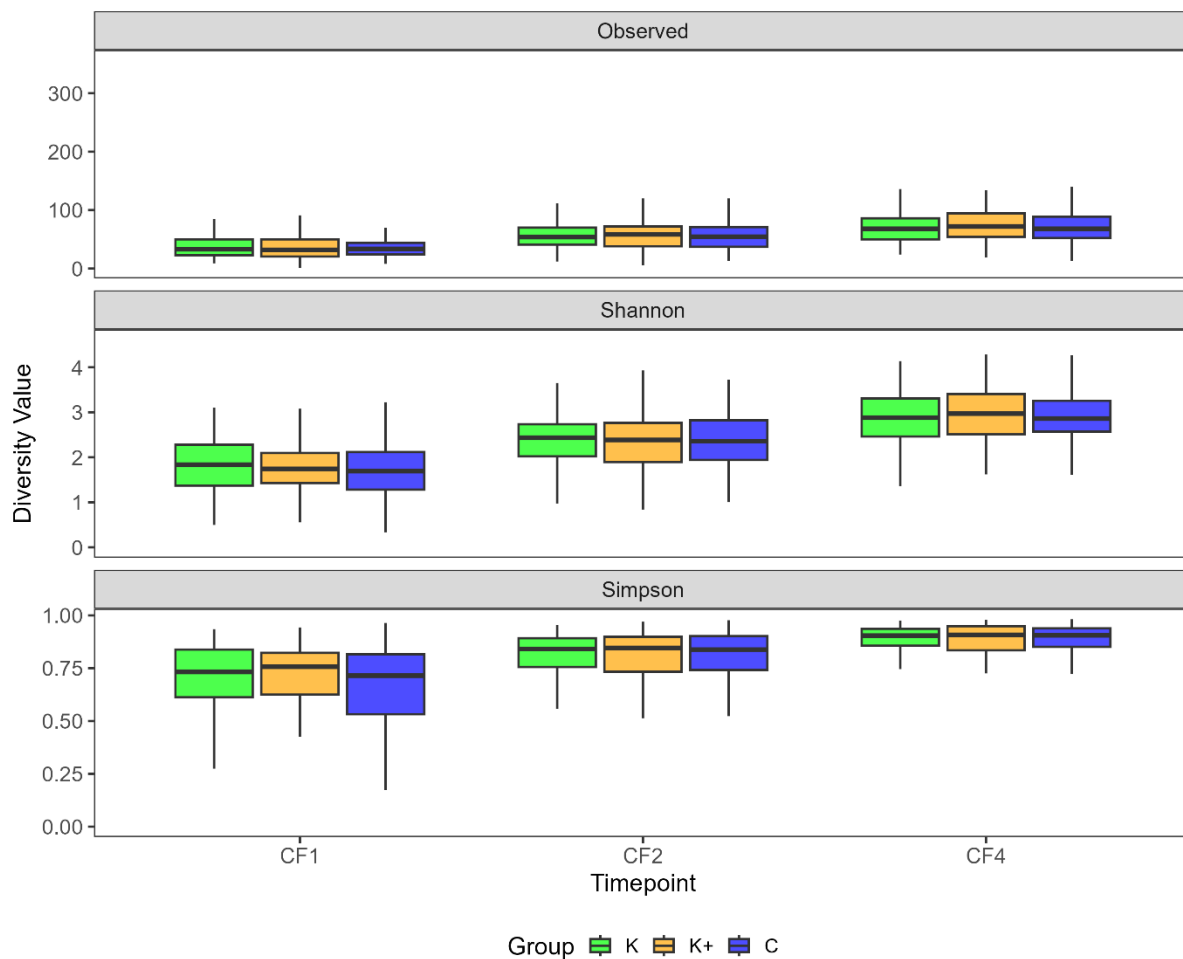


Figure 4.1. Alpha diversity metrics across complementary feeding timepoints and intervention groups. Box plots show Shannon diversity, Simpson diversity, and observed genus richness at CF1 (4-6 months), CF2 (8 months), and CF4 (10 months) for three intervention groups (K = kūmara (green), K+ = kūmara + green banana extract (orange), C = control (blue)).

Despite strong temporal effects, there was no evidence that the interventions altered diversity trajectories. The Timepoint $\times$ Group interaction was non-significant for all diversity metrics (Shannon: $F = 0.67$, $p = 0.62$; Simpson: $F = 0.89$, $p = 0.47$; Observed richness: $F = 0.93$, $p = 0.45$), indicating parallel developmental trajectories across intervention groups. Consistent with this, there was no main effect of intervention group (Shannon: $F = 0.11$, $p = 0.89$; Simpson: $F = 0.49$, $p = 0.61$; Observed: $F = 0.002$, $p = 0.10$). Estimated marginal means at CF4 was nearly identical across groups (K: 2.91 ± 0.07 ; K+: 2.95 ± 0.07 ; C: 2.91 ± 0.07 ; all EMM $\pm$ SE). Individual trajectory plots

corroborated the model results, showing broadly consistent increases in Shannon diversity across time within each group, with substantial overlap in trajectories between groups (Figure 4.2).

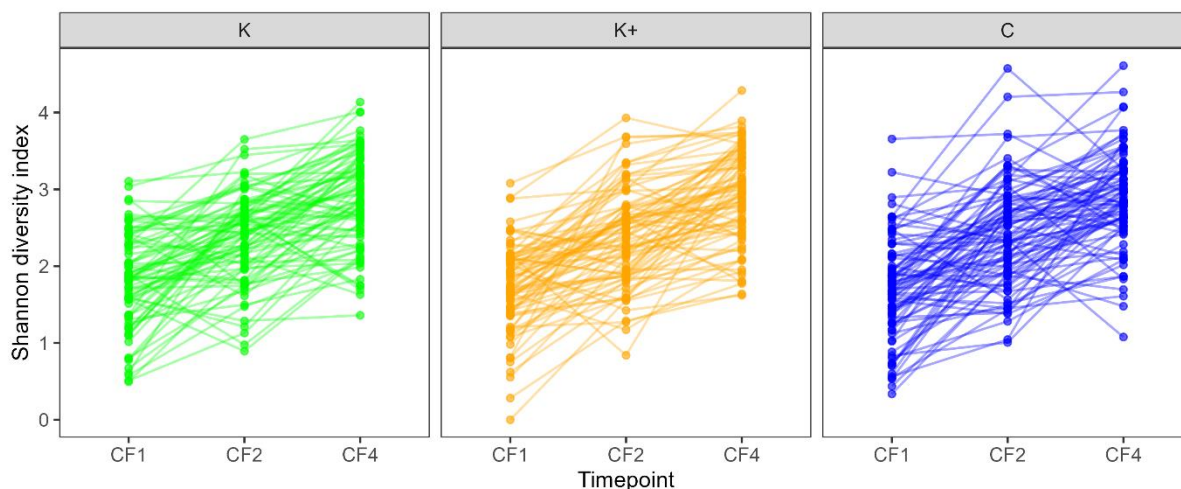


Figure 4.2. Individual Shannon diversity trajectories show parallel microbiota maturation across intervention groups. Each line represents one participant with complete longitudinal data ($n = 249$). Groups: K = kūmara (green), K+ = kūmara + green banana extract (orange), C = control (blue).

Beta diversity analysis using Aitchison distance on CLR-transformed genus-level profiles demonstrated pronounced temporal restructuring of the infant gut microbiota during complementary feeding. Permutational multivariate analysis of variance with blocked permutations (10,000 permutations, stratified by participant) revealed a strong main effect of timepoint (PERMANOVA: $F \approx 15.75$, $R^2 \approx 0.04$, $p < 0.001$, Appendix G), indicating that age-related development accounted for approximately 4% of the total compositional variance.

Principal coordinates analysis revealed progressive temporal separation along the first principal coordinate, with samples shifting consistently from CF1 to CF4 (Figure 4.3 Panel A). PC1 explained 7.9% of the total variance, while PC2 explained 4.1%, reflecting substantial within-timepoint heterogeneity typical of infant gut microbiota data.

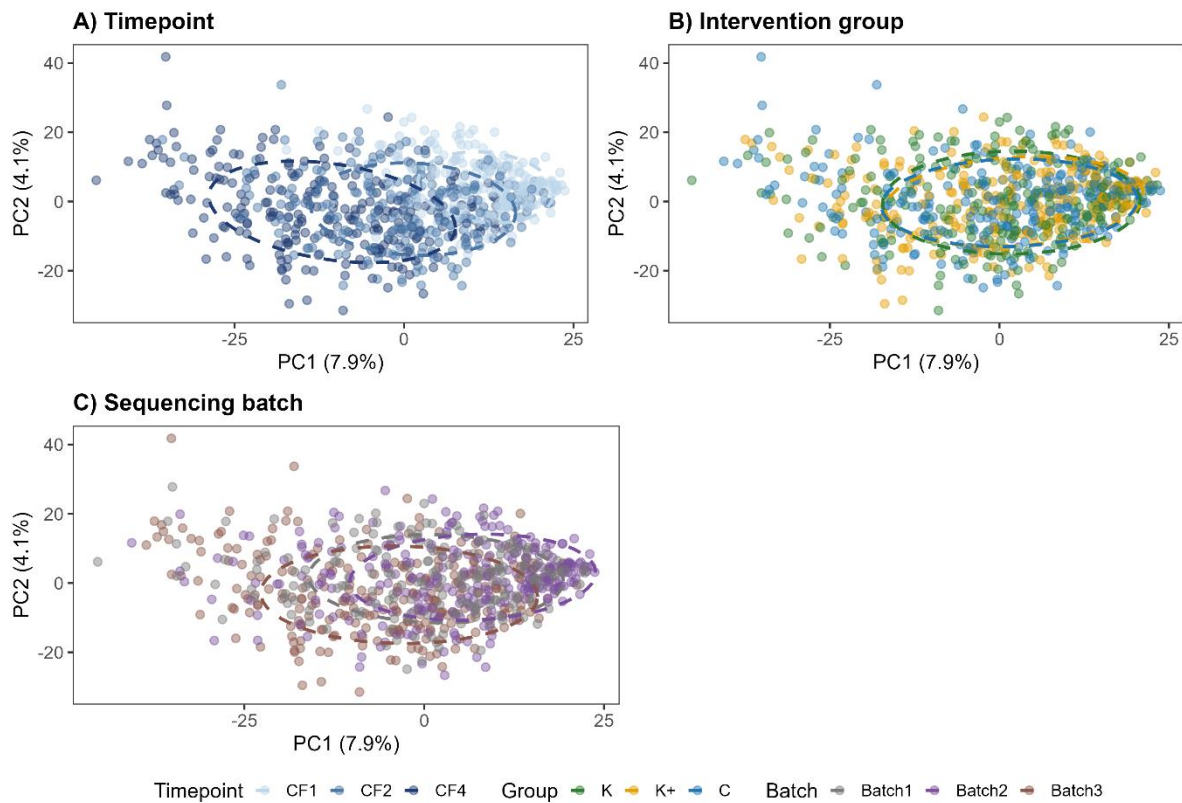


Figure 4.3. PCoA of Aitchison distance based on CLR-transformed genus-level profiles from participants with complete longitudinal data ($n = 747$ samples, 249 participants). Panels show ordination coloured by (A) timepoint, (B) intervention group, and (C) sequencing batch. Ellipses represent 68% confidence regions. PC1 explains 7.9% of the variance and captures developmental progression across complementary feeding stages, while PC2 explains 4.1% of the variance and reflects within-timepoint heterogeneity.

In contrast, complementary feeding interventions did not influence overall community composition. The main effect of intervention group was non-significant with a negligible effect size ($F \approx 1.2$, $R^2 \approx 0.003$, $p = 1.000$), and ordination plots demonstrated complete overlap between groups across all timepoints (Figure 4.3 Panel B). No evidence of a Timepoint $\times$ Group interaction was observed, indicating that compositional changes over time occurred in parallel across intervention groups.

Sequencing batch accounted for a small but statistically detectable proportion of variance ($R^2 \approx 0.01$ – 0.02 , $p < 0.01$) and was therefore retained as a covariate; however, batch effects were minor relative to temporal effects and did not alter the overall conclusions (Figure 4.3 Panel C).

Tests for homogeneity of multivariate dispersion confirmed that temporal effects reflected genuine compositional differences rather than within-group variability (PERMDISP timepoint: $F = 158.44$, $p < 0.001$). Dispersion did not differ between groups ($F = 0.20$, $p = 0.821$). Together, these findings suggest that the strong timepoint effect observed reflects both compositional restructuring and developmental changes in microbiota heterogeneity, while the absence of intervention effects is not attributable to differences in dispersion.

4.5.2 Taxonomic composition

Having established robust temporal diversity increases with no intervention effects, we next examined whether these patterns were reflected in underlying taxonomic composition.

4.5.2.1 Temporal changes in taxonomy

The gut microbiota composition showed clear temporal changes following complementary food introduction (Figure 4.4). At the phylum level, the baseline community (CF1) was dominated by Actinomycetota (49%), followed by Firmicutes (25%), Bacteroidota (13.5%), and Proteobacteria (11%). After complementary foods were introduced, Firmicutes increased steadily (33% at CF2; 42.5% at CF4), while Actinomycetota decreased to 39% by CF4. Proteobacteria showed a substantial decline to 3% by CF4, and Bacteroidota remained stable across timepoints. Minor phyla, such as Verrucomicrobia, showed a transient rise at CF2 before returning to baseline abundance at CF4.

These phylum-level shifts reflected broad changes within key genera. At baseline, the community was strongly dominated by *Bifidobacterium* (43.6%), accompanied by *Bacteroides*, *Clostridium*, *Phocaeicola*, and *Klebsiella*. After 2 months of complementary food introduction (CF2), *Bifidobacterium* remained dominant but decreased slightly, while several Firmicutes genera, including *Blautia*, *Anaerostipes*, *Veillonella*, *Mediterraneibacter*, and *Lacticaseibacillus*, increased approximately two-fold. Notably, *Faecalibacterium* expanded dramatically from near-zero to 0.8% at CF2. Conversely, early-life Proteobacteria such as *Klebsiella* and *Escherichia* declined sharply.

By CF4, these trends had strengthened. *Bifidobacterium* remained the most abundant genus (31.7%), but Firmicutes-associated genera continued to increase: *Blautia* rose to 6%, *Anaerostipes* to 2.5%, and *Faecalibacterium* to 3%. Collectively, Lachnospiraceae genera (*Blautia*, *Anaerostipes*, *Mediterraneibacter*) doubled in abundance from CF2 to CF4. Meanwhile, *Bacteroides*, *Klebsiella*, *Escherichia*, *Akkermansia*, *Enterococcus*, and *Streptococcus* all declined.

At the species level, the community was dominated throughout the study by *Bifidobacterium longum* and *B. breve*, which remained abundant and relatively stable across all timepoints. Several Firmicutes species exhibited substantial growth: *Ruminococcus gnavus* increased more than four-fold (3% to 11.7%), becoming one of the most abundant species by CF4. Both *Veillonella parvula* (3.6% to 8.1%) and *Veillonella dispar* (0.17% to 2.8%) expanded sharply, with *V. dispar* increasing more than twelve-fold at CF2. *Erysipelatoclostridium ramosum* nearly doubled by CF4 (2% to 3.7%). Several less abundant Firmicutes species also exhibited pronounced growth, including *Flavonifractor plautii* (~2.9-fold), *Streptococcus thermophilus* (~40.7-fold), and *Clostridium innocuum* (~3.8-fold). *Streptococcus salivarius* increased from 1% at CF1 to 2.3% at CF2 and CF4.

Clostridium neonatale showed one of the most pronounced declines across the study period. It was highly abundant at baseline (16.1%), but its abundance dropped sharply to 4.3% at CF2 and then to just 1% at CF4, representing an overall 93% decrease from baseline. Early-life Proteobacteria decreased in line with genus-level trends, with *Escherichia coli* falling from 12% at baseline to 4.8% at CF4.

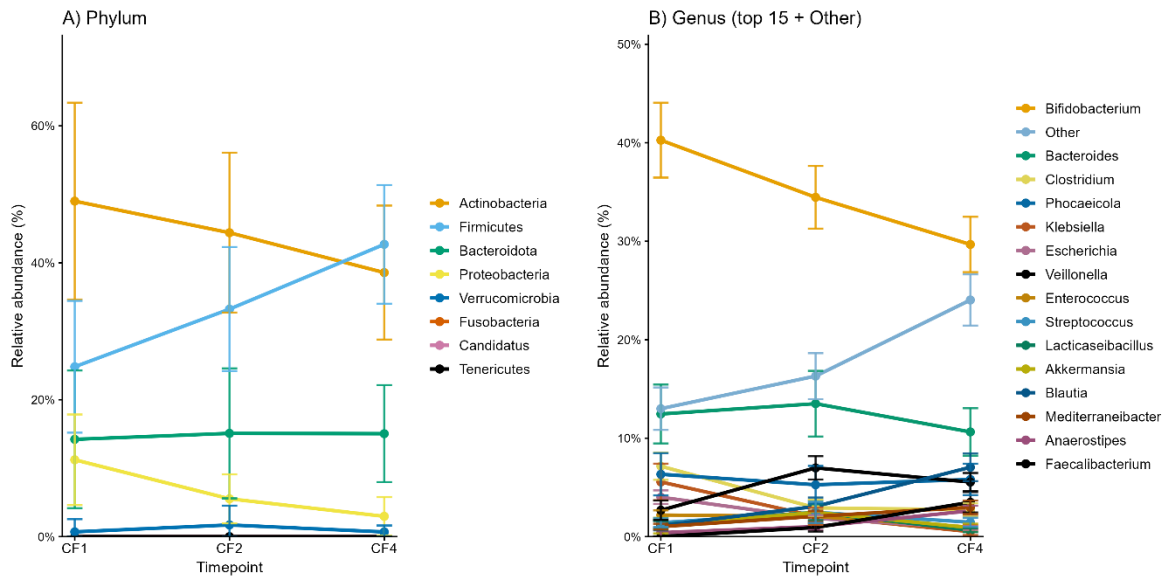


Figure 4.4. Taxonomic composition shifts during complementary feeding across phylogenetic levels. Temporal trajectories show relative abundance at (A) phylum, and (B) genus levels across CF1, CF2, and CF4.

4.5.2.2 Intervention group taxonomy changes

Intervention groups exhibited highly similar phylum-level profiles and temporal trajectories (Figure 4.5). At baseline (CF1, before complementary feeding had commenced), all groups showed comparable composition. Actinomycetota dominated in all groups (A: 47.3%; B: 46.9%; C: 54%), followed by Firmicutes (A: 26.5%; B: 25%; C: 23%), Bacteroidota (A: 13.5%; B: 14.5%; C: 12.6%), and Proteobacteria (A: 12.0%; B: 12.4%; C: 9.4%). By CF4, all groups showed comparable increases in Firmicutes (A: 41%; B: 45%; C: 42%) and decreases in Actinomycetota (A: 41%; B: 36%; C: 39%) and Proteobacteria (A: 3.5%; B: 2.9%; C: 2.6%). The largest between-group differences at CF4 were modest, including a 5% difference in Actinomycetota (A versus. B) and a 3% difference in Firmicutes (B versus. A/C). These small differences were substantially smaller than the overall temporal shifts observed across all groups.

Genera-level profiles were similarly consistent between groups. *Bifidobacterium* remained the dominant genus throughout, with a maximum difference of 4.0% between groups at CF4 (A versus. B). Other dominant genera showed only 0.2–1.7% differences at CF4. The largest genus-level

difference occurred in the “Other” category (5%), reflecting variability in low-abundance taxa rather than meaningful shifts in dominant genera.

Species-level patterns also revealed high similarity across intervention groups. The two dominant species, *Bifidobacterium longum* and *B. breve*, differed by at most 6% at CF4. Other abundant species showed even smaller differences, including *Veillonella parvula* (3%) and *Ruminococcus gnavus* (2%). All remaining species differed by <1.7% between groups.

4.5.2.3 Differential abundance

Differential abundance testing was performed to identify genera with significant temporal changes and intervention group effects after controlling for batch effects and longitudinal sampling.

4.5.2.3.1 Temporal effects on genus abundance

Genus-level differential abundance testing identified substantial temporal changes across the complementary feeding period. Overall, 22 genera changed significantly from CF1 to CF2, 5 genera from CF2 to CF4, and overall 28 genera from CF1 to CF4, indicating that the most dramatic restructuring occurred during the introductory complementary feeding period with continued but more limited changes during the establishing period (Supplementary File 4).

Early changes between CF1 and CF2 were characterised by strong increases in early-successional Firmicutes. *Limosilactobacillus* showed the largest expansion (33.8-fold, $q = 1.41 \times 10^{-2}$), followed by *Faecalibacterium* (8.5-fold, $q = 1.54 \times 10^{-4}$) and *Lachnospira* (6.8-fold, $q = 1.89 \times 10^{-4}$). Highly significant increases were also observed in *Veillonella* (4.2-fold, $q = 1.63 \times 10^{-11}$), *Megasphaera* (1.5-fold, $q = 1.58 \times 10^{-12}$), and *Blautia* (2.3-fold, $q = 3.41 \times 10^{-7}$), indicating rapid stimulation of SCFA-producing taxa following complementary food introduction.

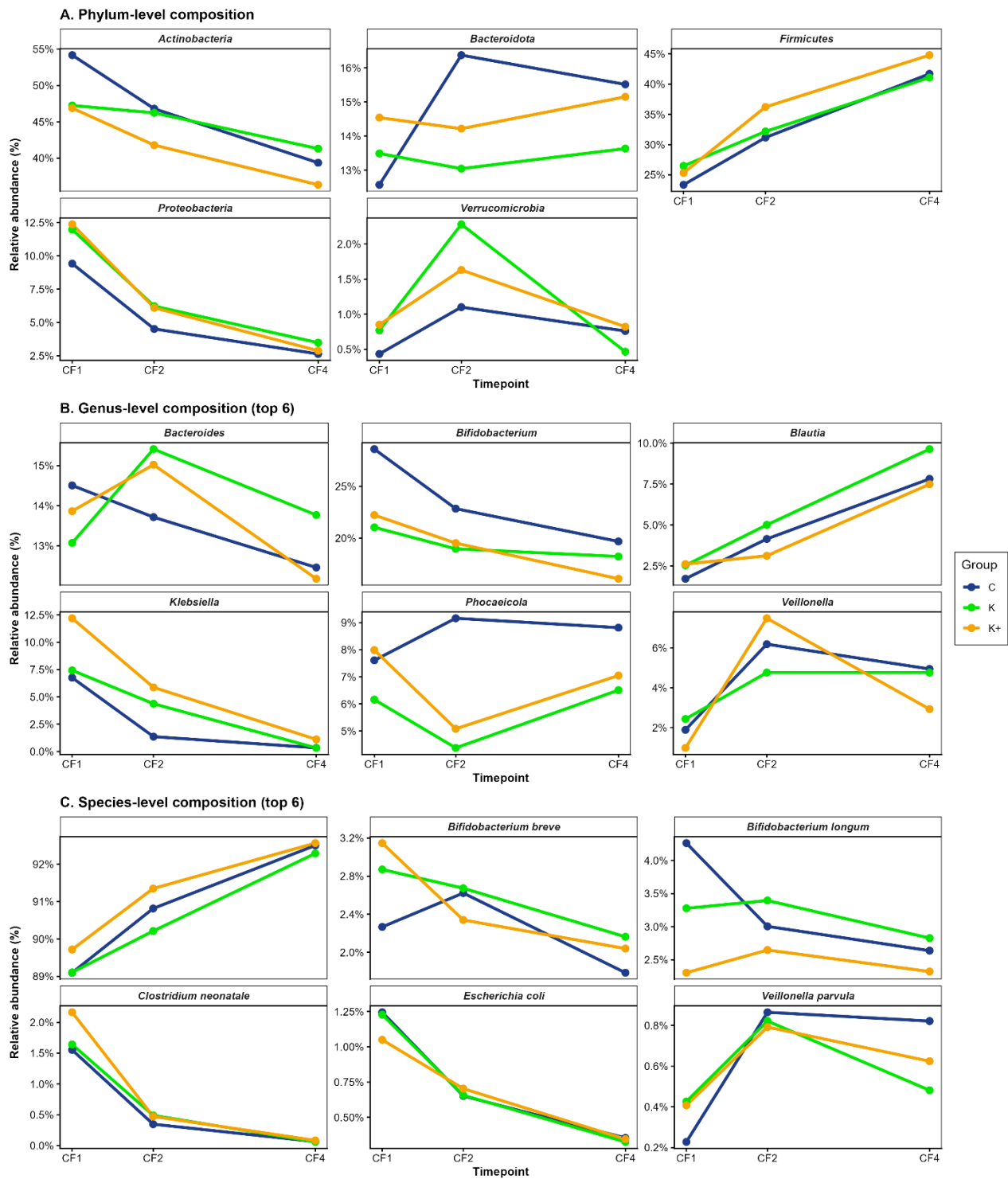


Figure 4.5. Relative abundance at (A) phylum, (B) genus (top 6), and (C) species (top 6) levels for *K* (*kūmara*, orange), *K+* (*kūmara* + green banana extract, blue), and *C* (control, green) at CF1, CF2, and CF4.

In contrast, several Lachnospiraceae genera showed marked early declines, including *Roseburia* (0.07-fold, 93% decrease, $q = 9.83 \times 10^{-4}$), *Hungatella* (0.13-fold, 87% decrease, $q = 9.16 \times 10^{-4}$),

Tyzzerella (0.25-fold, $q = 1.99 \times 10^{-2}$), and *Anaerostipes* (0.49-fold, $q = 1.86 \times 10^{-8}$). Additional decreases were observed in *Intestinibacter* (0.51-fold, $q = 2.04 \times 10^{-10}$), *Sellimonas* (0.41-fold, $q = 5.39 \times 10^{-3}$), and *Enterocloster* (0.53-fold, $q = 3.46 \times 10^{-3}$).

