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Foods to optimise the infant colonic microbiome for our lifelong health and well-being

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

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Abstract

Diet is a key factor in shaping the composition and function of the colonic microbiota, which in turn has a strong influence on human health. The transition from breastmilk to solid foods (weaning) is critical for the maturation of colonic microbes. However, this period is often overlooked in diet-microbiota research, and the effects of many complementary foods on the infant colonic microbiota remain uncharacterised. This thesis aimed to identify foods that support the development of the colonic microbiota in weaning infants using a combined *in silico* and *in vitro* approach.

Computational modelling tools are emerging tools to rapidly and inexpensively study interactions between dietary compounds and gut microbial communities, generating preliminary insights that can be further evaluated experimentally. A metagenome-scale community metabolic model was employed to predict the effects of 89 foods commonly introduced to New Zealand weaning infants on infant colonic microbiota composition and function, using faecal sample data as a proxy. Foods with the greatest impact on short-chain fatty acid production when combined with breastmilk were identified, notably foods rich in fibre and polyphenols.

The effects of these identified foods on the colonic microbes of New Zealand weaning infants were further evaluated *in vitro* through food digestion and faecal fermentation. Foods were tested individually or combined with infant formula, other foods, or both, resulting in 53 samples. Blackcurrants, raspberries, and strawberries resulted in the greatest microbial production of acetate and propionate, also increasing the relative abundance of saccharolytic bacterial genera. Similarly, black beans, when combined with infant formula, resulted in the greatest butyrate production and increased the relative abundance of bacteria specialising in degrading complex plant polysaccharides. These foods are promising candidates for future intervention trials involving weaning infants.

The knowledge generated by this thesis can guide the design of future diet-colonic microbiota studies, ultimately contributing to improved nutritional recommendations and food formulations for weaning infants. Additionally, by assessing the predictive accuracy of the metagenome-scale community metabolic model, this thesis highlighted the limitations of current modelling approaches but also their potential in accelerating diet-colonic microbiota investigations when combined with traditional methodologies.

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"If I have seen further, it is by standing on the shoulders of giants." – Isaac Newton, 1675

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List of Abbreviations

AGORA: assembly of gut organisms through reconstruction and analysis repository

ANOVA: analysis of variance

BCFAs: branched-chain fatty acids

COBRA: constraint-based reconstruction and analysis

FBA: flux balance analysis

GEMs: genome-scale metabolic models

GIT: gastrointestinal tract

HSD: honestly significant difference

LFC: log-fold changes

MGCMs: metagenome-scale community metabolic models

MICOM: microbial community

ODE: ordinary differential equation

PERMANOVA: permutational multivariate analysis of variance

RoB2: revised Cochrane risk-of-bias tool for randomised trials

SCFAs: short-chain fatty acids

VMH: virtual metabolic human

List of Publications

Geniselli da Silva V, Roy NC, Smith NW, Wall C, Mullaney JA, McNabb WC. Mathematical models of the colonic microbiota: an evaluation of accuracy using *in vitro* fecal fermentation data. *Front Nutr* (2025) 12:1623418. doi: 10.3389/fnut.2025.1623418 [Chapter 7]

Geniselli da Silva V, Roy NC, Smith NW, Wall C, Mullaney JA, McNabb WC. Dietary patterns influencing the human colonic microbiota from infancy to centenarian age: a narrative review. *Front Nutr* (2025) 12:1591341. doi: 10.3389/fnut.2025.1591341 [Chapter 2]

Silva VG da, Mullaney JA, Roy NC, Smith NW, Wall C, Tatton CJ, McNabb WC. Complementary foods in infants: an in vitro study of the faecal microbial composition and organic acid production. *Food Funct* (2025) 16:3465-3481. doi: 10.1039/D5FO00414D [Chapter 6]

Geniselli da Silva V, Tonkie JN, Roy NC, Smith NW, Wall C, Kruger MC, Mullaney JA, McNabb WC. The effect of complementary foods on the colonic microbiota of weaning infants: a systematic review. *Crit Rev Food Sci Nutr* (2024) 16:1–16. doi: 10.1080/10408398.2024.2439036 [Chapter 3]

da Silva VG, Smith NW, Mullaney JA, Wall C, Roy NC, McNabb WC. Food-breastmilk combinations alter the colonic microbiome of weaning infants: an in silico study. *mSystems* (2024) 9:e00577-24. doi: 10.1128/msystems.00577-24 [Chapter 5]

Chapter 1: Introduction

Around 400 years before the Common Era, the Greek physician Hippocrates emphasised the connection between nutrition and health with his famous quote: "Let food be thy medicine and medicine be thy food." Subsequent research has supported this link and demonstrated that dietary compounds interact not only with the human body directly, but also indirectly via its resident microbes (1–3). The human gastrointestinal tract (GIT) is inhabited by a diverse microbial community that feeds on nutrients unabsorbed by the host and on endogenous nutrients, ultimately exerting a critical influence on host nutrition and physiology (4). The term gut microbiota broadly describes the entire microbial community within the GIT, while colonic microbiota specifically pertains to the microbes that reside in the colon or large intestine. Additionally, the colonic microbiome refers to the entire theatre of activity within the colon, including the collection of microbial genes (metagenome) but also other factors that define the colonic ecosystem, such as structural elements, microbial metabolites, signalling molecules, and environmental conditions.

Research on colonic microbiota is often conducted using faecal samples as a proxy (5). These studies have demonstrated that the colonic microbiota plays a crucial role in various host physiological processes, although the precise mechanisms by which colonic commensals (microorganisms inhabiting the human large intestine) influence host physiology remain poorly understood. For instance, alterations in colonic microbiota composition and function have been observed in individuals with non-communicable diseases, such as obesity, type 2 diabetes, and colorectal cancer, when compared with healthy individuals (6–8). Moreover, colonic microbes can influence host neurocognitive functions through the gut-brain axis (9).

Evidence suggests that resident microbes can compete with pathogens, thereby limiting their colonisation in the colon (10). Additionally, colonic microbes ferment dietary compounds, producing bioactive metabolites. Notably, short-chain fatty acids (SCFAs) are primarily generated from the fermentation of non-digestible carbohydrates, but also arise from amino acid metabolism (11,12). The most abundant SCFAs in the colon are acetate, propionate, and butyrate, which confer numerous host health benefits [see review (13)]. For instance, acetate supports gut epithelial barrier integrity, propionate contributes to regulating appetite, and butyrate serves as an energy source for colonocytes, among other benefits (14–16). Branched-chain fatty acids (BCFAs), produced from the fermentation of branched-chain amino acids, are another group of microbial metabolites of interest (11). Although their effects on human physiology are not fully understood, BCFAs are considered biomarkers of

protein fermentation, which also generates potentially harmful metabolites like ammonia, hydrogen sulphide, and nitrogen derivatives (17).

Observational studies have demonstrated that multiple factors influence the colonic microbiota, including host-intrinsic characteristics such as age and health status, as well as lifestyle conditions like diet, sanitation, geographic location, and antibiotic use (4,18–20). Consequently, the composition of the colonic microbiota is dynamic and personalised, varying within the same individual over time (intra-individual) and between different individuals (inter-individual) (21,22). As a result, defining biomarkers for a balanced microbiota versus an unbalanced state is a major challenge. However, colonic microbes are functionally redundant, and healthy individuals tend to share similar colonic microbial gene functions, despite differences in taxonomic composition (4,23,24). Therefore, researchers often focus on the function of colonic microbiota rather than its composition, as this approach is more informative from a host health perspective.

Diet is a key factor under host control that influences the colonic microbiota, rapidly altering its composition and function (25). The number of investigations assessing the interactions between dietary compounds and the colonic microbiota has recently increased. However, most of these investigations have focused on adults, and knowledge gaps remain regarding the effects of dietary choices on infants' colonic microbes during the transition from breastmilk to solid foods (weaning). Longitudinal studies have demonstrated that weaning is crucial for the maturation of the colonic microbiota, as it potentially establishes long-lasting microbial communities that can influence the development of diseases in infancy and later life (26–29).

The interaction between dietary compounds and colonic microbes is typically evaluated using animal models, human trials, or *in vitro* food digestion and faecal fermentation. While these approaches are indispensable in advancing our understanding of how dietary compounds affect colonic microbes and subsequent host health outcomes, they are often limited by resource constraints and ethical considerations (30). Therefore, complementary methodologies have been proposed to accelerate microbiota research, including mathematical models (31). Modelling tools offer promising avenues for preliminary investigations: by using existing data, they can rapidly and inexpensively generate hypotheses *in silico*, which can then be further evaluated experimentally.

However, only a few studies have employed mathematical models to explore how dietary choices influence the colonic microbiota during weaning (32,33), and significant knowledge gaps remain regarding the most effective complementary feeding strategies to support colonic microbes in this decisive stage of life. In this context, this thesis combined traditional *in vitro* food digestion and

fermentation assays with mathematical modelling to investigate a critical yet often overlooked topic in colonic microbiota research: the effects of complementary foods on the composition and function of the colonic microbiota in weaning infants.

1.1. Thesis structure

The literature review highlights the need for a better understanding of how dietary choices influence the colonic microbiota of weaning infants, as well as the potential of mathematical models to provide insights into interactions between dietary compounds and colonic microbes. In alignment with this, Chapter 3 systematically evaluates clinical evidence on the effects of complementary foods on the colonic microbiota during weaning. Given the limited number of studies on this topic, the primary goal of this thesis was to employ a combined *in vitro* and *in silico* approach to characterise the effects of common foods on the colonic microbiota of weaning infants, aiming to identify foods that support the adequate development of the infant microbiota.

To determine the most appropriate modelling tool for this task, Chapter 4 presents a qualitative assessment of various modelling strategies. Chapter 5 describes the *in silico* screening of the effects of complementary foods on the colonic microbiota of New Zealand weaning infants, focusing on microbial function. Foods and food combinations with the greatest predicted influence on SCFA production were identified and then evaluated *in vitro*, as reported in Chapter 6. An additional objective of this thesis was to assess the predictive accuracy of the modelling tool in identifying foods that support the infant colonic microbiota *in vitro*. Chapter 7 compares *in silico* predictions with *in vitro* measurements, offering experimental validation. The structure of this thesis is illustrated in Figure 1.1.

Thesis structure

Chapter 2

Literature review

- Influence of dietary patterns on the human colonic microbiota across different life stages
- Aiming to identify knowledge gaps and research opportunities on the study of the interaction between dietary compounds and colonic microbes

Chapter 3

Systematic review

- On the effect of complementary foods on the colonic microbiota of weaning infants
- Aiming to identify foods supporting the development of the infant colonic microbiota

Chapter 4

Assessment of modelling tools

- Qualitative evaluation of three modelling strategies and five different models
- Aiming to identify the most appropriate modelling tool

Chapter 5

***In silico* simulations**

- Predicted effects of more than 150 commonly weaning foods and food combinations on the infant colonic microbiota
- Aiming to identify foods promoting the greatest impact on SCFA production

Chapter 6

***In silico* experiments**

- Digestion and faecal fermentation of the 13 identified foods with the greatest impact on the infant colonic microbial function *in silico*
- Foods tested individually or combined with infant formula, other foods, or both
- Aiming to identify foods promoting the growth of saccharolytic bacteria and SCFA production

Chapter 7

Modelling predictive accuracy assessment

- Evaluation of the agreement between *in silico* predicted and *in vitro* measured SCFA production
- Aiming to provide experimental validation of the model's predictions

Chapter 8

General discussion

- Summary of the key findings and conclusions
- Acknowledgement of the thesis limitations and future research perspectives

Figure 1.1. Overview of the thesis structure.

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We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Vitor Geniselli da Silva
Name and title of main supervisor:	Professor Warren McNabb
In which chapter is the manuscript/published work?	Chapter 2

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Vitor Geniselli da Silva (student): Conceptualisation, Formal Analysis, Visualisation, Writing – original draft, Writing – review & editing.
Nicole Roy (co-supervisor): Conceptualisation, Funding acquisition, Supervision, Writing – review & editing.
Nick Smith (co-supervisor): Supervision, Writing – review & editing.
Clare Wall (co-supervisor): Funding acquisition, Supervision, Writing – review & editing.
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Chapter 2: Literature review[1]

2.1 Abstract

Our dietary choices not only affect our body but also shape the microbial community inhabiting our large intestine. The colonic microbiota strongly influences our physiology, playing a crucial role in both disease prevention and development. Hence, dietary strategies to modulate colonic microbes have gained notable attention. However, most diet-colonic microbiota research has focused on adults, often neglecting other key life stages, such as infancy and older adulthood. In this narrative review, we explored the impact of various dietary patterns on the colonic microbiota from early infancy to centenarian age, aiming to identify age-specific diets promoting health and well-being by nourishing the microbiota. Diversified diets rich in fruits, vegetables, and wholegrains, along with daily consumption of fermented foods, and moderate amounts of fish and lean meats (two to four times a week), increase colonic microbial diversity, the abundance of saccharolytic taxa, and the production of beneficial microbial metabolites. Most of the current knowledge of diet-microbiota interactions is limited to studies using faecal samples as a proxy. Future directions in colonic microbiota research include personalised *in silico* simulations to predict the impact of diets on colonic microbes. Complementary to traditional methodologies, modelling has the potential to reduce the costs of colonic microbiota investigations, accelerate our understanding of diet-microbiota interactions, and contribute to the advancement of personalised nutrition across various life stages.

2.2 Introduction

The human GIT hosts a diverse and dynamic microbial community, including bacteria, archaea, fungi, and viruses, which play key roles in host health and well-being. Most microbes are found in the large intestine or colon, with an estimated concentration of 10^{11} cells/mL (34), although estimated colonic microbial abundance can vary depending on the sample site, analytical method used, and host physiology. Faecal samples are non-invasive proxies to study the relationship between colonic microbes and human health, particularly microbial composition and function. These analyses have revealed the impact of colonic commensals on host nutrition, metabolism, and the immune and neurological systems (35–41). However, many crucial aspects of this relationship remain unknown.

[1] This chapter has been published as Geniselli da Silva V, Roy NC, Smith NW, Wall C, Mullaney JA, McNabb WC. Dietary patterns influencing the human colonic microbiota from infancy to centenarian age: a narrative review. *Front Nutr* (2025) 12:1591341. doi: 10.3389/fnut.2025.1591341

Defining what constitutes a healthy colonic microbiota composition and function is an ongoing challenge (42,43).

One of the major challenges in investigating the colonic microbiota is its substantial compositional variability, which occurs both within the same individual over time and between individuals (22,44). This variation limits our ability to predict how each individual's microbiota may respond to interventions and their subsequent impact on host health. Colonic microbes interact with one another and the host, forming a dynamic network influenced by various individual and environmental factors. These factors mainly include dietary habits, host health status, genetics, age, gender, geographical location, lifestyle behaviours, and antibiotic use (45).

Among the factors under host control, diet is key. Dietary compounds not absorbed by the small intestine reach the colon, are fermented by colonic microbes, and produce metabolites that influence host physiology. Dietary interventions can rapidly alter the composition and function of the colonic microbiota (21). For instance, non-digestible carbohydrates have prebiotic properties: their consumption promotes the growth of saccharolytic microorganisms and increases the production of beneficial SCFAs (46,47). In contrast, a diet lacking non-digestible carbohydrates and with excessive intake of protein and fat from animal origin increases the abundance of pathogens and the production of potentially deleterious molecules (48–50). Recently, phytochemicals, bioactive compounds found in plants, have attracted attention for their potential prebiotic effect, as well as antioxidant and anti-inflammatory properties (51).

However, the long-term effect of dietary patterns on colonic microbes remains unclear, including the persistence of diet-induced alterations in the colonic microbiota when changing dietary exposures. Furthermore, diet-colonic microbiota research has predominantly focused on adults (ages 18 to 65), limiting our understanding of how diet influences colonic microbes in other life stages. Notably, dietary patterns during infancy (under three years) and older adulthood (over 65 years) differ from those in adulthood, likely influencing the composition and function of the colonic microbiota and highlighting the need for more investigations specific to these age groups (52,53). Literature reviews on the influence of diet on colonic microbiota across the human lifespan are scarce and often do not fully cover all life stages (54,55). Considering these knowledge gaps, this narrative review examined how different dietary patterns influence the human colonic microbiota across early infancy, weaning, adulthood, older adulthood, and centenarian age. This review aimed to identify age-appropriate diets for microbiota modulation, providing insights into promoting health and well-being.

2.3 Search strategy

A narrative review was conducted to evaluate the impact of dietary patterns on the human colonic microbiota across infancy (under three years), adulthood (between 18 and 65 years), older adulthood (between 65 and 100 years), and centenarian age (over 100 years). Articles were primarily identified through searches in the PubMed and Google Scholar databases using the terms "gut microbiota", "diet", and "infant OR adult OR older adult OR centenarian". Additional records evaluating the effect of dietary patterns on the colonic microbiota were identified by replacing "diet" with terms referring to specific dietary patterns (e.g., "gut microbiota" AND "western diet" AND "infant OR adult OR older adult OR centenarian"). Only studies involving humans and published in English were identified. No specific inclusion and exclusion criteria were applied. Articles were prioritised based on their publication date, giving preference to the most recent studies. When available, meta-analyses, systematic reviews, and randomised controlled trials were prioritised.

2.4 Principal colonic microbes and produced microbial metabolites

The colonic microbiota is dynamic, and its composition varies throughout life. Its development is proposed to have an initial phase until the first 14 months of postnatal life, followed by a transitional period between 15 and 30 months, reaching stability after 31 months (56). Multiple factors influence the composition and function of colonic commensals over life, resulting in a unique profile of colonic microbes for each individual (45,56). Despite this variability, certain taxa and gene functions prevail in healthy adults based on faecal data, suggesting the existence of core microbial functional groups (4,57). The principal microbial taxa in the colonic microbiota and their associated microbial metabolites are summarised in Supplementary Table 2.1 and Supplementary Table 2.2, respectively.

To date, the mechanisms by which colonic microbes influence host health remain unclear. Under favourable conditions, colonic microbes may support host homeostasis by competing with pathogens for resources, producing anti-microbial metabolites, and modulating host immune responses (58,59). Conversely, disruptions in the colonic microbiota can favour the growth of pathogens and the production of pro-inflammatory metabolites, compromising the integrity of the colonic epithelial barrier. This allows luminal molecules and microbes to enter the bloodstream, potentially triggering excessive host immune responses. Over time, these responses may lead to a chronic inflammatory state and increased disease risk. Additionally, it is plausible that certain diseases may alter the colonic environment, disrupting the colonic microbiota and creating a vicious cycle that perpetuates disease.

2.5 Influence of dietary patterns on colonic microbes

2.5.1 Infancy

During the first months of postnatal life, the colonic microbiota composition is mainly affected by the gestational age, mode of delivery, and type of feeding (human milk versus infant formula) (56,60,61). Systematic literature reviews suggest that full-term pregnancy, natural birth, and breastfeeding provide greater opportunities for the infant colonic microbiota to thrive (62,63). In contrast, pre-term pregnancy, C-section, and formula-feeding are associated with disruptions of the microbial community (56,61). Regarding feeding in early infancy, breastmilk contains oligosaccharides that promote the growth of the genus *Bifidobacterium* and support beneficial microbial cross-feeding interactions (64,65). In addition, breastmilk is not sterile and contains microbes that can colonise the infant's colon, as well as bioactive compounds like antibodies and lactoferrin, which reduce pathogen colonisation (66,67).

In contrast, infant formulas are predominantly made with bovine milk, have higher protein content, and are often supplemented with fructooligosaccharides and galactooligosaccharides to provide prebiotic effects. While infant formulas meet the nutritional requirements for infant development, they fail to mimic the bifidogenic effect of human milk. Systematic reviews have found that, compared to breastfed infants, formula-fed infants exhibit a lower faecal abundance of the genus *Bifidobacterium*, an increased abundance of pathogens, and a higher expression of microbial genes associated with amino acid metabolism (68,69).

Exclusive breastfeeding is recommended for the first six months of life (70). Nutrients from complementary foods reaching the colon unabsorbed mature the infant’s colonic microbiota towards a more adult-like configuration (Table 2.1). Non-digestible carbohydrates are preferentially fermented by colonic microbes, producing SCFAs and gases (Supplementary Table 2.2), and their availability in the colon limits the fermentation of other dietary compounds (71,72). *In vitro* evidence suggests that carbohydrate fermentation primarily occurs in the proximal colon, while other macronutrients are metabolised in the distal colon (73,74) (Figure 2.1). Notably, SCFAs are molecules that exert well-documented health benefits, and their reduced faecal levels are frequently observed in preterm infants, adults with autoimmune, metabolic, and gastrointestinal diseases, and older adults with neurological disorders (46,75–78). However, excessive fermentation of rapidly fermented fibres can increase gas production in individuals with functional gastrointestinal disorders, leading to bloating, pain, and discomfort (79).

Table 2.1. Impact of complementary foods on the colonic microbiota of weaning infants.

Food category	Food intervention trials	Observational studies	*In vitro* faecal fermentations	References
Meats (e.g., beef, pork)	↑Clostridiales, *Clostridium XIVa* ↓*Enterobacteriaceae* ↑alpha diversity (Chao1 and Shannon indexes)	↓*Bacteroides* ↑alpha diversity (Shannon index)	Not evaluated	(80–85)
Dairy (e.g., yoghurt, cheese)	Not evaluated	↓*Bacteroides, Clostridiaceae* ↑alpha diversity (Shannon index)	↑*Bifidobacterium, Lactobacillus, Enterococcaceae* ↓*Enterobacteriaceae* ↑acetate, propionate, butyrate	(80,82,86,87)
Infant cereals (e.g., wholegrain and refined cereals)	↑Bacteroidales, *Bacteroides* ↓*Enterobacteriaceae, Escherichia-Shigella*	↑alpha diversity (richness and Shannon index)	↑*Bacteroidaceae, Prevotellaceae, Ruminococcaceae* ↑acetate	(81,85,88–90)
Fruits and vegetables (e.g., apple, berries, carrot)	Not evaluated	↑alpha diversity (Shannon index)	↑*Bifidobacterium, Lactobacillus, Streptococcus, Ruminococcus, Faecalibacterium* ↓*Clostridium, Enterobacteriaceae* ↑acetate, propionate	(86,90,91)
Sweets (e.g., cakes, desserts, chocolates)	Not evaluated	↓*Bifidobacterium, Clostridium cluster IV*	Not evaluated	(3)

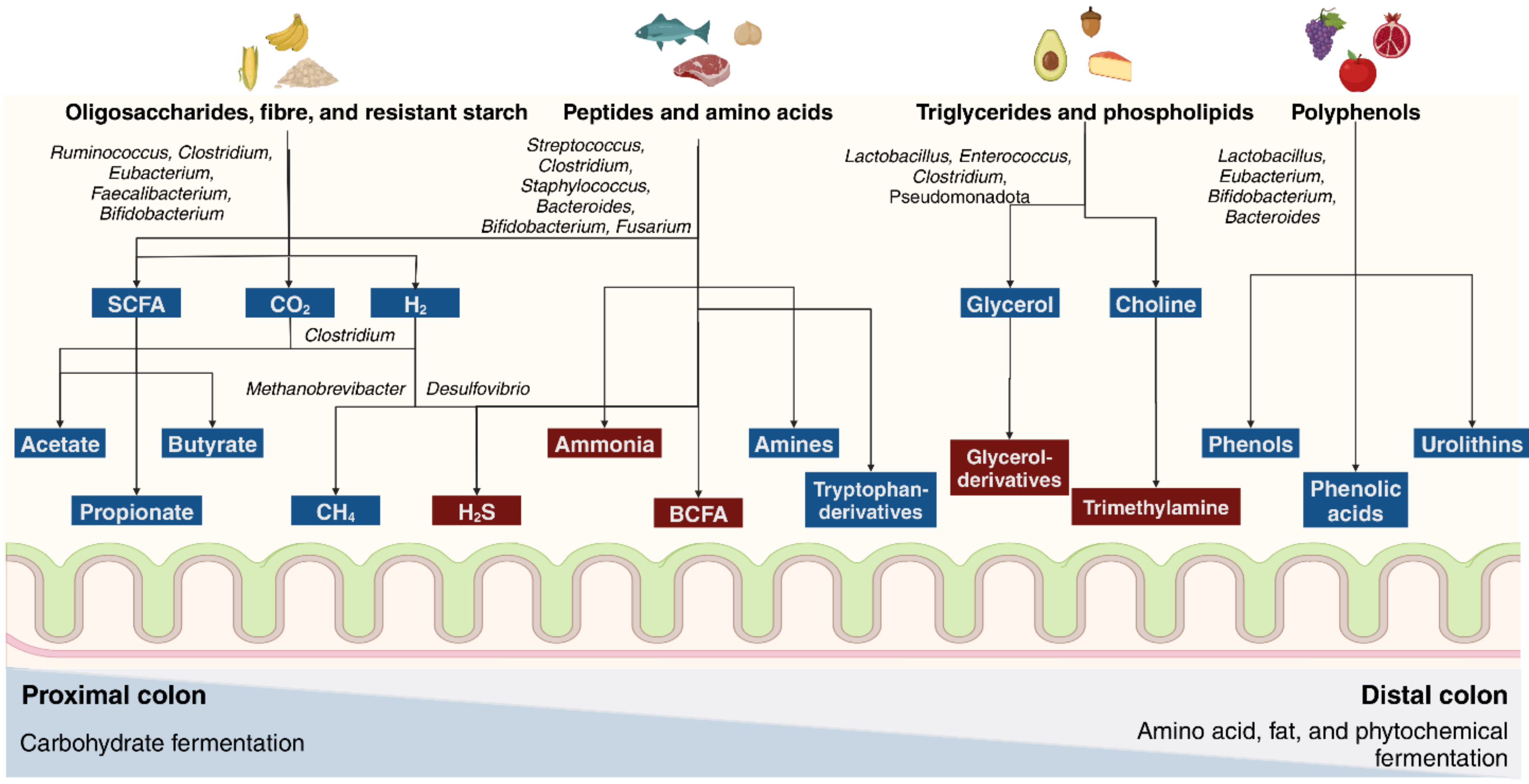

Figure 2.1. Fermentation of dietary compounds by the colonic microbiota. Metabolites in blue benefit the host, while those in red are potentially deleterious. A decrease in carbohydrate fermentation is observed from the proximal to the distal colon, mainly producing SCFAs and gases. On the other hand, the fermentation of amino acids, fats, and phytochemicals increases from the proximal to the distal colon. Microbial fermentation of amino acids produces SCFAs, BCFAs, biogenic amines, hydrogen sulphide (H_2S), and ammonia. Fermentation of dietary fats is involved in trimethylamine production, whereas the fermentation of polyphenols leads to the generation of multiple bioactive molecules.

Longitudinal evidence demonstrated that colonic microbial diversity and stability increase during weaning, and genes associated with carbohydrate degradation, vitamin biosynthesis, and production of SCFAs are enriched (3). The abundances of the genera *Bifidobacterium* and *Lactobacillus* decrease, while the phyla Bacillota and Bacteroidota and the genera *Clostridium* and *Bacteroides* are enriched (3,26,92). Furthermore, the dominant fungal species *Debaryomyces hansenii* is replaced by *Saccharomyces cerevisiae* and the viral richness and diversity decrease (93,94).

Diet-induced microbiota changes during infancy may have long-lasting effects, influencing susceptibility to diseases later in life (27–29). Weaning is crucial for the colonic microbiota development, influencing health and well-being later in life. Although many studies have characterised the influence of feeding type (breastmilk versus infant formula) on the development of the infant colonic microbiota (56,61,68,69), the impact of complementary foods on colonic microbes of weaning infants remains underexplored. A recent systematic review of interventional trials assessing the effects of complementary foods on faecal microbiota composition in weaning infants found that wholegrain cereals and meats increased the faecal abundance of SCFA-producing bacterial taxa and increased microbial richness (95). However, the review was limited to only seven clinical trials characterising the microbiota using 16S rRNA gene sequencing, highlighting the scarcity of food interventions in this field.

Another notable knowledge gap is the limited understanding of how maternal diet influences the infant's colonic microbiota. Maternal intake of plant-based dietary compounds during pregnancy has been reported to influence the neonatal faecal microbiota (96), while increased maternal dietary diversity and consumption of fermented foods during pregnancy were associated with lower faecal alpha diversity in infants (97). Additionally, the mother’s diet affects the microbial and nutritional composition of breastmilk, which may impact the colonic microbes of breastfed infants (98). A scoping review identified associations between maternal dietary patterns and infant faecal microbiota composition. Maternal consumption of seafood, fermented dairy, fruits and vegetables, and nuts was linked to an increased abundance of beneficial taxa in the infant faecal microbiota (63). Conversely, maternal intakes of artificial sweeteners and high-fat diets were associated with negative alterations in the infant microbiota (63).

Artificial sweeteners, along with emulsifiers, thickeners, stabilisers, preservatives, and colourants, are chemicals added during food production to extend shelf-life or modify sensory properties. Although little is known about the effect of food additives on colonic microbes, a systematic review of randomised controlled trials, encompassing participants from infants to older adults, found that maltodextrin impacts the composition of the faecal microbiota, notably the

abundance of the genera *Lactobacillus* and *Bifidobacterium* (99). Furthermore, another systematic review, including *in vivo* and *in vitro* studies, reported that exposure to colourants, sweeteners, emulsifiers, and preservatives is linked with perturbations in the colonic microbiota and adverse health effects (100). These findings highlight the need for further research and support recommendations to reduce the consumption of ultra-processed foods (101).

Another topic that requires further research is the interplay between micronutrients and the infant colonic microbiota. Vitamins are mostly absorbed in the upper small intestine, while minerals typically have lower bioavailability and reach the colon in greater amounts (102). Colonic microbes influence micronutrient levels by synthesising vitamins and modulating mineral absorption (103,104). In turn, micronutrients affect their composition and function. For instance, a systematic review of observational human studies reported that vitamin B12 supplementation may increase the alpha diversity of the faecal microbiota in adults and older adults, but not in infants (105). In contrast, iron supplementation in infants, commonly used to combat malnutrition, may increase the faecal abundance of pathogens like the *Escherichia-Shigella* group, as observed in randomised controlled trials profiling the microbiota using 16S rRNA gene sequencing (106,107).

Current nutritional guidelines recommend introducing infants to a diverse range of complementary foods (101,108). Consistently, faecal microbiota observational studies have shown that greater dietary diversity during weaning is associated with increased microbial diversity and richness (80,109). Importantly, dietary diversity in infancy is essential to prevent nutrient deficiencies often linked with monotonous diets, which can contribute to perturbations in the colonic microbiota and subsequent elevated risk of disease (110). In line with these recommendations, various fruits, vegetables, and wholegrains are recommended for weaning infants (101,108,111). These foods are sources of vitamins, minerals, complex carbohydrates and phytochemicals, leading to beneficial alterations in colonic microbes (Table 2.1). Phytochemicals have recently attracted attention in colonic microbiota research for their ability to support the growth of beneficial taxa and inhibit pathogens (112), in addition to having anti-inflammatory and antioxidant properties. Polyphenols are the most prominent phytochemicals, with less than 10 % absorbed in the small intestine and the majority reaching the distal colon, where they may be converted into more bioactive and bioavailable molecules by colonic microbes (113,114). This microbial conversion is associated with various health benefits to the host, such as cardioprotective and anti-metabolic syndrome effects, as observed in adults (115,116).

Wholegrain infant cereal interventions in weaning infants increased the faecal abundance of SCFA-producing bacteria, such as the genus *Bacteroides*, while reducing the abundance of pathogens

belonging to the family *Enterobacteriaceae*, as determined using 16S rRNA gene sequencing (81,88). Consistently, observational trials profiling the faecal microbiota of weaning infants by 16S rRNA sequencing, positively correlated the intake of complex carbohydrates with increased faecal alpha diversity and abundance of *Lachnospiraceae* and *Ruminococcaceae* families (82,90). These findings were further confirmed by a longitudinal trial that used shotgun metagenomic sequencing to evaluate the composition of the infant faecal microbiota during the first year of life. The study highlighted the key role of complex carbohydrates in supporting the maturation of the colonic microbiota in weaning infants, as evidenced by an increased faecal alpha diversity and abundance of SCFA-producing bacterial genera (85). *In vitro* faecal fermentations using inoculum from weaning infants reported that wholegrain cereals and numerous fruits and vegetables promoted the growth of the *Ruminococcaceae* and *Prevotellaceae* families to the detriment of the *Enterobacteriaceae* family, leading to acetate production after 24 hours of fermentation (86,89,91). An *in silico* investigation recently identified berries as promising candidates for increasing the production of acetate and propionate by the infant's faecal microbiota when consumed with breastmilk (117). Similarly, a 24-hour *in vitro* fermentation study using weaning infant inoculum found that berries increased acetate and propionate production (118).

Nutritional guidelines also recommend moderate consumption (one serving per day) of lean meats and fermented dairy for infants to meet protein and micronutrient requirements (101,108,111). Introducing protein-rich foods to infants increases their colonic availability of amino acids, as their protein digestion and absorption are not yet fully developed (119). In the colon, the fermentation of unabsorbed amino acids and small peptides produces SCFAs (approximately 30 % of protein mass is converted to SCFAs), BCFAs, tryptophan derivatives, and biogenic amines, but also pro-inflammatory metabolites, such as hydrogen sulphide (H_2S) and nitrogen derivatives (11,120). Consistently, clinical trials demonstrated that introducing protein-rich foods to weaning infants increases their faecal abundance of SCFA-producing taxa. For instance, pureed beef interventions in infants increased faecal abundance of *Clostridium XIVa* members, reduced the abundance of the *Enterobacteriaceae* family, and increased alpha diversity, as determined using 16S rRNA sequencing (81,83,84). Similarly, an observational study profiling the infant faecal microbiota using 16S rRNA amplicon sequencing reported a positive association between meat intake and increased faecal alpha diversity (80). For dairy products, their consumption by weaning infants was negatively associated with the abundance of the *Bacteroides* genus and the *Clostridiaceae* family, according to an observational study that profiled the faecal microbiota of infants aged 6 to 24 months using 16S rRNA

gene sequencing (87). In turn, the fermentation of bovine milk for ten hours using faeces from infants at weaning age increased the abundance of taxa from the *Bifidobacterium* genus *in vitro* (86).

An observational trial profiling the microbiota of weaning infants by 16S rRNA amplicon sequencing reported that dietary patterns characterised by the consumption of foods rich in protein and rich in fibre, such as meats, cheese, and wholegrain bread, have been positively correlated with the increased faecal abundance of taxa from the *Lachnospiraceae* family, decreased abundance of taxa from the *Bifidobacteriaceae* family, and increased microbial alpha diversity (80). On the other hand, the consumption of diets rich in fat but low in carbohydrates has been associated with reduced faecal levels of SCFAs and increased abundance of pathogens in post-weaning infants (121). Dietary fats are normally well-digested and absorbed, with less than 5 % of ingested fat reaching the colon (122). Importantly, their influence on colonic microbes depends on their type: as concluded by a systematic review of adult studies, saturated fatty acids are associated with reduced colonic microbiota richness and diversity, whereas polyunsaturated fatty acids do not exhibit this effect (123). Furthermore, certain fatty acids have anti-microbial activity and can reduce the abundance of pathogenic taxa (124). In this context, the Mediterranean diet, rich in polyunsaturated fatty acids from olive oil, was recently adapted to children (aged 3 and older) to help prevent obesity and cardiometabolic diseases in infancy and later life. The adapted diet emphasises the daily consumption of fruits, vegetables, legumes, nuts, wholegrains, olive oil, and dairy, along with weekly consumption of fish, eggs, and meat (125).

2.5.2 Adulthood

The complete maturation of the colonic microbiota is believed to occur around three years old (126). Nevertheless, evidence suggests its continued functional and compositional development during childhood and adolescence (18,127). Compared to other life stages, more is known about the influence of dietary patterns on the colonic microbiota of adults. This topic has been extensively reviewed in recent publications (1,128,129). Therefore, only the impact of common diets on the colonic microbes of adults is briefly discussed here (Table 2.2). However, it is important to acknowledge that definitions of dietary patterns vary considerably across the literature, particularly in relation to the frequency of specific food consumption (130), which may influence the colonic microbiome and complicate comparisons across studies.

Observational trials reported that Western diets (rich in fat, protein of animal origin, and simple sugars, and low in complex carbohydrates) were associated with increased faecal abundance of the genera *Alistipes*, *Bacteroides*, and *Penicillium*, and fewer methanogenic archaea, resulting in higher

expression of microbial genes related to bile acid metabolism, amino acid fermentation, and production of BCFAs (25,131,132). Adherence to this dietary pattern is linked with increased susceptibility to chronic inflammatory, intestinal, metabolic, neurological, and cardiovascular diseases (133). Consistently, diets high in protein but low in carbohydrates (30 % protein, 35 % carbohydrate, and 35 % fat as calories) and high-fat diets (40 % fat as calories) have been linked with perturbations in the colonic microbiota and adverse health outcomes in adults (17,49,134). These findings are supported by a systematic review concluding that increased intake of saturated fatty acids, predominantly found in animal-based foods, is associated with reduced colonic microbiota richness and diversity in adults (123). Recently, diets rich in industrially processed food and low in fibre from plants have been associated with colonic microbiota perturbations, an altered profile of plasma metabolites, and an increased risk of disease development, including metabolic disorders and colorectal cancer (50,135).

On the other hand, diets predominantly based on plants, like vegan and vegetarian diets, are associated with a higher faecal abundance of saccharolytic taxa and enrichment of microbial genes involved in complex carbohydrate degradation and production of SCFAs (25,132). Notably, colonic microbiota alterations in adults due to adherence to plant-based diets have been linked to protective effects against metabolic and cardiovascular diseases (136). Recently, adherence to a plant-based African heritage diet and consumption of a traditional fermented beverage were associated with lower levels of circulating inflammatory biomarkers in healthy adults (137). These effects may be explained by the high content of non-digestible carbohydrates and polyunsaturated fatty acids in these diets. A meta-analysis concluded that non-digestible carbohydrates support the growth of saccharolytic commensals and stimulate beneficial microbial cross-feeding interactions in adults, producing SCFAs (12). Additionally, the consumption of polyunsaturated fatty acids has been linked with beneficial alterations in colonic microbiota composition in both observational and interventional trials (138,139).

However, a cross-sectional study found no differences in the faecal concentration of SCFAs and BCFAs between vegans and omnivores (who consumed at least three servings of meat per week) (140). Furthermore, omnivores had higher faecal alpha diversity measured by the Shannon index (140). Consistent with these findings, flexitarian diets (primarily plant-based but including occasional meat, fish, and dairy) have been associated with increased faecal alpha diversity and abundance of beneficial taxa in adults (2,141,142). These findings suggest that moderate consumption of animal products (e.g., at least three times per week, as indicated by the studies cited) alongside a plant-rich diet may offer additional benefits to the colonic microbiota.

Table 2.2. Impact of various dietary patterns on the adult colonic microbiota.

Diet	Definition	Microbial composition	Microbial function	Observed effect of diet on host health	References
Western diet	High intake of protein, fat, and sugars. Low consumption of complex carbohydrates	↑*Bacteroides, Alistipes, Penicillium* ↓*Methanobrevibacter*	↑Amino acid and bile acid metabolism and production of nitrogen derivatives and BCFAs	Adherence to a Western diet increased risk factors for metabolic and cardiovascular diseases compared to a prudent dietary pattern in healthy adults (n = 42)	(25,131–133)
Plant-based diet	Exclusive or predominant consumption of plant-based foods (e.g., vegan or vegetarian diets)	↑*Prevotella, Faecalibacterium, Ruminococcus, Candida, Methanobrevibacter*	↑Degradation of complex carbohydrates and SCFA production	A meta-analysis of observational trials found that adherence to a vegetarian diet was linked with a lower risk of cardiovascular diseases and cancer in healthy adults (n = 47757 and 38033, respectively)	(25,131,132,143,144)
Mediterranean diet	High consumption of fruits, vegetables, and olive oil. Moderate of fish, dairy, and lean meats	↑*Bacteroides, Prevotella, Faecalibacterium*	↑Acetate and propionate	A meta-analysis linked adherence to the Mediterranean diet with a reduction of all-cause mortality in adults and older adults (n = 225600)	(145,146)
Fermented foods diet	High consumption of fermented foods (e.g., yoghurt, kimchi, cheese, bread)	↑*Lactobacillus* ↑alpha diversity	↑conjugated linoleic acid	Decreased biomarkers of inflammation in healthy adults under a 10-week intervention with a fermented foods diet compared to baseline values (n = 18)	(147,148)
Low FODMAP diet	Low consumption of rapidly fermentable carbohydrates and polyols	↓*Bifidobacterium* No changes in microbial diversity	No changes in SCFA and BCFA production	Alleviates pain and discomfort in patients suffering from irritable bowel syndrome. May have negative health outcomes	(149,150)
Low-gluten diet	Low consumption of gluten-containing foods	↓*Bifidobacterium, Dorea, Veillonellaceae* No changes in microbial diversity	↓Degradation of carbohydrates	Alleviates symptoms in patients with celiac disease or gluten sensitivity. May have negative health outcomes	(151–153)
Ketogenic diet	High consumption of fat and protein, and restricted consumption of carbohydrates	↓*Bifidobacterium, Eubacterium, Faecalibacterium, Roseburia*	↓total SCFAs, acetate, butyrate	Alleviates symptoms in patients with epilepsy and induces fat loss. A systematic review suggested that adherence to Ketogenic diets may increase risk for obesity, type 2 diabetes, and depression	(154,154)

In this context, the Mediterranean diet is a prime example of a healthy dietary pattern. This diet is typical of countries around the Mediterranean basin, varying according to the region. It traditionally consists of a high consumption of fruits, vegetables, and olive oil (at least two servings per day), daily intake of dairy (two servings), along with a moderate intake of seafood and fish (two or more servings per week), and occasional consumption of lean meats (no more than two servings per week) (155). A recent systematic review of observational and interventional trials found that following the Mediterranean diet increased the alpha diversity of the colonic microbiota in adults (145). It also increased the abundance of the genera *Faecalibacterium*, *Prevotella*, and *Bacteroides* and the production of SCFAs, particularly acetate and propionate (145). Additionally, adherence to the Mediterranean diet has been associated with lower mortality rates (146). Similarly, consuming fermented foods, including yoghurt, kefir, kimchi, and sourdough bread, is encouraged to provide health benefits. Adults consuming diets enriched in fermented foods (at least three servings per week) showed an increased faecal abundance of various *Lactobacillus* species, as well as increased production of conjugated linoleic acid (147). Furthermore, the intake of fermented foods has been associated with increased faecal alpha diversity and lower levels of circulating cytokines (148).

These observations highlight the importance of a balanced and diversified diet to nourish the adult colonic microbiota, aligning with current nutritional recommendations (101). Conversely, restrictions on macronutrients, notably carbohydrates, are associated with perturbations in the colonic microbiota, raising concerns about the potential long-term deleterious effects of such restrictions (156). Restricted diets are typically recommended for managing pre-existing medical conditions. For instance, the low FODMAP diet, characterised by low consumption of rapidly fermentable carbohydrates, is recommended for individuals with disorders of gut-brain interaction (formerly known as functional gastrointestinal disorders) to alleviate discomfort (149). However, a systematic review and a meta-analysis of interventional studies concluded that this diet reduces the faecal abundance of the genus *Bifidobacterium* in adults with irritable bowel syndrome (150).

Similarly, gluten-free or low-gluten diets are recommended for individuals with coeliac disease or gluten sensitivity. In healthy adults, adherence to these diets has been associated with decreased faecal abundance of the genera *Bifidobacterium* and *Dorea* and the family *Veillonellaceae*, and decreased expression of microbial genes involved in carbohydrate degradation (151,152). The ketogenic diet is characterised by high fat intake (70 to 80 % of total energy), moderate protein consumption (10 to 20 % of total energy), and restriction of carbohydrates (less than 10 % of total energy). Variants of this diet are sometimes recommended for individuals with epilepsy (for example, the medium-chain triglyceride diet) or those following a rapid weight-loss strategy (157). A

systematic review concluded that this diet decreases the faecal abundance of the *Bifidobacterium* genus and potentially reduces the abundance of butyrate-producing genera, leading to reduced levels of acetate and butyrate (154). These alterations in the colonic microbiota may contribute to an increased risk of obesity, type 2 diabetes, and depression (154). Further evidence supports these adverse outcomes, as adherence to the ketogenic diet reduced glucose tolerance in healthy adults (158).