From CF2 to CF4, only five genera showed significant abundance changes. The most dramatic shifts involved the decline of *Actinomyces* (0.04-fold, 96% decrease, $q = 1.38 \times 10^{-3}$) and *Rothia* (0.08-fold, 92% decrease, $q = 1.59 \times 10^{-3}$). Milk-associated Firmicutes also declined, including *Lacticaseibacillus* (0.22-fold, $q = 3.66 \times 10^{-2}$) and *Enterococcus* (0.27-fold, $q = 3.36 \times 10^{-2}$). In contrast, *Faecalibacterium* continued to increase (2.1-fold, $q = 3.95 \times 10^{-2}$), reflecting ongoing expansion of butyrate-producing capacity.

Over the full study period (CF1 to CF4), the cumulative effects demonstrated sustained expansion of fibre-degrading taxa, with *Limosilactobacillus* (18.7-fold, $q = 2.02 \times 10^{-8}$), *Faecalibacterium* (16.7-fold, $q = 6.31 \times 10^{-12}$), *Lachnospira* (6.4-fold, $q = 2.74 \times 10^{-8}$), and *Blautia* (3.1-fold, $q = 2.65 \times 10^{-21}$) showing the largest increases, alongside progressive enrichment in Bacteroidota (*Bacteroides* 1.9-fold, *Phocaeicola* 1.2-fold). Conversely, the most dramatic overall decreases were observed in Actinomycetota (*Actinomyces* 0.01-fold, 99% decrease; *Rothia* 0.03-fold, 97% decrease), *Klebsiella* (0.06-fold, $q = 1.70 \times 10^{-2}$), and fibre-associated Lachnospiraceae including *Roseburia* (0.10-fold, $q = 1.38 \times 10^{-9}$) and *Hungatella* (0.09-fold, $q = 3.75 \times 10^{-5}$). These diverse temporal patterns are illustrated in Figure 4.6.

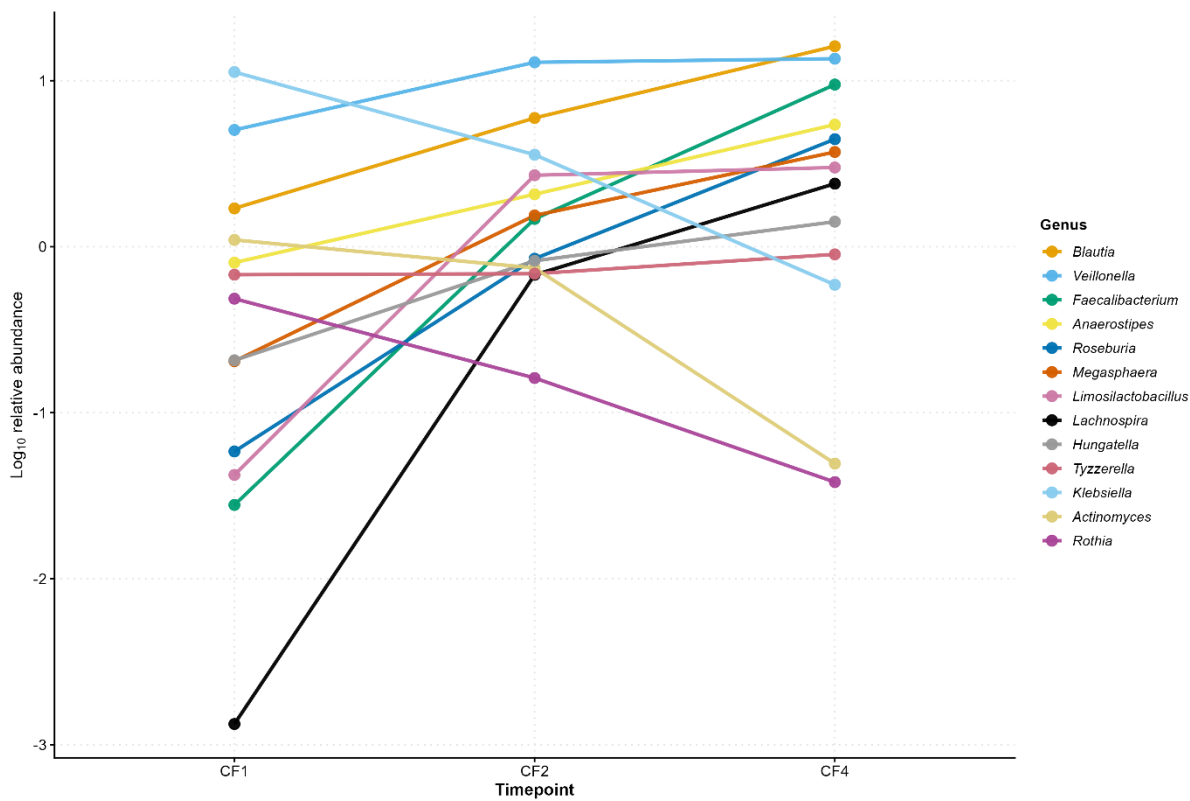


Figure 4.6. Temporal trajectories of differentially abundant genera during complementary feeding.

4.5.2.3.2 Intervention effects on genus abundance

All intervention groups exhibited temporal restructuring. Two Group $\times$ Timepoint interaction analyses were conducted: a main analysis spanning CF1, CF2, and CF4 (reference: Control at CF1; 106 significant associations, $q < 0.05$), and a focused analysis of the establishing period from CF2 to CF4 (reference: Control at CF2; 32 significant associations), enabling comparison of intervention-specific trajectories across developmental stages (Supplementary Files 5). These temporal and intervention-associated shifts are summarised in Figure 4.7). All fold changes reported reflect model-derived effect sizes from log-transformed, normalised genus abundances, representing relative differences in genus-level responses between groups over time rather than absolute changes in microbial abundance.

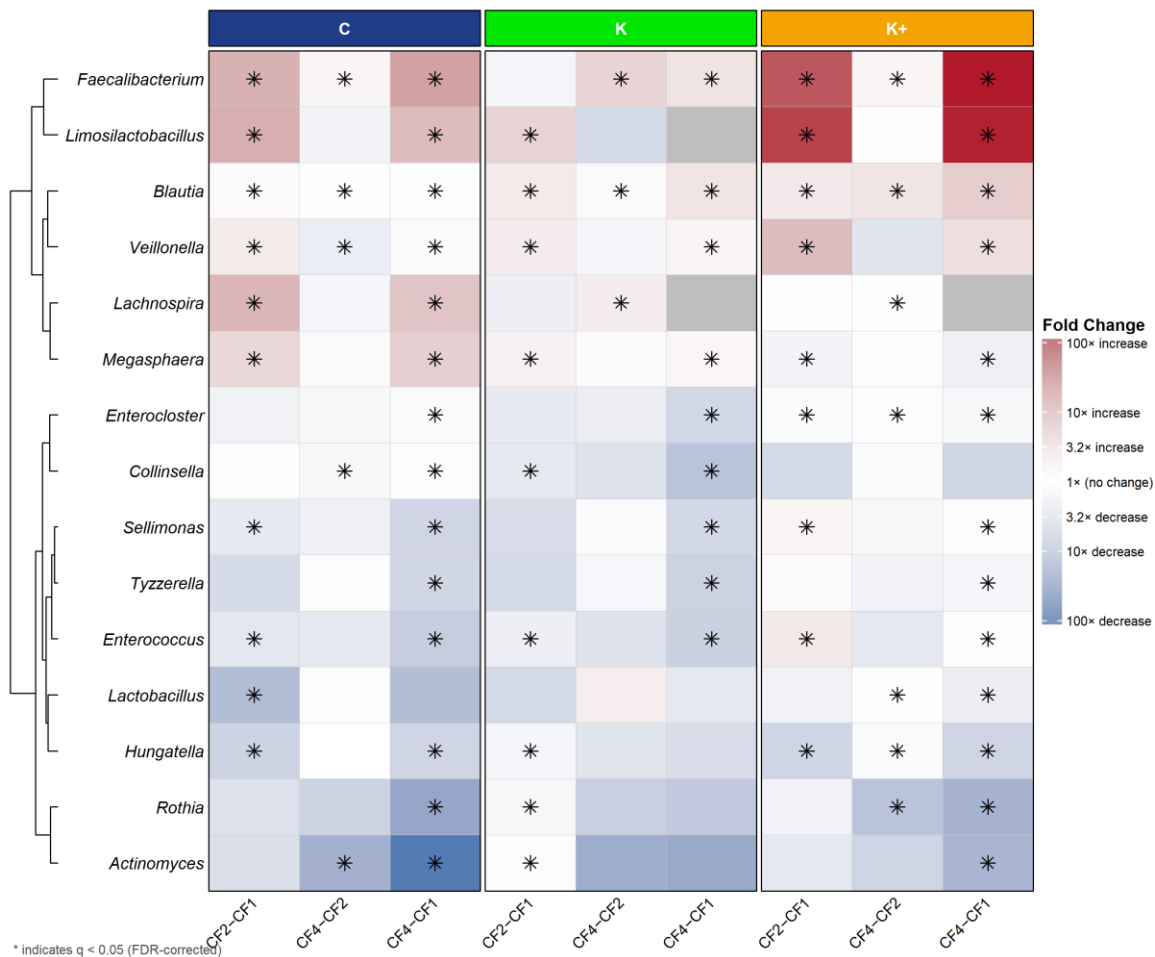


Figure 4.7. Genus-level effect sizes (fold changes) across complementary feeding (CF1 to CF2, CF2 to CF4, CF1 to CF4) by intervention group (C = Control, K = kūmara, K+ = kūmara + GBE).

During the introductory complementary feeding period (CF1 to CF2, 6 to 8 months of age), all groups demonstrated substantial metabolic restructuring, though with distinct taxonomic patterns. The Control group showed 16 significant modelled changes, characterised by large positive effect sizes in saccharolytic fermenters including *Limosilactobacillus* (27-fold increase), *Lachnospira* (22-fold increase), and *Faecalibacterium* (25-fold increase), alongside more modest increases in *Megasphaera*, *Veillonella*, and *Blautia*. Concurrently, several taxa showed negative modelled effect sizes, including *Anaerostipes* (4-fold decrease), *Lactobacillus* (26-fold decrease), *Hungatella* (10-fold decrease), and multiple Lachnospiraceae genera (*Sellimonas*, *Mediterraneibacter*, *Enterococcus*), alongside *Clostridium* and *Intestinibacter*. This pattern reflects the typical transition toward carbohydrate-fermenting communities during initial complementary food introduction.

The kūmara intervention group (K) displayed a broadly similar but attenuated response (15 significant changes). Enrichment of *Veillonella* (3-fold increase), *Megasphaera* (2-fold increase), and *Blautia* (3-fold increase) mirrored Control, although *Blautia* enrichment was stronger than in Control (3-fold increase versus 1.3-fold increase), while *Limosilactobacillus* expansion was markedly reduced (8-fold increase versus 27-fold increase). The K group uniquely showed early positive modelled effects for *Lacticaseibacillus* (1.5-fold increase) and *Streptococcus* (1.2-fold increase). Declines were observed in early colonisers including *Intestinibacter*, *Enterococcus*, *Anaerostipes*, and *Hungatella* (all ~2-fold decreases), alongside reductions in several Actinomycetota genera (*Actinomyces*, *Rothia*, *Eggerthella*, *Collinsella*; 1.2- to 4-fold decreases), indicating selective modulation of early microbial succession.

In contrast, the kūmara plus green banana extract group (K+) exhibited the most divergent early pattern (13 significant changes). *Veillonella* showed an exceptional positive effect size (18-fold increase), far exceeding both Control and K (3-fold increases). Similarly, *Limosilactobacillus* had a marked positive effect size (235-fold increase versus 27-fold increase in Control), and *Faecalibacterium* (182-fold increase versus 25-fold increase in Control). *Blautia* increased to a similar extent as in K (3-fold increase). Uniquely, *Megasphaera* declined (2-fold decrease), contrasting with its increase in both other groups. Additional increases in *Enterococcus*, *Sellimonas*, and *Mediterraneibacter* (2–3-fold increases) opposed declines observed in Control, while decreases in *Hungatella*, *Anaerostipes*, *Intestinibacter*, and *Clostridium* indicated substantial restructuring of early metabolic networks rather than simple acceleration of maturation.

As shown in Figure 4.7, responses diverged further between CF2 and CF4. Thirty-two significant Group × Timepoint interactions were detected, reflecting sustained and increasingly differentiated modelled intervention trajectories.

The Control group exhibited 11 significant changes, consistent with gradual maturation. *Faecalibacterium* (1.6-fold increase), *Roseburia* (1.4-fold increase), and *Anaerostipes* (1.02-fold

increase) increased modestly, while *Blautia* showed a slight decline (1.05-fold decrease). Early colonisers continued to decrease, most notably *Actinomyces* (40-fold decrease). *Veillonella* declined (3-fold decrease) following earlier expansion, indicating biphasic dynamics typical of microbiota consolidation.

The K group showed 9 significant changes but with substantially larger magnitudes for several fibre-associated taxa. *Faecalibacterium* showed a 7.2-fold greater modelled increase, greatly exceeding Control (1.6-fold increase), while *Anaerostipes* showed strong positive modelled effects during the establishing complementary feeding period (2.1-fold increase versus 1.02-fold increase in Control). *Roseburia* enrichment was also greater (3.2-fold increase versus 1.4-fold increase). *Blautia* increased modestly (1.4-fold increase), opposite to Control's decline. Notably, *Lachnospira* showed K-specific enrichment (2.5-fold increase), absent in both Control and K+. Declines in *Klebsiella* (5.9-fold decrease), *Faecalimonas* (3-fold decrease), and *Clostridium* (1.4-fold decrease) exceeded those observed in Control.

The K+ group exhibited the most extensive late-stage response (12 significant changes). *Blautia* showed enrichment (3.6-fold increase) far exceeding both K (1.4-fold increase) and Control (1.05-fold decrease). *Anaerostipes* recovered moderately (1.7-fold increase), while *Roseburia* increased modestly (1.4-fold increase), similar to Control. In contrast, *Faecalibacterium* showed a more modest late-stage effect size (1.7-fold increase), substantially less than in K (7.2-fold increase), despite its earlier dramatic expansion. *Lachnospira* declined slightly (1.1-fold decrease), opposing its strong increase in K. Marked reductions in *Rothia* (20-fold decrease), *Bifidobacterium* (1.6-fold decrease), *Mediterraneibacter* (1.9-fold decrease), and *Klebsiella* (5.4-fold decrease) suggest altered competitive dynamics.

Across CF2 to CF4, several genera showed consistent involvement in microbiota maturation, with direction and magnitude of change differing by intervention group. *Anaerostipes*, *Blautia*, *Faecalibacterium*, and *Roseburia* responded across all groups, with fold-change magnitudes

consistently greatest under the kūmara intervention and most variable under kūmara plus green banana extract. In contrast, *Lachnospira* exhibited opposite responses between K (2.5-fold increase) and K+ (1.1-fold decrease), indicating intervention-specific effects.

By 10 months of age (CF4), analysis across the entire complementary feeding period (CF1 to CF4) revealed several additional patterns not captured in period-specific analyses. Several genera showed significant cumulative modelled changes despite not appearing in either individual period analysis. In the Control group, *Tyzzerella* declined substantially (9-fold decrease), while *Phocaeicola* increased modestly (1.05-fold increase), neither of which showed significant changes during specific periods. The K group exhibited additional cumulative changes in *Enterococcus* (11-fold decrease), *Bacteroides* (2-fold increase), *Collinsella* (20-fold decrease), and *Streptococcus* (4-fold decrease), the latter revealing a biphasic pattern with early increase during CF1 to CF2 followed by late-stage decline. The K+ group showed the most extensive cumulative effects (24 significant changes), with sustained enrichment of *Limosilactobacillus* (295-fold) and *Faecalibacterium* (305-fold) from their dramatic CF1 to CF2 expansion, alongside cumulative declines in *Actinomyces* (34-fold decrease) and sustained *Megasphaera* reduction (2-fold decrease).

Cumulative analysis confirmed biphasic dynamics for several key genera. *Anaerostipes* showed net minimal change in K (1.1-fold increase cumulative) despite substantial CF1 to CF2 decline offset by CF2 to CF4 recovery, while Control maintained cumulative decline (4-fold decrease) and K+ achieved net recovery (1.6-fold increase cumulative). Similarly, *Roseburia* in Control showed net cumulative decrease (7-fold) despite CF2 to CF4 increase, revealing early decline that dominated overall trajectory. These findings demonstrate that intervention effects manifest through complex, modelled temporal dynamics, with some taxa responding primarily during the introductory complementary feeding period (*Limosilactobacillus*, *Lachnospira*), others showing later amplification (*Faecalibacterium* in K, *Blautia* in K+), and several exhibiting biphasic patterns requiring complete period assessment to characterise fully.

4.5.3 Microbial pathway abundance

A PERMANOVA using Bray–Curtis dissimilarities and participant-stratified permutations (complete cases only) was performed to assess the overall functional potential of the microbiota across the complementary feeding period. This analysis revealed a statistically significant temporal effect ($R^2 = 0.035$, $F = 13.7$, $p < 0.001$), indicating that functional pathway composition shifted as complementary feeding progressed. A small but statistically significant group effect was also detected ($R^2 = 0.003$, $F = 1.26$, $p < 0.0001$), suggesting subtle intervention-related differences in overall pathway composition. However, the Group $\times$ Timepoint interaction was not significant ($R^2 = 0.003$, $F = 0.52$, $p = 0.86$), indicating that intervention groups followed similar temporal trajectories of functional development. Multivariate dispersion was homogeneous across groups ($F = 0.75$, $p = 0.48$), validating the PERMANOVA assumptions. These findings provide context for subsequent MaAsLin3 analyses, which identify individual pathway-level changes and intervention-specific effects.

4.5.3.1 Temporal pathway changes during the introductory complementary feeding period

Analysis of functional pathway abundance between CF1 and CF2 revealed substantial metabolic restructuring of the infant gut microbiota, with 1,397 significant associations ($q < 0.05$) across 284 unique pathways (Supplementary File 6). Of these, 772 pathways (55%) showed higher CPM and 625 (45%) showed lower CPM at CF2 relative to CF1, indicating coordinated but taxon-specific functional adaptation during early complementary feeding. Major functional categories affected included nucleotide metabolism (342 pathways; 186 higher, 156 lower), amino acid metabolism (250 pathways; 140 higher, 110 lower), other biosynthesis pathways (192 pathways; 108 higher, 84 lower), cofactor and vitamin metabolism (140 pathways; 85 higher, 55 lower), central carbon metabolism (110 pathways; 61 higher, 49 lower), and cell wall and structural components (98 pathways; 52 higher, 46 lower).

Nucleotide metabolism showed the clearest pattern of expansion–contraction. De novo purine and pyrimidine biosynthesis pathways showed higher relative abundance (CPM) at CF2 (inosine-5'-

phosphate biosynthesis II: 16.6-fold higher; UMP biosynthesis I–III: 11.6-fold higher), consistent with increased DNA/RNA synthesis during rapid microbial proliferation. In contrast, salvage pathways showed lower relative abundance (CPM) (pyrimidine nucleobase salvage: 9.7-fold lower; adenosine nucleotide biosynthesis I–II: 8.6-fold lower), indicating a shift from recycling to active synthesis.

Amino acid metabolism exhibited selective enhancement. Branched-chain amino acid biosynthesis increased markedly (L-isoleucine biosynthesis III: 12.3-fold higher CPM; L-valine: 11.1-fold higher; L-lysine VI: 11.9-fold higher), reflecting elevated capacity for essential amino acid production to support cell growth. Conversely, aromatic amino acid pathways (superpathway of aromatic amino acids: 8.8-fold lower CPM; L-histidine biosynthesis: 8.8-fold lower) and L-arginine biosynthesis (7.9-fold lower) declined, indicating shifts in taxon-specific anabolic priorities.

The largest single shift occurred in lipid metabolism: fatty acid biosynthesis initiation showed higher relative abundance (CPM) than any other pathway (20.9-fold higher), alongside elevated production of unsaturated fatty acids (cis-vaccenate: 17.5-fold higher CPM; gondoate: 16-fold higher), consistent with accelerated membrane synthesis in expanding taxa. However, the same initiation module also showed lower CPM in certain taxa (9.1-fold lower), demonstrating opposing metabolic strategies across different microbial groups.

Central carbon metabolism showed coordinated enhancement of fermentation capacity. Pyruvate fermentation to acetate and lactate increased substantially (14.3-fold higher CPM), alongside glycolysis IV (10.4-fold higher) and 2-oxobutanoate degradation (10.7-fold higher), indicating prioritisation of readily fermentable substrates. Meanwhile, pathways associated with complex carbohydrate breakdown showed lower relative abundance (CPM) (pentose phosphate pathway I/II: 5.8-fold and 7.9-fold lower; glycolysis IV: 5.8-fold lower), reflecting reduced reliance on polysaccharide degradation.

Cofactor and vitamin metabolism expanded broadly. Folate transformations II (9-fold higher CPM) and multiple heme b biosynthesis routes (8.7-fold, 4.9-fold, 4.1-fold, 5.1-fold, and 5.5-fold higher) increased, supporting enhanced enzymatic and redox capacity. Targeted reductions were also evident, including coenzyme A biosynthesis (7.8-fold lower CPM), folate transformations II (7-fold lower), and phosphopantothenate biosynthesis I (6.9-fold lower), suggesting streamlined cofactor investment in certain taxa.

Cell wall and structural pathways showed both expansion and contraction. UDP-N-acetyl-D-glucosamine biosynthesis increased substantially (pathway I: 16-fold higher CPM; pathway II: 13.9-fold higher), supporting peptidoglycan synthesis for growing microbial populations. In contrast, full peptidoglycan biosynthesis routes showed lower relative abundance (CPM) (mycobacteria-type: 7.7-fold lower; UDP-N-acetylmuramoyl-pentapeptide: 7.5-fold lower), suggesting reduced investment in energetically demanding structural components.

Other biosynthetic functions underwent extensive restructuring. The largest increases occurred in selenium-containing amino acid biosynthesis (18.5-fold higher CPM), preQ₀ biosynthesis (10.2-fold higher), the branched-chain amino acid superpathway (9.6-fold higher), and molybdopterin biosynthesis (8.2-fold and 5.6-fold higher). These shifts point to enhanced production of specialised cofactors and tRNA modifications that support growth under changing nutrient conditions. Conversely, queuosine biosynthesis showed lower relative abundance (CPM) (9.2-fold and 2.9-fold lower), alongside strong decreases in CDP-diacylglycerol biosynthesis I/II (both 7.5-fold lower), L-ornithine biosynthesis (7.5-fold lower), and moderate reductions in dTDP-L-rhamnose biosynthesis (4.7-fold and 3-fold lower).

Overall, pathways showed both increases and decreases within the same functional module, driven by opposing responses across different taxa. Notably, the top 50 increasing and top 50 decreasing pathways were dominated by amino acid biosynthesis functions, underscoring the central role of nitrogen metabolism in early complementary feeding adaptation.

4.5.3.2 Temporal changes during the establishing complementary feeding period

Analysis of functional pathway abundance during the CF2 to CF4 complementary feeding period revealed a contrasting pattern to the earlier complementary feeding changes, with 1,669 significant pathway associations ($q < 0.05$) across 346 unique pathways. Of these, 559 pathways (33%) showed higher CPM while 1,110 (67%) showed lower CPM at CF4 compared to CF2, representing metabolic consolidation rather than expansion. Major functional categories affected included nucleotide metabolism (325 pathways; 110 higher, 215 lower), amino acid metabolism (303 pathways; 115 higher, 188 lower), other biosynthesis pathways (232 pathways; 74 higher, 158 lower), cofactor and vitamin metabolism (162 pathways; 63 higher, 99 lower), central carbon metabolism (133 pathways; 46 higher, 87 lower), and degradation pathways (124 pathways; 30 higher, 94 lower).

Nucleotide metabolism showed a reversal of the CF1 to CF2 pattern. De novo purine and pyrimidine biosynthesis pathways predominantly showed lower relative abundance (CPM) (superpathway of guanosine nucleotides I/II: 4.7-fold and 3.4-fold lower; adenosine deoxyribonucleotides biosynthesis II: 4-fold lower), while salvage pathways showed higher CPM (superpathway of pyrimidine ribonucleosides salvage: 3.7-fold higher). This shift from active biosynthesis to nucleotide recycling indicates stabilisation of microbial populations following the rapid expansion observed during early complementary feeding.

Amino acid metabolism displayed selective maintenance rather than broad enhancement. L-glutamate and L-glutamine biosynthesis showed the largest increase (4.8-fold higher CPM), alongside sustained branched-chain amino acid production (L-valine: 2.2-fold higher) and increased amino acid catabolism (L-histidine degradation I: 3.5-fold higher). In contrast, essential amino acid biosynthesis pathways showed lower relative abundance (CPM) (L-lysine biosynthesis VI: 3.7-fold lower; superpathway of L-lysine, L-threonine, and L-methionine biosynthesis: 3.3-fold lower), reflecting reduced reliance on microbial amino acid production as dietary sources became established.