2.5.3 Older adulthood

The colonic microbiota remains stable during adulthood until it undergoes modifications in its composition and function at around 65 years old, when changes in lifestyle and physiology occur with ageing. Metabolism and physical activity levels decrease, antibiotics are used more frequently, the diet becomes less diversified, colon motility decreases, and faecal retention time increases (159). Observational evidence suggests that older adults have high inter-individual variation in the colonic microbiota, which is strongly influenced by their health status (160).

Healthy older adults have faecal microbiota similar to that of healthy young adults, although observational studies suggest increased diversity of methanogenic archaea and lower viral richness (161–163). On the other hand, unhealthy ageing (e.g., frailty or chronic diseases) is associated with a reduced abundance of the genera *Bifidobacterium*, *Faecalibacterium*, and *Eubacterium*, and an increased abundance of the family *Enterobacteriaceae* and genera *Streptococcus, Clostridium, Penicillium, Candida,* and *Aspergillus* (164–166). In addition, the expression of microbial genes synthesising vitamins and fatty acids is reduced (165). These alterations in the colonic microbiota are associated with chronic inflammation and an increased risk of morbidity and mortality (167).

A recent systematic review evaluated the effect of different dietary patterns on the faecal microbiota of older adults, including a total of 38 intervention trials, most of which profiled the microbiota using 16S rRNA sequencing (168). Diets rich in plant foods and including animal products in moderation (such as daily consumption of dairy and lean meats or fish two to four times per week) increased the faecal abundance of saccharolytic taxa and the production of SCFAs (168). Similarly, an observational study that evaluated the faecal microbiota of older adults using shotgun metagenomic sequencing found that adherence to the healthy plant-based diet index was associated with a greater faecal abundance of bacterial saccharolytic species, along with enriched pathways for the biosynthesis of branched-chain amino acids (169). These results may be explained by the high content of non-digestible carbohydrates and phytochemicals in these diets. For instance, greater consumption of dietary fibre among older adults was associated with an increased faecal abundance

of the order Clostridiales, which includes butyrate-producing bacteria, and increased expression of pathways involved in polysaccharide degradation, according to an observational study employing shotgun metagenomic sequencing (170). Furthermore, an intervention trial reported that adherence to a polyphenol-rich diet (total polyphenols around 1300 mg/day) increased the faecal abundance of butyrate-producing bacteria, as profiled by 16S rRNA sequencing, and reduced blood pressure in older adults (171).

In contrast, the systematic review of intervention trials found that high intakes of fat, protein, and simple sugars but low consumption of complex carbohydrates were associated with the growth of opportunistic pathogens, production of pro-inflammatory toxins, and frailty (168). Furthermore, an intervention study that profiled the faecal microbiota of older adults using 16S rRNA sequencing found that diets rich in fat but low in carbohydrates have been associated with decreased abundance of the genus *Bifidobacterium* (172). Consistently, a longitudinal study in older adults linked higher red meat intake to increased plasma concentration of trimethylamine N-oxide, a microbial metabolite associated with cardiovascular risk (173). These observations are consistent with results observed for young adults, highlighting the importance of a diversified diet primarily composed of fruits, vegetables, and whole cereals to nourish beneficial colonic microbes (Table 2.3). Additionally, evidence from a longitudinal study of more than 100,000 participants followed over 30 years indicates that long-term consumption of diversified diets is associated with longevity. Diets rich in plant-based foods, such as fruits, vegetables, wholegrains, and nuts, and including animal-based foods in moderation, like low-fat dairy, were associated with healthy ageing, defined as survival to the age of 70 years with intact cognitive, physical, and mental functions, and without chronic diseases (174).

However, increased protein consumption appears to be beneficial in older adults, who typically have slower protein digestion and absorption compared to younger individuals (175). For instance, a cross-sectional analysis of the faecal microbiota of older men, characterised using 16S rRNA gene sequencing, revealed that higher protein intake was associated with increased faecal alpha diversity (176). From a broad health perspective, a meta-analysis of observational trials concluded that protein intake was negatively associated with frailty in older individuals (177). Therefore, a daily intake of 1.2 g protein/kg bodyweight, compared to the standard recommendations of 0.66-0.80, has been proposed to support good health and maintain functionality in older populations (178), without evidence of adverse effects on colonic microbiota composition and function (179).

Table 2.3. Impact of various dietary patterns on the colonic microbiota of older adults.

Diet	Definition	Microbial composition	Microbial function	Observed effect of diet on host health	Reference
Western diet	High consumption of processed meats and refined grains	↑*Alistipes, Desulfovibrio, Ruminococcus* ↓*Faecalibacterium, Prevotella*	↑Amino acid metabolism ↓SCFA production	Adherence to a Western diet was associated with increased body mass index compared to a Prudent diet in older adults (n = 517)	(48)
High-protein diet	Consumption of protein higher than the recommended dietary intake (> 0.8 g protein/kg bodyweight/day)	No changes in taxa abundance or microbial diversity	No changes in the production of organic acids	No changes in appetite after six-month intervention compared to control (habitual diet) in community-dwelling older adults (n = 47)	(179,180)
Prudent diet	High consumption of fruits, vegetables, nuts, fish, and chicken	↑*Clostridium, Faecalibacterium, Lachnospira* ↓*Desulfovibrio, Ruminococcus*	↑Complex carbohydrate degradation and SCFA production	Adherence to a Prudent diet was associated with reduced body mass index compared to a Western diet in older adults (n = 517)	(48)
Mediterranean diet	High consumption of fruits, vegetables, and olive oil. Moderate of fish, dairy, and lean meats	↑*Roseburia, Eubacterium, Faecalibacterium* ↓*Ruminococcus torques*	↑SCFA and BCFA production ↓Secondary bile acids, p-cresol, ethanol	Reduced frailty and chronic inflammation, and improved cognitive function after one-year intervention compared to control (habitual diet) in non-frail or pre-frail older adults (n = 612)	(181)
Polyphenol-rich diet	High consumption of polyphenol-rich foods (e.g., berries, pomegranate, green tea, dark chocolate)	↑*Faecalibacterium, Butyricicoccus, Ruminococcaceae* ↓*Streptococcus, Enterobacteriaceae*	Not evaluated	Increased serum concentration of indole 3-propionic acid and decreased of zonulin after eight-week intervention compared to the control diet (low-polyphenol diet) in older adults with increased intestinal permeability (n = 51)	(171,182)

In line with this recommendation, a narrative review suggested that high-fibre diets enriched with protein from legumes, dairy, and lean meats could promote a balanced colonic microbiota (eubiosis), also contributing to muscle synthesis and overall metabolic health in older adults (183). In this context, complex carbohydrates are important to mitigate excessive protein fermentation in the colon and the consequent production of deleterious metabolites, such as hydrogen sulphide and nitrogen derivatives (11,120). As demonstrated by intervention trials, protein intakes exceeding the recommended dietary allowance did not alter faecal microbiota composition, characterised using 16S rRNA sequencing, or function in older adults when accompanied by a prudent diet (179,180).

2.5.4 Centenarian age

Recently, colonic microbiota investigations have focused on centenarians (individuals aged 100 or older) as models for healthy ageing. Compared to younger controls, observational studies demonstrated that centenarians exhibit higher faecal bacterial and viral diversity (184–186). A systematic review of 27 observational studies suggests that their high faecal microbial diversity and abundance of health-promoting taxa contribute to healthy ageing and longevity (187).

In terms of microbial composition, the genera *Alistipes*, *Parabacteroides*, *Clostridium*, and *Methanobrevibacter* are enriched in the faeces of healthy centenarians, while the butyrate-producing species *Faecalibacterium prausnitzii* and *Eubacterium rectale* are depleted (184,186,188). However, no age-related changes have been observed in the faecal fungal microbiota (189). Concerning functionality, centenarians have lower faecal butyrate concentrations but higher levels of BCFAs, ammonium, and secondary bile acids compared to younger controls (184). Furthermore, their faecal microbiome is enriched in genes associated with SCFA production from amino acids, secondary bile acid metabolism, and the degradation of xenobiotics, plant-based fats, and tryptophan (190–192). In contrast, they have fewer genes involved in carbohydrate and animal fat metabolism (190–192).

Few investigations have assessed the interaction between diet and colonic microbes in centenarians, with current knowledge primarily derived from longitudinal and crossover studies. Overall, adherence to a diverse, plant-rich diet has been associated with a higher abundance of microbial taxa linked to longevity, as observed in Italian, Chinese, and South Korean centenarians (190,193–196). Additionally, the consumption of fermented soybean paste was positively associated with the distinct faecal microbiota composition of South Korean centenarians, according to an observational study that profiled the microbiota using 16S rRNA gene sequencing (196). A cross-sectional study of Estonian centenarians found that cereal consumption and lower adherence to Western dietary patterns were linked to longevity (186). These observations highlight the importance

of a prudent, plant-rich diet for healthy ageing, aligning with dietary patterns that support a balanced colonic microbiota across life stages.

Interestingly, Estonian centenarians were also more exposed to animals and experienced lower sanitary conditions during their childhood (186). Similarly, early-life exposure to animals has been shown to contribute to the development of the faecal microbiota in infancy (56). Taken together, these findings suggest that early exposure to environmental microbes may play a crucial role in supporting the long-term balance of colonic microbiota throughout life.

2.6 Dietary recommendations for nourishing a balanced colonic microbiota

The evidence gathered in this chapter suggests that diets promoting a balanced colonic microbiota in infants post-weaning, adults, older adults, and centenarians share similar compositions (Figure 2.2). These diets are diverse and primarily based on plant foods, such as fruits, vegetables, and wholegrains. These foods are rich in complex carbohydrates, polyunsaturated fatty acids, and polyphenols, which support the growth of saccharolytic microbes, for example, taxa from the genera *Bifidobacterium*, *Faecalibacterium*, *Prevotella*, *Eubacterium*, *and Ruminococcus*, and the production of beneficial metabolites, such as SCFAs and conjugated fatty acids (12,112,139). Additionally, polyunsaturated fatty acids and polyphenols have anti-inflammatory and antioxidant properties, and their metabolism by colonic microbes may confer further benefits to the host (112,197). For instance, a two-month intervention in adults consuming cereals enriched with polyphenols, dietary fibre, and omega-3 fatty acids increased the faecal abundance of *Bacteroides* species while reducing faecal calprotectin levels, a biomarker for intestinal inflammation (198).

In addition to plant-based foods, favourable diets also include moderate consumption of animal-based foods, such as fish and lean meats (two to four servings per week). These foods are rich sources of essential amino acids and micronutrients, supporting colonic microbial diversity and contributing to meeting nutritional needs (140). Their consumption is particularly important for infants and older adults to promote physiological development and reduce disease risk (177). Daily consumption of fermented foods, such as fermented dairy, is also encouraged, as they contain lactic acid bacteria, supporting colonic eubiosis (147). Importantly, the intake of animal foods should be paired with the consumption of complex carbohydrates to mitigate excessive microbial fermentation of animal protein and fat. Notably, these recommendations for nourishing the colonic microbiota align with current dietary guidelines (101).

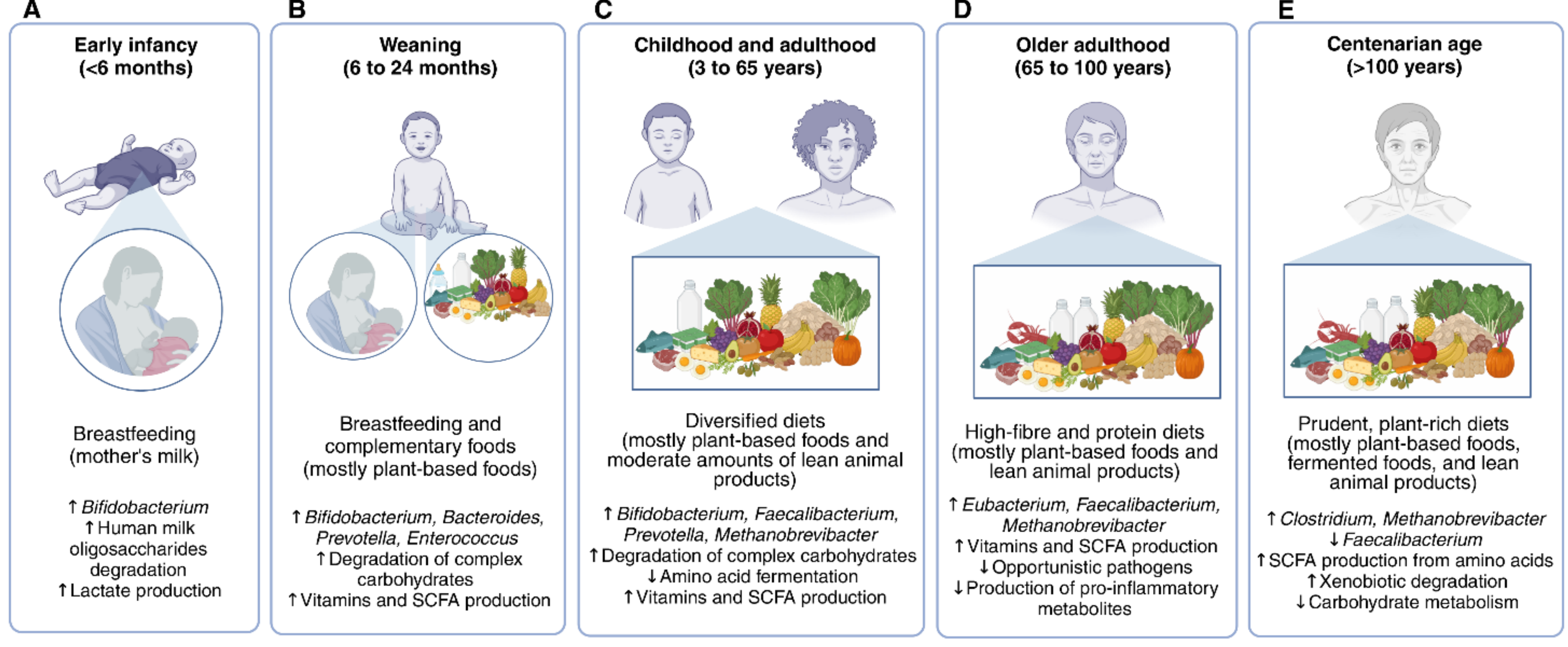

Figure 2.2. Favourable diets for nourishing the colonic microbiota in different life stages. In early infancy (A), breastfeeding is the optimal dietary strategy, but mother's milk no longer meets all the necessary nutritional requirements once the infant reaches the weaning period. In this stage (B), diets should combine breastfeeding with complementary foods, mainly fruits, vegetables, whole cereals, and animal products in moderation. The colonic microbiota matures as the introduction of solid foods increases and breastmilk or infant formula decreases, achieving an adult state in which breastmilk is no longer consumed. Children, adolescents, and adults have similar diets for promoting a balanced microbiota (C). These are rich in non-digestible carbohydrates, phytochemicals, and polyunsaturated fatty acids found in fruits, vegetables, nuts, pulses and wholegrains, also containing moderate amounts of fish, fermented dairy products, and lean meats. A higher intake of protein is recommended for older adults, particularly from dairy and lean meats (D). Centenarians have a similar dietary pattern to that of older adults (E).

2.7 Limitations and future perspectives in diet-colonic microbiota research

Most of the knowledge on diet-colonic microbiota interaction in humans comes from studies using faecal samples as proxies. Compared to biopsies, faecal sampling is a non-invasive and cost-effective approach (199). However, they predominantly represent microbial communities from the distal colon, providing limited information about microbes attached to the colon's mucosa or inhabiting other parts of the GIT (200). As a result, diet-induced changes observed in faecal microbial function and composition may not accurately reflect microbial alterations throughout the entire large intestine. Furthermore, current culture and sequencing techniques are unable to fully characterise the human colonic microbiota, particularly bacterial species that are uncultured or are less prevalent in well-studied populations (201). Ultimately, due to technical limitations, our current understanding of how dietary patterns influence colonic commensals remains largely inferred and may not fully capture the complexity of host-microbe interactions.

Diet-colonic microbiota research has traditionally focused on adults. Hence, there is an opportunity for further research to expand our knowledge of diet-microbiota interactions in other life stages, such as infancy and older adulthood. Notably, weaning plays a critical role in colonic microbiota development (80). Investigating the impact of complementary feeding on host-microbiota associations early in life is a promising field to promote health and well-being and prevent diseases. Similarly, the continuous rise in human life expectancy urges greater comprehension of the relationship between colonic microbiota and ageing. The confounding effects of diseases on colonic microbes often challenge research on older populations. Therefore, studies involving both healthy and unhealthy older adults are necessary to deepen our understanding of how diet-microbiota modulations can support functionality during ageing.

Among dietary compounds, the effects of complex carbohydrates on colonic microbes are well-characterised, whereas less is known about protein, fatty acids, polyphenols, micronutrients, and food additives. Notably, polyphenols and polyunsaturated fatty acids have been associated with health benefits, in which colonic microbes seem to play a critical role (112,139). A better understanding of the impact of these nutrients on the colonic microbiota is needed, including in the long term. Future research should also investigate how dietary compounds interact with each other and their combined effects on the microbiota. Increased knowledge of the role of various nutrients in shaping colonic microbes, alongside individual factors (e.g., age, health status, activity level), will help researchers and medical professionals tailor nutritional recommendations based on individual needs.

These challenges highlight the need for complementary methods to study the influence of dietary patterns on the colonic microbiota. Randomised clinical trials are the gold standard approach to measure the effect of food interventions on colonic microbes and resulting host health outcomes. They are also useful for validating findings from animal models and *in vitro* studies. However, clinical trials are time- and resource-consuming, are prone to confounding factors, and rely on participant compliance and the accuracy of food questionnaires, limiting their feasibility (30). Furthermore, dietary assessment tools vary across microbiome studies, highlighting the need for standardised methods to improve comparability between studies targeting different age groups.

A promising strategy to address these limitations is to combine traditional methodologies with mathematical modelling. Mathematical models can investigate hypotheses that cannot be efficiently evaluated *in vitro* or *in vivo*, using a fraction of the time and cost of traditional approaches. *In silico* pipelines for predicting the effect of diets on personalised microbial communities have already been proposed (31,202). Ongoing development and validation of these models could expand colonic microbiota research to traditionally underrepresented populations. For instance, *in silico* approaches were used to predict compositional and functional changes in the colonic microbiota of infants, children with different clinical conditions, healthy adults, and adults with Crohn's disease according to the diet (117,203–206). Moreover, models are flexible and can create personalised simulations based on input data, contributing towards personalised nutrition. Ultimately, integrating mathematical models with traditional approaches can reduce the costs of colonic microbiota research and accelerate our understanding of the relationship between diet, colonic microbes, and host health.

2.8 Conclusions

Colonic microbes ferment non-absorbed dietary compounds, producing bioactive metabolites that influence host physiology. Therefore, identifying dietary patterns that support colonic eubiosis across different life stages is crucial for promoting host health and well-being. The evidence gathered in this chapter suggests that diets nourishing the colonic microbiota throughout human life are primarily composed of plant-based foods and include daily consumption of fermented foods, such as dairy products, and moderate amounts of fish and lean meats (two to four times a week). However, most diet-colonic microbiota investigations have focused on adults, neglecting weaning infants and older adults. Notably, weaning is a critical period for colonic microbiota development, setting the foundation for later life. In older adulthood, colonic microbes have a crucial role in maintaining functionality and promoting healthy ageing. The limited understanding of how diets influence colonic microbes of infants and older adults is a significant barrier to using colonic microbiota modulation strategies to promote health. Further investigation of the long-term effects of dietary patterns on

colonic microbes across different life stages is necessary to overcome some of the current limitations in diet-colonic microbiota research.

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Vitor Geniselli da Silva
Name and title of main supervisor:	Professor Warren McNabb
In which chapter is the manuscript/published work?	Chapter 3

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Vitor Geniselli da Silva (student): Conceptualisation, Data curation, Formal Analysis, Investigation, Visualisation, Writing – original draft, Writing – review & editing.
Nicole Roy (co-supervisor): Conceptualisation, Funding acquisition, Supervision, Writing – review & editing.
Nick Smith (co-supervisor): Conceptualisation, Supervision, Writing – review & editing.
Clare Wall (co-supervisor): Conceptualisation, Funding acquisition, Supervision, Writing – review & editing.
Jane Mullaney (co-supervisor): Conceptualisation, Supervision, Writing – review & editing.
Warren McNabb (main supervisor): Conceptualisation, Project administration, Supervision, Writing – review & editing.

Please select one of the following three options:

(●) **The manuscript/published work is published or in press**

Please provide the full reference of the research output:
Geniselli da Silva V, Tonkie JN, Roy NC, Smith NW, Wall C, Kruger MC, Mullaney JA, McNabb WC. The effect of complementary foods on the colonic microbiota of weaning infants: a systematic review. Crit Rev Food Sci Nutr (2024) 16:1 – 16. doi: 10.1080/10408398.2024.2439036

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Chapter 3: Systematic review of the effects of complementary foods on the colonic microbiota of weaning infants[2]

3.1. Abstract

The transition from breastmilk to solid foods (weaning) is a decisive stage in the development of the colonic microbiota. However, little is known about how complementary foods influence the composition and function of the colonic microbiota in infants. This systematic review collected evidence of the effect of individual foods on the colonic microbiota of weaning infants (4-12 months old) using five databases: PubMed, CENTRAL, Scopus, Web of Science, and ScienceDirect. A total of 3625 records were examined, and seven randomised clinical trials met the review's eligibility criteria. Altogether, 983 participants were enrolled, and plant-based foods, meats, and dairy products were used as interventions. Wholegrain cereal increased the faecal abundance of the order Bacteroidales in the two included studies. Pureed beef increased the faecal abundances of the genus *Bacteroides* and the *Clostridium* XIVa group, as well as microbial richness in two of the three included studies. However, the conclusions of this review were limited by the small number of studies included. No conclusions could be drawn about the impact of complementary foods on faecal metabolites. Further clinical trials assessing the effect of dietary interventions on both faecal microbial composition and function are needed to fill this knowledge gap in infant nutrition.