Fatty acid and lipid metabolism showed the most dramatic reversal from early feeding. While CF1 to CF2 exhibited the largest functional increase in fatty acid biosynthesis initiation (20.9-fold higher CPM), CF2 to CF4 displayed substantial decreases across multiple lipid pathways, including *cis*-vaccenate biosynthesis (4-fold lower CPM), the superpathway of unsaturated fatty acids biosynthesis (2.9-fold lower), mitochondrial fatty acid biosynthesis initiation (3-fold lower), saturated fatty acid elongation (2.9-fold lower), anaerobic oleate biosynthesis IV (2.9-fold lower), and fatty acid β -oxidation I (3.2-fold lower). Lipid IVA biosynthesis (*E. coli* and *Pseudomonas*) also declined (both 3-fold lower). Despite this overall downward trend, a subset of pathways showed modest increases (fatty acid biosynthesis initiation: 1.2-fold to 1.9-fold higher CPM; gondoate biosynthesis: 1.2-fold higher; phospholipid biosynthesis I: 1.3-fold higher), suggesting that specific taxa maintained or upregulated lipid synthesis while the broader community reduced lipid metabolic activity.

Central carbon metabolism showed a bidirectional response. Major reductions occurred in core carbohydrate degradation and fermentation pathways, including the pentose phosphate pathway (oxidative and non-oxidative branches: 3.3-fold and 2.6-fold lower CPM), glycolysis I/II (both 2.6-fold lower), mixed acid fermentation (2.6-fold lower), pyruvate fermentation to acetate and lactate (2.7-fold lower), partial TCA cycle activity (3-fold lower), and (S)-lactate conversion to propionate, acetate, and hydrogen (2.7-fold lower). In parallel, some pathways showed higher relative abundance (CPM), notably the pentose phosphate pathway non-oxidative branches I and II (2.7-fold and 2.4-fold higher), methanogenesis from acetate (2.3-fold higher), glycolysis III (1.7-fold higher) and IV (2-fold higher), butanoate fermentation (1.6-fold higher), and *Clostridium acetobutylicum* acidogenic fermentation (1.6-fold higher). These bidirectional changes reflect taxon-specific redistribution of central carbon flux rather than a uniform community-wide shift.

Cell wall and structural pathways exhibited bidirectional modulation between CF2 and CF4, indicating selective remodelling of microbial envelopes. Several key peptidoglycan biosynthesis and UDP-N-acetyl-D-glucosamine biosynthesis pathways showed lower relative abundance (CPM) (5-fold to 2.2-fold lower), including peptidoglycan maturation and multiple meso-diaminopimelate-

containing peptidoglycan biosynthesis pathways, suggesting reduced investment in energetically costly cell wall assembly for certain taxa. Conversely, the same pathways also displayed coordinated increases (2.2-fold to 1.8-fold higher CPM), particularly in peptidoglycan maturation, UDP-N-acetylmuramoyl-pentapeptide, and mycobacterial peptidoglycan biosynthesis, reflecting selective reinforcement of structural components in other taxa. This pattern highlights taxon-specific remodelling, with the microbiota balancing structural maintenance and metabolic efficiency during the later stages of complementary feeding.

Other biosynthetic functions showed similar bidirectional restructuring. Increases were observed in dTDP- β -L-rhamnose biosynthesis (2.4-fold higher CPM), CDP-diacylglycerol biosynthesis I/II (2.3-fold higher), ADP-L-glycero- β -D-manno-heptose biosynthesis (2.2-fold higher), 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I (2.2-fold higher), queuosine biosynthesis I (2.2-fold and 2-fold higher), and preQ₀ biosynthesis (2-fold higher). In contrast, decreases occurred in the same and related pathways, including 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I (4.7-fold lower CPM), polyisoprenoid biosynthesis (4-fold lower), seleno-amino acid biosynthesis (3.7-fold lower), GDP-mannose biosynthesis (3.7-fold to 3.5-fold lower), CDP-diacylglycerol biosynthesis I/II (3.4-fold lower), and phosphatidylglycerol biosynthesis (3.4-fold lower). This pattern reflects dynamic, taxon-specific allocation of metabolic resources, consistent with maturation and functional diversification of the gut microbiota during the later stages of complementary feeding.

4.5.3.3 Pathway abundance comparisons between intervention groups in the introductory complementary feeding period (CF1 to CF2)

Group $\times$ Timepoint interaction analysis identified 2,160 significant pathway-level differences across intervention groups (Control: 61.8% higher CPM; Kūmara: 71.4% higher; Kūmara + green banana extract: 62.3% higher). All groups showed net increases in pathway abundance at CF2, consistent with the expansion phase observed in overall temporal analysis, though the magnitude and intensity

of enrichment differed markedly across groups, with the kūmara group demonstrating the strongest metabolic expansion.

The control group showed marked increases in amino acid biosynthesis pathways, with the strongest gains in L-valine biosynthesis (16-fold higher CPM), L-threonine biosynthesis (15.2-fold higher), and L-isoleucine biosynthesis III/I (14.2-fold and 12.9-fold higher). These coordinated increases, along with the broader branched-chain amino acid superpathway (10.1-fold higher), indicate a pronounced early enhancement of protein-building capacity. Parallel gains in nucleotide biosynthesis, including the 5-aminoimidazole ribonucleotide pathway (12.9-fold higher CPM) and de novo purine nucleotide synthesis I (10-fold higher), suggest increased microbial replication and biomass production between CF1 and CF2. Increases in cell wall precursor biosynthesis, notably UDP-N-acetylglucosamine I/II (12.9-fold and 11-fold higher CPM) and peptidoglycan precursors (12.9-fold higher), further reinforce this growth-associated signature.

Conversely, several pathways in the control group showed lower relative abundance (CPM). Reductions were concentrated in nucleotide salvage and deoxyribonucleotide synthesis, including pyrimidine salvage (12.9-fold lower), deoxyribonucleotide formation (both 11.6-fold lower), and multiple de novo adenosine and guanosine nucleotide routes (10.3-fold to 11.6-fold lower). Amino acid metabolism also showed selective decreases: L-histidine biosynthesis (9.6-fold lower CPM), L-methionine biosynthesis (9.3-fold lower), and L-arginine biosynthesis II (8.5-fold lower). Additional declines included coenzyme A biosynthesis (13.2-fold lower), assimilatory sulphate reduction (12.9-fold lower), and (S)-propane-1,2-diol degradation (8.6-fold lower). These reductions indicate a restructuring away from salvage-based energy and nucleotide strategies as the microbiota adjusts to new dietary components.

The kūmara group displayed strong increases in pathways linked to lipid and cell-wall precursor biosynthesis. The largest positive shift was in fatty acid biosynthesis initiation (19.9-fold higher CPM), supported by increases in cis-vaccenate (17.1-fold higher) and gondoate biosynthesis (14.6-

fold higher), indicating enhanced microbial membrane-building activity. Parallel rises in UDP-N-acetylglucosamine production (17.8-fold and 11.6-fold higher CPM) point toward increased synthesis of peptidoglycan precursors, consistent with active cell-wall remodelling during microbial growth. Broader biosynthetic processes also showed higher relative abundance (CPM), including chorismate biosynthesis (12-fold higher) and multiple nucleotide metabolism pathways such as inosine monophosphate biosynthesis (11-fold higher), de novo guanosine nucleotide formation (10.9-fold higher), and UMP biosynthesis (all 9.6-fold higher). These increases collectively suggest a period of rapid metabolic expansion and biomass accumulation following the introduction of kūmara.

In contrast, the strongest decreases in the kūmara group centred on purine precursor formation, particularly the two steps of 5-aminoimidazole ribonucleotide biosynthesis (22-fold and 8.2-fold lower CPM), indicating a pivot away from early-stage purine synthesis despite increases in downstream nucleotide pathways. Several amino acid pathways also showed lower relative abundance (CPM), including L-valine biosynthesis (14-fold lower) and S-adenosyl-L-methionine salvage (13.2-fold lower), along with reductions in peptidoglycan biosynthesis variants (7.2-fold lower), suggesting selective downregulation of specific structural routes even as other cell-wall precursors increased. Additional decreases were observed in deoxyribonucleotide biosynthesis (6.4-fold lower) and aromatic amino acid biosynthesis (6.3-fold lower), highlighting a broader reorganisation of anabolic priorities during early microbiota adaptation to kūmara.

The kūmara + green banana group's extensive functional activation spanned fermentation, amino acid biosynthesis, nucleotide metabolism, lipid metabolism, and cofactor biosynthesis. Several pathways exhibited exceptionally high fold changes, surpassing all other groups both in magnitude and breadth of functional coverage. The largest increase was observed for pyruvate fermentation to acetate and lactate II (109-fold higher CPM), suggesting a marked enhancement in fermentative metabolism and end-product generation. This group also demonstrated substantial enrichments in amino acid biosynthesis, including L-lysine biosynthesis VI (90-fold higher CPM) and selenium-containing amino acid biosynthesis (86-fold higher), reflecting an upregulation of anabolic capacity.

Complementary increases in fatty acid biosynthesis initiation (mitochondrial) (79-fold higher) point to enhanced membrane lipid production, while several core nucleotide metabolism pathways, particularly salvage and de novo inosine/guanosine biosynthesis, showed higher relative abundance (CPM) (55-fold to 77-fold higher). These collectively indicate higher cellular turnover, synthesis, and energetic activity within the microbial community.

While the overall trend in the kūmara + green banana group was towards metabolic enhancement, several pathways showed lower relative abundance (CPM), revealing targeted downregulation rather than uniform upscaling. The most pronounced reductions were observed in L-arginine biosynthesis II (acetyl cycle) (23.4-fold lower), L-valine biosynthesis (19.5-fold lower), and multiple de novo nucleotide biosynthesis routes (18.2-fold to 18.9-fold lower). Interestingly, decreases in de novo purine and pyrimidine synthesis coincided with increases in nucleotide salvage pathways, suggesting a metabolic rebalancing that prioritises energy-efficient recycling pathways over energetically costly de novo synthesis. This pattern is consistent with microbial communities adapting to substrate availability and maximising metabolic efficiency.

4.5.3.4 Pathway abundance comparisons between intervention groups in the establishing complementary feeding period (CF2 to CF4)

Analysis revealed 1,522 significant pathway-level differences across intervention groups at the CF4 timepoint (Control: 62.6% higher CPM; Kūmara: 54.1% higher; Kūmara + green banana: 56.1% higher). In contrast to the CF1 to CF2 expansion phase where 61-71% of pathways showed higher CPM, the CF2 to CF4 period showed more balanced directional changes (54-63% higher), reflecting metabolic consolidation and selective pathway regulation. Across all groups, nucleotide and amino acid metabolism remained the dominant functional categories, with cofactor & vitamin, carbohydrate, central carbon, fatty acid/lipid, and cell wall/structural pathways also showing notable shifts.

Shared patterns across groups included broad enhancement of amino acid metabolism, particularly L-glutamate and L-glutamine biosynthesis, which showed higher relative abundance (CPM) in all

groups (Control: 6.5-fold higher; Kūmara: 4.9-fold higher; Kūmara + green banana: 3.4-fold higher), indicating a core metabolic adaptation supporting protein synthesis during late complementary feeding. Fatty acid and lipid metabolism showed consistent downregulation, with *cis*-vaccenate biosynthesis showing lower CPM in all groups (Control: 6.5-fold lower; Kūmara: 3.1-fold lower; Kūmara + green banana: 2.4-fold lower), highlighting reduced investment in unsaturated fatty acid production. Central carbon metabolism, particularly the pentose phosphate pathway, showed strong upregulation in the kūmara group (6-fold higher CPM), supporting enhanced NADPH and ribose-5-phosphate generation for biosynthetic processes. Cofactor and vitamin metabolism exhibited group-specific patterns, with thiamine phosphate formation notably showing higher relative abundance (CPM) in the kūmara (5.7-fold higher) and control (4-fold higher) groups, but less so in the kūmara + green banana group, reflecting differential investment in cofactor biosynthesis.

The control group displayed the most consistent increases (62.6% of pathways), reflecting a natural maturation trajectory. Nucleotide metabolism was broadly maintained, with guanosine ribonucleotide *de novo* biosynthesis (3.7-fold higher CPM), UMP biosynthesis (3-fold higher), and queuosine biosynthesis (3.7-fold higher) moderately upregulated. Amino acid metabolism was robust, including L-glutamate/glutamine (6.5-fold higher) and moderate L-threonine biosynthesis (3.3-fold higher), supporting protein biosynthetic capacity. Cofactor & vitamin pathways showed the highest proportion of increases among all groups (77.6%), suggesting substantial investment in cofactor production. Carbohydrate metabolism changes were selective, with D-galactose degradation showing lower CPM (4.2-fold lower), while lipid and fatty acid pathways showed lower relative abundance (CPM), including *cis*-vaccenate (6.5-fold lower), gondoate (5.5-fold lower), and mitochondrial fatty acid biosynthesis initiation (5.2-fold lower).

The kūmara group exhibited a balanced profile (54.1% higher CPM), with strong amino acid and central carbon metabolism enhancement. Nucleotide metabolism showed moderate upregulation, including inosine-5'-phosphate biosynthesis II (4.7-fold higher CPM). Amino acid pathways, particularly L-valine (5.5-fold higher) and L-glutamate/glutamine (4.9-fold higher), showed higher

relative abundance (CPM), while cofactor & vitamin pathways were also enhanced (60.5% higher). Central carbon metabolism was strongly upregulated, notably the pentose phosphate pathway (6-fold higher CPM). Carbohydrate metabolism showed mixed patterns, with glycogen biosynthesis showing higher CPM (4.1-fold higher) and sucrose degradation showing lower CPM (4.9-fold lower). Lipid metabolism showed lower relative abundance (CPM), including cis-vaccenate biosynthesis (3.1-fold lower), indicating reduced unsaturated fatty acid production.

The kūmara + green banana group demonstrated a distinctive profile (56.1% higher CPM), with pronounced emphasis on nucleotide metabolism and secondary metabolic pathways. Pyrimidine ribonucleosides salvage showed higher relative abundance (CPM) (6.3-fold higher), indicating prioritization of nucleotide recycling over de novo synthesis, and tRNA charging pathways showed higher CPM (4.5-fold higher), supporting enhanced translational capacity. Amino acid metabolism showed selective changes: L-glutamate/glutamine biosynthesis showed higher CPM (3.4-fold higher), but L-valine biosynthesis showed lower CPM (3.3-fold lower), reflecting a metabolic shift toward nucleotide and cofactor processing rather than broad amino acid expansion. Carbohydrate metabolism showed moderate increases, including dTDP and beta L-rhamnose biosynthesis (2-fold and 4.3-fold higher CPM) and glycogen biosynthesis (1.9-fold higher), all lower in magnitude than the kūmara group. Lipid metabolism showed lower relative abundance (CPM), with fatty acid biosynthesis initiation (3.8-fold lower), fatty acid and beta oxidation I (2.8-fold lower), and cis-vaccenate (2.4-fold lower) downregulated.

From CF1 to CF4, the infant gut microbiota exhibited progressive functional maturation, with nucleotide and amino acid metabolism consistently dominating. Early complementary feeding (CF1 to CF2) was characterised by rapid, broadly distributed increases in pathway abundance, reflecting general biosynthetic capacity development. In contrast, CF2 to CF4 shifts were more selectively regulated, representing consolidated metabolic sophistication. Intervention-specific trajectories were evident: the kūmara group emphasised central carbon metabolism, amino acid biosynthesis, and polysaccharide storage; the kūmara + green banana group prioritised nucleotide salvage and selective

metabolic efficiency, with targeted increases in pyrimidine recycling and tRNA charging pathways; and the control group maintained moderate, broad-spectrum increases across core metabolic functions, reflecting natural microbiota maturation. These patterns indicate that while nucleotide and amino acid metabolism underpin microbiota development, interventions modulate the magnitude and focus of functional shifts during later complementary feeding stages.

4.5.3.5 Genus contributions to metabolic shifts

Analysis of genus contributions across functional categories revealed a consistent core of metabolically active taxa (Figure 4.8). *Veillonella* dominated nucleotide (383 pathways, 25.1%), amino acid (266 pathways, 19.8%), and cofactor metabolism (135 pathways, 24.6%), with 96% of its 1,151 pathways increasing across timepoints. *Blautia* contributed to amino acid metabolism (165 pathways) and carbohydrate fermentation (73 pathways), showing a mix of increasing (54%) and decreasing (46%) pathways. *Erysipelatoclostridium* (494 pathways) and *Enterococcus* (328 pathways) were exclusively associated with decreasing nucleotide pathways, while *Bifidobacterium* (202 pathways) contributed mainly to amino acid and carbohydrate metabolism, with 16% of pathways increasing. Other genera, including *Streptococcus*, *Megasphaera*, and *Bacteroides*, showed predominantly increasing contributions in nucleotide metabolism, highlighting coordinated activity among multiple genera.

Group-level comparisons indicated similar genus representation across interventions (Kūmara : 15 genera, 491 pathways; Kūmara + green banana extract: 14 genera, 532 pathways; Control: 12 genera, 570 pathways), though pathway distribution differed (Figure 4.9). The control group exhibited more concentrated metabolic activity per genus, while Groups K and K+ showed broader, more distributed contributions. Temporally, CF2 was characterised by widespread increases across most genera, whereas CF4 showed a more balanced pattern with both increases and decreases, reflecting consolidation of functional activity. *Veillonella* maintained strong nucleotide contributions through

CF4, while *Bifidobacterium* retained carbohydrate fermentation pathways but showed decreases in nucleotide pathways, indicating temporal shifts in genus-specific functional emphasis.

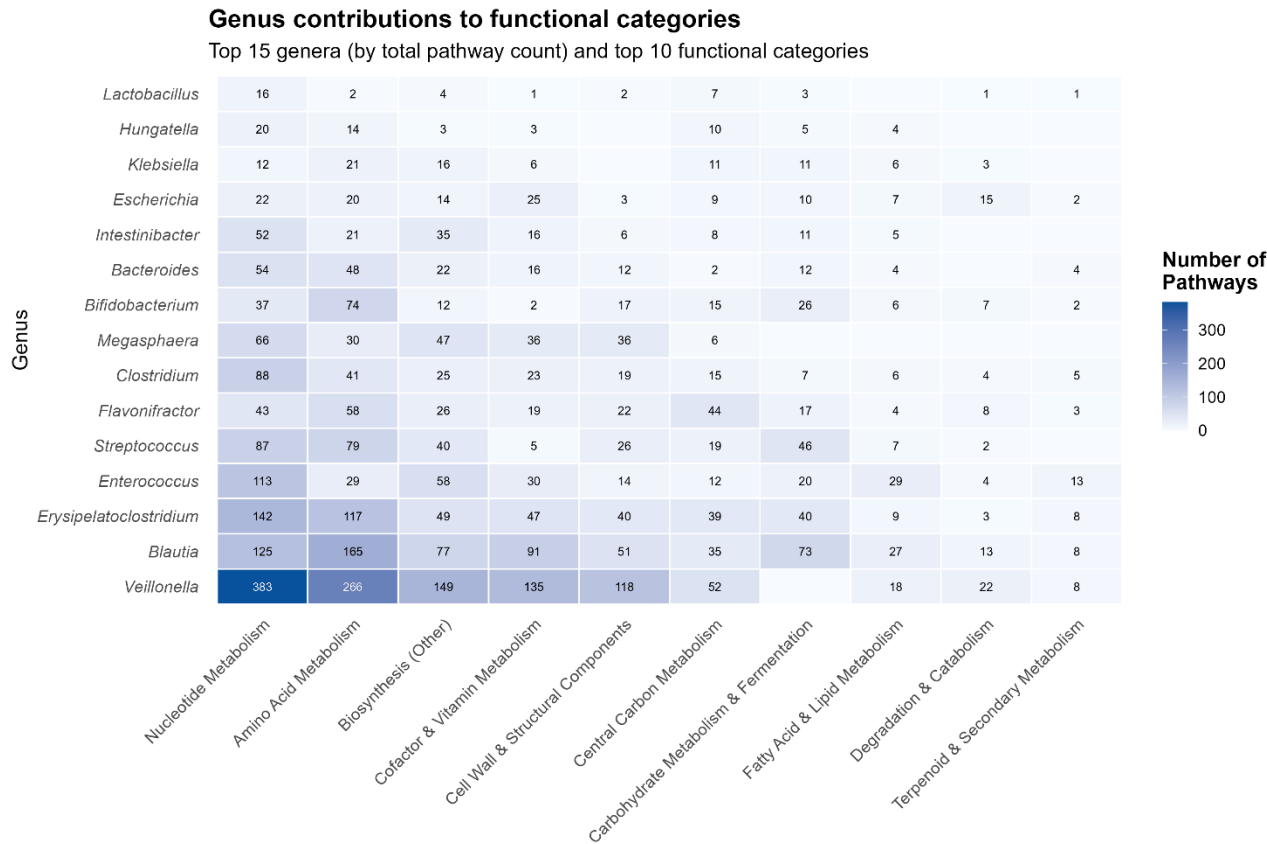


Figure 4.8. Heatmap of the top 15 genera contributing to major functional categories based on total pathway counts.

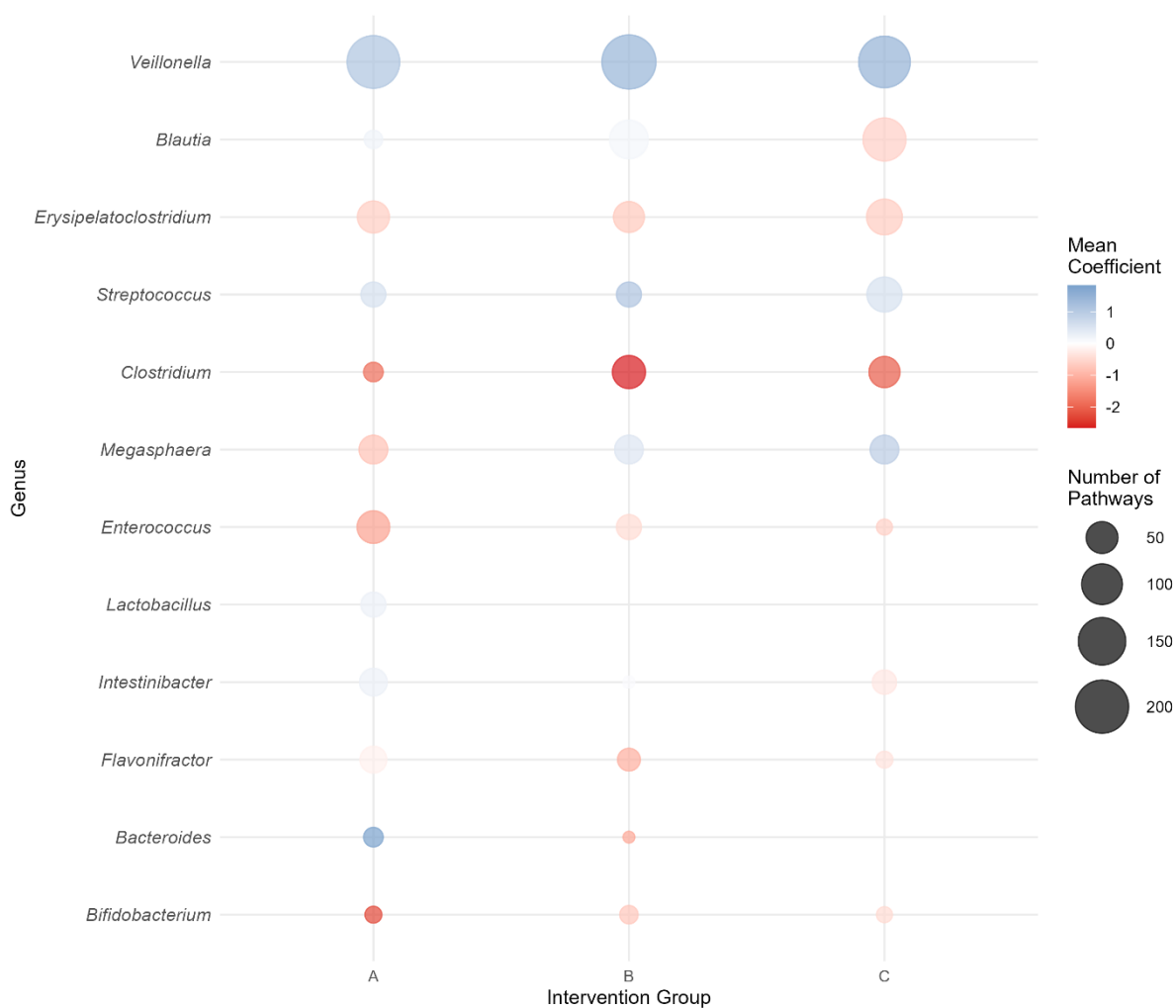


Figure 4.9. Differential Genus Contributions Across Groups at CF4: Functional Impact (Colour) and Pathway Count (Dot Size).

4.5.3.6 VIP score analysis

Variable Importance in Projection (VIP) scores from sparse Partial Least Squares Discriminant Analysis (sPLS-DA) were used to prioritise pathways by discrimination power, complementing the univariate MaAsLin3 results.

For the temporal analysis, temporal VIP scores correlated positively but modestly with MaAsLin3 effect sizes across pairwise comparisons: CF1 versus CF2 $r = 0.16$ ($p = 7.2 \times 10^{-17}$), CF2 versus CF4 $r = 0.15$ ($p = 9.2 \times 10^{-17}$), and CF1 versus CF4 $r = 0.29$ ($p = 1.2 \times 10^{-68}$). The strongest concordance for CF1 to CF4 reflects cumulative, consistent developmental change. The top temporal discriminators

(by VIP) were dominated by amino-acid biosynthesis and fermentation pathways. These top temporal VIP pathways showed coherent increases in log₂ fold-change across feeding: for example, the L-glutamate/glutamine pathway showed higher CPM in CF2 to CF4 (Control +1.87; Kūmara +1.59; Kūmara + green banana extract +1.22), and pyruvate to butanoate increased in both CF1 to CF2 (+0.81) and CF2 to CF4 (+1.01), consistent with progressive acquisition of fermentation capacity.