3.2. Introduction

The human colon is home to a diverse microbial community called the colonic microbiota. Microbes from the mother's vaginal canal and faeces are the first to populate the newborn's colon, followed by microbes found in the environment (207). In human studies, the colonic microbiota is mainly characterised using faecal samples (208). Multiple factors belonging to the host and the environment continuously shape the colonic microbiota during infancy (18,56). According to this complex and not yet fully understood interplay of factors, colonic commensals influence the host's physiology. A balanced colonic microbiota is linked with disease prevention and overall longevity (45,209). Conversely, imbalances in the microbiota or dysbiosis are associated with gastrointestinal and metabolic diseases, among other pathologies (75,210).

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[2] This chapter has been published as Geniselli da Silva V, Tonkie JN, Roy NC, Smith NW, Wall C, Kruger MC, Mullaney JA, McNabb WC. The effect of complementary foods on the colonic microbiota of weaning infants: a systematic review. *Crit Rev Food Sci Nutr* (2024) 16:1–16. doi: 10.1080/10408398.2024.2439036

In the early stages of life, breastmilk promotes the growth of microbes capable of metabolising human milk oligosaccharides, notably bifidobacteria (211). At around six months of age, the introduction of complementary foods and the simultaneous reduction in breastmilk intake create conditions for other microbes to thrive (80,109). At this stage, the infant's gastrointestinal system is not yet fully developed (212). Macronutrients from complementary foods, especially complex carbohydrates and proteins, can reach the colon undigested. This promotes the growth of a myriad of new colonic commensals specialised in degrading these nutrients to produce, among other metabolites, SCFAs, like the genera *Bacteroides*, *Clostridium*, *Prevotella*, and *Ruminococcus* (3,56,61).

Dietary changes during the weaning period gradually increase faecal microbial richness and diversity, supporting the complete maturation of the colonic microbiota, which typically stabilises by two to three years of age (126,213). At this stage, the infant microbiota is more stable, resembling the adult microbiota in composition and function (126). Acetate, propionate, and butyrate become the major SCFAs produced by colonic microbes, primarily contributing to gut health while also providing other benefits to the host (214). Changes in the colonic microbiota that occur during weaning may persist in the long term, ultimately influencing host health later in life (29,215). Therefore, weaning represents a unique opportunity to establish beneficial interactions between the host and its colonic microbes.

Nevertheless, microbiota investigations traditionally focus on early infancy or adulthood, neglecting the weaning period. Limited knowledge exists about the effects of individual complementary foods on the colonic microbiota of weaning infants, and the question of the best complementary feeding practices for nourishing colonic commensals in this crucial stage of life remains unanswered. This systematic review investigated how complementary foods affect the composition and function of the colonic microbiota of infants (4-12 months old) to shed light on this knowledge gap in infant nutrition. Changes in faecal microbial diversity, relative abundance of bacterial taxa, and SFCA and BCFA concentrations were identified in response to clinical interventions with complementary foods.

3.3. Materials and methods

3.3.1. Search strategy

This systematic review was conducted following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (216) and was registered on PROSPERO (CRD42023438679). The eligibility of studies was assessed using the PICOS (Population,

Intervention, Comparator, Outcome, and Study design) criteria (Table 3.1). Studies were identified using five databases: PubMed, CENTRAL, Scopus, Web of Science, and ScienceDirect. Search terms were related to food, infant, weaning, and microbiota, as follows: (food OR diet OR feeding OR nutrition) AND (bowel OR gut OR colon* OR feces OR faeces OR fecal OR faecal OR gastrointestinal OR intestinal OR stool) AND (microb* OR bacteria OR microorganism* OR micro-organism*) AND (infancy OR "early life" OR "early-life" OR infant* OR baby OR babies OR toddler* OR child*) AND (weaning OR complementary OR supplementary).

3.3.2. Eligibility criteria

The search strategy was tailored to each database to identify articles in English published up to October 2024 (Supplementary Table 3.1). The inclusion criteria were randomised controlled trials and crossover studies that reported the effect of complementary foods on the colonic microbiota of healthy infants at weaning age (4-12 months old) as primary or secondary outcomes. The main outcomes were changes in the faecal microbial composition and/or production of microbial organic acids, measured by comparing the intervention group with the control group at the end of the intervention.

No restrictions were placed on the infant's mode of delivery (vaginal or caesarean delivery), feeding strategy before the introduction of solids (breastmilk or infant formulas), geographical location, or ethnicity. Studies were excluded if the infants were unhealthy (diagnosed with chronic diseases, including gastrointestinal, metabolic, or neurological conditions) or not at the defined age. Interventions with supplements containing prebiotics or probiotics, isolated food compounds, and food additives were excluded. Trials evaluating foods that naturally contain potential prebiotics or probiotics (for instance, fermented foods) were included. Studies evaluating the impact of infant formulas on the colonic microbiota of infants were excluded. Systematic and narrative reviews were also excluded.

Table 3.1. PICOS criteria for inclusion of studies.

Parameter	Criteria
Population	Healthy infants at weaning age (4 to 12 months old)
Intervention	Use of any complementary food
Comparator	Placebo control or any other complementary food used as a control Participants not exposed to the intervention or consuming their habitual diets
Outcome	Changes in colonic microbiota composition (alpha and beta diversity scores, relative abundance of bacterial taxa) and/or function (production of short-chain or branched-chain fatty acids)
Study design	Randomised controlled trials

3.3.2. Data extraction

Records were identified using the described search strategy. Titles and abstracts of studies were independently screened by two review authors (VG, JT) using the web application PICO Portal (PICO Portal, USA). Studies potentially meeting the inclusion criteria were selected for full-text eligibility assessment, which was independently performed by two reviewers (VG, JT). Disagreements in judgment were resolved by discussion with a third reviewer (WM). A standardised form was used to extract data from the included studies by two independent reviewers (VG, JT). Extracted information included study design (length, sample size, sample collection points, methods), study population (age, nationality, gender, inclusion criteria), details of the intervention (food intervention, quantity, control), and outcomes (changes in alpha and beta diversity scores, changes in the relative abundance of microbial taxa, production of SCFAs and BCFAs).

3.3.3. Risk of bias assessment

The risk of bias in selected studies was independently assessed by two reviewers (VG, JT) using the Revised Cochrane risk-of-bias tool for randomised trials (RoB2) (217). The following elements of the studies were assessed: the randomisation process, deviations from the intended interventions, missing outcome data, a measure of the outcome, and selective reporting of results. Scores for each element were generated by the RoB2 algorithm, resulting in "low risk of bias", "some concerns", or "high risk of bias". Conflicting scores between reviewers were resolved by discussion to reach a consensus.

3.4. Results

3.4.1. Characteristics of identified studies

A total of 3625 unique studies were identified after removing duplicates, of which 3618 were excluded for various reasons (Figure 3.1), such as not meeting age and health criteria, use of probiotics and/or prebiotics, inappropriate study design, or lack of a discernible independent effect of complementary foods on the infant colonic microbiota. Of these, seven food intervention trials met the eligibility criteria and were included in this systematic review: six randomised controlled trials (81,83,84,218–220) and one randomised crossover trial (88).

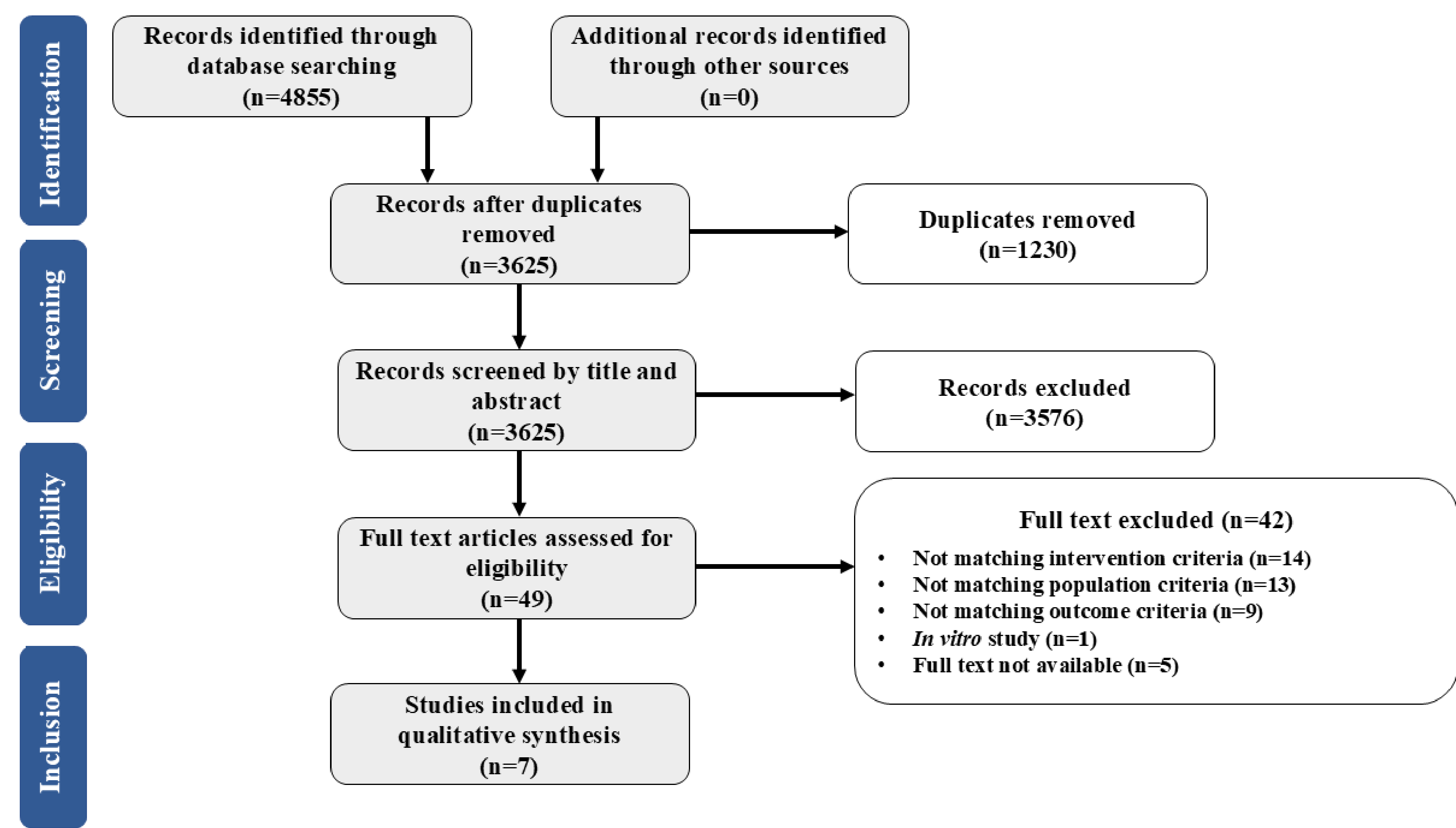

Figure 3.1. PRISMA flow diagram of literature search.

Study characteristics are summarised in Table 3.2. A total of 983 infants were enrolled in the identified studies (enrolment ranged from 45 to 355 participants), and their mean age at enrolment was approximately six months, ranging from four to nine months old. Trial intervention times ranged from two weeks to seven months, with three months being the most common trial length (3 out of 7 trials). Studies were conducted in Africa (Mali and Malawi), North America (USA and Canada), Europe (Spain) and Central America (Nicaragua), with one study conducted simultaneously in two different countries: Nicaragua and Mali (218).

The majority of the identified trials (5 out of 7) used plant-based foods as an intervention (81,83,88,218,219), including legumes (beans and cowpea, which is also known as black-eyed peas), rice bran, and commercial cereal products. Two studies used wholegrain cereals as an intervention (81,88). The effect of pureed meats (beef, pork, and poultry) on the colonic microbiota of infants was assessed by three studies (81,83,84) while dairy products (yoghurt, cheese, whey protein, and bovine colostrum) were used in two trials (84,220). One study assessed the combination of bovine colostrum with egg powder (220). In most studies (4 out of 7), other intervention products were used as controls (81,83,84,88), while two trials used a mixture of corn-soy flour (219,220) and one study used the absence of an intervention (218).

All the identified studies used faeces to represent the colonic microbiota of infants. Faecal samples were collected at least three times in most of the trials (4 out of 7) (81,84,218,219), while three studies collected the samples only at baseline and at the end of the intervention (83,88,220). The colonic microbiota composition was determined by 16S ribosomal RNA amplicon sequencing in all studies, mainly through amplifying the V3-V4 regions, while one study amplified all nine variable regions (220). Bioinformatic tools varied between studies; none used the same pipeline and reference database. QIIME (221) and its updated version, QIIME2 (222), were the most commonly used pipelines (3 out of 7 trials) (83,88,219), while SILVA (223) was the most employed reference database (4 out of 7 trials) (84,88,218,220).

Table 3.2. Characteristics of identified studies.

Reference	Study design	Intervention	Population	Inclusion criteria	Antibiotic usage during the trial	Infant diet during the trial	Dietary intake assessment	Microbiota analyses	Risk of bias
Bierut et al. (2021)	Randomised, blinded, placebo-controlled trial (3-month intervention time). Collection of faecal samples at baseline and at the end of the intervention (9 and 12 months of age)	Bovine colostrum (5.7 g) with egg powder (4.3 g) twice daily. Corn/soy flour (15 g) was used as a control	Malawian healthy breastfed infants (9-12 months old, 41 % female) (n = 277 enrolled)	Infants aged 9 months living in the Limera and Masenjere village clusters without acute malnutrition or other medical conditions affecting normal growth	Not reported	It is unknown whether infants consumed other foods, breastmilk, or infant formula during the trial	Survey of animal source food consumption and use of food supplements for malnutrition. Assessment at the entry of the study, during the intervention (weeks 2, 4, 8, and 12), and after the intervention (weeks 20 and 32)	DNA sequencing by 16S ribosomal RNA (all 9 variable regions) using Illumina MiSeq platform. Bioinformatics using MVRSION pipeline and SILVA 132 database (n = 263 analysed at the end of the intervention)	Low risk
Krebs et al. (2013)	Randomised controlled trial (3-month intervention time). Collection of faecal samples at 5, 6, 7, 8, and 9 months of age	Wholegrain iron-fortified cereal, iron and zinc-fortified cereal, or pureed beef (1-2 servings/day)	American healthy breastfed infants (5-9 months old, 60 % female) (n = 45 enrolled)	Infants from the Denver metropolitan region, with a gestational age of 37-42 weeks, vaginally delivered, exclusively breastfed, without chronic conditions, and aged between 5-6 months old	Not reported	In addition to the intervention, fruits, vegetables, teething biscuits, and unfortified cereals were allowed *ad libitum*. There was no consumption of infant formula	Use of a 3-day food record questionnaire. Monthly assessment	DNA sequencing by 16S ribosomal RNA (V1-V3 regions) pyrosequencing using a genome sequencer FLX System. Bioinformatics using the Infernal RNA alignment tool and the RDP classifier database (n = 14 analysed at 5 to 9 months of age)	Some concerns

Ordiz et al. (2020)	Randomised, double-blind, controlled trial (6-month intervention time). Collection of faecal samples at the baseline, 6.5, 7.5, 9, 10.5, and 12 months of age	Common bean or cowpea (80-120 kcal/d). Corn-soy blend flour (80-120 kcal/d) was used as a control	Malawian healthy breastfed infants (6-12 months old, 48 % female) (n = 355 enrolled)	Healthy infants aged between 5.5 and 6.5 months living in the Limera and Masenjere village clusters without acute malnutrition or chronic non-infectious diseases	103 courses of antibiotics were recorded (22 % in infants from the Limela and 81 % in infants from Masenjere villages). It was not reported whether there was different antibiotic usage between interventions and controls	In addition to the intervention, infants were breastfed *ad libitum* and consumed corn porridge as the primary complementary food. It is unknown whether they consumed infant formula	Two 24-hour dietary recalls were conducted in a random subset of 50 participants	DNA sequencing by 16S ribosomal RNA (V4 region) using Illumina MiSeq platform. Bioinformatics using QIIME pipeline and Greengenes 13.8 database (n = 236 analysed at ≤ 6-time points)	High risk
Plaza-Diaz et al. (2021)	Randomised, triple-blind, cross-over trial (14-week intervention time). Collection of faecal samples at the baseline and the end of the intervention (7 weeks)	50 % wholegrain low sugar (12 g/100g) cereal or 0 % wholegrain high sugar (24 g/100g) cereal. Parents decided how much cereal to consume	Spanish healthy infants (4-7 months old, 48 % female) (n = 48 enrolled)	Infants with a gestational age of 37-42 weeks, weight at birth > 2500 g, aged between 4-5 months, not breastfed since at least 4 months old, not yet introduced to complementary foods, and without antibiotic usage in the last 15 days	No use of antibiotics during the intervention period	In addition to the intervention, infants consumed infant formula and other foods. There was no consumption of human milk	Two non-consecutive days weighed dietary record. Assessment at baseline and after 1, 4, and 7 weeks of intervention	DNA sequencing by 16S ribosomal RNA (V3-V4 regions) using Illumina MiSeq platform. Bioinformatics using QIIME2 and DADA2 pipelines and SILVA 132 database (n = 43 analysed at the baseline and 7 weeks of intervention)	Low risk

Qasem et al. (2017)	Randomised controlled trial (2-4 weeks intervention time according to parents' discretion). Collection of faecal samples at the baseline and the end of the intervention	Iron-fortified cereal, iron-fortified cereal with raspberry, or pureed beef. The consumed amount of intervention was not reported	Canadian healthy breastfed infants (4-6 months old, 50 % female) (n = 87 enrolled)	Infants aged between 4-6 months old with birth weight > 2500 g and more than 37 gestational weeks, exclusively breastfed, not yet introduced to solid foods, and without medical conditions	Not reported	In addition to the intervention, infants consumed breastmilk, but it is unknown whether they consumed infant formula or other foods	Use of a 3-day food record questionnaire	DNA sequencing by 16S ribosomal RNA (V3 to V4 regions) using Illumina MiSeq platform. Bioinformatics using QIIME pipeline and Greengenes 13.5 database (n = 56 analysed at the baseline and the end of intervention)	Some concerns
Tang et al. (2023)	Randomised controlled trial (7-month intervention time). Collection of faecal samples at 5, 10, and 12 months of age	Meat (pureed beef, pork, and poultry) or dairy-based (yoghurt, cheese, and whey protein powder) foods (3 g/kg/day)	American healthy formula-fed infants (5-12 months old, 50 % female) (n = 71 enrolled)	Infants from Denver (Colorado, USA), full-term, exclusively formula-fed, aged between 3-5 months, and without chronic diseases	Two infants had antibiotics during the intervention (one from each group)	In addition to the intervention, infants consumed infant formula, cereal, fruit, and vegetables *ad libitum*. There was no consumption of breastmilk	Use of a 3-day food record questionnaire. Assessments at 5, 10, and 12 months of age	DNA sequencing by 16S ribosomal RNA (V3 to V4 regions) using Illumina MiSeq platform. Bioinformatics using SINA pipeline and SILVA 115 database (n = 59 analysed at 5 months and n = 57 at 12 months of age). SCFAs analysis by GC-MS	Low risk

Zambrana et al. (2019)	Randomised controlled trial (6-month intervention time). Collection of faecal samples at 6, 8, and 12 months of age	Rice bran (1-5 g/day). The absence of the intervention was used as a control	Nicaraguan and Malian healthy infants (6-12 months old, 45 % and 50 % female, respectively) (n = 50 and 50 enrolled, respectively). Malian infants were breastfed. 96 % of Nicaraguan infants in the control group and 83 % in the rice bran group were breastfed	Infants aged between 4-5 months old, without diarrhoea episodes and antibiotics used in the last month, without prior hospitalisations, allergies, or immune-compromising conditions	Of the 47 Nicaraguan infants, 25 used antibiotics (14 in the control and 11 in the intervention group). Of the 48 Malian infants, 27 used antibiotics (14 in the control and 13 in the intervention group)	In addition to the intervention, infants consumed other foods like staple grain porridges, soups, milk, fruits, juices, eggs, and fish. Nicaraguan infants also consumed breastmilk and infant formula, while Malian infants only breastmilk during the study	Daily dietary records (measuring the consumption of the intervention as none, half, or all)	DNA sequencing by 16S ribosomal RNA (V4 region) using Illumina MiSeq sequencer. Bioinformatics using mothur pipeline and SILVA 128 database (n = 48 Malian and 47 Nicaraguan analysed at 8 and 12 months of age). Metabolome analysis by UPLC-MS (at 8 months of age)	Some concerns

DADA: Divisive Amplicon Denoising Algorithm. GC: gas chromatography. MS: mass spectrometry. MVRSION: Multiple 16S Variable Region Species-Level IdentificatiON. QIIME: Quantitative Insights Into Microbial Ecology. SINA: SILVA Incremental Aligner. UPLC: ultrahigh-performance liquid chromatography. RDP: Ribosomal Database Project.

Among the seven trials, only two assessed the metabolite production of the colonic microbiota (84,218). Zambrana et al. (2019) characterised the non-targeted metabolite profile of faecal samples at two months of intervention by ultrahigh-performance liquid chromatography-tandem mass spectrometry. Tang et al. (2023) measured faecal SCFAs and BCFAs at three different time points using gas chromatography-mass spectrometry. Out of the seven identified studies, three were found to have some concerns of bias (81,83,218), three had a low risk of bias (84,88,220), and one had a high risk of bias (219). The main source of bias in most studies was potential selection in reporting results, which corresponds to domain five of the RoB2 tool (Supplementary Figure 3.1).

3.4.2. Influence of plant-based foods on the colonic microbiota of weaning infants

Commercial cereals were the most used plant-based foods, being used as intervention products in three of the identified trials (81,83,88). Wholegrain cereals were used in two studies (81,88). Plaza-Diaz et al. (2021) evaluated the influence of cereals made with wheat, corn, rice, oats, barley, rye, sorghum, and millet on the colonic microbiota of Spanish infants not breastfed since at least four months of age. Two formulations were used during seven weeks of intervention, one rich in wholegrains (50 %) and the other rich in sugar (0 % wholegrains and 24 g of sugar/100g). In addition to the intervention, infants consumed infant formula and other foods. There was no consumption of human milk. Of the 48 enrolled participants, 43 had their faeces analysed, 18 in the sugar-rich cereal group and 25 in the wholegrain cereal group. Infants did not use antibiotics during the intervention period. A permutational multivariate analysis of variance demonstrated that the cereal type had a small but significant effect on the faecal microbial composition of the samples (p = 0.029), explaining 2 % of the variance between samples. After the intervention, infants who consumed wholegrain cereal had a lower relative abundance of the *Escherichia-Shigella* genus compared to those who consumed sugar-rich cereal. Longitudinal changes in the relative abundance of microbial taxa were also observed. The wholegrain cereal group showed an increase in the relative abundances of *Bacteroides* and *Lachnoclostridium* genera compared to the baseline. Both intervention groups had a decrease in the abundance of the *Enterococcus* genus and an increase in the abundance of the *Veillonella* genus by the end of the intervention. However, there were no differences in faecal microbial alpha diversity scores when comparing the end of the intervention values to the baseline values for both cereal groups (Table 3.3).

Table 3.3. Changes in the colonic microbiota composition and function according to complementary food intervention.

Reference	Complementary food	Microbiota composition				Microbiota function
		Changes between baseline and at the end of the intervention		Changes between groups at the end of the intervention		
		Diversity indexes	Relative abundance (order, family, or genus)	Diversity indexes	Relative abundance (order, family, or genus)	Short-chain and branched-chain fatty acids
Plaza-Diaz et al. (2021)	0 % wholegrain high-sugar cereal	No changes in Shannon, Pielou's evenness, Simpson's, and inverse Simpson's indices	↑*Veillonella* ↓ *Bifidobacterium, Enterococcus*	No changes in Shannon, Pielou's evenness, Simpson's, and inverse Simpson's indices between groups	↓*Escherichia-Shigella* (wholegrain cereal compared to high-sugar cereal)	Not evaluated
	50 % wholegrain, low-sugar cereal	No changes in Shannon, Pielou's evenness, Simpson's, and inverse Simpson's indices	↑*Veillonella, Bacteroides, Lachnoclostridium* ↓*Escherichia-Shigella, Enterococcus*			Not evaluated
Krebs et al. (2013)	Wholegrain iron-fortified cereal	No changes in the Schao1 diversity index	↑Bacteroidales, *Clostridium group XIVa* ↓*Bifidobacterium,* Lactobacillales, *Enterobacteriaceae*	No changes in the Schao1 diversity index between groups	↑Bacteroidales ↓*Bifidobacterium, Rothia,* Lactobacillales (wholegrain iron-fortified cereal compared to other interventions) ↑ *Clostridium group XIVa* (pureed beef compared to other interventions)	Not evaluated
	Iron- and zinc-fortified cereal	No changes in the Schao1 diversity index	↑*Clostridium group XIVa* ↓Bacteroidales			
	Pureed beef	No changes in the Schao1 diversity index	↑*Clostridium group XIVa* ↓*Enterobacteriaceae*			

Qasem et al. (2017)	Iron-fortified rice cereal	No changes in the Chao1 richness estimator and Shannon diversity index	No changes reported	↑Chao1 richness estimator (pureed beef compared to other interventions)	No changes between groups reported	Not evaluated
	Iron-fortified rice cereal with raspberry	↑Chao1 richness estimator, Shannon diversity index	No changes reported			
	Pureed beef	↑Chao1 richness estimator No changes in the Shannon diversity index	No changes reported			
Ordiz et al. (2020)	Common bean	No changes in Faith's Phylogenetic diversity and weighted UniFrac distances	No changes reported	No changes in Faith's Phylogenetic diversity and weighted UniFrac distances between groups	↑*Bifidobacterium* ↓*Prevotella* (cowpea compared to other intervention groups)	Not evaluated
	Cowpea	No changes in Faith's Phylogenetic diversity and weighted UniFrac distances	No changes reported			
	Corn-soy blend flour (control)	No changes in Faith's Phylogenetic diversity and weighted UniFrac distances	No changes reported			

Zambrana et al. (2019)	Rice bran (absence of intervention was used as control)	No changes reported	No changes reported	No changes in alpha diversity indices (Observed, Shannon, Inverse Simpson, and Richness; Malian and Nicaraguan cohorts)	Malian cohort: ↑*Lactobacillus, Alloprevotella* ↓*Bifidobacteriaceae, Clostridium, Terrisoporobacter* Nicaraguan cohort: ↑*Paraprevotella, Phascolarctobacterium, Veillonella, Bifidobacterium* ↓*Lachnospiraceae, Alisonella*	Not evaluated
Tang et al. (2023)	Pureed meats (beef, pork, and poultry)	↑Chao1 richness, Evenness, and Shannon Diversity estimators	↑*Blautia, Bacteroides, Ruminococcaceae, Coprococcus, Anaerostipes, Faecalibacterium, Roseburia,* Clostridiales, *Ruminococcus, Dorea, Lachnospira, Haemophilus* ↓*Escherichia, Enterobacteriaceae, Enterococcus, Klebsiella, Akkermansia*	Changes in beta diversity between groups. Increased Chao1 richness estimator for the meat group	↑Akkermansia (dairy compared to the meat group)	No changes were observed between groups. Increased faecal butyrate for the meat group at the end of the intervention, as compared to the baseline
	Dairy products (yoghurt, cheese, and whey protein)	↑Evenness and Shannon Diversity estimators No changes in the Chao1 richness estimator	↑*Blautia, Bacteroides, Ruminococcaceae, Coprococcus, Anaerostipes, Faecalibacterium, Roseburia,* Clostridiales, *Ruminococcus, Dorea, Lachnospira, Haemophilus, Akkermansia* ↓*Escherichia, Enterobacteriaceae, Enterococcus, Klebsiella*			
Bierut et al. (2021)	Bovine colostrum and egg powder mixture	Not evaluated	Not evaluated	No changes in beta diversity between groups (weighted UniFrac distances)	Changes were reported only at the species level	Not evaluated
	Corn and soy flour (control)	Not evaluated	Not evaluated			

In a three-month trial, Krebs and colleagues (2013) allocated exclusively breastfed American infants (5 months old) to either wholegrain iron-fortified cereal, iron- and zinc-fortified cereal, or pureed beef. In addition to the intervention, fruits, vegetables, teething biscuits, and unfortified cereals were allowed *ad libitum*. There was no consumption of infant formula. The study monitored the use of antibiotics during the trial but did not report it. Faecal samples were collected and analysed monthly for a small subset of 14 infants (out of 45 enrolled), with four in the wholegrain cereal, six in the zinc-fortified cereal, and four in the pureed beef group. Despite the small sample size, significant changes ($p < 0.05$) in microbial composition were observed between the intervention groups at the end of the study. Infants consuming wholegrain iron-fortified cereal had reduced abundances of the *Bifidobacterium* genus and the Lactobacillales order and an increased abundance of the Bacteroidales order compared to the other infants (Table 3.3). No longitudinal changes in the Chao1 species richness estimator were observed for any of the three intervention groups.

In a similar study, Qasem and co-authors (2017) evaluated the influence of iron-fortified rice cereal, iron-fortified rice cereal with raspberry, or pureed beef on the colonic microbiota of exclusively breastfed Canadian infants (4 to 6 months old). In addition to the intervention, infants consumed breastmilk, but it is unknown whether they consumed infant formula or other foods. The trial lasted from two to four weeks according to the parents' discretion. Of the 87 enrolled infants, 82 completed the study, and 56 had their faeces analysed (18 in the iron-fortified cereal, 19 in the iron-fortified cereal with raspberry, and 19 in the pureed beef group). The use of antibiotics during the intervention was not reported. In contrast to the findings of Krebs et al. (2013), there were no differences in the microbial composition between intervention groups (permutational analysis of variance, $p = 0.22$), nor were longitudinal changes in the relative abundance of microbial taxa found after correction for multiple comparisons. On the other hand, the Chao1 richness index increased for the iron-fortified rice cereal with raspberry and pureed beef groups at the end of the intervention (Table 3.3).

Ordiz and colleagues (2020) evaluated the effect of a 6-month legume supplementation on the colonic microbiota of breastfed Malawian infants. In addition to the intervention, infants were breastfed *ad libitum* and consumed corn porridge as the primary complementary food. It is unknown whether they consumed infant formula. The trial recruited 355 participants and divided them into three intervention groups: common bean, cowpea, or a mixture of corn and soy flour (control). The use of antibiotics was recorded (103 times), but it was not reported whether there was different antibiotic usage between interventions and controls. Of the 291 infants who completed the trial, 236 had their faeces analysed (82

in the cowpea group, 78 in the common bean group, and 76 in the control group). No changes in Faith's Phylogenetic Diversity or weighted UniFrac distances were observed between groups at the end of the study, nor were longitudinal changes in diversity scores observed for each intervention. However, infants who consumed cowpea had increased relative abundances of the genus *Bifidobacterium* and decreased abundances of the genus *Prevotella* compared to those who consumed common bean or corn-soy flour.

Zambrana et al. (2019) studied the impact of rice bran on the colonic microbiota of predominantly breastfed six-month-old infants from Nicaragua and Mali. In addition to the intervention, infants consumed other foods like staple grain porridges, soups, milk, fruits, juices, eggs, and fish. Nicaraguan infants also consumed breastmilk and infant formula, while Malian infants only breastmilk during the study. Of the 100 enrolled participants (50 in each country), 47 Nicaraguan and 48 Malian infants completed the six-month intervention. Notably, 52 infants received antibiotics during the trial: 25 Nicaraguan infants (14 in the control and 11 in the intervention group) and 27 Malian infants (14 in the control and 13 in the intervention group). No changes in alpha diversity indices (Observed, Shannon, Inverse Simpson, and Richness) between intervention and control groups were observed for either cohort. However, infants from different locations had distinct microbiota compositions at eight and twelve months of age (the faecal microbial composition was not measured at the study's baseline). At the end of the intervention (12 months old), Nicaraguan infants who consumed rice bran had increased abundances of the genera *Paraprevotella*, *Phascolarctobacterium*, *Veillonella*, and *Bifidobacterium*, and decreased abundances of the family *Lachnospiraceae* and the genus *Allisonella* compared to the control group. Malian infants consuming rice bran had increased abundances of the genera *Lactobacillus* and *Alloprevotella* but decreased abundances of the *Bifidobacteriaceae* family, and *Clostridium* and *Terrisoporobacte*r genera compared to the control group.

Existing evidence on the effects of plant-based foods on the colonic microbiota of weaning infants indicates no changes in microbial alpha diversity following the consumption of commercial infant cereals, legumes, or rice bran. This finding was consistent across all five trials that used plant-based foods as an intervention. Among the evaluated foods, wholegrain cereal promoted a longitudinal increase in the faecal abundance of bacteria from the order Bacteroidales in two studies. Additionally, one study reported that consumption of wholegrain cereal decreased the faecal abundance of the genus *Escherichia-Shigella,* which includes potential pathogens.

3.4.3. Influence of meats on the colonic microbiota of weaning infants

Three studies compared the influence of pureed meat versus other foods on the infant colonic microbiota (81,83,84). Krebs et al. (2013) allocated American breastfed infants to pureed beef or iron-fortified cereals during a three-month intervention. Fruits, vegetables, teething biscuits, and unfortified cereals were allowed *ad libitum*, while there was no consumption of infant formula during the study. The study monitored the use of antibiotics during the trial but did not report it. No changes in the Chao1 richness index were observed between groups, nor were there longitudinal changes in the microbial richness due to pureed beef consumption. On the other hand, infants who consumed pureed beef showed an increase in the relative abundances of the group *Clostridium XIVa* and a decrease in the family *Enterobacteriaceae* compared to baseline values. Consumption of pureed beef also promoted an increased abundance of the *Clostridium VIXa* group compared to the other intervention groups.

A similar study design was used by Qasem et al. (2017) who introduced either pureed beef or iron-fortified cereals to Canadian breastfed infants for two to four weeks. During the trial, infants consumed breastmilk, but it is unknown whether they consumed infant formula or other foods. The use of antibiotics during the intervention was not reported. Unlike the findings of Krebs et al. (2013), the authors reported that infants consuming pureed beef showed a longitudinal increase in microbial alpha diversity measured by the Chao1 richness index when compared to their baseline. At the end of the study, the pureed beef intervention group also exhibited a higher Chao1 index compared to the other intervention groups. However, no changes in the relative abundance of microbial taxa were observed due to pureed beef consumption.

Tang and co-authors (2023) characterised differences in the colonic microbiota of American formula-fed infants consuming pureed meats (beef, pork, and poultry) or dairy-based foods from five to twelve months of age. In addition to the intervention, infants consumed infant formula, cereal, fruit, and vegetables *ad libitum*. There was no consumption of breastmilk. One infant in each group used antibiotics during the intervention. Of the 71 infants enrolled in the trial, 64 completed it, and 57 had their faecal composition and function assessed (27 in the meat group and 30 in the dairy group). A permutational analysis of variance indicated differences in microbial composition between intervention groups at the end of the study ($p = 0.014$). Alpha diversity indexes differed between groups at 12 months, with infants in the meat group having a higher Chao1 richness estimator ($p = 0.002$). A longitudinal increase in the Chao1 estimator was also reported for infants consuming pureed meats (Table 3.3).

The consumption of pureed meats drove longitudinal changes in the relative abundance of microbial taxa, with increased abundances of the genera *Bacteroides*, *Faecalibacterium*, and the family *Ruminococcaceae*, while the genus *Escherichia* and the family *Enterobacteriaceae* decreased. Only the genus *Akkermansia* differed between intervention groups, having a lower abundance at the end of the study for infants who consumed meat. The study also evaluated the concentration of SCFAs in infants' faecal samples, reporting an increase in butyrate concentration in the meat group from five to twelve months (1.75-fold change), but no changes between groups (84).

Current evidence suggests that meat consumption, particularly pureed beef, increases the microbial alpha diversity of the colonic microbiota in weaning infants. This result was observed in two of the three studies using pureed beef as an intervention, compared to baseline values or other interventions. Furthermore, two trials reported that pureed beef consumption increased the faecal abundance of SCFA-producing bacteria, including the group *Clostridium XIVa* and the genera *Faecalibacterium* and *Bacteroides*. In contrast, pureed beef intervention decreased the faecal abundance of the family *Enterobacteriaceae* in two trials.

3.4.4. Influence of dairy products on the colonic microbiota of weaning infants

Only two identified trials used dairy products as an intervention (84,220). Bierut et al. (2021) assessed the influence of consuming a mixture of bovine colostrum and egg powder on the colonic microbiota of Malawian breastfed infants (9 months old). It is unknown whether infants consumed other foods, breastmilk, or infant formula during the trial. A mixture of corn and soy flour was used as a control in the three-month intervention. Of the 277 enrolled infants, 267 completed the trial, and 263 infants had their faecal microbial composition analysed at the end of the intervention (12 months old). The use of antibiotics during the trial was not reported. A permutational analysis of variance reported no changes in weighted UniFrac distances between control and intervention groups ($p = 0.374$; Table 3.3). Changes in microbial relative abundance were reported only at the species level. As compared to the control group, infants who consumed bovine colostrum with egg had higher abundances of *Clostridium perfringens*, *Streptococcus thermophilus*, *Megamonas rupellensis*, *Megasphaera paucivorans*, and *Eubacterium sp*., while a lower abundance of *Lactobacillus sp.*

More changes in the faecal microbial composition were observed in the study conducted by Tang and colleagues (2023), who assigned five-month-old American infants, exclusively formula-fed, to dairy foods (yoghurt, cheese, and whey protein) or pureed meats. Infants consumed infant formula and other

foods *ad libitum* but not breastmilk. One infant in each group used antibiotics during the intervention. At the end of the seven-month intervention, infants allocated to the dairy group had an increased faecal relative abundance of the genus *Akkermansia* compared to infants who consumed pureed meats. Dairy product consumption also promoted longitudinal increases in the abundances of the genus *Bacteroides* and the order Clostridiales and decreases in the abundances of the genus *Escherichia* and the family *Enterobacteriaceae*, among other changes (Table 3.3). Increases in alpha diversity scores (Evenness and Shannon Diversity) were reported for the dairy group when comparing values at the baseline and the end of the intervention. On the other hand, no longitudinal changes in the concentration of faecal SCFAs and BCFAs were observed for the dairy group compared to its baseline, nor between intervention groups at the end of the study.

Limited evidence exists regarding the influence of dairy foods on the colonic microbiota of weaning infants. One study reported that a mixture of bovine colostrum and egg powder increased the faecal abundance of the genus *Eubacterium* while decreasing the abundance of *Lactobacillus* compared to the control group. Another study found that the consumption of whey, cheese, and yoghurt increased the abundance of the genus *Akkermansia* compared to the control. Additionally, these dairy foods increased the faecal microbial richness compared to baseline values.

3.5. Discussion

This systematic review examined the effect of complementary foods on the composition and function of the colonic microbiota of infants at weaning age (4 to 12 months). Only a few clinical trials have evaluated the influence of early-life nutrition on the development of colonic microbes as infants transition from breastmilk to complementary foods. Seven studies met the inclusion criteria for this review, providing examples of the effect of plant-based foods, meats, and dairy foods on infant faecal microbial composition.

The evidence collected by this review suggests that the consumption of complementary foods by infants promotes the colonic development of microbial taxa capable of metabolising complex carbohydrates and proteins, notably the genus *Bacteroides* (74,224). At the same time, the abundance of microbes commonly found in faeces in the early months of life, such as the family *Enterobacteriaceae* and the genus *Escherichia*, decreases. This effect was observed for all the sources of foods included in this review (plant-based foods, meats, or dairy products), suggesting a natural progression in the development of the colonic microbiota as the infant ages, which is not associated with consuming a

specific food item. This finding aligns with previous studies (80,225). However, it is important to acknowledge that the evidence collected in this systematic review lacked detailed dietary intake information regarding the intake of breastmilk, infant formula intake, and other complementary foods.

This review also suggests that plant-based complementary foods have a lesser impact on the diversity of the infant colonic microbiota compared to meats and dairy products. At the end of all five studies using plant-based foods as intervention, no changes in faecal microbial diversity were observed between intervention and control groups. On the other hand, longitudinal increases in microbial richness were observed in two out of three trials that used pureed meats, and in one trial out of two that used dairy products. In agreement with this finding, the consumption of meats and cheeses was reported to be associated with increased faecal alpha diversity in infants, likely due to their relatively higher protein content (80).

Commercial infant cereals were the most studied complementary plant-based foods (n = 3). Two studies reported that wholegrain cereal interventions during the complementary feeding period promoted the increased abundance of microbial taxa commonly found in adult faeces, like the order Bacteroidales, while reducing the abundance of potential pathogens, such as the genus *Escherichia-Shigella*, compared to baseline values. Similar outcomes were observed *in vitro* through the fermentation of wholegrain commercial cereals using a faecal inoculum from weaning infants, showing an increase in the abundance of the *Bacteroidaceae* family and a decrease in the abundance of the *Enterobacteriaceae* family (89).

These changes in microbial taxa can likely be attributed to the high content of non-digestible carbohydrates in wholegrain cereals, particularly dietary fibre. Dietary fibres support the development of the colonic microbiota as they reach the colon undigested and are preferentially fermented by colonic commensals, producing, among other products, organic acids that ultimately impact host physiology (226,227). For instance, the SCFAs acetate, propionate, and butyrate have been extensively studied for their health benefits, including providing energy to colonocytes and supporting intestinal barrier function, among others (214).

Longitudinal studies linking dietary patterns with the colonic microbiota of infants reported that fibre consumption, including the intake of infant cereals, is positively correlated with increased faecal concentrations of propionate at six months and alpha diversity at twelve months (3,90). Another study positively correlated complex carbohydrate consumption with the faecal abundances of the SCFA-producing genera *Ruminococcus* and *Lachnospira* in infants aged six to twelve months (82). Although

there are no current recommendations for daily dietary fibre intake for infants in their first year of life, nutritional guidelines highlight the importance of consuming fibre-rich foods, such as wholegrains, fruits, and vegetables, as part of a healthy diet in early infancy (101,108,228).

Dietary guidelines for infants also recommend the consumption of lean meats to provide protein and micronutrients, particularly iron and zinc (101,108). Pureed beef was the most studied meat in the identified trials (n = 3). Its effect on faecal microbial diversity scores and relative abundances of microbes in weaning infants varied between studies. Nevertheless, the evidence collected in this review suggests that pureed beef beneficially increases the faecal abundance of butyrate-producers, like the genus *Bacteroides* and members of the *Clostridium XIVa* group, while reducing the abundance of the family *Enterobacteriaceae* (2 out of 3 studies). Pureed beef also increased microbial richness in infant faeces in two studies, a parameter positively associated with a more stable colonic microbiota and negatively associated with biomarkers for disease development in adults (229–231).

Observed changes in the faecal microbial composition due to pureed beef consumption are likely due to its high protein and iron content. Although the human body is efficient at digesting and absorbing protein, approximately 10 % of consumed protein reaches the adult colon unabsorbed, where it is fermented by proteolytic bacteria producing SCFAs and BCFAs (11,232,233). Longitudinal trials positively correlated animal protein consumption with increased faecal abundance of the *Bacteroides* genus and alpha diversity in weaning infants (3,80,82). In contrast, iron supplementation was associated with increased faecal abundance of the genera *Escherichia-Shigella* and *Clostridium* in weaning infants (107). *In vitro* faecal fermentation studies using adult inoculum demonstrated that consuming beef promotes the development of taxa from the genera *Clostridium* and *Peptoclostridium,* leading to the production of acetate, propionate, butyrate, isobutyrate, and isovalerate (234,235).

Neither of the two studies evaluating faecal metabolites reported changes in the concentration of BCFAs due to the intervention of complementary foods. Currently, the effect of BCFAs on host physiology is not well characterised. These organic acids are considered markers of protein fermentation (11), which in turn also produces potentially deleterious metabolites like ammonia and p-cresol (236,237). For instance, ammonia is neurotoxic when present at high levels in the blood, while high doses of p-cresol are toxic to colonic epithelial cells (238,239). In this context, moderation is key. Evidence in adults has shown that excessive consumption of animal protein, concomitant with insufficient fibre

intake, disrupts the colonic microbiota (17), but when consumed as part of a balanced diet, it beneficially increases the colonic microbial diversity (140).