Intervention group VIPs revealed time-dependent shifts in the pathways that best separate interventions. At CF1, group discrimination was dominated by pathways linked to *Bifidobacterium* and baseline composition, indicating compositional differences at baseline. By CF2 the principal discriminators shifted toward *Veillonella*-associated and polyamine pathways, reflecting emergence of mid-feeding metabolic guild differences. By CF4 the strongest group discriminators were carbohydrate and vitamin biosynthesis pathways, indicating that late-feeding group differences reflect established metabolic specialisation rather than only compositional variation.

4.6 Discussion

This comprehensive shotgun metagenomic analysis of the SUN Study cohort represents a detailed examination of gut microbiota compositional and functional changes during the complementary feeding transition in healthy infants and the effects of kūmara-based complementary foods. Three principal findings emerged from this randomised controlled trial. First, gut microbiota maturation during the approximately 4-month period of complementary feeding followed robust, developmentally programmed trajectories characterised by rising diversity, declining *Bifidobacterium* dominance, emergence of Firmicutes taxa, and extensive functional metabolic reorganisation. Second, comprehensive functional pathway analysis revealed a biphasic pattern: early metabolic expansion (CF1 to CF2) followed by consolidation (CF2 to CF4), with nucleotide, amino acid, and lipid metabolism showing particularly dynamic regulation. Third, while the kūmara and kūmara + green banana extract interventions did not substantially alter overall diversity or taxonomic

trajectories, they contributed to measurable pathway-level functional differences, indicating subtle but detectable effects on metabolic capacity.

4.6.1 Temporal effects dominate microbiota development during the complementary feeding period

The strong temporal effects observed across all analytical levels underscore the central role of developmental maturation in shaping the infant gut microbiota during complementary feeding. The rise in Shannon diversity echoes consistent patterns reported in recent studies describing clear increases in α - and β -diversity following solid food introduction (Dikareva et al., 2025; Homann et al., 2021; Stinson et al., 2025; Vallès et al., 2014b). These studies also document characteristic taxonomic transitions: declining Bifidobacteriaceae dominance and emergence of adult-like taxa including Lachnospiraceae, Ruminococcaceae, and *Clostridium* species.

The longitudinal consistency in our cohort, with 89% retention providing complete data, enabled robust within-participant comparisons showing these trajectories occur with notable regularity regardless of intervention group. The compositional changes observed, Actinomycetota declining from 68.9% to 32.4% and Firmicutes rising from 21.5% to 48.1%, closely mirror findings from multiple studies. Parkin et al. (2024) reported similar shifts in Australian infants, with both feeding modes showing decreasing *Bifidobacterium* after introducing solids. Shi et al. (2024) and others (Cerdó et al., 2018; de Muinck & Trosvik, 2018) likewise describe parallel increases in Bacteroidota and Firmicutes during later infancy. The EDJA cohort further demonstrated that dietary fibres, including resistant starch, accelerate this transition in some infants but do not fundamentally alter the trajectory (Lalli et al., 2025).

4.6.2 Functional metabolic reorganisation during the complementary feeding period

The shift from metabolic expansion (CF1 to CF2: 55% of pathways showed higher CPM) to metabolic consolidation (CF2 to CF4: 67% showed lower CPM) is consistent with early complementary feeding being associated with broad functional diversification as the community adapts to new substrates,

followed by selective refinement. An important caveat: these pathway abundance changes reflect genetic capacity, the presence of pathway genes in the microbial community, rather than confirmed metabolic activity. The actual flux through these pathways would require metatranscriptomic or metabolomic measurement. While most infant microbiota studies have focused on taxonomy, emerging shotgun metagenomic and metatranscriptomic work is mapping how microbial metabolism evolves during infancy (Bokulich et al., 2016; Bottino et al., 2024; Hugenholtz et al., 2017; McKeen et al., 2022; C. J. Stewart et al., 2016).

Studies using KEGG Orthology (KO) annotations show shifts in carbohydrate utilisation and energy metabolism during the transition from exclusive milk feeding. However, KO analyses quantify individual gene families, whereas our MetaCyc pathway-based approach captures multi-step metabolic processes. Analysis of pathway changes across functional categories revealed that many pathways showed bidirectional changes within the same metabolic module, for example, some amino acid biosynthesis routes increased while others decreased at CF2, indicating pathway-specific rather than uniformly coordinated regulation.

Amino acid metabolism emerged as one of the most dynamically regulated functional categories, with both the top 50 increasing and decreasing pathways during early feeding dominated by amino acid biosynthesis routes. At the genetic potential level, branched-chain amino acid biosynthesis pathways showed marked increases (L-isoleucine III: 12.3-fold higher CPM; L-valine: 11.1-fold higher; L-lysine VI: 11.9-fold higher) between CF1 and CF2. These increases in pathway gene abundance occurred concurrently with rising diversity (Shannon index: 1.47 to 2.44) and genus richness (60 to 111 genera). While this parallel enrichment of biosynthetic genes and community expansion could reflect metabolic preparation for growth, our shotgun metagenomic data cannot determine whether these pathway genes were actively transcribed or metabolically utilised during this period. Subsequent reductions in essential amino acid biosynthesis pathway abundance by CF4 occurred as diversity plateaued, patterns that align with reports identifying amino acid metabolism as a dynamic functional category in developing infant microbiotas (Baumann-Dudenhoeffer et al., 2018), though

the functional significance of these genetic changes remains to be confirmed through metabolomic or metatranscriptomic approaches.

The reversal in nucleotide metabolism, early increases in de novo purine and pyrimidine biosynthesis (inosine-5'-phosphate II: 16.6-fold higher CPM; UMP biosynthesis: 11.6-fold higher at CF2) followed by later increases in salvage pathways (pyrimidine salvage: 3.7-fold higher CPM at CF4), is consistent with a transition from active proliferation to stabilisation. Although it cannot confirm whether observed pathway enrichments represent active metabolic processes or dormant genetic potential, the magnitude (>1,000 significantly changing pathways), coordination (consistent directional patterns across related pathways), and temporal consistency (early expansion followed by late consolidation) of changes suggest functional reorganisation rather than simple taxonomic turnover. Cerdó et al. (2018), examined functional potential in infants of obese versus normal-weight mothers and reported that healthy infants (born to normal-weight mothers) were relatively enriched in pathways associated with microbial growth and biosynthesis, such as amino-acid and nucleotide metabolic functions, compared with infants exposed to maternal obesity, who showed more stress- and inflammation-related pathways (e.g., LPS biosynthesis, sulphur metabolism). Although their study did not investigate complementary feeding specifically, the enrichment patterns in healthy infants are broadly consistent with the early-feeding biosynthetic expansion observed in this study.

Other functional studies in younger infants have identified feeding-mode differences in carbohydrate metabolism and cellular defence pathways (Di Guglielmo et al., 2022; M. Wang et al., 2025), although these comparisons largely concern breastfed versus formula-fed infants rather than the complementary feeding period. Nevertheless, they further illustrate that microbial functional potential is sensitive to nutritional context.

Dramatic changes in lipid metabolism were observed during the complementary feeding period. Fatty acid biosynthesis showed the largest single pathway increase during early feeding (fatty acid biosynthesis initiation: 20.9-fold higher CPM). However, substantial decreases occurred during late

feeding, with cis-vaccenate showing up to 6.5-fold lower CPM and fatty acid biosynthesis initiation showing up to 5.2-fold lower at CF4. This pattern is consistent with elevated membrane biosynthesis during early community restructuring, followed by reduced relative investment in lipid biosynthesis at later stages.

4.6.3 Absence of intervention effects

This study found that the kūmara and kūmara + green banana extract interventions did not substantially alter the overall trajectories of infant gut microbiota development. Across all groups, strong temporal changes in diversity and taxonomic composition occurred during complementary feeding, consistent with normal maturational processes. However, the interventions contributed to distinct functional profiles, as evidenced by PERMANOVA analysis showing a small but statistically significant group effect ($R^2 = 0.003$, $F = 1.26$, $p < 0.0001$). Differential abundance analysis identified pathway-level differences, with 1,522-2,160 significantly altered pathways across intervention groups depending on developmental stage, indicating that while the interventions did not dramatically redirect trajectories, they influenced metabolic capacity.

These findings can be considered in the context of the pilot study by McKeen et al. (2022), which tested the kūmara intervention. In that study, infants consuming kūmara showed typical age-related increases in diversity and expected taxonomic shifts during complementary feeding, with no large-scale impact on overall functional potential. Our results extend this work by demonstrating that, while the interventions did not broadly redirect microbiota maturation, they were associated with measurable pathway-level changes.

Several factors likely explain the limited intervention effects. First, robust developmental programming during complementary feeding may be sufficiently strong that specific food interventions have minimal impact relative to background dietary diversification. All participants introduced standard complementary foods alongside study products, and this broader diversification may have masked specific effects of kūmara or green banana extract. Second, the high baseline

Bifidobacterium abundance (68.9% Actinomycetota at CF1) may have resulted in limited headroom for detectable increases in relative abundance during the intervention period.

Overall, these findings suggest that while modest whole-food interventions can shape aspects of functional potential, the broader developmental trajectory of the infant gut microbiota during complementary feeding remains largely age-driven and resilient to specific dietary modulation.

4.6.4 Methodological Considerations and Strengths

The randomised controlled design with substantial sample size (n=279, 789 samples) provides robust statistical power. Shotgun metagenomics enabled species-level taxonomic integrated assessment of microbial functional potential alongside taxonomic composition within a single analytical framework. High retention of participants (89% complete longitudinal data) further enabled within-subject trajectory analysis with appropriate repeated measures modelling and blocked permutations strategies, strengthening inference regarding temporal and intervention-driven changes.

The study was informed by the "Nourish to Flourish" pilot feasibility study (McKeen et al., 2022), which demonstrated that daily kūmara powder consumption was safe, well-tolerated, and feasible in infant complementary feeding. Insights from the pilot guided critical design choices in the full-scale trial, including the delivery format, intervention duration, and sample collection protocols. This evidence-based, iterative approach increases confidence that the intervention findings reflect genuine biological outcomes rather than implementation failures.

Integration of Variable Importance in Projection analysis with differential abundance testing provides a data-driven framework for prioritising pathways among thousands of significant changes. VIP scores validated through correlation with effect sizes confirmed high-VIP pathways represent biologically meaningful changes. Our use of MetaCyc pathways provides more interpretable results than KO-based annotations, capturing complete metabolic processes rather than isolated gene functions. The comprehensive functional pathway analysis examining >3,000 MetaCyc pathways

across 14 categories provides comprehensive detail on metabolic reorganisation during complementary feeding.

4.6.5 Limitations

Functional pathway abundance detection indicates that the microbial community harbours the genes for a given function but does not confirm that these pathways are transcriptionally active or contributing directly to metabolite production. Definitive confirmation of pathway activity would require metatranscriptomic and/or metaproteomic approaches, which were beyond the scope of this thesis but represent an important direction for future work.

The 4-month intervention, while covering the early complementary feeding period (6–10 months), may be insufficient to detect longer-term or persistent microbiota restructuring. Infant gut microbial development is highly dynamic during this period, and the intervention-related effects may only emerge after prolonged exposure of stabilisation of dietary patterns later in infancy.

Microbiota profiling was based on faecal samples, which primarily reflect microbial communities and metabolic activity in the distal colon and may not capture processes occurring in other regions of the gut. Microbial composition and functional activity vary along the gut, particularly in early life, where the small intestine and proximal colon play key roles in nutrient digestion, absorption, and early host-microbe signalling during complementary feeding. Consequently, important microbial responses to dietary substrates may occur upstream and remain undetected in faecal-based analyses. This spatial limitation should be considered when interpreting functional and metabolic outcomes.

4.6.6 Implications for Infant Feeding Guidelines and Future Research

These findings have important implications for translating microbiota science into practical feeding recommendations, particularly given the extensive development work preceding this trial. The "Nourish to Flourish" pilot study (McKeen et al., 2022) established that kūmara-based interventions are feasible, acceptable, and safe for infant feeding, providing a strong foundation for the current

efficacy trial. The efficacy findings in both the pilot and SUN Study do not negate this work and kūmara remains a nutritious, appropriate complementary food rich in vitamins, minerals, and fibre.

These findings confirm that kūmara and kūmara with green banana extract are appropriate complementary foods that support normal microbiota maturation without disrupting the robust developmental trajectories observed during early feeding. While the interventions did not dramatically redirect microbiota development, which followed age-appropriate maturation patterns across all groups, they contributed to functional differences. Given that optimal microbiota development targets during complementary feeding remain incompletely defined, the observation that these whole food interventions support rather than disrupt natural maturation is reassuring from both a practical feeding perspective and a biological understanding of developmental resilience. Rather, the robust developmental programming during early complementary feeding may limit the influence of specific dietary components when introduced alongside diverse complementary foods. While comparing whole food supplementation within the context of varied complementary feeding reflects real-world infant feeding practices, it may yield smaller effect sizes than studies using more controlled or restrictive dietary backgrounds.

Future research directions include: (1) Integrating targeted metabolomic profiling with functional pathway analysis to directly quantify microbial metabolic outputs, particularly organic acids such as short-chain fatty acids, branched-chain fatty acids, and other fermentation products. Direct metabolite measurements provide critical validation of whether predicted pathways translate into realised metabolic activity. Organic acid analysis and integration with dietary data and microbial composition and functional potential will be addressed in Chapter 5 of this thesis; (2) Extending follow-up beyond 10 months into the second year of life to determine whether the metabolic consolidation pattern continues and whether late-emerging intervention effects become apparent as the gut microbiota continues to mature and diversify; (3) Examining intervention effects in populations with different baseline microbiotas, particularly infants with dysbiosis risk factors (caesarean delivery, antibiotic

exposure, formula feeding without probiotics), in whom dietary modulations may exert stronger or more detectable effects.

The VIP analysis framework developed here provides a valuable template for prioritising pathways in future mechanistic studies. Rather than attempting to investigate thousands of significant pathway changes, researchers can focus on the top 20-50 discriminating pathways that most powerfully separate developmental stages or intervention groups. For example, the identification of L-glutamate biosynthesis as the top temporal discriminator suggests that nitrogen cycling efficiency could serve as a biomarker of microbiota maturation, warranted for validation in independent cohorts. Similarly, the dual discriminators, pathways showing high VIP for both temporal and intervention comparisons, represent prime candidates for studies linking diet, microbiota, and health outcomes.

4.7 Conclusions

This large, statistically powered randomised controlled trial provides a detailed characterisation of gut microbiota development during the complementary feeding period and the potential modulatory effects of kūmara-based complementary foods. Across taxonomic, functional, and multivariate analyses, temporal maturation dominated microbiota trajectories. Infants followed a robust, developmentally programmed shift from a milk-adapted, *Bifidobacterium*-rich community to a more diverse, metabolically versatile assemblage enriched in Firmicutes and Bacteroidota. This transition was accompanied by extensive functional reorganisation, with early complementary feeding marked by broad metabolic expansion and later feeding characterised by consolidation and refinement of biosynthetic capacity.

Against this strong maturational backdrop, the kūmara and kūmara + green banana extract interventions did not meaningfully alter diversity trajectories or overall community composition. Functional analyses did, however, identify subtle but measurable pathway-level differences between intervention groups, indicating that while the interventions did not redirect developmental trajectories, they contributed to distinct functional signatures. These findings suggest that moderate

whole-food supplementation during complementary feeding may influence aspects of microbial functional potential but is insufficient to shift the dominant age-driven patterns of microbiota development.

This study confirms that kūmara remains a nutritionally appropriate complementary food. Methodologically, this study's integration of shotgun metagenomics, stringent longitudinal design, and comprehensive pathway-level analysis represents a significant advance in understanding functional maturation during complementary feeding. The identification of key discriminating pathways, particularly in amino-acid, nucleotide, and lipid metabolism, provides a mechanistic basis for future work linking microbial function to diet and infant health outcomes.

Overall, this research demonstrates that complementary feeding represents a period of rapid, structured microbiota maturation that is resilient and responds to modest dietary modulation. Future studies incorporating metabolomic profiling, longer follow-up, and stratified analyses of at-risk infant populations may determine how specific dietary interventions can meaningfully shape microbial function during this critical developmental window.

Chapter 5

Dietary, Microbial, and Functional Determinants of Organic Acid Production During Complementary Feeding in the SUN Study

5.1 Abstract

Organic acids produced through microbial fermentation are key metabolic outputs of the infant gut microbiota during complementary feeding. In the SUN Study, faecal organic acids were quantified in 242 infants across three timepoints (CF1: baseline; CF2: 6–8 months; CF4: 8–10 months) using targeted liquid chromatography–tandem mass spectrometry (LC–MS/MS). Dietary intake was assessed at CF2 and CF4 using 3-day food records. Temporal patterns and intervention effects were evaluated using mixed-effects models, and associations with gut microbial taxonomic composition and functional metabolic pathways derived from shotgun metagenomic sequencing were examined.

Dietary patterns of macronutrients and dietary fibre were consistent across groups, but resistant starch differed significantly across groups, particularly in the kūmara and green banana extract group, which received an additional ~0.35g/day from the intervention product. However, no intervention effects on organic acid fresh weight concentrations or proportions were observed (all $p > 0.05$).

Across the cohort, the proportional contribution of acetate, propionate, and butyrate increased from 59.3% at CF1 to 91.6% at CF4, with the largest shift occurring between CF1 and CF2. This transition was accompanied by marked declines in succinate, lactate, and formate. Although resistant starch intake differed significantly between intervention groups, particularly in infants receiving kūmara plus green banana extract, no intervention effects on organic acid fresh weight concentrations or proportions were detected.

Integration with microbiome data identified a limited number of genus–organic acid associations, most prominently between *Bacteroides* and succinate and *Bifidobacterium* and acetate. Functional pathway abundance explained a greater proportion of variation in organic acid profiles than

taxonomic composition, particularly for relative organic acid proportions. Pathway–organic acid relationships were conserved across intervention groups.

Together, these findings show that faecal organic acid profiles undergo a pronounced age-related transition during early complementary feeding, while interventions did not measurably alter organic acid production within this complementary feeding period.

5.2 Introduction

Organic acids produced through microbial fermentation of dietary substrates are key metabolic outputs of the infant gut microbiota during early life. These metabolites reflect the combined influence of dietary substrate availability, microbial community composition, and microbial metabolic activity, and are commonly used as functional indicators of gut microbial metabolism during infancy. The introduction of complementary foods represents a major dietary transition, introducing new fermentable substrates that can alter patterns of organic acid production as the gut microbiota develops.

The faecal organic acid pool during infancy is diverse and includes short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, as well as other organic acids, including lactate, succinate, formate, and branched-chain fatty acids (BCFAs). SCFAs are the dominant end-products of saccharolytic fermentation in the colon and have well-established roles in intestinal barrier function, immune modulation, and host energy metabolism (Blaak et al., 2020; Y. Yao et al., 2022). In contrast, organic acids such as lactate and succinate can be produced as end-products by some bacterial taxa and as transient metabolites in cross-feeding pathways involving other members of the gut microbiota, depending on community composition and metabolic context. BCFAs and other minor organic acids are primarily derived from amino acid fermentation and tend to increase as dietary protein intake rises during complementary feeding.

Longitudinal studies indicate that organic acid profiles undergo marked developmental change during infancy, particularly during the transition from exclusive milk feeding to complementary feeding

(Bridgman et al., 2017; Norin et al., 2004; Tsukuda et al., 2021). This transition is commonly characterised by increases in total SCFAs and the emergence of detectable butyrate following the introduction of solid foods. Concurrently, organic acids that are prominent earlier in life, including lactate and succinate, often decline in relative contribution as microbial communities capable of converting these metabolites become more prevalent. However, most infant studies sample relatively few timepoints and use heterogeneous analytical approaches, limiting detailed characterisation of organic acid trajectories across the complementary feeding window .

Dietary resistant starch (RS) represents a fermentable carbohydrate with the potential to influence organic acid production by gut bacteria (Zheng et al., 2026). In adults, RS supplementation consistently increases faecal butyrate concentrations and promotes the growth of starch-degrading and butyrate-producing taxa (Martínez et al., 2010; Upadhyaya et al., 2016; Venkataraman et al., 2016; Vital et al., 2018). These effects are mediated by RS-degrading specialists such as *Ruminococcus bromii* and *Eubacterium rectale*, which harbour specialised starch-degrading enzymes and cross-feed with butyrate-producing bacteria. In contrast, the impact of RS exposure during infancy remains poorly understood, with only limited *in vitro* and small-scale studies available (Gopalsamy et al., 2019; Ordiz et al., 2020). The infant gut microbiota differs substantially from the adult microbiota in both composition and functional capacity, and the extent to which RS can modulate organic acid production during complementary feeding may be constrained by developmental stage, dose, or microbial metabolic potential.

A further limitation in the current literature is the lack of integrative analyses linking microbial composition, functional potential, and metabolic outputs (Bikel et al., 2015; Knight et al., 2018; Sirangelo, 2018). Multiple microbial taxa can contribute to the production of the same organic acid through distinct biochemical pathways, meaning that taxonomic associations provide limited insight into underlying metabolic processes. Advances in shotgun metagenomic sequencing now enable direct quantification of microbial metabolic pathway abundance, allowing integration of taxonomic composition, functional capacity, and measured metabolic outputs. Such integrative approaches are

particularly valuable during infancy, when microbial communities and metabolic profiles are rapidly changing.

The present chapter addresses these gaps by characterising faecal organic acid profiles across complementary feeding in a large cohort of New Zealand infants participating in the SUN Study, a randomised dietary intervention trial. By combining targeted organic acid measurements, detailed dietary intake assessment, and shotgun metagenomic analysis of microbial taxonomic composition and metabolic pathway abundance, this chapter examines temporal changes in organic acid production, evaluates the impact of whole-food prebiotic interventions, and investigates associations between diet, microbiota, microbial metabolic potential, and organic acid profiles during complementary feeding.

5.3 Aims

The overarching aim of this chapter was to characterise faecal organic acid production during complementary feeding and across intervention groups, and to examine relationships between dietary intake, the gut microbiota, microbial metabolic pathways, and organic acid profiles. Specific objectives were to: (i) characterise temporal changes in faecal organic acid concentrations and proportional composition across the complementary feeding period; (ii) assess whether the kūmara-based intervention influences organic acid profiles; (iii) assess dietary intake patterns, including resistant starch exposure, to contextualise intervention effects; and (iv) determine which dietary and microbial factors are most strongly associated with organic acid profiles during complementary feeding.

5.4 Methods

Details of the overall study design, participant recruitment, intervention allocation, and faecal sample collection are provided in Chapter 4.4.

5.4.1 Dietary Assessment

Infant dietary intake was assessed using 3-day food records collected at CF2 and CF4. Parents or caregivers were instructed to record all foods and beverages consumed by the infant over three consecutive days, including study intervention products, coinciding with faecal sample collection. Food records were analysed using FoodWorks (version 10; Xyris Software, Brisbane, Australia), with nutrient values derived from the New Zealand Food Composition Database (FOODfiles 2018; version 01). Resistant starch (RS) content of the intervention product (kūmara and green banana extract) was determined by laboratory analysis as detailed in Chapter 4 (7 g RS per 100 g). Based on the prescribed 5 g daily serving, the intervention provided approximately 0.35 g RS per day to participants in the K+ group.

Breastmilk intake was estimated from recorded breastfeeding duration (minutes) and converted to volume using an established conversion factor of 10 mL per minute of active feeding, as described in the published protocol (Wall et al., 2024).

Dietary intake data were summarised as average daily intakes across the 3-day recording period. Daily energy intake was calculated and reported as kJ/day. Nutrient variables included energy and 12 dietary components: protein, total fat, available carbohydrate, sugars, starch, total dietary fibre, resistant starch, soluble non-starch polysaccharides, insoluble non-starch polysaccharides, oligosaccharides, and fructo-oligosaccharides.

5.4.2 Organic Acid Quantification

Faecal short-chain fatty acids (SCFAs) and organic acids were quantified from faecal material processed alongside samples used for DNA extraction (see Chapter 4, Section 4.4.3. and 4.4.4. for sample storage and handling procedures). A total of 100 mg of homogenised faecal material was used for analysis.

Targeted quantification was performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) on an AB Sciex QTRAP 5500 system equipped with a Turbo V™ ion source and an

atmospheric pressure chemical ionisation (APCI) probe (Sciex, Concord, Ontario, Canada), coupled to an Exion UHPLC system (Shimadzu, Kyoto, Japan). The analytical method was adapted from Han et al. (2015), with modifications described by Fristedt et al. (2024) and Cooney & Trower (2017). Briefly, faecal samples were spiked with ^{13}C -labelled internal standards and extracted in acidified water. Following centrifugation, supernatants were derivatised with 3-nitrophenylhydrazine and analysed by LC–MS/MS in negative ionisation mode. Quantification was achieved using calibration curves generated from authentic standards across physiologically relevant concentration ranges. Quality control samples were included in each analytical batch to monitor inter-batch reproducibility, with coefficients of variation maintained below 15%.

The following organic acids were quantified: acetate (AA), propionate (PA), butyrate (BA), isobutyrate (IBA), valerate (VA), isovalerate (IVA), 2-methylbutyrate (MBA_2), caproate (CA), succinate (SucA), lactate (LA), formate (FA), 3-methylvalerate (MVA_3), and 4-methylvalerate (MVA_4). Concentrations are reported as mmol kg^{-1} faeces.

5.4.3 Statistical Analysis of Dietary Data

Between-group differences in nutrient intakes were assessed using Kruskal-Wallis tests (non-parametric due to skewed distributions), with post-hoc pairwise comparisons using Dunn's test with Bonferroni correction. Analyses were conducted separately for CF2 and CF4 timepoints, and for both absolute and energy-adjusted intakes. Energy-adjusted intakes were calculated as nutrient density (grams per 1000 kcal, where $1 \text{ kJ} = 0.239 \text{ kcal}$) to account for variations in total energy intake.