Longitudinal trials in weaning infants observed that dairy consumption was negatively correlated with the faecal abundances of the *Bacteroides* genus and the *Enterobacteriaceae* family, and cheese intake was associated with increased faecal microbial alpha diversity (3,80,82,87). In adults, a trial comparing high versus low consumption of milk, cheese, and yoghurt reported an increased relative abundance of the species *Streptococcus thermophilus* for participants consuming higher amounts of dairy foods (240). However, no changes in faecal microbial diversity were observed between groups (240). This result aligns with the findings of Bierut et al. (2021) who observed no changes in microbial diversity but an increased abundance of *S. thermophilus* in weaning infants consuming a mixture of bovine colostrum and egg powder.

A recent systematic review of the impact of dairy products on the human colonic microbiota reported no changes in the microbial diversity in response to dairy interventions, but increased abundances of the *Lactobacillus* and *Bifidobacterium* genera in three out of seven studies included (241). These genera include members that have benefits on children's gastrointestinal health, suggesting that dairy consumption positively impacts the colonic microbiota of weaning infants (242). The effect of dairy products on colonic microbes, compared to other complementary foods, may be explained by the presence of lactose, which fosters the growth of lactic acid bacteria and lactate production, contributing to SCFA production through cross-feeding interactions (243). Whey protein also promotes the growth of the *Bifidobacterium* and *Lactobacillus* genera, stimulating SCFA production (244).

In addition, fermented dairy products, like cheese and yoghurt, are also a source of living microorganisms and microbial metabolites, the consumption of which may support a balanced colonic microbiota (245). Ultimately, changes in the colonic microbiota promoted by dairy products may benefit host health, as their consumption has been linked to a reduced risk of developing chronic diseases, such as type 2 diabetes and obesity, as reported in meta-analyses involving children and adults (246,247). Dairy foods (rather than whole milk as a drink) are recommended for weaning infants in the first year of life as part of a nutritionally balanced diet, providing protein, vitamins, and minerals, particularly calcium and riboflavin (101,108).

This chapter systematically reviewed literature on the impact of complementary foods on the colonic microbiota of infants aged 4 to 12 months. Although often understudied in microbiota investigations, the

weaning period plays a crucial role in the maturation of colonic microbes. The description of the evidence collected here sheds light on how a few complementary foods introduced to infants at the early stages of weaning affect their faecal microbial composition, contributing to filling a current knowledge gap in infant nutrition. Another strength of this chapter was the inclusion of randomised controlled trials, which are the gold standard methodology for assessing outcomes of dietary interventions.

It is important to emphasise that although this review concentrated on the effects of individual complementary foods, introducing weaning infants to a diversified complementary diet is crucial for meeting their nutritional requirements and supporting the adequate maturation of their colonic microbiota. As recommended by dietary guidelines, diversity is a key factor in healthy complementary feeding patterns (101,108,228). Infants consuming a diverse diet are less likely to develop allergies and atopic diseases (248). In turn, infants who lack a varied diet at weaning are more prone to nutrient deficiencies, ultimately compromising their health status later in life (249,250). Regarding the colonic microbiota, longitudinal investigations have shown that increased dietary diversity during weaning is associated with higher microbial diversity and richness, contributing to stabilising the microbiota (80,109).

One limitation of this chapter is that only seven trials satisfied the eligibility criteria. This was due to the scarcity of interventions assessing the impact of complementary foods on the colonic microbiota of weaning infants. However, a search strategy limited to only papers published in English excluded potentially eligible records in other languages, which may have contributed to the small number of included studies. Due to the lack of available data for the same food intervention, meta-analyses of the effect of complementary foods on the microbiota of weaning infants could not be performed. Instead, a narrative discussion of the results of the included trials was performed. The limited data on the same food interventions also compromised the synthesis capacity of this systematic review, highlighting the urgent need for more randomised controlled trials to evaluate the relationship between complementary foods and the colonic microbiota of weaning infants.

One study reported considerable antibiotic usage during the trial, with 52 out of 95 infants receiving antibiotics (218). Additionally, three studies did not provide information about antibiotic usage during the intervention period (81,83,220). Antibiotics are known to alter the colonic microbiota composition, potentially confounding the effects of the dietary intervention (251). One trial included in this review had an intervention time lower than one month and may not have been long enough to capture persistent

changes in the colonic microbiota (83). Although there are no absolute guidelines about the duration of food intervention trials in microbiome research, evidence in adults suggests that short-term dietary interventions lead to rapid but temporary modifications in faecal microbial composition (25,252).

Most of the identified trials (4 out of 7) compared the effect of one intervention against another in the absence of a relatively inert food used as a control (81,83,84,88). This approach makes it difficult to isolate the individual impact of each food on the colonic microbiota and may lead to confounding results and limited conclusions. One study analysed the faecal microbial composition of a small subset of 14 participants from a total of 45 enrolled infants, reducing its statistical power and potentially leading to false conclusions (81). Additionally, three trials characterised the colonic microbiota of weaning infants as a secondary outcome, and did not analyse faecal samples from all infants or collection time points (81,88,220).

Infants under six months of age were enrolled in four trials (81,83,84,88), including infants at four months (83,88). This contrasts with current complementary feeding recommendations, which recommend exclusive breastfeeding for around the first six months (101,108). Although this recommendation is flexible and can be adapted to each infant's needs, the colonic microbiota develops rapidly during the first year of life, and even a difference of a few months in age can lead to distinct compositions among infants (56,225,253). Nevertheless, parents may introduce their infants to complementary foods earlier than recommended. As observed by the Feeding Infants and Toddlers Study (2016), the largest dietary intake survey in infants and toddlers performed in the USA, only 15 % of infants aged 4-5.9 months were exclusively breastfed (254). Furthermore, the introduction of solids between four and six months is recommended for infants at risk of developing food allergies (101). Given this scenario, this chapter included trials involving infants aged at least four months. This is justified because it is likely that infants will be introduced to complementary foods at around four months of age.

Importantly, Krebs et al. (2013) and Qasem et al. (2017) reported contrasting effects on the faecal microbial composition of infants for the same food intervention, either pureed beef or infant cereals. Regarding the effect of the pureed beef intervention, Krebs and co-authors (2013) observed changes in the abundance of microbial taxa but no changes in microbial diversity, while the latter noticed alterations in the microbial richness but not in the relative abundance of microbes (83). These contrasting results may be due to other key factors influencing the colonic microbiota in early postnatal life, such as mode of delivery, type of feeding (breastmilk versus infant formula), maternal diet, and geographical location

(62,63). The influence of these factors on colonic microbes reduces as the infant ages but is still evident in the early stages of weaning (26,255).

In addition, several other host factors affecting colonic microbes, such as the circadian cycle and intestinal transit time, were not considered in the selected studies (225,256,257). Notably, while all studies included in this review assessed the dietary intake of participants, it is not evident whether changes in dietary intake over time, particularly for breastmilk or infant formula consumption, were accounted for in the microbiota analyses. This limitation is especially relevant for infant formula, as its consumption may increase during weaning as a replacement for breastmilk.

In the example above, Krebs and co-authors (2013) recruited only vaginally delivered American infants, while Qasem and colleagues (2017) recruited Canadian infants delivered vaginally, by caesarean, and through other modes. It is worth noting that the trials included in this review were conducted in different parts of the world and that the geographical location of the infants will have influenced the colonic microbiota composition, as evidenced in infants and adults (26,258). The colonic microbiota also varies between individuals and within the same individual over time, potentially reacting differently to the same dietary intervention for each person (22,259). Furthermore, infants normally consume complementary foods alongside other foods, often in mixed meals, which may influence the digestion and absorption of nutrients and subsequent microbiota response.

In addition, methods to characterise colonic microbes were heterogeneous among studies, limiting the comparison of the results. All trials used 16S rRNA sequencing but amplified different hypervariable regions, leading to potential disagreements in microbial taxa identification and resolution (260). Different bioinformatic pipelines and reference databases were used, which are likely to affect the taxonomic assignment and the estimation of the relative abundance and diversity scores of the microbial community (261,262). Identified trials used faecal samples to assess the colonic microbiota due to the ease of collection, transportation, and storage, and their non-invasive and cost-effective nature. However, faecal samples mainly reflect the microbial composition of the distal colon, failing to fully represent microbial communities free in the colonic lumen or attached to the colonic mucosa and other parts of the colon (200).

Only one trial evaluated the influence of complementary foods on the production of both SCFAs and BCFAs by the colonic microbiota of infants (84). The lack of metabolite production analyses in most of the included studies is a crucial limitation of the existing data that raises questions about the potential

impact of observed changes in taxonomic composition on the overall functionality of the microbial community. It is important to note that evaluating the impacts of dietary interventions on the colonic microbiota requires more than just analysing microbial composition. Ultimately, understanding the functional aspects of the microbiota is more relevant for host health. Despite interindividual variations in the microbial composition, the colonic microbiota of healthy individuals has similar functions (4). On the other hand, imbalances in the production of microbial metabolites are associated with colonic dysbiosis and increased risk of disease (29,263,264). Further research, particularly studies using a multi-omics approach to assess both the impact of foods on microbial composition and function, is necessary to better understand how introducing complementary foods affects the colonic microbiota in infants.

3.5. Conclusions

In conclusion, this chapter systematically included seven food intervention trials that assessed the influence of plant-based foods, meats, or dairy products on the colonic microbiota of weaning infants. The evidence collected suggests a natural progression in the increasing abundance of colonic microbes capable of metabolising complex carbohydrates and proteins as infants age, which does not seem to be related to the consumption of any specific complementary food. Two studies evaluated wholegrain cereals as an intervention, whereas three other trials investigated the effects of pureed beef. These foods increased the faecal abundance of microbes producing SCFAs compared to baseline values, with this effect observed in two trials for each food. Additionally, pureed beef increased faecal microbial richness in two studies. However, the conclusions of this chapter are limited by the small number of included studies and their varying methodologies and reported outcomes. Further research assessing the impact of complementary foods on both the composition and function of the colonic microbiota in weaning infants is essential to fill this knowledge gap in infant nutrition.

Chapter 4: Qualitative assessment of modelling tools for predicting the effects of dietary compounds on the human colonic microbiota

4.1. Abstract

Traditional *in vitro* and *in vivo* approaches for studying the colonic microbiota are time- and resource-consuming. Mathematical models are rapid, high-throughput, and inexpensive tools that can complement the understanding of the relationship between diet and colonic microbes. This chapter evaluated five modelling tools predicting the effect of dietary compounds on colonic microbial communities. The ordinary differential equation (ODE)-based models, microPop and microPopGut, were characterised by easy customisation and low computational requirements. However, their reductionistic approach limited their ability to represent the complexity and diversity of the human colonic microbiota. These models simulated dietary effects at low resolutions, considering only variations in total protein and carbohydrate content while overlooking the role of individual micronutrients and other macronutrients. A major drawback of ODE-based models is their reliance on microbial kinetic parameters, which are often unknown or poorly characterised experimentally, leading to assumptions or model fitting. In contrast, metagenome-scale community metabolic models (MGCMs) represent microbial community metabolism using genome information from individual microorganisms, thereby eliminating the need for predefined microbial kinetic parameters. These models are suitable for simulating the influence of detailed dietary fluxes on personalised representations of the colonic microbiota. The model MICOM stood out for its user-friendly workflows and for containing all necessary tools, from data processing to results visualisation, in a single package. In qualitative agreement with experimental data, MICOM predicted higher fluxes of SCFAs in the colonic microbiota of healthy adults consuming a high-fibre diet compared to those on a Western diet. The MICOM model is a valuable tool for generating insights into how dietary compounds affect the colonic microbiota, aiding in the design of future *in vitro* or *in vivo* experiments.

4.2. Introduction

The relationship between colonic microbes and host health has prompted investigations into how diet, a key modulator of colonic commensals, shapes the composition and function of the colonic microbiota (25,131). Most colonic microbiota investigations have been based on *in vitro* and *in vivo*

approaches, including faecal fermentation, animal models, and clinical trials (25,219,265–267). These traditional methods have established fundamental knowledge of the colonic microbiota, including proposed mechanisms of action and potential host health outcomes. However, they are limited by technical, resource, and ethical constraints (30). To address these challenges, complementary computational tools have been developed. Mathematical models show promise as an initial source of investigation, generating hypotheses that traditional methods can further evaluate. They use pre-existing data to conduct simulations, thereby reducing the cost and time required for colonic microbiota research.

Initial *in silico* models of gut microbial communities focused on simulating the metabolism of individual microorganisms or microbial communities composed of a few members (268–270). While their reductionistic approach limited their ability to fully represent the colonic microbiota and human physiology, the assumptions they proposed paved the way for more complex frameworks. Among these, ODE-based models and genome-scale metabolic models (GEMs) are the current most used models to assess the influence of dietary compounds on microbial communities.

ODE-based models characterise the metabolism of distinct phenotypes within a microbial community, describing resource exchange between colonic microbes and the system through ODEs. These models require the definition of microbial kinetic parameters, such as growth rates and metabolite production rates under different substrates (271,272). ODE-based models can effectively represent *in vitro* data, have low computational costs, and allow the investigation of cross-feeding interactions between members of the microbial community. However, experimentally determining the necessary microbial parameters remains a major challenge for building ODE-based models. This limitation restricts their application to small and well-categorised microbial communities (273–275).

In contrast, GEM-based models use genome information from microorganisms to infer their metabolism. These models employ metabolic reconstructions to describe the biochemical reactions present in a microbial community, creating a metabolic network represented by a system of linear equations that is solved by flux balance analysis (FBA). Constraints and assumptions are then applied to predict optimal fluxes of substrates and microbial biomass [see review (276)]. MGCMs extend this concept to large microbial communities (31,277). These models have increased in popularity in recent years due to the publication of metabolic reconstructions of thousands of colonic microbial strains, facilitating their implementation (278,279). However, little is known about the experimental validation of MGCMs, and their predictive accuracy depends on the quality of metabolic reconstructions (280,281).

Although mathematical models have the potential to accelerate the study of microbial communities, studies evaluating the influence of dietary compounds on the human colonic microbiota *in silico* are scarce (117,203,280,282,283). Expanding the use and development of modelling approaches could generate insights to guide experimental research, ultimately enhancing the understanding of how dietary compounds influence colonic commensals and contributing to better nutritional recommendations. In this context, this chapter aimed to evaluate the suitability of pre-existing mathematical models in predicting how diet shapes the composition and function of the human colonic microbiota. Characteristics of different modelling tools, including ODE-based and GEM-based models, were qualitatively assessed.

4.3. Materials and methods

4.3.1. Software

Five mathematical models were investigated. Two ODE-based models: microPop (274) and microPopGut (275); and three GEM-based models: BacArena (284), The COnstraint-Based Reconstruction and Analysis (COBRA) Toolbox (202,277), and Microbial Community (MICOM) (31). These models were selected for being open-source and free of charge, employing various modelling strategies, and being released in recent years and/or regularly updated.

Models and additional software required for their execution were installed according to the developers' instructions, which are available on GitHub channels (see Appendix 3). For microPop, microPopGut, and BacArena, R (version 4.2.1) and RStudio (Posit) were used. For the COBRA Toolbox, MATLAB (version 2020b, MathWorks) was used. For MICOM, Python (version 3.9) and the integrated development environment Spyder (version 5) were employed. The numerical solvers GUROBI (version 10.0.1, Gurobi Optimisation) and CPLEX Optimisation Studio (version 22.1.0, IBM) were used under academic licences. The code and data used in the simulations described in this thesis are available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/Chapter%204.

4.3.2. Workflow, modelling assumptions, and simulation conditions

4.3.2.1. MicroPop

MicroPop is an R package that models the biomass and production of metabolites by microbial communities by solving a system of ODEs. The microbial community is defined by a reductionistic approach that focuses on identifying key members, known as microbial functional groups. To operate, the user must define parameters for each microbial functional group, including microbial growth rates with different substrates, as well as rates of resource uptake and metabolite production (274). Simulations have a customisable duration and account for the influence of total protein and total carbohydrate, including fractions of non-starch polysaccharide and resistant starch. The initial colonic concentration (g/L) and inflow (g/L/d) for these dietary compounds must be defined.

The package has default conditions to model the adult colonic microbiota, which consists of 10 microbial functional groups and a reduced list of produced microbial metabolites, including SCFAs (285). MicroPop was initially executed using the default conditions. The initial concentrations (g/L) and inflows (g/L/d) of protein, resistant starch, and non-starch polysaccharides were then linearly scaled to simulate the effects of a high-protein, low-fibre diet or a high-fibre, low-protein diet on the colonic microbiota. Due to the limited data in the literature concerning the concentration of unabsorbed dietary compounds in the human large intestine, values for the representative diets were assumed as follows:

- Default conditions: 5.90 g/L and g/L/d of protein, 1.62 g/L and g/L/d of non-starch polysaccharides, and 5.60 g/L and g/L/d of resistant starch;
- High-protein-low-fibre diet: 8.90 g/L and g/L/d of protein, 1.22 g/L and g/L/d of non-starch polysaccharides, and 3.00 g/L and g/L/d of resistant starch;
- High-fibre-low-protein diet: 2.90 g/L and g/L/d of protein, 2.02 g/L and g/L/d of non-starch polysaccharides and 8.20 g/L and g/L/d of resistant starch.

4.3.2.2. MicroPopGut

MicroPopGut is an updated version of microPop that uses the same default conditions as its predecessor (microbial functional groups and substrates) but focuses on predicting the concentrations of major SCFAs in three segments of the human colon: ascending, transverse, and descending (275). Like microPop, microPopGut allows users to modify the total protein and carbohydrate amounts reaching the colon, as well as the fraction of carbohydrates that are resistant starches. To assess how the model

responds to variations in the content of these dietary compounds, the values of these parameters were chosen (based on assumptions) to simulate the effect of three different diets, as follows:

- Western diet (default diet): 10 g/d of protein, 50 g/d of carbohydrates, 78 % resistant starch;
- High-protein, low-fibre diet: 30 g/d of protein, 30 g/d of carbohydrates, 30 % resistant starch;
- High-fibre, low-protein diet: 5 g/d of protein, 55 g/d of carbohydrates, 90 % resistant starch.

4.3.2.3. BacArena

BacArena combines two modelling strategies, GEM-based and agent-based modelling, to simulate the spatial and temporal variations of microbial communities. It is an R package that contains a default microbial community composed of seven bacterial species, representing the adult colonic microbiota (284,286). A diet designed through flux variability analysis is also provided as a default. This diet contains all the necessary substrates for the growth of the representative colonic microbial community, with the amount of dietary compounds set as 0.001 mmol (for essential nutrients) or 0.0001 mmol (for non-essential nutrients).

The model was initially executed using the default conditions. Then, to better represent the microbial diversity found in the human colon, five species involved in key functions of the colonic microbiota, such as SCFA metabolism, mucus degradation, and methanogenesis (287–290), were added to the simulations. Metabolic reconstructions of these microbial species (*Akkermansia muciniphila, Eubacterium rectale*, *Methanobrevibacter smithii*, *Prevotella copri*, and *Ruminococcus bromii*) were imported from the Assembly of Gut Organisms through Reconstruction and Analysis repository (AGORA, version 1.03) (278). Simulations with the resulting microbial community were then performed under the default diet.

4.3.2.4. The COBRA Toolbox

The COBRA Toolbox is open-source software executed in MATLAB that contains several functions for modelling microbial communities using genome-scale metabolic reconstructions (202,277). One of the main advantages of the software is its extensive documentation, tutorials, and active community forum (Appendix 3). The tutorial "Simulation of Growth of Human Gut Microbes on Different Diets" exemplifies how the toolbox can be used to predict microbial growth under various dietary conditions. Using its default conditions, the tutorial compares the growth of colonic microbes using Western and

high-fibre diets as inputs, in which metabolic reconstructions were obtained from the AGORA repository (278) and dietary fluxes from the Virtual Metabolic Human (VMH) database (291).

Another tutorial is the "Creation, interrogation, and analysis of personalised microbiota models from the AGORA models through metagenomic data integration", also known as Microbiome Modelling Toolbox (202). It builds personalised microbial communities and predicts the production of microbial metabolites based on dietary inputs. The tutorial was used to compare the influence of a European average diet and a high-fibre diet, both obtained from the VMH database (291), on a microbial community corresponding to the average relative abundance of ten selected microbial strains from four healthy adults (292).

4.3.2.5. MICOM

MICOM is a Python package that creates and analyses personalised simulations for microbial communities under different dietary conditions in a user-friendly manner (31). It contains pre-existing workflows for designing dietary fluxes representative of host diets and predicting their influence on the growth rates of colonic microbes and fluxes of microbial metabolites. The package is compatible with AGORA metabolic reconstructions (278) and the VMH database (291), allowing straightforward customisation of inputs needed to execute its pre-defined workflows.

MICOM was used to simulate the influence of two VMH diets (a European average diet, representative of the Western dietary pattern, and a high-fibre diet) on the genera present in the faeces of four adult healthy individuals (293). Dietary fluxes, imported from the VMH database, were processed using a predefined workflow for designing new *in silico* media, which identifies and dilutes dietary compounds absorbed by human intestinal cells while also adding host-secreted bile acids and mucin cores. Finally, missing essential substrates for microbial growth, such as vitamins, micronutrients, and amino acids, were automatically identified and added to the media in minimal amounts.

4.3.3. Model assessment

The following characteristics were qualitatively assessed to identify the most suitable model: user-friendliness, computational intensity, experimental validation, prediction accuracy, and the ability to capture dietary influence. In the user-friendliness category, the ease of operating the model and modifying the simulation conditions was evaluated. Models that required extensive knowledge of bioinformatics and/or an extensive literature search to modify default simulation conditions were

considered unsatisfactory. Computational intensity was assessed based on the time necessary to simulate on a standard desktop personal computer (Windows 10, processor 12th Gen Intel(R) Core(TM) i5-1235U, installed RAM 16.0 GB). As a quantitative criterion, models that required more than four hours to complete a simulation were considered unsatisfactory.

Ideally, models have been previously validated using *in vitro* or *in vivo* data, satisfying the validation criteria. However, this does not apply to GEM-based models, in which experimental validation is a current technical challenge [see review (294)]. To assess the prediction quality of the models, the microbial growth and metabolite production predicted *in silico* were qualitatively compared with *in vitro* or *in vivo* data. When quantitative comparison between *in silico* simulations and *in vitro* or *in vivo* data was possible, models in which predicted values differed by more than 50 % from experimental values were considered unsatisfactory.