5.4.4 Statistical Analysis of Organic Acid Temporal Dynamics and Intervention Effects

All statistical analyses were performed in R (version 4.3.0). Organic acid data were processed to generate both FW concentrations (mmol kg^{-1} faeces) and relative abundances, expressed as the percentage contribution of each organic acid to the total quantified organic acid pool. Relative abundances were used to assess compositional shifts in the faecal metabolome during complementary

feeding, while concentration-based analyses were used to support the interpretation of changes in organic acid production.

Samples were screened for completeness, and only participants with faecal samples available at all three timepoints (CF1, CF2, CF4) were retained for within-participant analyses (complete-case approach). This resulted in a final analytical cohort of 242 infants (Group K: Kūmara, $n = 81$; Group K+: Kūmara and green banana extract, $n = 77$; Group C: Control, $n = 84$). For each organic acid, descriptive statistics were summarised as median and interquartile range (IQR) at each timepoint and for each intervention group. Within-participant percentage changes from baseline were calculated for CF1 to CF2, CF2 to CF4, and CF1 to CF4 to characterise individual organic acid trajectories during complementary feeding.

Temporal changes in organic acid concentrations and relative abundances were assessed using paired Wilcoxon signed-rank tests comparing timepoints within participants (CF1 versus CF2, CF2 versus CF4, and CF1 versus CF4). To account for multiple testing across organic acids, p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR), with statistical significance defined as $q < 0.05$.

To formally evaluate intervention effects while accounting for repeated measures, linear mixed-effects models (LMMs) were fitted separately for each organic acid outcome using the lme4 package. Models included Participant ID as a random intercept and fixed effects for Group, Timepoint, and their interaction ($\text{Group} \times \text{Timepoint}$). Type III analysis of variance with Satterthwaite's approximation was used to assess fixed effects. Resulting p-values were corrected for multiple comparisons across all organic acids using the Benjamini–Hochberg FDR procedure ($q < 0.05$). Estimated marginal means (EMMs) and model-predicted trajectories were derived using the emmeans package to visualise group-specific temporal patterns.

5.4.5 Integrated Microbial-Diet-Metabolic Analyses

Taxonomic and functional profiling of the infant gut microbiota was performed as described in detail in Chapter 4. Briefly, genus-level taxonomic profiles and functional pathway abundances were obtained from shotgun metagenomic sequencing using the MetaPhlAn4 and HUMAnN3 pipelines. For the analyses presented in this chapter, taxonomic data were aggregated at the genus level, and functional outputs were summarised as MetaCyc pathway abundances. These datasets were integrated with faecal organic acid profiles to examine temporal associations between microbial composition, metabolic potential, and organic acid production during complementary feeding.

All analyses were conducted in R (version 4.3.0). Data processing and wrangling were performed using the tidyverse, mixed-effects modelling was implemented using lme4 and lmerTest, estimated marginal means were derived using emmeans, and visualisations were generated using ggplot2 and pheatmap.

5.4.5.1 Genus–Organic Acid Associations

To minimise associations driven by low-abundance features, taxa were pre-filtered before analysis. Only genera present in more than 20% of samples and with a mean relative abundance greater than 0.1% were retained.

Associations between genus-level relative abundance and individual organic acids were assessed using linear mixed-effects models (LMMs), fitted separately for each genus–organic acid pair. Genus relative abundances were centred and scaled (z-scored) before modelling to facilitate interpretation of standardised coefficients and improve numerical stability. Models included Participant ID as a random intercept to account for repeated measures, with fixed effects for genus abundance, timepoint, and their interaction (genus $\times$ timepoint). P-values were adjusted for multiple testing across all genus–organic acid pairs using the Benjamini–Hochberg false discovery rate (FDR), with statistical significance defined as $q < 0.05$.

To assess the specificity of observed associations, partial correlation analyses were conducted, controlling for total organic acid concentration. This approach distinguished organic acid-specific associations from correlations driven by overall fermentative activity. Associations were classified as organic acid-specific, where partial correlations remained significant after adjustment, non-specific, where only zero-order correlations were significant, or non-associated, where neither correlation was significant.

All genus-organic acid analyses were performed using both concentration-based measures, expressed per gram of faecal fresh weight (FW concentration, mmol kg⁻¹ faeces), and relative abundances (percentage of total quantified organic acids). Concentration-based measures reflect organic acid content relative to the mass of faecal material analysed but may be influenced by inter-sample variation in water content, as samples were not dried prior to analysis. Relative abundance measures are inherently robust to such dilution effects and capture compositional shifts within the organic acid pool. The use of both metrics enabled differentiation between taxa associated with overall organic acid production capacity versus those primarily influencing organic acid profile composition. Standardised effect sizes (β coefficients) were compared across FW concentration- and proportion-based models to assess consistency of genus-organic acid associations across analytical scales.

5.4.5.2 Pathway–Organic Acid Associations

Metabolic pathways were filtered to retain those present in at least 20% of samples, with a mean abundance ≥ 0.0001 copies per million (CPM) and a maximum abundance ≥ 0.001 CPM. Pathway annotations were obtained from MetaCyc, and pathways were grouped into broad functional classes (e.g. carbohydrate metabolism, fermentation, biosynthesis) using keyword-based classification to aid interpretation.

To identify pathways exhibiting developmental dynamics, temporal variation in pathway abundance was assessed using linear mixed-effects models with Timepoint as a fixed effect and Participant ID as a random intercept. Marginal R^2 values, representing the variance explained by Timepoint, were

calculated, and pathways with marginal $R^2 > 0.1$ and a Timepoint p-value < 0.05 were considered to display meaningful temporal variation and were prioritised for organic acid integration analyses. Associations between individual organic acids and metabolic pathways were evaluated using linear mixed-effects models analogous to the genus-level analyses. P-values were adjusted for multiple testing across all pathway–organic acid comparisons using the Benjamini–Hochberg FDR procedure, with significant main effects ($q < 0.05$) retained for downstream interpretation. All pathway-organic acid analyses were performed separately for both FW concentrations (mmol/kg faeces) and relative abundances (percentage of total organic acids) to distinguish overall fermentative capacity from compositional balance.

5.4.5.3 Variance Partitioning Analysis

To quantify the relative explanatory power of taxonomic composition and functional pathway abundance for predicting organic acid profiles, variance partitioning was performed using redundancy analysis (RDA) implemented in the *vegan* package in R. Organic acid concentrations and relative abundances were modelled separately against genus-level taxonomic profiles and MetaCyc pathway abundances.

Variance was partitioned into four components: (1) unique variance explained by taxonomy, (2) unique variance explained by pathways, (3) shared variance explained by both, and (4) unexplained residual variance. Statistical significance of each component was assessed using permutation tests (999 permutations), with significance defined as $p < 0.001$.

5.4.5.4 Dietary Intake–Organic Acid Associations

Associations between dietary intake and faecal organic acid profiles were assessed to evaluate whether background diet or intervention-related nutrient exposure directly influenced organic acid production during complementary feeding.

For each dietary variable–organic acid pair, associations were evaluated using non-parametric correlation analyses conducted separately at CF2 and CF4. Organic acid outcomes were examined as both FW concentrations (mmol kg^{-1} faeces) and relative abundances (percentage of total organic acids). Spearman’s rank correlation coefficients were calculated to account for non-normal distributions.

To control for multiple testing across all dietary–organic acid comparisons, p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR), with statistical significance defined as $q < 0.05$. Analyses were performed separately by intervention group to assess whether diet–organic acid coupling differed across dietary contexts.

5.4.5.5 Dietary Intake, Microbiota, and Organic Acid Associations

For integrated multi-layer analyses, dietary, taxonomic, functional, and organic acid datasets were jointly filtered to reduce sparsity and limit multiple-testing burden. Dietary variables comprised daily intakes of energy and 12 nutrients derived from 3-day food records (as described in Section 5.4.1). For this integrated multi-layer analysis, taxa and pathways were further restricted to the top 25 genera and top 50 pathways by mean relative abundance to reduce computational burden while retaining features.

Pairwise associations were then assessed separately for diet–organic acid, genus–organic acid, and pathway–organic acid relationships at CF2 and CF4 within each intervention group. To formally compare the strength of associations across intervention groups and analytical layers, correlation coefficients were compared using Fisher’s r-to-z transformation. Resulting p-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR), with significance defined as $q < 0.05$.

5.5 Results

5.5.1 Dietary Intake During Complementary Feeding

Analysis of 3-day food records showed that mean daily energy intake did not differ significantly between groups at either timepoint (CF2: 2725–2915 kJ/day [651–697 kcal]; CF4: 3221–3306 kJ/day [770–790 kcal]; all $p > 0.05$; Table 5.1). Macronutrient intakes, including protein and total carbohydrate, were also similar across groups at both timepoints.

A modest difference in total fat intake was observed at CF2 ($p = 0.05$), with the kūmara and green banana extract group consuming slightly less fat than the Control group (mean difference ≈ 2.5 g/day, corresponding to $\sim 7\%$ of total fat intake). This difference was not present at CF4 and was small relative to overall fat consumption, indicating a transient and nutritionally minor variation rather than a sustained dietary divergence. Dietary fibre intake excluding resistant starch increased from 3.7–4.1 g/day at CF2 to 6.1–7.0 g/day at CF4 but did not differ significantly between groups at either timepoint (CF2: $p = 0.96$; CF4: $p = 0.48$). Other fibre fractions, including soluble and insoluble non-starch polysaccharides, oligosaccharides, and fructo-oligosaccharides, also showed no significant group differences (all $p > 0.05$).

Background dietary resistant starch (RS) intake from non-intervention complementary foods was uniform across all groups at both timepoints. At CF2, mean RS intake from background diet was 0.07 ± 0.03 g/day in the Kūmara group and 0.04 ± 0.23 g/day in the Control group, with similar values observed at CF4. In contrast, the Kūmara + green banana extract group exhibited higher total RS intake at both timepoints (CF2: 0.30 ± 0.16 g/day; CF4: 0.34 ± 0.25 g/day), closely matching the expected RS contribution from the intervention product (~ 0.35 g RS/day from a 5 g daily serving containing 7 g RS per 100 g).

Between-group differences in RS intake remained statistically significant when expressed relative to energy intake (g/1000 kcal) at both CF2 and CF4 (both $p < 0.001$). Post-hoc analyses demonstrated

significant differences between all intervention groups at both timepoints (all pairwise comparisons $p < 0.001$; Dunn's test with Bonferroni correction).

5.5.2 Organic Acid Analyses

5.5.2.1 Temporal Changes of Organic Acids

Fresh weight concentrations of all major short-chain fatty acids increased significantly across the complementary feeding period (Figure 5.1, top panels). Acetate concentrations increased from a median of 20.4 to 37.4 mmol/kg faecal fresh weight (FW) between CF1 and CF4 ($p < 0.001$), representing an 83% increase. Propionate showed a more pronounced relative increase, rising from 2.3 to 7.6 mmol/kg FW (222% increase, $p < 0.001$). Butyrate exhibited the largest relative change, increasing from 0.6 to 3.5 mmol/kg FW (444% increase, $p < 0.001$), though remaining at lower FW concentrations than acetate or propionate throughout the study period.

When expressed as proportions of total quantified organic acids, major SCFAs (acetate, propionate, and butyrate) increased from 59.7% to 91.7% between CF1 and CF4 ($p < 0.001$). Within this major SCFA pool (Figure 5.1, bottom panels), acetate dominated the profile at CF1 (median 51.8% of total organic acids), with lower contributions from propionate (6.3%) and butyrate (2.1%). By CF2, the profile had shifted substantially, with acetate increasing to 64.8%, propionate to 14.1%, and butyrate to 4.2%. These relative distributions remained broadly similar from CF2 to CF4 (65.0% acetate, 14.3% propionate, and 6.9% butyrate), indicating that the largest compositional change occurred during the introductory complementary feeding period (CF1 to CF2).

Table 5.1. Dietary intake during complementary feeding at CF2 (8 months) and CF4 (10 months), presented as mean $\pm$ SD (n) by intervention group for energy, macronutrients, dietary fibre, and resistant starch, with p-values from Kruskal–Wallis tests.

CF2					
		Group K	Group K+	Group C	p value
Energy	(kJ/day)	2849.3 ± 871.0	2725.1 ± 749.4	2915.1 ± 567.5	0.13
Protein	(g/day)	17.5 ± 8.2	16.9 ± 7.1	18.2 ± 7.3	0.34
Carbohydrate	(g/day)	69.7 ± 20.7	67.5 ± 20.3	73.5 ± 16.2	0.1
Total fat	(g/day)	35.1 ± 12.5	33.5 ± 10.9	36.0 ± 7.9	0.05
Dietary fibre	(g/day)	4.1 ± 4.3	3.8 ± 2.9	3.7 ± 2.7	0.96
Resistant starch	(g/day)	0.1 ± 0.0	0.3 ± 0.2	0.0 ± 0.2	<0.001
CF4					
Energy	(kJ/day)	3221.2 ± 1119.8	3228.4 ± 942.6	3305.6 ± 811.9	0.61
Protein	(g/day)	24.2 ± 11.2	25.5 ± 11.7	25.0 ± 9.8	0.78
Carbohydrate	(g/day)	82.6 ± 32.5	83.2 ± 26.9	84.6 ± 24.2	0.59
Total fat	(g/day)	35.6 ± 14.2	35.8 ± 14.2	37.5 ± 10.4	0.23
Dietary fibre	(g/day)	6.8 ± 4.4	7.0 ± 4.8	6.1 ± 4.1	0.48
Resistant starch	(g/day)	0.1 ± 0.1	0.3 ± 0.2	0.1 ± 0.3	<0.001

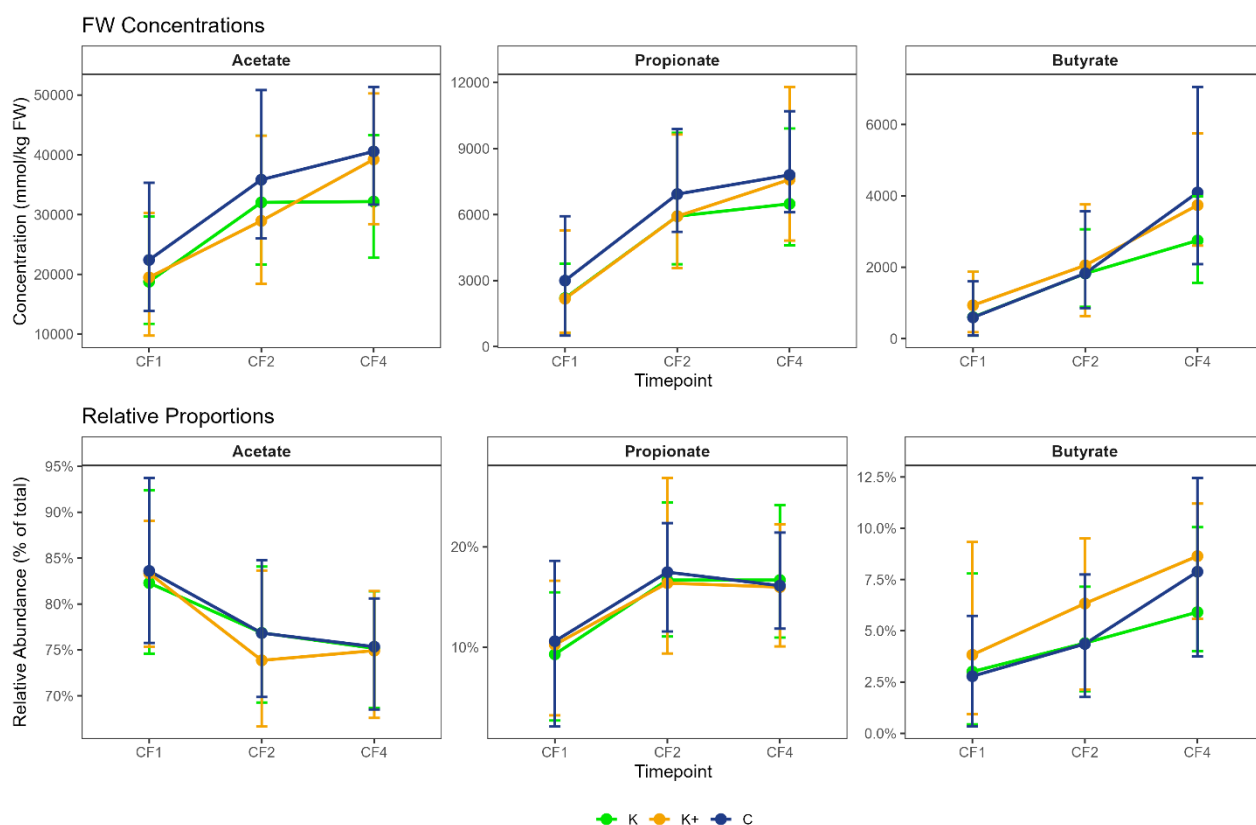


Figure 5.1. Temporal trajectories of major short-chain fatty acids: FW concentrations and relative proportions across intervention groups.

The reciprocal increase in major SCFAs was accompanied by pronounced declines in several non-SCFA organic acids, which together comprised approximately 40% of total organic acids at CF1 but declined to less than 9% by CF4. Succinate represented the largest single non-SCFA component at CF1 (median 11.9% of total organic acids) but decreased markedly to 0.4% by CF4 ($p < 0.001$). This decline reflected both a substantial reduction in FW concentration (from 4.3 to 0.2 mmol/kg FW, 95% decrease; $p < 0.001$) and a proportional shift as acetate, propionate, and butyrate came to dominate the organic-acid pool. Formate and lactate showed similar declining patterns. Formate decreased from 4.2% to 1.1% of total organic acids ($p < 0.001$), while lactate declined from 3.8% to 0.4% ($p < 0.001$). Elevated levels of these metabolites are characteristic of early-life gut microbial metabolism, when facultative anaerobes and primary fermenters predominate.

Branched-chain SCFAs (isobutyrate, isovalerate, and 2-methylbutyrate), which derive primarily from bacterial fermentation of branched-chain amino acids, increased modestly across timepoints, rising from 0.25% to 2.0% of total organic acids ($p < 0.001$). This increase occurred alongside the dietary transition from milk to complementary foods containing higher proportions of protein. However, branched-chain SCFAs remained a small proportion of the total organic-acid pool throughout the study period. Other minor organic acids, including hexanoate/caproate and methylvalerate isomers, remained near the detection limit and showed no consistent temporal patterns. Together, these results demonstrate that the primary shift in organic-acid composition occurred between CF1 and CF2, characterised by increasing proportions of acetate, propionate, and butyrate alongside concurrent declines in succinate, lactate, and formate. This compositional transition reflects the development of mature cross-feeding networks during early complementary feeding, where some metabolites are efficiently converted into terminal SCFAs rather than accumulating in the colonic environment. This shift represents a key marker of gut microbiota metabolic maturation during infancy.

5.5.2.2 Organic Acids Across Groups

In FW concentrations, median acetate ranged from 19.5 to 20.9 mmol/kg FW across groups, propionate from 2.2 to 2.5 mmol/kg FW, and butyrate from 0.6 to 0.7 mmol/kg FW, with no significant between-group differences (all $p > 0.05$). When expressed as relative proportion, major SCFAs (acetate, propionate, and butyrate) collectively comprised 56–65% of total quantified organic acids across groups, with acetate representing the largest single component (48–54%).

The remaining 35–44% was comprised primarily of succinate (10–14% across groups), formate (3–5%), and lactate (3–5%). Branched-chain SCFAs and other minor organic acids were detectable at low baseline abundances (<1% combined).

Following the introduction of complementary foods, all intervention groups exhibited nearly identical trajectories for both FW concentrations and relative abundances (Figure 5.2 and Table 5.2). Between CF1 and CF2 (introductory complementary feeding period), acetate concentrations increased by 9.8–15.8 mmol/kg FW across groups, propionate by 2.8–3.4 mmol/kg FW, and butyrate by 0.8–1.1 mmol/kg FW, with no significant differences between groups in the magnitude of these increases (all adjusted $p > 0.05$). During the CF2 to CF4 period (establishing complementary feeding), acetate increased by a further 2.7–9.8 mmol/kg FW, propionate by 0.7–1.2 mmol/kg FW, and butyrate by 0.8–1.9 mmol/kg FW, again with no significant between-group differences (all adjusted $p > 0.05$). Across the complete study period (CF1 to CF4), cumulative increases in major SCFA concentrations were similar across all groups: kūmara group median increase of 19.9 mmol/kg FW, kūmara + green banana group 26.3 mmol/kg FW, and control group 24.4 mmol/kg FW (Kruskal-Wallis adjusted $p = 0.162$).

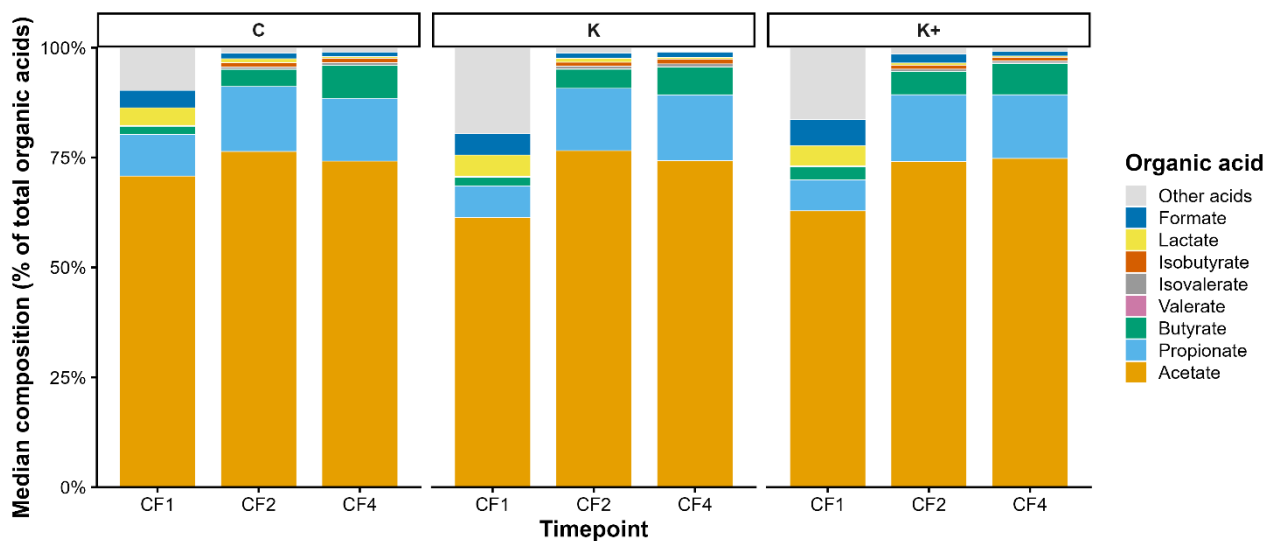


Figure 5.2. Median relative abundance of organic acids at CF1, CF2 and CF4 across each group (K: kūmara, K+: kūmara + green banana extract, C: control).

Compositionally, all groups showed parallel shifts characterised by declines in some organic acids and increasing proportions of major SCFAs. By CF2, major SCFAs had increased to 89–92% of total organic acids across all groups, with this proportion maintained through CF4 (90–93%). Succinate, which comprised 10–14% of total organic acids at baseline, declined to 0.3–0.5% by CF4 across all groups. Formate decreased from 3–5% to 1.1–1.2%, and lactate from 3–5% to 0.3–0.4%, with no significant between-group differences in the magnitude or timing of these declines (all adjusted $p > 0.05$). Branched-chain SCFAs increased modestly in all groups (from 0.2–0.3% to 1.8–2.2%) but remained a minor component of the organic acid pool throughout the study period.

Statistical comparisons of within-participant changes (delta values) between intervention groups revealed no significant differences in organic acid profiles after correction for multiple testing. Although unadjusted p-values suggested potential group differences for several FW concentration-based measures (butyrate: unadjusted $p = 0.004$; major SCFAs: unadjusted $p = 0.028$), none of these associations remained significant after false discovery rate correction for the 36 variables tested (butyrate adjusted $p = 0.079$; major SCFAs adjusted $p = 0.162$). Similarly, no relative abundance measures showed significant intervention effects after correction. Linear mixed-effects models

confirmed these findings, identifying timepoint as the overwhelmingly dominant driver of variation across all organic acid measures, with group effects contributing minimally to model variance once temporal effects were accounted for.

Table 5.2. Median relative abundances (median $\pm$ IQR) at CF2 and CF4 with percentage change from baseline (CF1) for all measured organic acids, by intervention group (K: kūmara, K+: kūmara + green banana extract, C: control). Values shown are medians across complete cases; percent change = median (CF2 or CF4) versus median (CF1).