The capability of models to represent the impact of host diets on the colonic microbiota was assessed in two ways. Dietary resolution refers to the extent of the list of dietary compounds considered in the simulations. Models based on overly reductionist strategies, for example, those that only use total macronutrients instead of different types of carbohydrates, amino acids, and micronutrients, were considered unsatisfactory. Lastly, dietary influence was evaluated, which measures whether the model generates different outcomes under distinct dietary conditions. Quantitatively, models in which the predicted production of microbial metabolites changed by less than 10 % when diets with varying compositions were used in the simulations were considered unsatisfactory. This ensured that the models were able to represent the rapid response of colonic microbes to changes in host diet observed *in vivo* (25).

4.4. Results

The characteristics of the evaluated models are shown in Table 4.1. Overall, ODE-based models were characterised by easier installation and operation, as well as lower computational intensity, compared to GEM-based models. On the other hand, due to their reductionistic approach, ODE-based models were suitable only for small-scale or simplified representations of microbial communities. They accounted for the influence of only a few dietary compounds, resulting in low dietary resolution. GEM-based models, although requiring additional software for operation and being less user-friendly, had a deep dietary resolution and predicted the influence of host diets on an extensive list of metabolites produced by colonic microbes. GEM-based models were selected as more promising candidates for

simulating the influence of diets on colonic microbes despite their notorious lack of experimental validation.

Table 4.1. Assessment of the models microPop, microPopGut, BacArena, The COBRA Toolbox, and MICOM.

Model (year of publication)	User-friendliness	Computation intensiveness	Validation	Prediction quality	Dietary influence	Dietary resolution
microPop (2018)	Easy to install, operate, and customise	It is not computationally intensive (simulations took around one minute to run). 10 microbial functional groups were included in the simulations	Predicted SCFA concentration was compared to *in vitro* continuous fermentation data (285), reporting similar profiles for butyrate concentration but not for acetate and propionate	Predicted concentration of butyrate was inconsistent with the literature, with a ratio of acetate, propionate, and butyrate of $2:1:4x10^{-6}$	Variations in total protein and dietary fibre content generated small changes (up to 11 %) in the concentration of SCFAs and predominant microbial functional groups	Has low resolution, simulating only the impact of total protein, non-starch polysaccharides, and resistant starch in the diet
microPopGut (2022)	Easy to install and operate, but less customisable than microPop	It is not computationally intensive (simulations took around 5 minutes to run). 10 microbial functional groups were included in the simulations	Predicted SCFA production was qualitatively compared with *in vivo* data (275). In agreement with experimental data, the model predicted increased butyrate production when increasing the amount of dietary fibre	In agreement with previous studies, the model predicted increased biomass of the *Bacteroides* genus with increased dietary protein content and increased SCFA production with increased dietary fibre content	Variations in total protein and carbohydrate content generated changes up to 37 % in the total SCFA production and 81 % in the biomass of microbial function groups	Has low resolution, accounting only for the effects of total protein, carbohydrates, and the resistant starch fraction
BacArena (2017)	Easy to install and operate, but difficult to customise	Becomes highly computationally intensive when simulating larger microbial communities (simulations took up to 10 hours to run with 12 microbes). 7 microbial species were included in the simulations	Predicted SCFA ratio was compared with *in vitro* data (286). Acetate ratio was consistent with experimental values (both ratios around 0.7), but the model predicted a higher ratio of butyrate (0.2 vs 0.1) and a lower of propionate (< 0.1 vs 0.2)	The ratio of predicted SCFAs was inconsistent with the literature. Under default simulation conditions, the proportion between acetate, propionate, and butyrate was approximately 10:100:1	Not evaluated (an extensive literature search is necessary to define the parameters necessary for changing diets in the simulation)	Has deep resolution, comprising the effect of various dietary substrates (e.g., micronutrients, amino acids, and sugars)

The COBRA Toolbox (2019)	Requires additional software for installation and operation. Simulation conditions (dietary fluxes and microbial communities) are easily customisable. Requires prior bioinformatics knowledge for operation	It is computationally intensive (simulations took around 3 hours to run). 10 microbial genera were included in the simulations	Predictions were not validated experimentally	The toolbox predicted SCFA fluxes that disagree with the ratio acetate:propionate:butyrate reported in the literature. Predicted fluxes of butyrate under both European average and high-fibre diets were zero, while fluxes of acetate and propionate were identical	The model predicted identical SCFA fluxes under both European average and high-fibre diets, contrasting previous studies that reported increased SCFA production with increased fibre consumption	Has deep resolution, using fluxes of various dietary compounds as input (approximately 80 different compounds incorporated in the simulations)
MICOM (2020)	Requires additional software for installation and operation. Pre-existing workflows facilitate its operation. Simulation conditions (dietary fluxes and microbial communities) are easily customisable	It is computationally intensive (simulations took around 2 hours to run). 18 microbial genera were included in the simulations	Predictions were experimentally validated using *ex vivo* faecal incubations with different dietary fibres. Satisfactory agreement between predicted and measured outcomes was reported for butyrate and propionate (280)	In qualitative agreement with the literature, the model predicted increased SCFA production on a high-fibre diet compared to a Western diet	Simulations under two different diets (European average and high-fibre diets) resulted in changes of up to 170 % in the predicted SCFA fluxes	Has deep resolution, using fluxes of various dietary compounds as input (approximately 170 different compounds used in the simulations)

4.4.1. ODE-based models

4.4.1.1. MicroPop

The influence of three diets (high-protein, low-fibre, high-fibre, low-protein, and the model's default diet) on a representative community of the adult colonic microbiota was simulated using MicroPop. In all dietary conditions, the microbial population was dominated by the *Bacteroides* functional group (Figure 4.1). Modifying the initial concentrations (g/L) and inflows (g/L/d) of protein, non-starch polysaccharides, and resistant starch resulted in small changes in the final concentration of the *Bacteroides* functional group, with a maximum increase of 11 %. In contrast, greater changes were observed in the concentrations of lactate, propionate, and butyrate producers, which varied up to 84 %. Compared to the default diet, the high-fibre, low-protein diet increased the concentration of all the microbial functional groups. In contrast, the high-protein, low-fibre diet had the opposite effect.

Similar results were observed for SCFA concentration (Table 4.2). The predicted concentrations of SCFAs increased with the high-fibre, low-protein diet and decreased with the high-protein, low-fibre diet. Butyrate showed the greatest variation, with a range of up to 76 %. In contrast, changes in dietary conditions had a modest impact on the final concentrations of acetate and propionate, with variations of up to 10 %. Acetate was the predominant SCFA (up to 2.99 g/L), followed by propionate (up to 1.67 g/L). In contrast, the predicted butyrate concentration was negligible, reaching up to 7.86×10^{-6} g/L.

Table 4.2. Predicted concentration of microbial functional groups and SCFAs after 24 hours of simulation under three dietary conditions using microPop.

Microbial functional groups (g/L)			
	Default diet*	High-protein-low-fibre diet*	High-fibre-low-protein diet*
Bacteroides	3.50	3.12	3.88
Lactate producers	8.85×10^{-9}	2.47×10^{-9}	1.29×10^{-8}
Propionate producers	1.72×10^{-8}	4.46×10^{-9}	2.58×10^{-8}
Butyrate producers1	7.72×10^{-7}	1.22×10^{-7}	1.38×10^{-6}
Butyrate producers2	3.37×10^{-6}	8.75×10^{-7}	4.46×10^{-6}
Butyrate producers3	1.80×10^{-8}	4.66×10^{-9}	2.70×10^{-8}
Acetogens	1.24×10^{-2}	1.15×10^{-2}	1.38×10^{-2}
Short-chain fatty acids (g/L)			
Acetate	2.72	2.47	2.99
Propionate	1.53	1.38	1.67
Butyrate	5.62×10^{-6}	1.37×10^{-6}	7.86×10^{-6}

*The initial concentration (g/L) and inflow (g/L/d) of protein, non-starch polysaccharides, and resistant starch were, respectively, 5.90, 1.62, and 5.60 for the default conditions, 8.90, 1.22, and 3.00 for the high-protein, low-fibre diet, and 2.90, 2.02, and 8.20 for the high-fibre, low-protein diet.

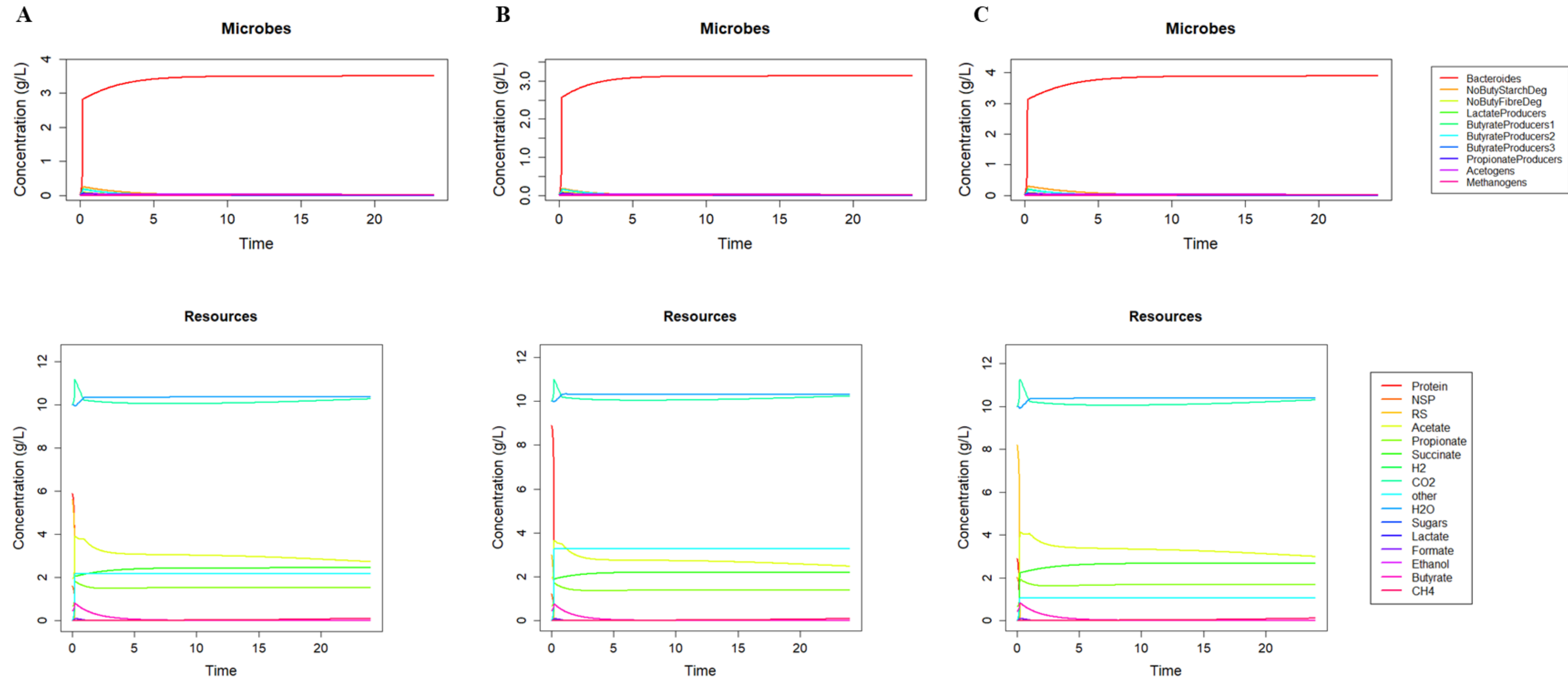

Figure 4.1. Simulations of microbial biomass and resource concentrations using microPop. Concentrations of microbial biomass and resources were simulated over 24 hours under three dietary conditions. The initial concentrations (g/L) and inflows (g/L/d) of protein, non-starch polysaccharides, and resistant starch were, respectively, 5.90, 1.62, and 5.60 for the default conditions (A), 8.90, 1.22, and 3.00 for the high-protein-low-fibre diet (B), and 2.90, 2.02, and 8.20 for the high-fibre-low-protein diet (C).

4.4.1.2. MicroPopGut

MicroPopGut predicted similar profiles for SCFA production in the distal colon across three dietary conditions: a default Western diet, a high-protein, low-fibre diet, and a high-fibre, low-protein diet. Acetate was the most produced SCFA, followed by butyrate and propionate (Figure 4.2). The high-protein, low-fibre diet resulted in the lowest total SCFA production at the end of the simulation (43.4 mM). In comparison, the default and high-fibre diets exhibited similar values of 69.3 and 73.7 mM, respectively (Table 4.3). Compared to the default diet, the high-fibre-low-protein diet increased butyrate production by 15.9 %. In contrast, the high-protein, low-fibre diet led to decreases in acetate, propionate, and butyrate by 45.1 %, 35.5 %, and 14.4 %, respectively.

Dietary changes resulted in variations in the biomass of only the microbial functional groups of the *Bacteroides* genus and lactate producers (Table 4.3). Compared to the default diet, the high-fibre-low-protein diet increased the biomass of lactate producers by 8.5 % and reduced the biomass of the *Bacteroides* genus by 16.6 %. In contrast, the high-protein-low-fibre diet increased the biomass of the *Bacteroides* genus by 81.7 % and reduced that of lactate producers by 64.3 %.

Table 4.3. Predicted biomass of microbial functional groups and production of SCFAs in the distal colon after 24 hours of simulation under three dietary conditions using microPopGut.

		Microbial functional group (g)	
	Default diet*	High-protein, low-fibre diet*	High-fibre, low-protein diet*
Bacteroides	1.75	3.18	1.46
Lactate producers	4.14	1.48	4.49
Propionate producers	1.74	1.30	1.81
Butyrate producers[1]	1.52	1.44	1.61
Butyrate producers[2]	1.91	1.94	1.88
Butyrate producers[3]	1.49	1.27	1.55
Acetogens	1.04	1.14	1.07
		Short-chain fatty acids (mM)	
Acetate	42.60	23.40	45.30
Propionate	13.50	8.70	13.10
Butyrate	13.20	11.30	15.30

*The inflow (g/d) of protein and carbohydrates, as well as the resistant starch fraction, were, respectively, 10, 50, and 78 % for the default conditions, 30, 30, and 30 % for the high-protein, low-fibre diet, and 5, 55, 90 % for the high-fibre diet.

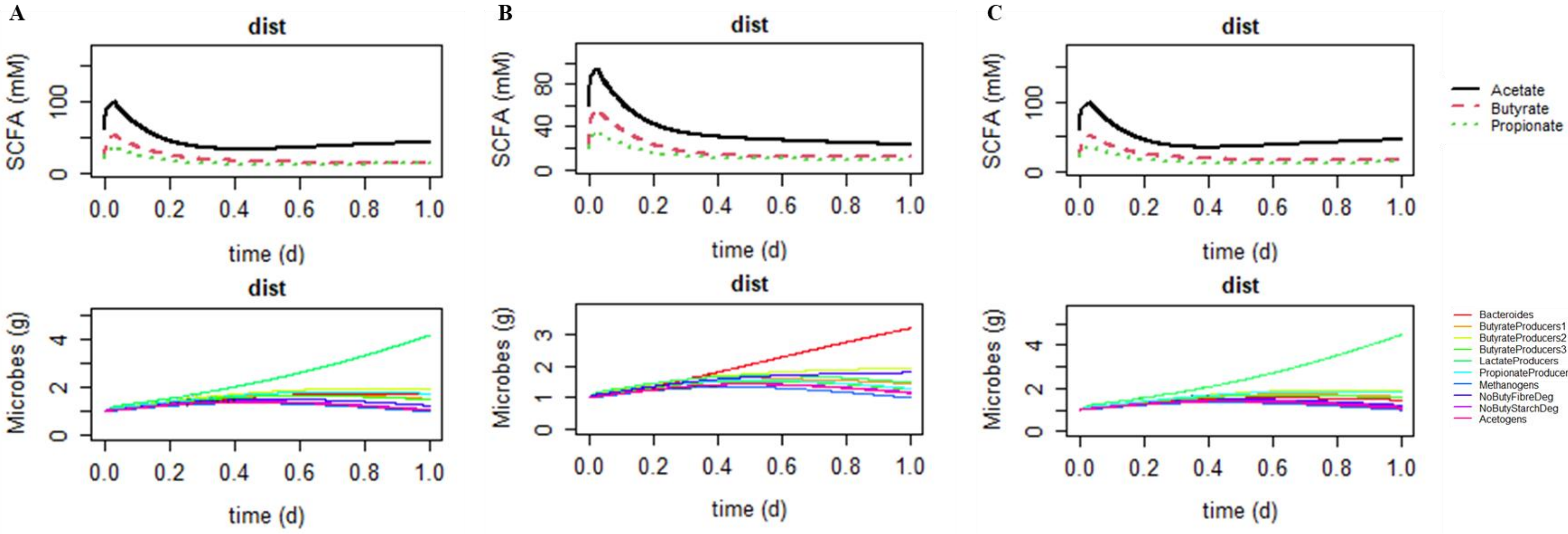

Figure 4.2. Simulations of microbial biomass and SCFA concentration in the distal colon using microPopGut. The microbial biomass and SCFA production were simulated over one day under three different conditions. The inflow (g/d) of protein and carbohydrates, as well as the resistant starch fraction, were respectively: 10, 50, and 0.78 for the default conditions (A), 30, 30, and 0.30 for the high-protein, low-fibre diet (B), and 5, 55, and 0.90 for the high-fibre diet (C).

4.4.2. GEM-based models

4.4.2.1. BacArena

Adding new taxa to the BacArena simulations altered the predictions for microbial abundance and metabolite production under the same dietary conditions (Figure 4.3). The relative abundance of *B. thetaiotaomicron* and *B. longum* decreased, while the abundance of *E. coli* and the *Blautia* genus increased. Butyrate production increased, while acetate and propionate production decreased (Table 4.4). After ten hours of simulated microbial growth, propionate was the most abundant SCFA. Incorporating additional taxa into the microbial community shifted the ratio of SCFAs by reducing the proportion of propionate and increasing that of butyrate. However, this also led to an increase in the model's processing time from two hours to ten hours.

Although it is possible to modify the dietary conditions in BacArena simulations, this was not undertaken in the present evaluation. The model's default dietary conditions include all the necessary substrates for the growth of the default microbial community, which were determined through flux variability analysis, along with associated estimates of their concentration and diffusion constants in the human colon. Modifying these conditions would require additional computational tools to identify and supply essential nutrients for microbial growth that may be absent under a new diet. Additionally, it would also demand an extensive literature search and/or assumptions to estimate the concentrations and diffusion parameters of diverse dietary compounds in the human colon. Given the limited literature in this area, such assumptions risk being overly speculative, which could compromise the credibility and accuracy of the results.

Table 4.4. Predicted production of SCFAs after ten hours of simulation of microbial growth using BacArena.

	Short-chain fatty acids (mmol)		
	Acetate	Propionate	Butyrate
Default microbial community*	1.75×10^{-7}	1.46×10^{-6}	1.80×10^{-8}
Modified microbial community*	1.04×10^{-7}	6.60×10^{-7}	3.73×10^{-7}

*The default conditions included *A. caccae*, *B. thetaiotaomicron*, *B. longum*, *B. producta, C. ramosum*, *E. coli*, and *L. plantarum*. The modified community included the addition of five new microbes: *A. muciniphila*, *E. rectale*, *M. smithii*, *P. copri*, and *R. bromii*.

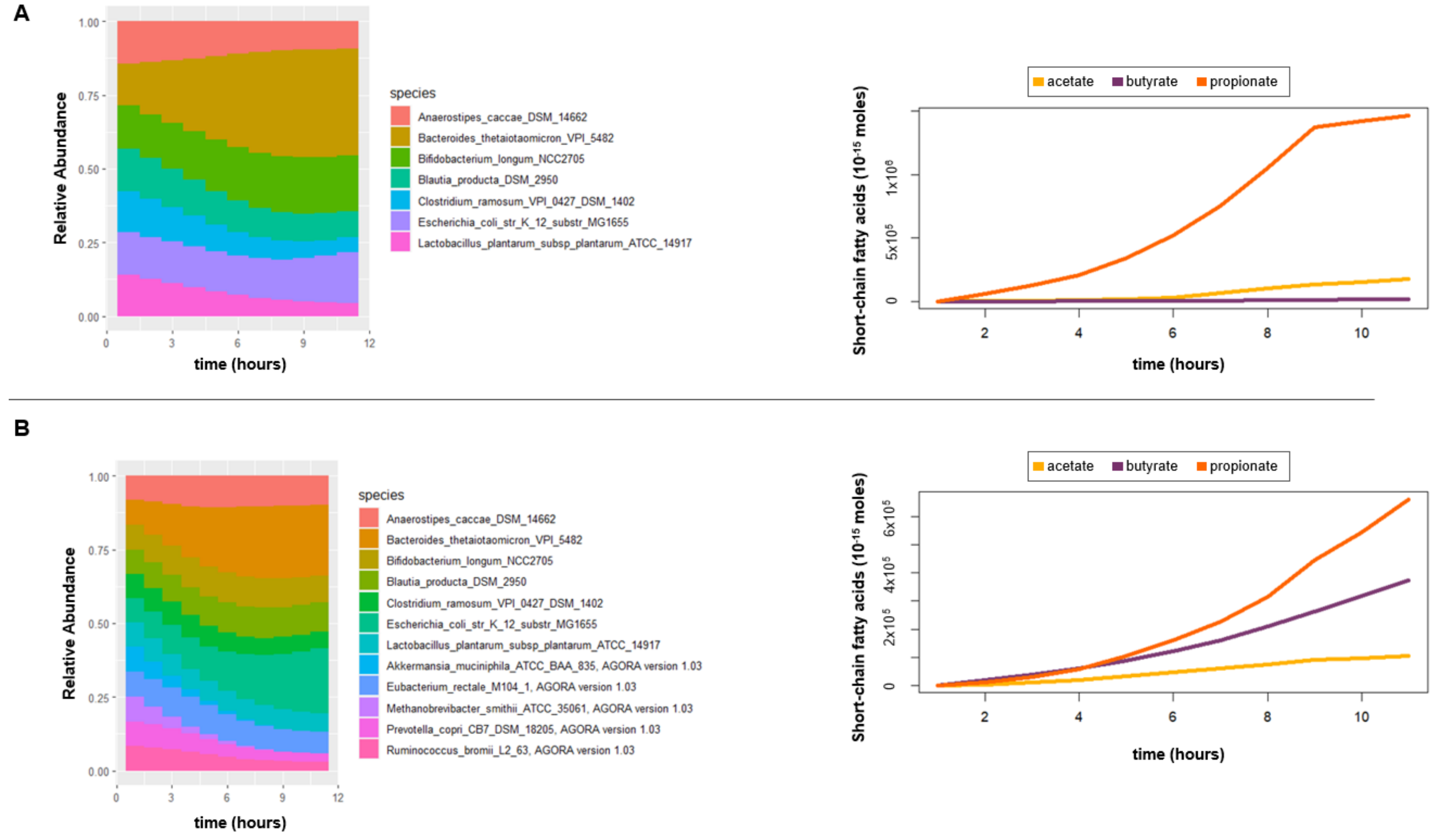

Figure 4.3. Prediction of microbial growth and SCFA production of the colonic microbiota using BacArena. The model was executed under the same dietary conditions using two microbial communities. The default conditions included *A. caccae, B. thetaiotaomicron, B. longum, B. producta, C. ramosum, E. coli,* and *L. plantarum* (A). The modified community included the addition of five new microbes: *A. muciniphila, E. rectale, M. smithii, P. copri, and R. bromii* (B).