Organic Acid	Group	CF2 Median $\pm$ IQR (% change)	CF4 Median $\pm$ IQR (% change)
Acetate (AA)	K	65.72 $\pm$ 25.12 (35%)	64.73 $\pm$ 20.83 (33%)
	K+	62.97 $\pm$ 18.06 (21%)	67.17 $\pm$ 17.92 (29%)
	C	64.02 $\pm$ 20.14 (20%)	65.08 $\pm$ 19.53 (19%)
Propionate (PA)	K	14.44 $\pm$ 12.21 (195%)	14.60 $\pm$ 13.22 (199%)
	K+	14.65 $\pm$ 11.98 (146%)	14.47 $\pm$ 12.21 (137%)
	C	12.83 $\pm$ 11.10 (86%)	13.68 $\pm$ 12.21 (94%)
Butyrate (BA)	K	4.01 $\pm$ 2.78 (126%)	5.60 $\pm$ 2.55 (229%)
	K+	5.03 $\pm$ 3.10 (106%)	7.42 $\pm$ 3.56 (205%)
	C	3.42 $\pm$ 2.35 (88%)	10.34 $\pm$ 3.91 (277%)
Isobutyrate (IBA)	K	0.84 $\pm$ 0.39 (666%)	1.14 $\pm$ 0.67 (942%)
	K+	0.92 $\pm$ 0.61 (785%)	0.84 $\pm$ 0.56 (709%)
	C	0.78 $\pm$ 0.68 (766%)	0.85 $\pm$ 0.70 (853%)
Isovalerate (IVA)	K	0.10 $\pm$ 0.17 (696%)	0.58 $\pm$ 0.32 (892%)
	K+	0.45 $\pm$ 0.62 (736%)	0.38 $\pm$ 0.45 (696%)
	C	0.42 $\pm$ 0.68 (576%)	0.44 $\pm$ 0.56 (788%)
Valerate (VA)	K	0.03 $\pm$ 0.05 (400%)	0.06 $\pm$ 0.12 (810%)
	K+	0.04 $\pm$ 0.06 (257%)	0.09 $\pm$ 0.18 (507%)
	C	0.03 $\pm$ 0.05 (190%)	0.06 $\pm$ 0.13 (400%)
Lactate (LA)	K	0.85 $\pm$ 1.23 (-84%)	0.38 $\pm$ 0.25 (-93%)
	K+	0.53 $\pm$ 0.68 (-86%)	0.29 $\pm$ 0.32 (-92%)
	C	0.64 $\pm$ 0.92 (-80%)	0.42 $\pm$ 0.39 (-88%)
Succinic acid (SucA)	K	21.80 $\pm$ 31.64 (-96%)	15.70 $\pm$ 12.10 (-97%)
	K+	23.36 $\pm$ 28.50 (-94%)	20.17 $\pm$ 15.20 (-97%)
	C	16.44 $\pm$ 21.80 (-91%)	14.20 $\pm$ 17.10 (-96%)
Formate (FA)	K	7.09 $\pm$ 5.16 (-79%)	6.84 $\pm$ 5.23 (-72%)
	K+	1.91 $\pm$ 2.40 (-58%)	1.21 $\pm$ 1.87 (-73%)
	C	1.77 $\pm$ 2.10 (-70%)	1.18 $\pm$ 1.65 (-72%)
Hexanoate/Caproate (CA)	K	0.003 $\pm$ 0.004 (186%)	0.004 $\pm$ 0.006 (239%)
	K+	0.004 $\pm$ 0.005 (43%)	0.005 $\pm$ 0.005 (79%)
	C	0.004 $\pm$ 0.006 (25%)	0.006 $\pm$ 0.007 (61%)
2-Methylbutyrate (MBA_2)	K	0.51 $\pm$ 0.42 (999%)	0.60 $\pm$ 0.49 (1175%)
	K+	0.39 $\pm$ 0.34 (432%)	0.42 $\pm$ 0.36 (472%)
	C	0.37 $\pm$ 0.33 (887%)	0.43 $\pm$ 0.41 (1047%)
3-Methylvalerate (MVA_3)	K	0 $\pm$ 0	0 $\pm$ 0
	K+	0 $\pm$ 0	0 $\pm$ 0
	C	0 $\pm$ 0	0 $\pm$ 0
4-Methylvalerate (MVA_4)	K	0 $\pm$ 0	0 $\pm$ 0
	K+	0 $\pm$ 0	0 $\pm$ 0
	C	0 $\pm$ 0	0 $\pm$ 0

5.5.3 Organic Acid and Taxonomic Composition Integration

5.5.3.1 Organic Acid and Genus Associations

Linear mixed-effects screening identified six organic-acid concentration variables and five proportional variables exhibiting strong temporal structure (marginal $R^2 > 0.1$). Among concentration-based models, butyrate showed the strongest temporal signal ($R^2 = 0.19$), followed by propionate ($R^2 = 0.16$), isobutyrate ($R^2 = 0.15$), and acetate ($R^2 = 0.15$). In contrast, relative-abundance models identified succinate as the most temporally variable organic acid ($R^2 = 0.18$), followed by acetate ($R^2 = 0.15$), propionate ($R^2 = 0.11$), and butyrate ($R^2 = 0.10$). Longitudinal modelling of these organic acids against genus-level taxonomic profiles identified a limited number of significant main effects in concentration-based models (12 of 258 tests, 4.7%). These associations are summarised in Figure 5.3, which displays the direction and magnitude of significant genus–organic acid relationships for both concentration and relative-abundance outcomes. Succinate accounted for nearly half of all significant concentration-based genus associations, with strong positive effects observed for *Bacteroides*, *Phocaeicola*, and *Parabacteroides*. Acetate concentration showed a positive association with *Bifidobacterium* and a negative association with *Clostridium*.

In contrast, relative-abundance models identified a substantially broader set of taxonomic associations (39 of 215 tests, 18.1%). Succinate proportions again exhibited the greatest number of significant associations, with positive relationships observed for *Bacteroides*, *Phocaeicola*, and *Parabacteroides*, and a negative association with *Bifidobacterium*. Acetate proportions were positively associated with *Bifidobacterium* and *Limosilactobacillus*, while butyrate proportions were positively associated with *Clostridium*.

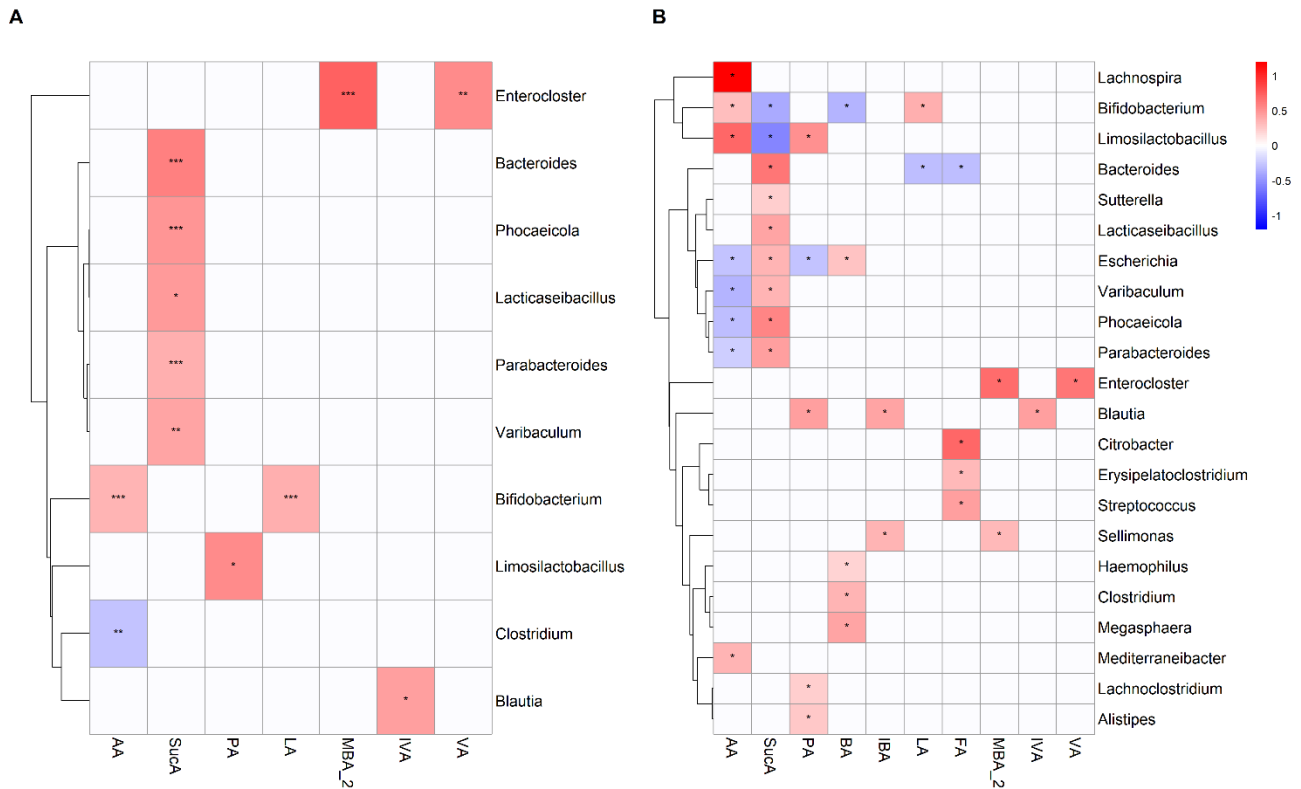


Figure 5.3. Genus-organic acid associations identified by linear mixed-effects models. Standardised coefficients (β -coef) for significant genus main effects ($q < 0.05$) are shown for organic acid concentrations (A) and relative abundances (B). Positive associations are shown in red and negative associations in blue. Asterisks indicate FDR-adjusted significance magnitude. Abbreviations: AA, acetate; SucA, succinate; PA, propionate; LA, lactate; MBA_2, 2-methylbutyrate; IVA, isovalerate; VA, valerate; BA, butyrate; IBA, isobutyrate; FA, formate.

5.5.3.2 Temporal Dynamics and Taxonomic Interactions

Temporal interaction modelling was used to determine whether associations between specific bacterial genera and organic acids remained stable across infancy or changed during complementary feeding. This approach extends the preceding analyses by assessing time-dependent restructuring of genus–metabolite relationships, rather than assuming constant associations across age.

Across the complementary feeding period, 29 genera exhibited significant time $\times$ genus interactions for one or more organic acids (FDR < 0.05). These interactions were more frequently detected and of greater magnitude in relative-abundance models than in FW concentration-based models, indicating

that age-related shifts in organic-acid composition were more strongly linked to changes in proportional microbial metabolism than to metabolite concentrations.

Succinate displayed the most prominent temporal interactions. Both concentration and relative-abundance models identified positive main effects for *Bacteroides* and *Phocaeicola*, alongside significant negative time interactions, indicating that associations between these genera and succinate weakened as complementary feeding progressed. This pattern is consistent with the marked decline in succinate proportion observed across timepoints and reflects reduced accumulation of this metabolite as microbial metabolic networks mature.

In contrast, BCFAs showed increasingly positive associations with specific genera over time, particularly in relative-abundance models. *Eggerthella* exhibited strengthening associations with isovalerate, 2-methylbutyrate, and isobutyrate, while *Alistipes* became more strongly associated with valerate. These patterns suggest a gradual emergence of protein fermentation pathways during later complementary feeding, consistent with increasing dietary protein exposure and microbial functional diversification.

Overall, these findings indicate that genus–organic acid relationships are dynamic across infancy, with early-life associations linked to metabolites that diminish over time, while associations related to branched-chain fatty acid production strengthen. This temporal restructuring aligns with the broader metabolic maturation observed in organic-acid profiles and reinforces the conclusion that microbial–metabolite interactions evolve in concert with developmental stage rather than remaining static across infancy.

5.5.3.3 Association Specificity

To distinguish metabolite-specific associations from those reflecting overall changes in fermentative output, partial correlation analyses were performed, controlling for total organic acid concentration or total proportional abundance, respectively.

Among concentration-based associations identified by linear mixed-effects models, approximately half remained significant after adjustment for total organic acid concentration. Succinate retained positive associations with *Bacteroides* (partial $\rho = 0.21$, $q = 0.006$) and *Phocaeicola* (partial $\rho = 0.17$, $q = 0.024$), while acetate remained positively associated with *Bifidobacterium* (partial $\rho = 0.21$, $q = 0.006$). In contrast, associations between lactate and *Bifidobacterium* and between isovalerate and *Blautia* were attenuated after adjustment.

Relative-abundance analyses retained a higher proportion of significant associations following adjustment. Acetate proportion showed strong positive associations with *Bifidobacterium* (partial $\rho = 0.32$, $q = 7.7 \times 10^{-6}$), while butyrate proportion exhibited negative associations with *Bifidobacterium* (partial $\rho = -0.23$, $q = 0.0026$) and positive associations with *Clostridium* and *Megasphaera* (partial $\rho \approx 0.17$ – 0.28 , $q < 0.01$). Succinate proportion displayed opposing genus-specific associations, with negative relationships observed for *Bifidobacterium* and positive relationships for *Bacteroides*, which remained significant after adjustment.

5.5.3.4 Intervention Group Effects

The relationships between metabolic pathways and organic acid production were the same across all intervention groups. No statistically significant differences were found between the kūmara, kūmara + green banana, and control groups in how metabolic pathways related to organic acid concentrations (90 comparisons, all adjusted $p > 0.90$). This indicates that the dietary interventions did not influence how gut bacteria produce organic acids; all groups produced the same mix of organic acids through the same metabolic processes.

Comparison of genus–organic acid associations across intervention groups revealed no statistically significant group-specific differences in metabolic coupling after correction for multiple testing. This indicates that the core relationships linking microbial taxa to organic acid production were largely conserved across dietary groups.

5.5.4 Organic Acid and Functional Pathways Integration

5.5.4.1 Pathway Temporal Dynamics and Organic Acid Associations

Linear mixed-effects screening identified 217 of 291 MetaCyc pathways (74.6%) that changed significantly across complementary feeding, with 71 pathways (24.4%) showing substantial variance explained by time. The pathways exhibiting the strongest temporal effects were primarily involved in amino acid biosynthesis, particularly glutamate/glutamine biosynthesis (26% of variance explained by timepoint) and lysine biosynthesis (23% of variance explained by timepoint), consistent with progressive expansion of microbial anabolic capacity during infancy.

Next, associations between metabolic pathways and individual organic acids were examined to identify which functional processes were most closely linked to organic-acid production. In concentration-based models (mmol/kg faecal fresh weight), 260 significant pathway–organic acid associations were identified (12.0% of tested associations). Propionate exhibited the greatest breadth of associations, accounting for approximately one-quarter of all significant findings (24%). These associations were most frequently observed with amino acid biosynthesis and cell wall synthesis pathways, suggesting that propionate production is tightly coupled to broader anabolic and growth-related metabolic activity. Succinate accounted for a further 16% of significant concentration-based associations, predominantly involving amino acid metabolism pathways. This pattern is consistent with succinate's role as a major metabolic product of amino acid fermentation and an intermediate within central carbon metabolism during early life. In contrast, acetate and butyrate showed more restricted association profiles, indicating that their concentrations were linked to a narrower subset of functional pathways. Acetate was most frequently associated with carbohydrate and nucleotide metabolism pathways, while butyrate was preferentially associated with energy-related pathways, reflecting its role as a terminal fermentation product arising from specialised metabolic routes.

Relative-abundance models identified a larger number of pathway–organic acid associations overall, with 555 significant associations detected (26%). In these models, butyrate exhibited the highest

degree of pathway association, accounting for more than one-fifth of all significant findings (22%). Notably, relative-abundance models revealed extensive associations between butyrate and nucleotide metabolism pathways that were not detected in concentration-based analyses (Figure 5.4Figure 4.1A). This suggests that while FW butyrate concentrations may remain relatively constrained, the proportional contribution of butyrate to the organic-acid pool is closely linked to shifts in microbial biosynthetic and growth-related capacity.

Substantial overlap was observed between concentration-based and relative-abundance results. Of the significant associations identified, 221 (85% of concentration-based associations and 40% of relative-abundance associations) were detected in both analyses, indicating a core set of robust pathway–organic acid relationships. Acetate and propionate showed the strongest concordance between methods. In contrast, relative-abundance models identified many additional butyrate-associated pathways that were not evident in concentration-based analyses, indicating that changes in butyrate’s proportional contribution to the organic-acid pool were more sensitive to shifts in microbial functional capacity than changes in FW concentration (Figure 5.4B). Together, these results indicate that relative-abundance models capture broader compositional restructuring of microbial metabolism, while concentration-based models highlight more constrained relationships tied to metabolite production.

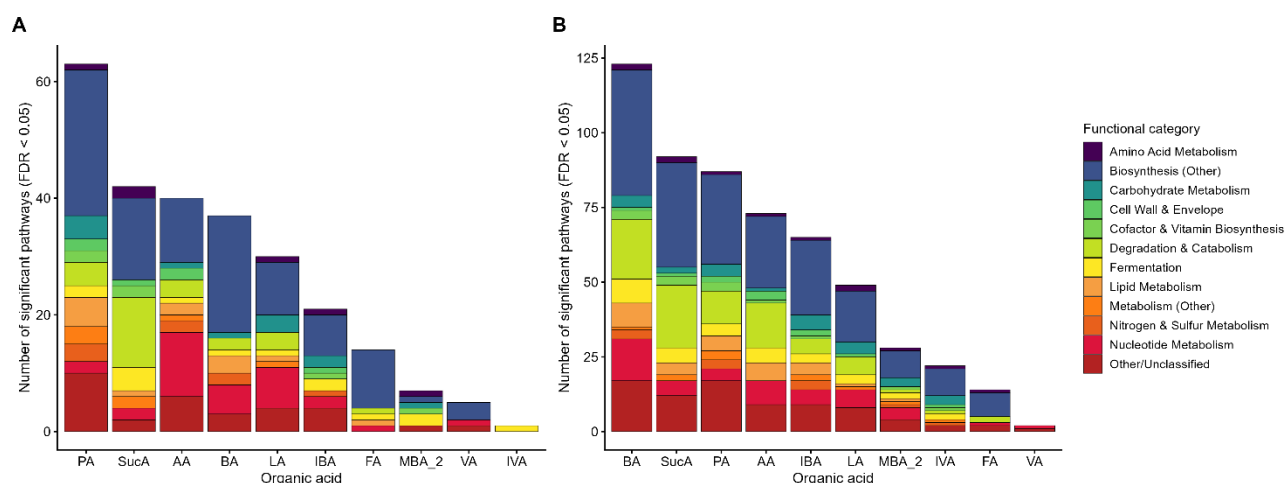


Figure 5.4. Stacked bar charts show the number of significant pathway associations ($FDR < 0.05$) for each organic acid based on concentration models (A) and relative-abundance models (B), grouped by functional category. Abbreviations: PA, Propionate; SucA, Succinate; AA, Acetate; BA, Butyrate; LA, Lactate; IBA, Isobutyrate; FA, Formate; MBA_2, 2-Methylbutyrate; VA, Valerate; IVA, Isovalerate.

5.5.4.2 Intervention Effects on Pathway–Organic Acid Relationships

Whether the dietary interventions altered the relationships between metabolic pathways and organic acids were tested. Using Fisher's Z transformation, pathway–organic acid correlations were compared between the three intervention groups (kūmara, kūmara + green banana extract, and control) for 90 pathway–organic acid pairs. No significant differences were detected between groups (0/90 comparisons significant after false discovery rate correction; all adjusted $p > 0.90$).

These findings indicate that the dietary interventions did not modify which metabolic pathways were associated with specific organic acids. Instead, pathway–organic acid relationships were consistent across all groups, indicating that these metabolic associations were not altered by the dietary interventions.

5.5.4.3 Functional, Taxonomic, and Dietary Contributions to Organic Acid Variation

Variance partitioning analysis showed that functional pathway abundance explained more variance in organic acid profiles than taxonomic composition, for both concentration-based and proportional outcomes. Functional pathways uniquely accounted for 8.5% of variance in organic acid concentrations and 12.1% of variance in relative composition, compared with 3.2% and 4.0% for taxonomic composition, respectively. Across individual organic acids, total explained variance ranged from 57% to 76% in concentration models, with pathway-based predictors consistently explaining a larger unique proportion than taxonomic predictors. The remaining 24–43% of variance was unexplained, reflecting additional influences such as host physiology, diet, and unmeasured microbial processes.

For relative organic acid composition, functional capacity again explained more variance than taxonomy. Functional pathways uniquely accounted for 12.1% of variance in relative abundances, compared with 4.0% for taxonomic predictors. Total explained variance ranged from 53% to 79% across individual organic acids in proportion models. This indicates that knowing what metabolic activities bacteria are performing (functional capacity) provides better insight into organic acid profiles than knowing which bacterial species are present (taxonomic composition), regardless of whether organic acids are measured as FW concentrations or relative proportions.

Building on the functional and taxonomic analyses above, we next examined whether dietary intake further explained variation in organic acid profiles. Correlation analyses were used to test relationships between dietary intake variables, bacterial genus abundances, metabolic pathway abundances, and organic acid concentrations at CF2 and CF4. These analyses were conducted separately within each intervention group to assess whether dietary interventions modified these relationships.

Across 3,168 pairwise correlation tests, no statistically significant differences in correlation strength were detected between intervention groups after false discovery rate correction ($q < 0.05$). This

indicates that the dietary interventions did not alter how diet, microbial composition, or microbial functional pathways related to organic acid production. Instead, similar patterns of association were observed across all groups.

5.6 Discussion

This chapter characterised faecal organic acid profiles during complementary feeding in the SUN Study and examined whether kūmara-based interventions influenced organic acid production. It further evaluated how dietary intake (including other complementary foods), gut microbial composition, and microbial metabolic pathway abundance relate to organic acid profiles during this developmental period. Four principal findings emerged. First, faecal organic acid profiles underwent a pronounced and coordinated temporal shift during early complementary feeding. Second, no intervention effects on organic acid composition were detected. Third, associations between organic acids and microbial metabolic pathways were more prevalent and explanatory than those based on taxonomic composition or dietary intake. Fourth, these pathway–organic acid relationships were conserved across intervention groups.

5.6.1 Temporal Maturation and Absence of Intervention Effects on Organic Acid Profiles

Faecal organic acid profiles in this cohort underwent a pronounced and coordinated transition during early complementary feeding, with the largest compositional changes occurring between CF1 (5–6 months age, baseline) and CF2 (8 months age, 2-months post-complementary feeding), followed by relative stabilisation through CF4 (10 months age, 4-months post complementary feeding). This temporal pattern was consistent across all intervention groups and was confirmed by linear mixed-effects modelling, indicating that age was the dominant driver of organic acid production during this period.

The most prominent change was an increase in the proportional dominance of the major short-chain fatty acids acetate, propionate, and butyrate (from 59.3% to 91.6% of total organic acids). This increase occurred alongside declining contributions from succinate, lactate, and formate, which were present at higher relative levels at CF1. These metabolites are commonly detected at higher levels in early infancy and are known end-products of specific microbial metabolic pathways that are more prevalent during milk-dominated feeding (Culp & Goodman, 2023; Louis & Flint, 2017; Tsukuda et al., 2021). Their decline across complementary feeding likely reflects changing microbial community structure and metabolic activity as dietary substrates diversify and microbial networks mature. While fresh weight concentrations of several organic acids increased with age, proportional analyses showed that changes in composition were driven primarily by the increasing contribution of major SCFAs to the total organic acid pool.

Consistent with this pattern, the most pronounced transition occurred shortly after the introduction of solid foods. This aligns closely with longitudinal studies identifying early complementary feeding as a key inflection point in infant gut fermentation. Multiple cohorts report that butyrate is typically absent or present at very low levels during exclusive milk feeding and emerges rapidly following the introduction of solid foods (Conta et al., 2021; Differding et al., 2020; Koenig et al., 2011; Shi et al., 2024; Sillner et al., 2021; Thompson et al., 2015). In the present study, butyrate more than doubled in relative abundance by CF2 and continued to increase thereafter.

In contrast to the major SCFAs, BCFAs increased modestly across timepoints but remained a minor component of the organic acid pool. This pattern is consistent with previous infant studies showing that BCFAs (including isobutyrate, isovalerate, and 2-methylbutyrate) emerge with increasing dietary protein intake and microbial proteolysis during later stages of complementary feeding, but typically remain at low relative abundance compared with saccharolytic fermentation products such as acetate, propionate, and butyrate (Differding et al., 2020; Laursen, 2021; Tang et al., 2019). Minor straight- and branched-chain organic acids often show large relative percentage changes driven by very low baseline levels, and their biological relevance in early infancy remains poorly characterised.

Notably, organic acid composition showed relatively limited change after CF2, despite continued dietary diversification and increasing solid food intake. Similar stabilisation of SCFA profiles following early weaning has been reported in longitudinal cohorts, where the most pronounced metabolic and compositional shifts occur soon after solid food introduction, followed by smaller incremental changes thereafter (Differding et al., 2020; Pham et al., 2022). Consistent with these findings, Stewart et al. (2018) reported that functional gene repertoires for SCFA production underwent rapid maturation during early complementary feeding (4–9 months) before reaching relative stability.

The absence of proportional intervention effects, despite clear dietary differences between groups, further underscores the dominant role of developmental stage in shaping organic acid composition during this window. Previous work suggests that infant microbiome assembly is shaped by early colonisation patterns and developmental constraints that limit responsiveness to modest environmental variation, leading to convergence toward similar functional profiles during late infancy Sprockett et al. (2018) Our findings are consistent with this framework, indicating that SCFA production capacity follows predictable age-related trajectories that are relatively insensitive to the dietary interventions tested between 8 and 10 months of age.

Importantly, this apparent resilience is likely specific to the magnitude and nature of dietary variation examined in the present study (Gopalsamy et al., 2019; Ordiz et al., 2020; Sprockett et al., 2018). Larger or qualitatively different dietary contrasts may still influence organic acid profiles during infancy, as suggested by cross-population studies reporting substantial differences in infant SCFA profiles between populations with markedly different diets, such as rural African versus European cohort (De Filippo et al., 2010; Roswall et al., 2021; Yatsunencko et al., 2012). Together, these findings suggest that while organic acid trajectories during complementary feeding are developmentally constrained, sufficiently large dietary contrasts can modify these trajectories, effects that were not observed for the interventions tested here.

5.6.2 Dietary Intake Patterns

Dietary intake was assessed at CF2 and CF4 using 3-day food records to characterise background complementary feeding patterns and quantify exposure to the intervention foods. Across all groups, total energy intake and macronutrient distribution were broadly comparable at both timepoints. Intakes of total carbohydrate, fat, and protein increased between CF2 and CF4, consistent with progressive dietary diversification during complementary feeding.

The resistant starch (RS) dose delivered by the kūmara-based interventions was deliberately selected to reflect physiologically realistic exposures for infants undergoing complementary feeding. RS intake differed significantly by intervention group. Infants in the kūmara + green banana extract group consumed an additional ~0.35 g/day of RS from the intervention product, resulting in a 5–8-fold higher RS intake compared with the control group and the kūmara-only group. Background dietary RS intake from non-intervention foods was uniformly low across all groups, remaining below 0.1 g/day at both CF2 and CF4 in the kūmara-only and control groups. This dose is orders of magnitude lower than those used in adult RS intervention studies, which typically administer 15–40 g/day and consistently report increases in faecal butyrate concentrations alongside enrichment of recognised butyrate-producing taxa, including *Ruminococcus bromii*, *Eubacterium rectale*, and *Faecalibacterium prausnitzii* (Baxter et al., 2018; Sobh et al., 2022; Venkataraman et al., 2016; N. P. West et al., 2013). However, the dose–response relationship for RS in early life remains poorly characterised.

Evidence from paediatric populations is extremely limited, with only one *in vivo* RS supplementation study conducted in young children. In that trial, Ordiz et al. (2015) administered approximately 1.2 g RS/day to Malawian children aged 3–5 years for four weeks and observed changes in microbiota composition, alongside alterations in faecal organic acid profiles and markers of gut inflammation. These responses differed from those typically reported in adults, highlighting the context-dependent nature of RS fermentation in early life. Complementary *in vitro* studies using infant faecal inocula

further demonstrate that RS is fermentable by infant microbiota, but fermentation is characterised primarily by increased acetate and propionate production, with limited butyrate formation (Gopalsamy et al., 2019; Y. Wang et al., 2019).

Beyond dose alone, the developmental stage of the infant gut microbiome is likely to constrain responsiveness to resistant starch (RS). Efficient RS fermentation requires specialised carbohydrate-active enzymes, including amylases, pullulanases, and debranching enzymes, whose abundance increases across the first year of life but remains substantially lower than adult levels throughout infancy (Ye et al., 2019). The pilot study informing the present study McKeen et al. (2022) demonstrated early expansion of carbohydrate metabolism capacity during complementary feeding, but also highlighted persistent functional immaturity. Consistent with this, recent metagenomic analyses show that even by one year of age, the infant gut microbiome remains functionally enriched for milk-derived glycan utilisation and comparatively limited in the enzymatic repertoire required for efficient degradation of starch and plant polysaccharides (Dikareva et al., 2025).

In addition, the infant gut microbiome during mid-infancy remains dominated by taxa that preferentially ferment simple carbohydrates, including *Bifidobacterium*, *Streptococcus*, and members of the *Enterobacteriaceae* (Parkin, Palmer, Verhasselt, Amenyogbe, Cooper, et al., 2024b; Stinson et al., 2025). Obligate anaerobes capable of degrading resistant polysaccharides and supporting butyrate production, such as *Bacteroides*, *Ruminococcus*, and *Faecalibacterium*, increase during complementary feeding but may not reach sufficient abundance or functional integration to efficiently metabolise RS until later in infancy (Dikareva et al., 2025). Consistent with this, butyrate production increased across all timepoints in all groups, with the most pronounced shift occurring between CF1 and CF2. While this indicates rapid establishment of butyrogenic activity following the introduction of complementary foods, the continued rise through CF4 suggests that full fermentative capacity may still be maturing during mid-infancy.

Taken together, these findings indicate that while the intervention successfully altered RS exposure, it reinforces the dominant role of developmental progression on organic acid production during mid-infancy and constrained responsiveness to dietary intervention.

5.6.3 Integrated Microbial-Diet-Metabolic Analysis

Associations between microbial metabolic pathways and organic acids were substantially more numerous and explanatory than those based on taxonomic composition alone. A large proportion of MetaCyc pathways exhibited significant associations with organic acids, particularly those involved in amino acid biosynthesis, central carbon metabolism, nucleotide metabolism, and cell wall synthesis. These associations were detected across both concentration-based and relative-abundance models, indicating consistent coupling between microbial metabolic capacity and organic acid profiles. Similar pathway-centric regulation of SCFAs has been reported in adult and paediatric studies, where functional gene content explains metabolite variation more effectively than taxonomic abundance alone (Differding et al., 2020; Laursen, 2021; Shi et al., 2024; Tsukuda et al., 2021).

Only a subset of genera showed consistent and biologically interpretable associations with organic acids. Succinate exhibited the strongest genus-level signal, with positive associations involving *Bacteroides* and closely related taxa across both concentration and relative-abundance models. This aligns with the well-established role of *Bacteroides* species in succinate production during polysaccharide fermentation and propionate synthesis via the succinate pathway (Louis & Flint, 2017; Reichardt et al., 2014).

Acetate showed positive associations with *Bifidobacterium*, consistent with acetate production via the “bifid shunt” during early infancy. However, partial correlation analyses demonstrated that this association was largely non-specific and attenuated after accounting for total organic acid output, indicating that *Bifidobacterium* contributes primarily to overall fermentative activity rather than exerting acetate-specific regulation. The maintenance of high acetate proportions despite declining *Bifidobacterium* abundance suggests functional redundancy, likely involving other acetate-producing

taxa such as *Blautia*, which increase during complementary feeding and harbour homoacetogenic pathways (Reichardt et al., 2018).

In contrast, butyrate displayed comparatively weak genus-level associations. This finding reflects the specialised and cooperative nature of butyrate production, which depends on metabolic cross-feeding between multiple taxa rather than on the abundance of individual butyrate-producing genera. Consistent with this, previous studies have shown that butyrate levels are better predicted by functional gene content than by taxonomic composition alone (Vieira-Silva et al., 2016). Together, these findings indicate that organic acid profiles are structured primarily by microbial metabolic capacity and, to a lesser extent, by taxonomic composition.

Direct associations between dietary intake and organic acids were comparatively rare across intervention groups, despite clear differences in resistant starch consumption. This pattern suggests that dietary substrates influenced organic acid profiles primarily through their effects on microbial composition and functional capacity, rather than through direct substrate–product relationships during the CF2 to CF4 period. Similar observations have been reported in infant cohorts, where complementary feeding reshapes microbiome structure and metabolic potential, yet correlations between individual nutrients and SCFA levels remain inconsistent and context-dependent (Differding et al., 2020; Laursen, 2021; Tsukuda et al., 2021).

5.6.4 Strengths and Limitations

The large sample size and dense temporal sampling enabled robust characterisation of SCFA maturation trajectories with sufficient statistical power to detect clinically meaningful effects. Previous infant SCFA studies have typically been limited to <50 participants or single/two timepoints, constraining temporal resolution.

The integration of faecal organic acid measurements with shotgun metagenomic profiles of microbial composition and functional pathway abundance enabled interpretation of organic acid variation in the

context of microbial metabolic potential. By relating taxa and pathways to organic acid profiles, this approach provided biologically coherent associations consistent with known microbial metabolic capabilities, allowing functional interpretation beyond descriptive correlations based on taxonomy or metabolites alone. While these analyses do not establish causality or directly measure pathway activity, they support plausible biological explanations for observed organic acid patterns during complementary feeding. Experimental validation would be required to confirm causal mechanisms.

Comprehensive dietary assessment using 3-day food records at multiple timepoints, including both absolute and energy-adjusted nutrient intakes, enabled evaluation of dietary RS exposure and potential confounding. The finding that background dietary RS was uniformly low across groups, with the K⁺ group receiving a well-defined supplement (0.35 g/day), establishes clear exposure contrasts and eliminates dietary confounding as an alternative explanation for null intervention effects.

Several limitations warrant acknowledgement. Firstly, a technical consideration related to the estimation of background dietary RS intake. National food composition databases, including FOODfiles 2018 (New Zealand Institute for Plant & Food Research, 2019) used in this study, often underestimate RS content, as RS quantification requires specialised analytical methods that are not routinely applied to all foods. Standard carbohydrate analyses typically measure total starch without distinguishing resistant fractions, and RS values are frequently absent or inferred from limited food categories. While RS content of the intervention product was determined by laboratory analysis, background dietary RS was derived from database values that may not fully capture variation introduced by food preparation and storage practices.

However, even allowing for systematic underestimation, the observed 5–8-fold difference in measured RS intake between groups represents a clear relative contrast. The persistence of null effects despite this difference supports the conclusion that RS supplementation had minimal impact on the faecal microbiota and organic acid profiles at this developmental stage.

Secondly, organic acid measurements were performed on faecal samples, which reflect distal colonic metabolism rather than proximal colon (where most fermentation occurs) or systemic circulation (where organic acids exert physiological effects). Faecal organic acids, therefore, provide an indirect measure of fermentative output and may underestimate production. Nonetheless, faecal sampling is the only non-invasive approach feasible in infant populations, and faecal organic acid profiles have been shown to reflect colonic metabolic processes in both adult and paediatric studies.

Finally, this cohort consisted of healthy, term-born New Zealand infants, which may limit generalisability to populations with different health status, environmental exposures, or feeding practices. However, this relative homogeneity is also a strength of the study. By minimising variation related to prematurity, underlying disease, and extreme dietary or environmental differences, the SUN Study provides a well-controlled context in which to characterise typical organic acid development during complementary feeding. Studies in more diverse or contrasting populations have reported substantial differences in infant gut microbiome composition and organic acid profiles related to diet, environment, and mode of delivery, suggesting that intervention responses may differ under markedly different conditions. Future studies in diverse settings will be important to assess the transferability of these findings.

5.6.5 Conclusions

This chapter demonstrates that faecal organic acid profiles undergo pronounced and coordinated maturation during early complementary feeding, with the largest compositional shifts occurring shortly after the introduction of solid foods. Across all intervention groups, organic acid trajectories were dominated by developmental time, with increasing contributions of acetate, propionate, and butyrate and declining relative abundance of metabolites more characteristic of early infancy.

Integration of organic acid profiles with microbial composition and functional pathway data indicates that organic acid regulation during this period is driven primarily by microbial metabolic capacity rather than by taxonomic composition or short-term dietary variation. Associations between

metabolic pathways and organic acids were more numerous and explanatory than those involving bacterial genera or dietary intake, highlighting the importance of functional maturation of the gut microbiota in shaping fermentative outputs.

Despite a clearly defined and sustained contrast in resistant starch exposure, no intervention effects on organic acid composition were detected. This suggests that organic acid maturation during mid-infancy follows developmentally constrained trajectories that are relatively insensitive to modest dietary manipulation. Together, these findings support a model in which complementary feeding initiates rapid restructuring of microbial metabolic networks, establishing a core fermentative configuration by mid-infancy that is shaped primarily by age-related microbial maturation rather than by specific complementary food composition.

Chapter 6

General Discussion

As infants transition from an exclusively milk-based diet to complementary foods around 6 months of age, the gut microbiota undergoes substantial changes in taxonomic composition, functional capacity, and metabolite production. Although diet is recognised as an important driver of microbiota development, evidence remains limited regarding the extent to which complementary foods can modulate these trajectories during early infancy.

Within this context, this thesis examined whether whole-food-based prebiotic interventions, introduced at physiologically realistic levels during early complementary feeding, could influence the developing infant gut microbiota. The interventions comprised kūmara (New Zealand sweet potato) alone or combined with green banana extract (GBE). Kūmara was selected as a low-risk complementary food with a favourable nutritional profile, providing complex carbohydrates and dietary fibre, with feasibility, acceptability, and tolerance in infants previously demonstrated in a Nourish to Flourish pilot study (McKeen et al., 2022). Preparation of kūmara through cooking and cooling with the skin on, followed by drying and milling, promoted retrograded starch formation, increasing dietary fibre content, particularly resistant starch, within a whole-food matrix. Green banana extract was included to increase exposure to fermentable carbohydrate, primarily resistant starch, allowing assessment of whether additional fermentable substrate could influence microbiota development beyond the effects of kūmara alone.

Controlled *in vitro* fermentation experiments (Chapter 3) investigated microbial responses across a wide range of kūmara–green banana extract ratios beyond what is feasible in a clinical setting. The SUN Study (Chapters 4 and 5) then evaluated kūmara alone and kūmara combined with GBE (85:15 ratio of kūmara to GBE), compared with standard complementary feeding, in a large randomised controlled trial. Shotgun metagenomics, faecal organic acid analysis, and dietary intake were integrated to assess microbiota maturation and intervention effects during the 6–10 month

complementary feeding period. This chapter synthesises findings from the *in vitro* and *in vivo* studies, situates them within the existing literature, and considers implications for infant nutrition and future research on gut microbiota development during complementary feeding.

6.1 Summary of Key Findings

Three principal findings emerged across the *in vitro* and *in vivo* components of this thesis. First, taxonomic composition showed limited response to dietary interventions. In the *in vitro* fermentation experiment, microbial community composition remained statistically indistinguishable across nine substrate mixtures spanning a wide range of kūmara–green banana extract ratios and resistant starch contents (1.2–30.5%), with no species reaching significance. Similarly, in the SUN Study, no genera differed significantly between intervention groups at any timepoint. Alpha diversity increased markedly with age (Shannon index 1.74 to 2.88), and taxonomic composition underwent substantial age-driven restructuring (PERMANOVA $R^2 = 0.04$, $p < 0.001$); however, these trajectories were parallel across intervention groups. Overall, the kūmara-based interventions did not measurably alter taxonomic structure beyond normal developmental trajectories.

Second, in contrast to taxonomic stability, functional metabolic capacity was responsive to both interventions and developmental stage. *In vitro*, 94 metabolic pathways exhibited significant associations with substrate mixtures, demonstrating broad metabolic reorganisation in response to substrate composition. The functional response was bidirectional and taxonomically concentrated: 96% of pathway effects involved suppression of biosynthetic pathways (nucleotide metabolism, amino acid biosynthesis, cell wall synthesis) in GBE-rich mixtures, overwhelmingly contributed by Enterobacteriaceae (94% of pathways), while six catabolic pathways showed enrichment in 100% GBE conditions. These effects were stronger in mixtures with higher GBE content and were minimal in mixtures containing low levels of GBE ($\leq 15\%$).

In vivo, functional pathway abundance changed significantly throughout the complementary feeding period (PERMANOVA $R^2 = 0.05$, $p < 0.001$). The largest compositional shifts occurred early in this

period. Between CF1 and CF2 (6–8 months of age), more than half of the detected pathways (55%) increased in relative abundance, reflecting broad reorganisation of functional pathway representation following the introduction of complementary foods. Between CF2 and CF4 (8–10 months of age), pathway-level changes were more selective, with most pathways (67%) decreasing in relative abundance, consistent with a narrowing of the functional profile toward a smaller set of dominant metabolic processes rather than continued broad change. This biphasic pattern reflects developmental maturation rather than dysfunction. Early expansion represents metabolic preparation for new substrates, while late consolidation indicates selective maintenance of core biosynthetic processes and a shift from energetically expensive *de novo* synthesis (e.g., nucleotides) to efficient salvage pathways, characteristic of a maturing, stabilising ecosystem. Intervention effects on functional capacity were detectable but modest. During the first two months of complementary feeding, group-specific differences emerged, with the kūmara and green banana extract group showing enhanced pyruvate fermentation pathways, nucleotide salvage metabolism, and amino acid biosynthesis. The kūmara group showed enrichment in carbohydrate metabolism pathways. By 4 months post commencement of complementary feeding, intervention differences shifted toward carbohydrate and vitamin biosynthesis pathways. However, these group-specific patterns did not override dominant age-driven trajectories, which explained substantially greater variance in overall functional capacity.

Third, faecal organic acid profiles changed markedly with age, characterised by increasing dominance of the three major short-chain fatty acids, acetate, propionate, and butyrate, which together rose from 59% to 92% of the total organic acid pool. This shift occurred alongside concurrent declines in succinate, lactate, and formate, which collectively decreased from approximately 30% to 5%. Organic acid profiles did not differ significantly between intervention groups at any timepoint, suggesting that developmental timing exerted a stronger influence than dietary variation within the range examined.

Integration of metagenomic and metabolomic data revealed that functional pathway abundance explained substantially more variation in organic acid profiles than taxonomic composition (12.1% versus 4%), a three-fold difference. Most strikingly, 94% of the statistical relationships between

individual bacterial genera and organic acids were mediated through metabolic pathways, indicating that organic acid production is shaped by coordinated microbial metabolism rather than by the presence of specific taxa.

Collectively, these findings reveal a consistent pattern across experimental scales: while infant gut microbiota possess intrinsic capacity for metabolic adaptation to dietary substrates (Chapter 3), this capacity is substantially constrained by developmental programming, background dietary exposure, and host context during early complementary feeding (Chapter 4). Metabolic output during this period is shaped more by coordinated functional pathway changes than by taxonomic composition (Chapter 5), indicating that functional pathway profiling provides greater insight into metabolic outputs than taxonomic surveys alone, with important implications for microbiome-metabolite research design.

6.2 Translation from *In Vitro* to *In Vivo*

The parallel *in vitro* fermentation experiments with the SUN Study provided a rare opportunity to examine how dietary effects on the infant gut microbiota translate from simplified experimental systems to real-world developmental contexts. Across both settings, taxonomic composition was relatively stable, whereas microbial metabolic function was responsive. This convergence supports the biological plausibility of whole-food-based prebiotic interventions influencing microbial metabolism without necessitating wholesale restructuring of microbial communities. However, the nature, magnitude, and specificity of functional responses diverged substantially between *in vitro* and *in vivo* contexts, highlighting key constraints on translational inference.

In vitro fermentations demonstrated clear metabolic responsiveness across a wide substrate gradient of kūmara and green banana extract ratios, confirming that the infant gut microbiota possesses the intrinsic capacity to adjust functional responses to these substrates. These responses involved coordinated changes across multiple biosynthetic pathways, including nucleotide metabolism, amino acid biosynthesis, and cell wall synthesis, with bidirectional effects indicating metabolic shifts between anabolic and catabolic states depending on substrate availability. In contrast, *in vivo*

functional changes were shaped primarily by developmental progression, with dietary effects appearing as modest, time-dependent deviations within a strongly age-structured trajectory. Functional signatures *in vivo* centred on amino acid biosynthesis, fermentation pathways, and cofactor metabolism, processes closely tied to microbial growth, biomass production, and metabolic integration within the host environment.

Several biological and contextual factors likely explain the divergence between *in vitro* and *in vivo* functional responses.

Substrate exposure differed in both magnitude and ecological context. The *in vitro* fermentation experiment tested a gradient of kūmara and green banana extract mixtures spanning a wide range of resistant starch contents (1.2–30.5%), enabling direct comparison of microbial responses across substrate compositions under controlled conditions. In contrast, the *in vivo* intervention provided a small, physiologically realistic increase in resistant starch intake (~0.35 g/day), which was further diluted by background complementary foods and, for many infants, ongoing breastmilk-derived substrates.

Microbial growth conditions also differed fundamentally between settings. *In vitro* batch fermentations allow microbes to respond directly to substrate availability in a simplified, closed system. *In vivo*, microbial metabolism occurs within a complex gut ecosystem, where growth and activity are constrained by host regulation, nutrient absorption, and continuous ecological turnover rather than driven solely by substrate supply (Isenring et al., 2023; Kang et al., 2022).

Developmental programming further constrained translational alignment. The *in vivo* study coincided with a tightly regulated window of microbiota maturation, during which strong age-related trajectories dominated microbiota composition and function. Within this window, dietary interventions acted on an already evolving system rather than initiating change *de novo*. Continued exposure to human milk oligosaccharides (HMOs) in infants likely further buffered intervention

effects by providing a dominant, structurally complex fermentable substrate that shaped community-wide metabolism independently of the intervention foods.

Taken together, these differences indicate that *in vitro* fermentation experiments identify microbial metabolic capabilities under static conditions, whereas *in vivo* outcomes reflect the extent to which these capabilities are realised within the complex, regulated, and developmentally constrained environment of the developing infant gut.

6.3 Comparison with Existing Studies

Across studies, solid food introduction is associated with increasing microbial diversity and progressive restructuring of community composition. Multiple studies report rising Shannon diversity throughout infancy, typically continuing until around two years of age . Following complementary feeding initiation, consistent taxonomic shifts include expansion of Lachnospiraceae, Ruminococcaceae, and Bacteroidota alongside declining *Bifidobacterium* abundance. The SUN Study exhibited the same patterns, with increasing diversity and characteristic enrichment of fibre-degrading genera such as *Faecalibacterium*, *Roseburia*, *Blautia*, and *Anaerostipes*, accompanied by declining *Bifidobacterium*, occurring uniformly across all intervention groups. Importantly, Bergstrom et al. (2014) reported that many taxonomic changes intensify between 9 and 18 months, indicating that microbiota maturation extends beyond the SUN Study intervention window (6–10 months).

Despite growing interest in the role of specific complementary foods, evidence for consistent diet-driven effects during infancy remains limited. Studies examining individual dietary components have yielded heterogeneous results. Thompson et al. (2015) reported associations between fruit and vegetable diversity and selected taxa, but these effects varied substantially between infants and did not translate into consistent community-level patterns. Homann et al. (2021), analysing Dutch and Canadian cohorts, similarly observed associations between dietary patterns and individual taxa, yet found overall microbiota structure dominated by age and inter-individual variation. Shi et al. (2024)

reported associations between complementary food introduction and several genera, including increases in *Blautia* and *Akkermansia* and declines in *Escherichia-Shigella*, but noted that temporal effects consistently outweighed dietary signals. Tang et al. (2022) found minimal microbiota effects of protein intake despite clear dietary contrasts between meat and dairy interventions. The SUN Study, showing minimal intervention effects despite defined differences in resistant starch exposure, fits squarely within this broader pattern.

Large-scale longitudinal studies provide particularly strong support for these conclusions. Age was identified as the largest single contributor to microbiota variation in the TEDDY study; however, its explanatory power was modest, accounting for just 4–8% of variance, while dietary factors explained less than 1% after adjustment for age and breastfeeding status (C. J. Stewart et al., 2018). Within this framework, breastfeeding emerged as the most influential dietary exposure, whereas individual solid food components showed weak, transient associations.

The EDIA (Early Dietary Intervention and Later Signs of Beta-Cell Autoimmunity) cohort provides important temporal context for interpreting these findings. Lalli et al. (2025) demonstrated that associations between plant-based fibre intake and microbiota composition became most apparent at around 9 months, coinciding with declining breastfeeding rates and the point at which complementary food fibre intake first exceeded estimated human milk oligosaccharide (HMO) exposure. These associations strengthened further by 12 months as breastfeeding continued to decline. In contrast, approximately 64% of SUN Study infants remained breastfed at 10 months. At this stage, HMOs likely accounted for 30–40% of total fermentable carbohydrate exposure, based on published estimates of breastmilk intake and HMO concentrations. At CF4, infants consumed 6.1–7.0 g/day complementary food fibre, with breastfed infants receiving an additional estimated 3.2–3.5 g/day from HMOs, for total daily fibre intake of 9–11 g/day. Within this dietary context, the ~0.35 g/day difference in resistant starch between intervention groups represented only ~2% of total fibre intake, limiting its capacity to drive detectable taxonomic divergence. The timing of the SUN Study

intervention, ending before a major decline in breastfeeding, therefore likely preceded the developmental window in which complementary food fibres exert stronger microbiota effects.

Organic acid profiles in the SUN Study also followed developmental patterns consistent with prior reports. Bridgman et al. (2017) and Tsukuda et al. (2021) documented progressive increases in short-chain fatty acids during the first year of life, with solid food introduction coinciding with increasing butyrate and propionate and declining lactate and succinate. The SUN Study observed the same trajectory, with the three major SCFAs increasing from 59% to 92% of the total organic acid pool between baseline and 10 months, and the most pronounced transition occurring shortly after complementary feeding commenced. These consistent findings across populations suggest that organic acid maturation follows a robust developmental programme that is relatively resistant to modest dietary variation during early complementary feeding.

The role of resistant starch as a prebiotic substrate during infancy warrants specific consideration given its prominence in the SUN Study interventions. *In vitro* work has demonstrated that infant faecal microbiota possesses resistant starch fermentation capacity even before weaning, with capacity increasing following complementary food introduction (Gopalsamy et al., 2019). Wang et al. (2019) further showed that microbiota from 12-month-old infants exhibit greater resistant starch fermentation capacity than those from 6-month-old infants, accompanied by increased expression of starch-degrading enzymes and enhanced butyrate production. The SUN Study intervention occurred during a period when specialised resistant starch-degrading capacity was still emerging, providing a plausible explanation for why clear differences in resistant starch intake did not translate into detectable changes in organic acid profiles.

Collectively, these comparisons indicate strong convergence between the SUN Study and existing literature: microbiota and metabolite maturation during complementary feeding is dominated by developmental timing, dietary effects are modest and inconsistent, and responsiveness to specific substrates is constrained by both developmental stage and broader dietary context. The SUN Study

demonstrates that even targeted whole-food prebiotic interventions capable of producing detectable functional pathway changes do not override these developmental patterns or alter metabolite production while HMOs remain a substantial component of total fermentable substrate exposure. These findings do not imply that diet is unimportant for infant microbiota development, but rather that its effects are conditional, shaped by timing, substrate context, and developmental state, and are more likely to manifest as subtle functional modulation than large-scale compositional changes during early complementary feeding.

6.4 Strengths and Limitations

A key strength of this work lies in its scale and design. The SUN Study included 279 infants, with high retention and 89% providing complete longitudinal data, enabling robust characterisation of temporal trajectories during the critical 6–10-month window. The availability of repeated measures within individuals substantially reduced inter-individual variability and strengthened inference regarding developmental change, which is particularly important in infant microbiome research where between-subject heterogeneity is high.

This study also extends findings from the Nourish to Flourish pilot study, which established the feasibility, acceptability, and tolerability of kūmara-based complementary feeding interventions and examined the microbiota effects. The current study's larger sample size and comprehensive functional profiling confirmed these observations while revealing subtle pathway-level effects not detectable in smaller-scale work.

A further strength is the integration of shotgun metagenomics with targeted faecal organic acid profiling. By jointly examining taxonomic composition, functional pathway abundance, and metabolic outputs, this work demonstrates that microbial functional capacity explains substantially more variation in organic acid profiles than taxonomic composition alone. This finding has broader methodological implications, highlighting the limitations of taxonomy-focused analyses for understanding microbiome–metabolite relationships and supporting the use of functional profiling

when metabolic outcomes are of interest. The fact that pathway–organic acid associations were similar across all intervention groups suggests these relationships are biologically meaningful rather than random.

The use of whole-food interventions delivered at physiologically realistic doses represents another important strength. Kūmara and green banana extract were incorporated into complementary foods in a form and quantity consistent with real-world infant feeding practices, enhancing translational relevance. This contrasts with many prebiotic studies that rely on purified substrates at doses well above those achievable through habitual dietary intake. The feasibility and acceptability of these interventions had already been established in a pilot study, providing a strong foundation for evaluating biological effects under realistic conditions.

The parallel *in vitro* fermentation experiment provided insight into underlying biological processes. The *in vitro* system showed what the infant gut microbiota can do metabolically when exposed to kūmara–green banana extract mixtures, whereas the *in vivo* study showed how this capacity is expressed within the constraints of infant development, host physiology, and diet.

Several limitations should be considered when interpreting the findings. This research employed MetaPhlAn for taxonomic profiling, which is optimised for bacterial and archaeal characterisation. While shotgun metagenomic data theoretically contain information on fungal and viral communities, comprehensive profiling of these components requires specialised bioinformatic pipelines. Given that bacteria represent the dominant fraction of the infant gut microbiome during this developmental period and are the primary mediators of dietary fibre fermentation, bacterial profiling was prioritised. Future research integrating fungal and viral profiling would provide a more complete picture of microbiome development and potential interactions between different microbial kingdoms in response to dietary interventions.

Dietary intake was assessed using 3-day food records at CF2 and CF4, which provide reasonable estimates of macronutrient, fibre, and breastmilk intake while remaining subject to day-to-day

variability and caregiver reporting accuracy. Resistant starch content of the intervention products was quantified analytically; however, background dietary resistant starch intake was estimated from food composition databases, which are known to underestimate resistant starch. Although this limits precision in estimating absolute resistant starch exposure, the consistent 5–8-fold difference between intervention and control groups supports the conclusion that null intervention effects are unlikely to be explained by dietary misclassification alone.

The intervention period concluded at 10 months of age, prior to the major decline in breastfeeding observed in many populations and before the developmental window in which complementary food fibres may become the dominant fermentable substrate. Continued breastfeeding during the study period likely buffered dietary intervention effects, particularly on taxonomic composition and organic acid profiles. Longer follow-up or interventions extending into later infancy may reveal effects not detectable within this timeframe.

While shotgun metagenomics provided detailed insight into functional potential, the products of gene expression and enzymatic activity were not measured. Consequently, pathway abundance reflects metabolic capacity rather than confirmed flux. Nonetheless, the coordinated and biologically coherent patterns observed across hundreds of pathways, alongside concordance with organic acid profiles, suggest genuine functional reorganisation rather than passive shifts in gene content.

Finally, the study population consisted predominantly of healthy, term-born infants with relatively stable early-life microbiota development, which may limit generalisability to infants at higher risk of dysbiosis. Intervention effects may be more pronounced in populations with disrupted or delayed microbiota maturation, or experiencing early-life illness. In such contexts, dietary interventions may have greater capacity to alter microbial metabolic trajectories than in healthy, developmentally resilient cohorts. Consistent with this, synbiotic intervention studies have demonstrated greater microbiota and metabolic responses in higher-risk infant populations compared with healthy cohorts,

highlighting the potential for increased responsiveness where baseline microbiota resilience is reduced (Zimmermann et al., 2025).

6.5 Implication for Infant Nutrition and Future Research

The findings of this thesis have important implications for how microbiome research is translated into infant feeding guidance and for the design of future dietary intervention studies during complementary feeding. Although the kūmara-based interventions did not produce detectable taxonomic or organic acid differences relative to control feeding, these null findings should be interpreted within the developmental and dietary context in which the study was conducted rather than as evidence against the nutritional value of the foods themselves. Kūmara remains a nutritious, culturally appropriate complementary food, and the absence of microbiota effects reflects developmental constraints rather than a lack of dietary quality.

Several priorities for future research emerge from this work. First, intervention studies should extend follow-up beyond 10 months of age, specifically into the 12–18 month period. Evidence from longitudinal cohorts indicates that associations between complementary food fibre and microbiota composition strengthen between 9 and 12 months as breastfeeding declines and complementary foods become the dominant source of fermentable substrate. Extending follow-up to 12–18 months would allow assessment of whether dietary interventions exert stronger taxonomic or metabolite effects once background human milk oligosaccharide exposure decreases and microbial starch-degrading capacity further matures.

Second, deeper functional resolution will be essential. While shotgun metagenomics enabled assessment of microbial metabolic potential, integration with metabolomics, metatranscriptomics, metaproteomics, or metabolic flux analysis would clarify whether observed pathway abundance changes reflect active metabolic engagement or latent capacity. Such approaches would strengthen causal inference regarding how dietary substrates are processed in vivo during infancy.

Third, replication in more diverse populations is warranted. Infants predisposed to dysbiosis, may show greater responsiveness to dietary interventions, and targeted studies in these populations are needed to assess differential benefit. These groups may represent contexts in which dietary modulation has a larger relative influence than observed in our study involving breastfed, healthy term infants.

Finally, future research should move toward integrative frameworks that link microbial function and metabolites to host outcomes. Incorporating immune, inflammatory, metabolic, and gut barrier markers would clarify whether modest functional changes in the microbiota translate into meaningful effects on infant health, even in the absence of large taxonomic shifts.

6.6 Conclusions

This thesis provides strong evidence that gut microbiota development during early complementary feeding (6–10 months of age) follows robust, developmentally patterned trajectories characterised by increasing diversity, taxonomic restructuring, and coordinated functional metabolic reorganisation. While whole-food-based prebiotic interventions produced measurable changes in microbial metabolic pathway composition, their effects during this period were modest relative to age-associated maturation and did not alter overall developmental trajectories when introduced alongside continued breastfeeding.

Integration of shotgun metagenomics with faecal organic acid profiling demonstrated that microbial functional pathway composition explains substantially more variation in metabolite profiles than taxonomic composition alone, and that most observed taxa–metabolite associations are mediated through shared metabolic pathways. This finding highlights the value of functional profiling for understanding microbial metabolism during infancy and suggests that taxonomic changes alone may provide an incomplete picture of microbiome–metabolite relationships.

Collectively, these findings support current dietary guidance that prioritises dietary diversity during complementary feeding, while indicating that targeted prebiotic interventions are likely to require careful consideration of developmental timing, substrate dose, and background fibre exposure, particularly from HMOs, to exert detectable effects on microbiota structure or metabolic output. Future research should extend follow-up into later infancy, when complementary foods represent a greater proportion of total fermentable substrate intake, and incorporate approaches that capture realised microbial function, such as metatranscriptomics and metaproteomics, to strengthen causal inference about functional responses. Understanding how developmental programming interacts with dietary composition and breastfeeding context will be critical for designing effective nutritional strategies to support healthy microbiota maturation during infancy.

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APPENDICES

Appendix A. *In vitro* digestion solutions (for 31 samples, over 4 batches with 1 spare per batch) for 20ml salivary volume.

Stock Solutions:

	Mass (g)		H ₂ O (mL)
KCl (0.5M)	7.46	plus	200
KH ₂ PO ₄ (0.5M)	2.04	plus	30
NaHCO ₃ (1M)	59.64	plus	710
NaCl (2M)	29.25	plus	250
MgCl ₂ (H ₂ O) ₆ (0.15M)	0.61	plus	20
(NH ₄) ₂ CO ₃ (0.5M)	0.288	plus	6
CaCl ₂ (1.5M)	1.1025	plus	25
Bile salts (0.01M)	5.47625	plus	81

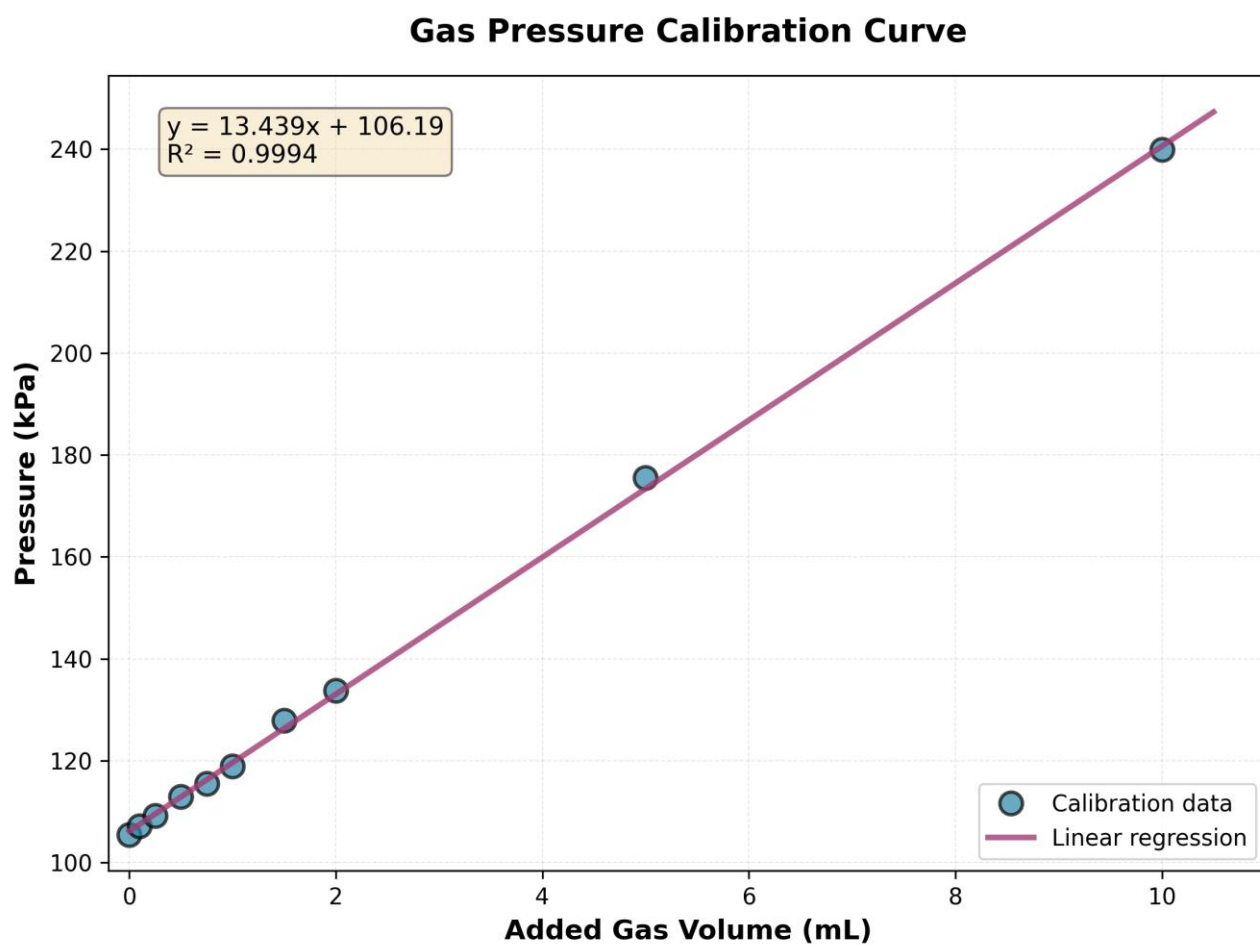
Electrolyte Salivary Fluid Solutions:

	eSSF (mL)	eSGF (mL)	eSIF (mL)
KCl (0.5M)	18.88	17.25	20.40
KH ₂ PO ₄ (0.5M)	4.63	2.25	2.40
NaHCO ₃ (1M)	8.50	31.25	127.50
NaCl (2M)	-	29.50	28.80
MgCl ₂ (H ₂ O) ₆ (0.15M)	0.63	0.83	3.30
(NH ₄) ₂ CO ₃ (0.5M)	0.08	1.25	-
Total	32.70	82.33	182.40
Plus			
H ₂ O (MilliQ)	467.30	917.67	1017.60
Final Volume (1.25x)	500.00	1000.00	1200.00

Enzymes Solutions:

	Mass (mg)		Solvent (mL)
Amylase	120	Plus	40 eSSF
Pepsin	2500	Plus	100 eSGF
Pancreatin	880	Plus	220 eSIF
Amyloglucosidase	1467		

Appendix B. *Gas pressure calibration curve. Data points represent measured pressure (kPa) following addition of known volumes of air (0-10 mL) to sealed fermentation tubes.*



Appendix C. Calibration data for quantification of fermentation gases (hydrogen and methane).

Hydrogen Calibration Data

Standard	Concentration (%)	Peak Area
Std 1	10.0	1539699
Std 2	5.0	819491
Std 3	2.5	126026
Std 4	1.0	199074
Air	0.0	0

Calibration equation: $y = 15,794,217.87x - 47,528.06$
 $R^2 = 0.9607$, $n = 5$
(x = concentration as decimal, e.g., 0.10 for 10%)

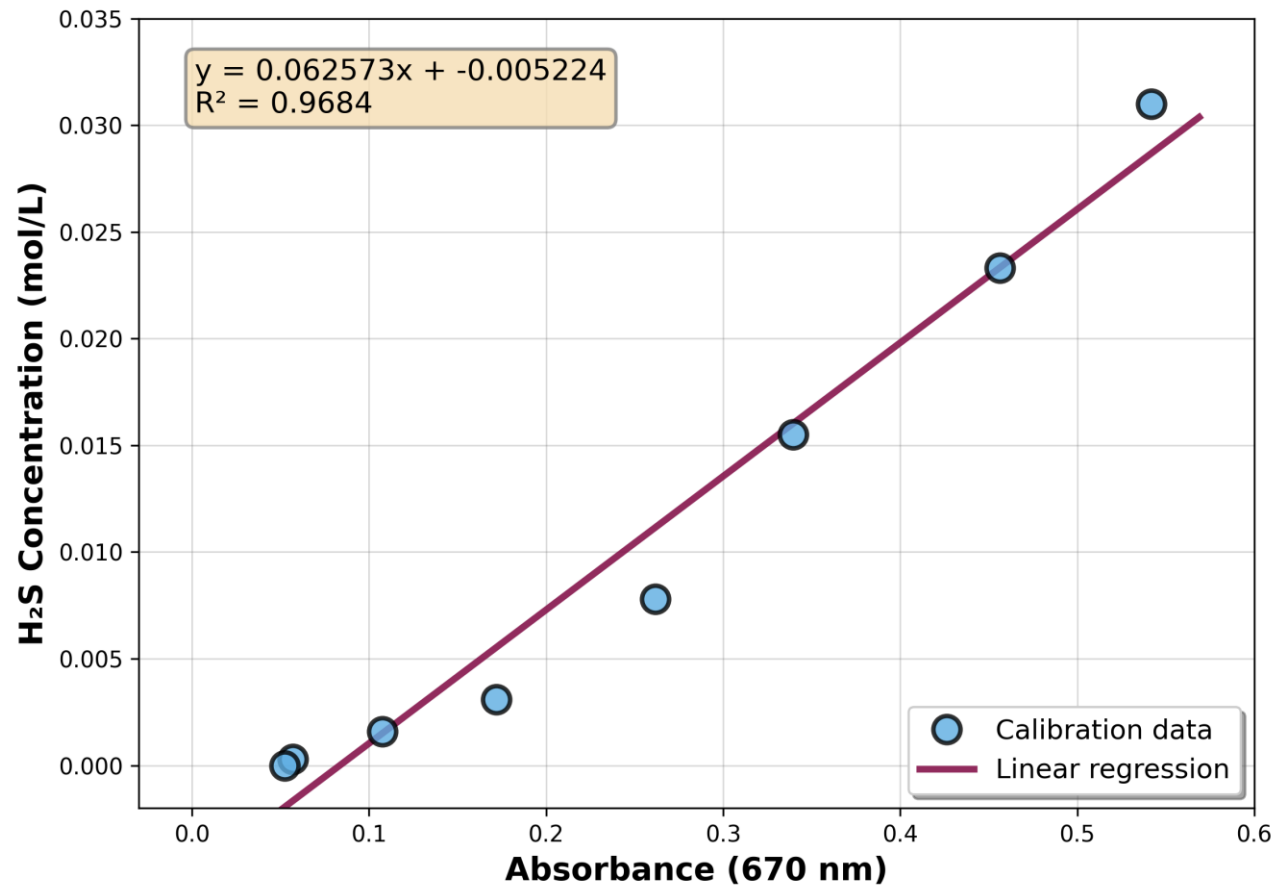
Methane Calibration Data

Standard	Concentration (%)	Peak Area
Std 1	20.0	16463057
Std 2	10.0	10754269
Std 3	5.0	4136908
Std 4	2.0	1119737
Air	0.0	0

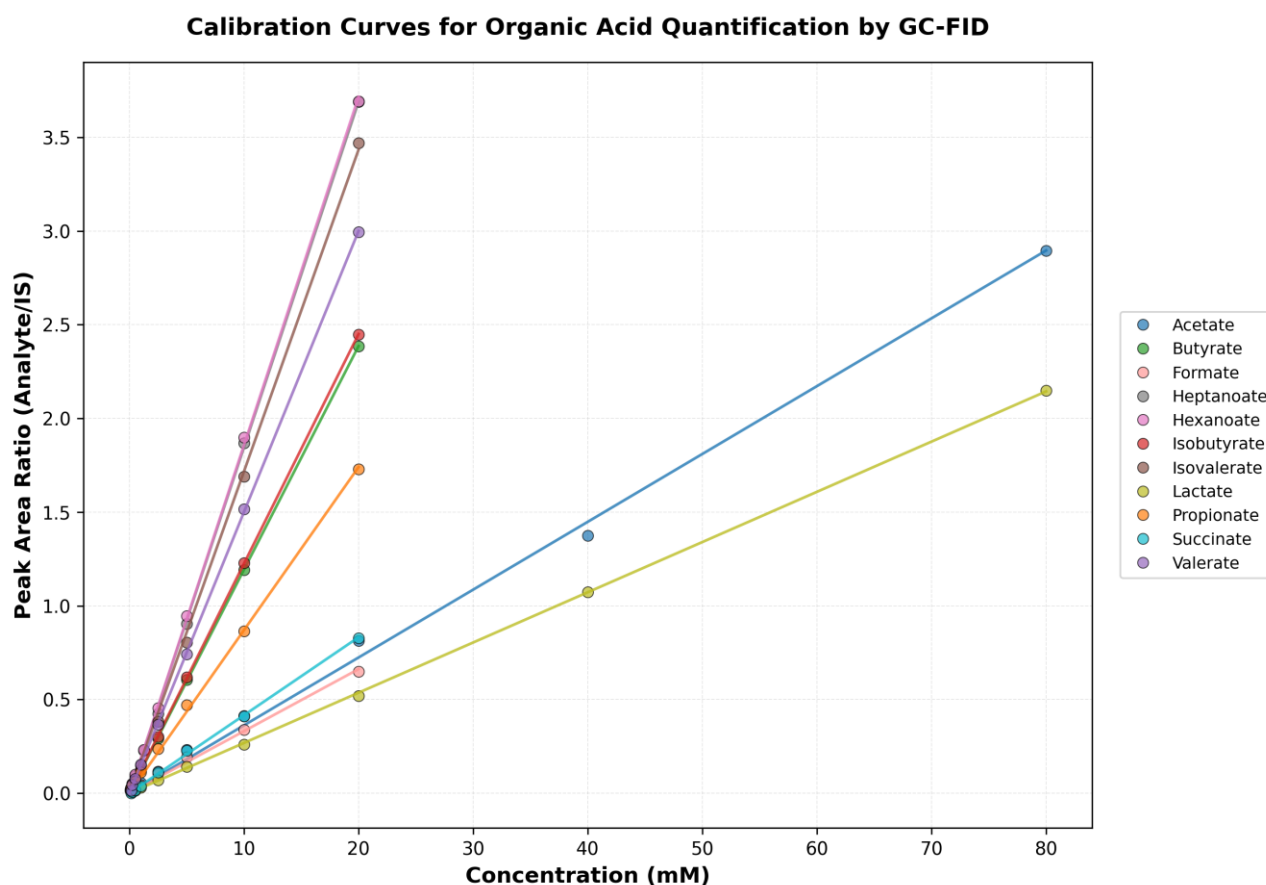
Calibration equation: $y = 173,589,812.93x + 1,664,676.04$
 $R^2 = 0.9047$, $n = 5$
(x = concentration as decimal, e.g., 0.20 for 20%)

Appendix D. Calorimetric quantification of sulphide production.

Hydrogen Sulphide Calibration Curve



Appendix E. Calibration curves for all organic acids quantified by GC-FID. Data points represent peak area ratios (analyte/internal standard) from triplicate injections at each concentration level. Lines show linear regressions. Internal standard: 4-methylvaleric acid (5 mM). Regression equations: Acetate: $y = 0.036x$, $R^2 = 0.998$; Butyrate: $y = 0.119x$, $R^2 = 0.100$; Formate: $y = 0.033x$, $R^2 = 0.997$; Heptanoate: $y = 0.185x$, $R^2 = 0.100$; Hexanoate: $y = 0.186x$, $R^2 = 0.100$; Isobutyrate: $y = 0.123x$, $R^2 = 0.100$; Isovalerate: $y = 0.172x$, $R^2 = 0.999$; Lactate: $y = 0.027x$, $R^2 = 0.100$; Propionate: $y = 0.087x$, $R^2 = 0.999$; Succinate: $y = 0.042x$, $R^2 = 0.999$; Valerate: $y = 0.150x$, $R^2 = 0.100$.



Appendix F. *Sugar composition of the different kumara and green banana extract mixture.*

Individual sugar levels are reported as percent mass/mass (% m/m); total sugar is expressed as grams per 100 g of sample (g/100 g). “<0.1” denotes values below the assay’s detection limit.

	Mixture								
	1 100/0	2 92.5/7.5	3 85/15	4 FreshAs	5 70/30	6 50/50	7 30/70	8 15/85	9 0/100
Fructose	1	1	1	0.9	0.9	0.7	0.6	0.5	0.3
Glucose	1.5	1.5	1.5	1.4	1.4	1.2	1.1	0.9	0.3
Lactose Anhydrous	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Lactose Monohydrate	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Maltose	36.8	22.2	24.9	22.6	23.6	14.5	15.8	7.7	<0.1
Sucrose	14.9	14.2	13.7	12.8	11.7	8.2	5.8	3.2	0.9
Galactose	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Total sugar	54.2	38.9	41.1	37.7	37.6	24.6	23.4	12.4	1.5

Appendix G. *Summary of alpha diversity metrics by timepoint and intervention group (mean $\pm$ SD for Shannon diversity, Simpson diversity, and observed genus richness at CF1, CF2, and CF4).*

Timepoint	Group	n	Shannon_mean	Shannon_SD	Simpson_mean	Simpson_SD	Observed_mean	Observed_SD
CF1	K	81	1.81	0.61	0.687	0.196	39.3	21.6
CF1	K+	82	1.73	0.56	0.692	0.186	37.4	21.9
CF1	C	86	1.7	0.66	0.655	0.215	36.6	18.8
CF2	K	81	2.38	0.57	0.801	0.137	58	24.5
CF2	K+	82	2.38	0.62	0.813	0.114	57.5	26.6
CF2	C	86	2.41	0.67	0.811	0.122	63.2	45.3
CF4	K	81	2.87	0.61	0.875	0.098	74	32.9
CF4	K+	82	2.92	0.6	0.881	0.082	76.6	31.6
CF4	C	86	2.88	0.63	0.881	0.09	74.3	38.4

Appendix H. *Post-hoc pairwise comparisons for alpha diversity metrics across complementary feeding timepoints (estimated marginal means contrasts for Shannon diversity, Simpson diversity, and observed richness).*

Metric	Contrast	Estimate	SE	df	t-ratio	p-value
Shannon	CF1 - CF2	-0.615	0.047	497.06	-13.03	4.05E-33
Shannon	CF1 - CF4	-1.157	0.054	591.49	-21.61	2.61E-76
Shannon	CF2 - CF4	-0.543	0.05	594.06	-10.92	6.29E-25
Simpson	CF1 - CF2	-0.125	0.012	499.35	-10.35	2.08E-22
Simpson	CF1 - CF4	-0.21	0.013	591.98	-15.54	1.89E-45
Simpson	CF2 - CF4	-0.085	0.013	591.09	-6.77	9.63E-11
Observed	CF1 - CF2	-20.25	2.21	496.82	-9.15	4.33E-18
Observed	CF1 - CF4	-41.84	2.52	591.17	-16.63	9.49E-51
Observed	CF2 - CF4	-21.59	2.34	594.06	-9.24	1.26E-18

Appendix I. *PERMANOVA results for genus-level beta diversity models (full model and reduced models testing timepoint and intervention group effects).*

Model	Term	df	Sum of Squares	R2	F statistic	p-value
Full model	Model	11	109230.37	0.057	4.05	<0.001
Full model	Residual	735	1803028.86	0.943	-	-
Full model	Total	746	1912259.23	1	-	-
Timepoint only	Timepoint	2	77657.94	0.041	15.75	<0.001
Timepoint only	Residual	744	1834601.28	0.959	-	-
Timepoint only	Total	746	1912259.23	1	-	-
Group only	Group	2	8016.2	0.004	1.57	1
Group only	Residual	744	1904243.03	0.996	-	-
Group only	Total	746	1912259.23	1	-	-