4.4.2.2. The COBRA Toolbox

The tutorial "Simulation of growth of human gut microbes on different diets" was executed to predict the microbial growth rates under a Western diet and a high-fibre diet (Figure 4.4). In addition to diet, the tutorial also predicted microbial growth according to the presence or not of oxygen (oxic and anoxic conditions, respectively). However, the tool predicted the overall community growth without specifying the growth of specific taxa or the production of microbial metabolites. To better understand the influence of the two diets on the function of colonic microbes, the tutorial "Creation, interrogation, and analysis of personalised microbiota models from the AGORA models through metagenomic data integration" was followed.

Unexpectedly, distinct dietary conditions generated very similar profiles of microbial metabolite production. Both the average European and high-fibre diets resulted in identical predicted fluxes for acetate, propionate, and isobutyrate (Table 4.5). Aside from the flux of isovalerate, which increased by 12 % for the high-fibre diet, no other changes were observed in the predicted fluxes of SCFAs under different dietary conditions. Most concerningly, predicted butyrate fluxes were zero under both diets, and the fluxes of isobutyrate and isovalerate had similar values to those of acetate and propionate.

Table 4.5. Predicted fluxes of SCFAs for the colonic microbiota of adults under two different diets using The COBRA toolbox.

Diet	Acetate (mmol/gDW.h)	Propionate (mmol/gDW.h)	Butyrate (mmol/gDW.h)	Isobutyrate (mmol/gDW.h)	Isovalerate (mmol/gDW.h)
European average diet	194.77	194.77	0	185.70	61.62
High-fibre diet	194.77	194.77	0	185.70	69.32

The European average diet and high-fibre diet were imported from the Virtual Metabolic Human database. Fluxes of organic acids are shown in mmol per gram (dry weight) per hour.

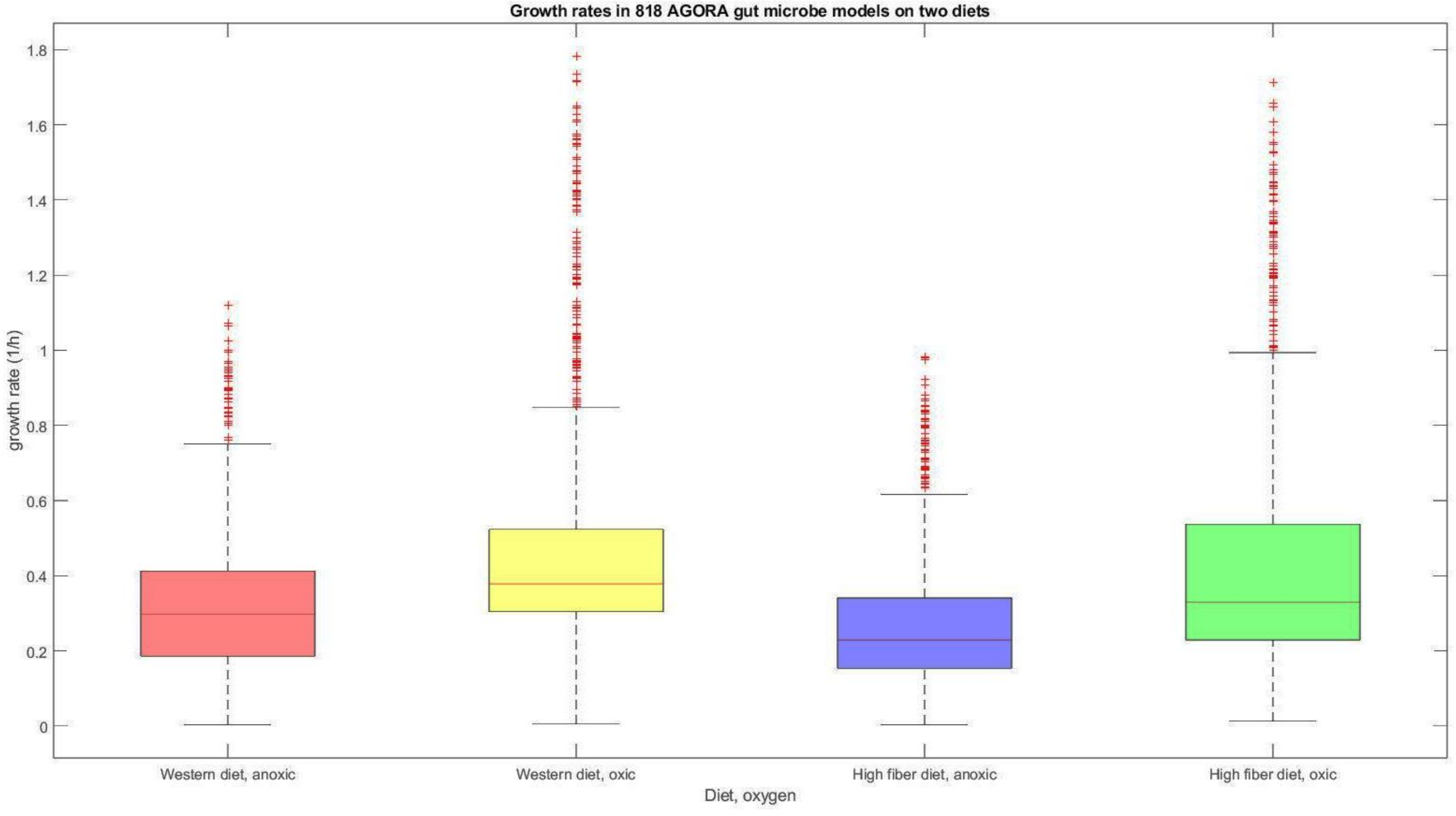

Figure 4.4. Microbial growth rates under different dietary and oxygen conditions using The COBRA Toolbox. Dietary fluxes for the Western and high-fibre diets were imported from the Virtual Metabolic Human database. The interquartile range of growth rates for individual microbes, represented by boxes, is shown for each diet and oxygen condition. Error bars indicate the upper and lower adjacent values, while outlier values are marked in red (+).

4.4.2.3. MICOM

The influence of European average and high-fibre diets on the colonic microbiota of four healthy adult individuals was simulated using MICOM. Faecal microbial relative abundance data were obtained from a study that evaluated the impact of faecal transplantation on the treatment of *Clostridium difficile* infection (293). The model predicted fluxes of an extensive list of metabolites (over 200) and the growth rates of the taxa composing the microbial communities (at least 18 genera). For ease of visualisation, only a selection of metabolites and the seven most abundant genera are shown here (Figure 4.5).

Higher microbial growth rates were predicted for the high-fibre diet compared to the European average diet. Under both diets, the *Bacteroides* genus had the highest growth rate. The high-fibre diet also led to an overall increase in SCFA and gas production. Notably, MICOM accounted for individual variations in SCFA fluxes and microbial growth rates. For instance, individual 3 showed the highest increase in total SCFA production on the high-fibre diet (170 % increase relative to the European average diet). In contrast, individual 4 exhibited no change in total SCFA flux (Table 4.6).

Table 4.6. Predicted fluxes of SCFAs for four individuals under European average and high-fibre diets using MICOM.

Diet	Individual	Acetate (mmol/gDW.h)	Propionate (mmol/gDW.h)	Butyrate (mmol/gDW.h)	Total SCFA (mmol/gDW.h)
European average diet	Individual 1	0.93	0.06	0.04	1.03
	Individual 2	0.45	0.16	0.03	0.65
	Individual 3	0.41	0.09	0.03	0.54
	Individual 4	0.21	0.09	0.02	0.32
High-fibre diet	Individual 1	1.84	0.07	0.04	1.95
	Individual 2	0.90	0.30	0.04	1.25
	Individual 3	1.13	0.27	0.07	1.46
	Individual 4	0.21	0.08	0.02	0.31

The European average diet and high-fibre diet were imported from the Virtual Metabolic Human database. Fluxes of short-chain fatty acids are shown in mmol per gram (dry weight) per hour.

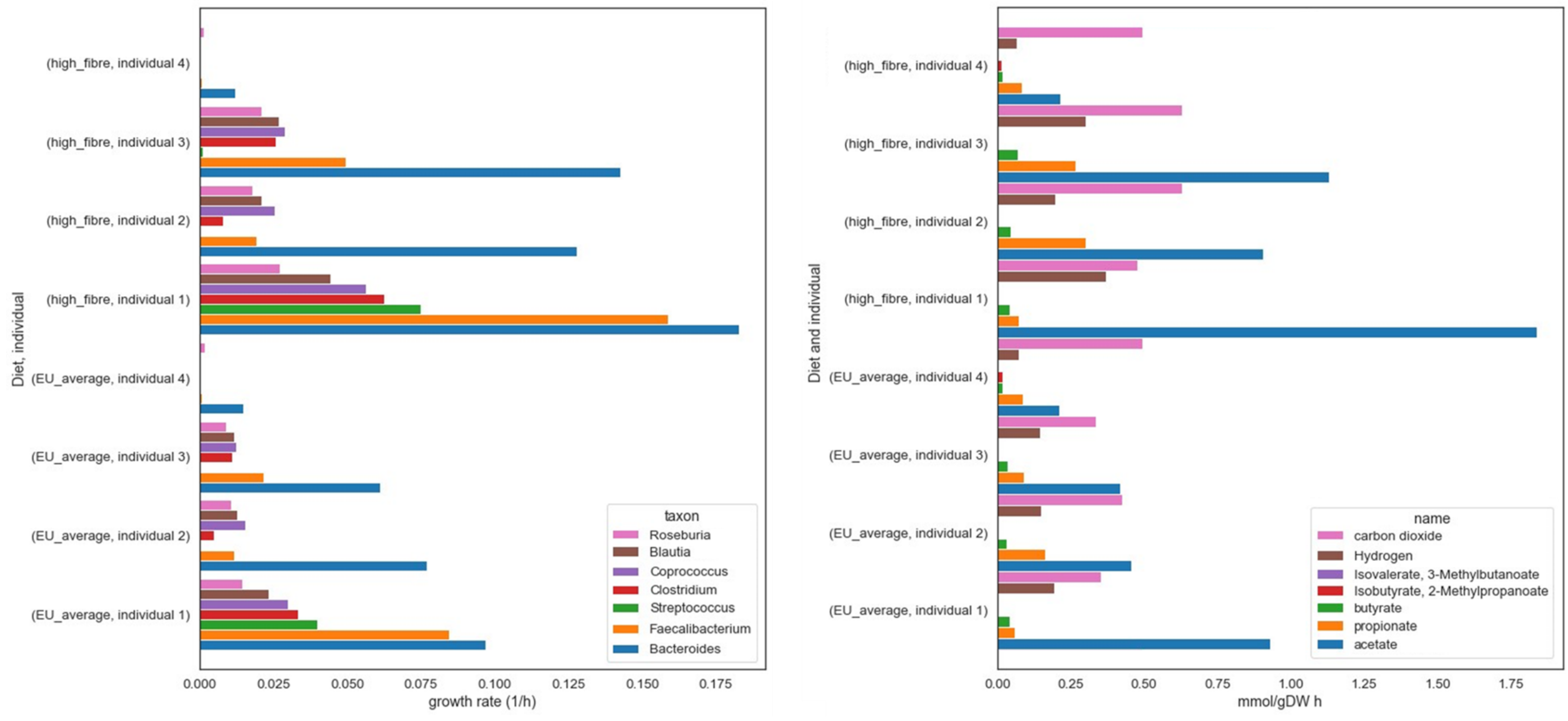

Figure 4.5. MICOM predictions of microbial growth rates and fluxes of microbial metabolites by the human colonic microbiota under different dietary conditions. Simulations were conducted using faecal microbial relative abundances of four healthy adult individuals under European average and high-fibre diets. Dietary fluxes were obtained from the Virtual Metabolic Human database.

4.5. Discussion

Consistent with the reductionistic approach of ODE-based models, predicting the dietary influence on colonic microbial communities using microPop and microPopGut was characterised by ease of use, ease of customisation, and low computational requirements. However, these models relied on default conditions that did not fully capture the compositional variability of the human colonic microbiota and the diversity of food compounds in human diets.

The default microbial community used by these models consisted of ten microbial functional groups, which reduced the computational cost of the simulations (285). This approach was supported by the functional redundancy of colonic microbes, meaning that the function of the colonic microbiota remained similar despite individual variations in composition (4,295). However, this reductionistic strategy has limitations. The proposed microbial functional groups were based on the colonic microbiota of healthy adults and did not account for inter-individual variations (22). Furthermore, they did not reflect the distinct microbiota of individuals in different life stages or with health conditions, such as weaning infants or individuals with gastrointestinal disorders (18,296).

These models simulated only the effects of total protein and carbohydrate concentrations in the colon on microbial communities (274,275). This approach oversimplifies human dietary patterns, which involve a wide range of amino acids, dietary fibres, fatty acids, micronutrients, and phytochemicals that have been shown to influence the composition and function of the colonic microbiota [see systematic reviews (12,112,123,297)]. Moreover, while databases provide information about the composition of foods and diets, data on the concentration of unabsorbed dietary compounds in the colon are scarce. This leads to the assumption of simulation inputs, reducing the objectivity of the model.

Under a high-protein, low-fibre diet and a high-fibre, low-protein diet, microPop predicted the dominance of the *Bacteroides* genus at the expense of other functional groups after 24 hours of simulation. This contrasts with the observed coexistence of several microbial genera at different abundances in the human colon (4,298). Increased production of total SCFAs was observed with higher colonic content of dietary fibre in the simulations, agreeing with findings from meta-analyses (12,299). However, the predicted concentration of butyrate was negligible compared to acetate and propionate, resulting in SCFA production ratios that disagree with experimental data (300).

Simulations using microPopGut aligned more closely with experimental literature. For instance, the model predicted increased total SCFA production, particularly butyrate, under a high-fibre, low-protein

diet compared to a high-protein, low-fibre diet. Increasing the protein content while decreasing the fibre content in the simulations also promoted the growth of the *Bacteroides genus*. This finding is consistent with the results of a continuous fermentation study comparing the impact of high-protein and high-fibre diets on the human colonic microbiota, using faeces as a proxy (301). However, the simulation time (one day) was not enough for the model to reach a steady state, suggesting that eventual changes in the microbial community may occur in longer simulations. Furthermore, the application of microPopGut using default conditions is limited due to its inadequacy in representing realistic human dietary patterns. Extensive development of the model is therefore necessary, notably defining new microbial functional groups and their growth behaviour on distinct dietary compounds.

For this purpose, microbial kinetic parameters under different substrates, which are largely unknown and challenging to obtain experimentally, need to be defined. Most colonic microbes are anaerobes, imposing technical challenges for their *in vitro* manipulation (302). Additionally, determining microbial kinetic parameters requires pure cultures, which hinders the cultivation of colonic microorganisms that rely on cross-feeding interactions to grow. The lack of definition of microbial parameters for building ODE-based models is currently a bottleneck for their development and application, which requires future improvements in microbial cultivation methodologies. Another possible approach, as described in the microPop publication (274), is to account for variations between microbial strains. This can be achieved by randomly varying the kinetic parameters of the microbial functional groups (stochastically generated values) and assigning multiple strains to each functional group. In this way, new microbial communities are created, offering greater relevance to the diversity of the colonic microbiota. Statistics can then be used to determine the average trait of each microbial functional group. However, this approach was not implemented here due to the evaluative nature of this chapter.

In turn, MGCMs were identified as more suitable for predicting the effect of human diets on gut microbial communities. Since these models use genomic information to mathematically represent the metabolism of colonic microbes, they do not rely on the experimental definition of microbial kinetic parameters, although microbial metabolic reconstructions benefit from manual curation using experimental data (303). In this way, MGCMs are a good alternative to ODE-based models, leveraging the vast amount of data generated by advances in DNA sequencing technologies. Furthermore, MGCMs can be continuously improved as more data becomes available and can incorporate individual data to create and analyse personalised microbial communities [see reviews (294,304)].

BacArena, a combined agent-based and GEM-based model, was unsuccessful in simulating the effect of different host diets on the colonic microbiota due to its limited applicability outside the default conditions. The model's small-scale default microbial community does not adequately represent the diversity of microbes found in the human colon (4) and adding new members to the simulation significantly increased its computational cost. Furthermore, modifying the dietary conditions of the simulations is not straightforward, requiring additional modelling tools and extensive literature searches or assumptions.

In contrast, the COBRA Toolbox and MICOM could simulate large-scale microbial communities at a reasonable computational cost. One key advantage of these models is their compatibility with the VMH database and the AGORA metabolic reconstructions (278,279), which facilitates the design of customised diets and personalised colonic microbial communities. These models operate similarly, requiring taxonomic data, metabolic reconstructions, and dietary fluxes as inputs to predict the microbial growth rates and fluxes of produced metabolites.

The COBRA Toolbox stood out for its extensive range of functions. Still, only a few are focused on large-scale microbial communities, and there is no single workflow that integrates all the necessary steps to simulate the effect of host diets on colonic microbes. Unexpectedly, the model predicted ratios of SCFAs and BCFAs that contradicted the literature (300). For instance, predicted fluxes of butyrate under both European average and high-fibre diets were zero, while fluxes of isobutyrate and isovalerate were similar to those of acetate and propionate. Moreover, identical fluxes of major SCFAs were predicted for both diets, despite the evidence for dietary fibre increasing colonic SCFA production (12,299). Similarly, another investigation using the COBRA Toolbox predicted equivalent major SCFA and isobutyrate fluxes under two distinct diets (European average and high-protein diets) (282). These results raise concerns about the capability of the toolbox to accurately differentiate the effects of distinct diets on the function of colonic microbes.

MICOM was characterised by its user-friendly design, which contained predefined workflows that facilitated its operation. In qualitative agreement with previous studies, the model predicted higher fluxes of SCFAs when increasing dietary fibre content in a diet (12,299). A key strength of the package is its ease of customisation, enabling simulations with personalised colonic microbial communities and dietary fluxes. By integrating all steps from data processing to visualisations within a single toolbox, MICOM surpasses the COBRA Toolbox in usability and was selected as the most suitable model for evaluating

how dietary compounds influence the composition and function of the colonic microbiota *in silico*. Similarly, a recent qualitative and quantitative evaluation of GEM-based tools recommended MICOM due to its accessibility and usability (305).

MICOM employs a compartmentalised modelling strategy, where each microbe is kept separate but shares a common extracellular space for resource exchange. Importantly, the model assumes a mass steady state condition, implying that there is no accumulation of substrate in the system. This assumption is intrinsic to the FBA strategy, as it is necessary to reduce the number of possible numerical solutions in the system (276). This is also justified by the trend of substrate concentrations in long-term simulations to reach a steady state, where they neither accumulate nor deplete (306).

The tool uses parsimonious FBA, a variation of standard FBA that optimises a biological objective while minimising the total sum of fluxes, as an optimisation technique to maximise microbial growth rates and predict fluxes of microbial metabolites while minimising enzyme usage. Hence, parsimonious FBA predicts optimal growth rates that are more realistic with biological systems, compared to standard FBA. MICOM also incorporates a user-defined trade-off between community growth and individual microbial growth (31), preventing dominant microorganisms from overgrowing at the expense of others. These strategies enhance the realism of colonic microbial community simulations, aligning them more closely with *in vitro* and *in vivo* observations. For instance, parsimonious FBA mimics how microorganisms maximise growth rates using minimal resources under evolutionary pressures (307). Meanwhile, the community trade-off, although a less mechanistic and more subjective approach, ensures that all taxa within a large microbial community can grow, which is consistent with human colonic microbiota observations *in vivo* (298).

However, MICOM's validation with experimental data is still in progress. Recent comparisons of the model's propionate and butyrate fluxes with *ex vivo* faecal incubation data reported significant positive correlations (280). Nevertheless, this validation was based on single dietary fibres as substrate, and the strength of the correlations varied between study designs. As a result, MICOM's accuracy in predicting realistic dietary patterns remains unvalidated. Validating any model with experimental data or an independent dataset is essential for ensuring accuracy. However, due to the complexity of GEM-based models and the nature of their predictions, finding suitable experimental data or designing experiments for their validation is challenging (308). As discussed in a recent review (309), microbial growth rates and metabolite fluxes are among the most challenging predictions to validate experimentally. For

example, individual microbial growth rates are typically measured in monoculture, which does not scale well to larger microbial communities (310). Measuring fluxes of metabolites is costly and laborious, requiring isotope-labelled substrates or repeated metabolomics analyses over time (311).

Furthermore, while the mass steady state assumption is justified by the trend of the colonic microbiota towards a stable composition and function (22,312), it may not fully capture the dynamic behaviour of colonic microbes, which rapidly change under environmental constraints such as diet (25,309). Dynamic FBA offers an alternative approach by allowing intracellular and extracellular variables to vary over time, enabling the prediction of temporal changes in metabolite fluxes and growth rates (313,314). However, dynamic FBA is traditionally applied to small-scale communities, as it becomes extremely computationally intensive for large microbial communities (315).

Another limitation is that the accuracy of GEM-based models depends on the quality of the metabolic reconstructions used in simulations (281). Although several tools have been developed to automatically generate these reconstructions (316,317), they rely on biochemical and genome databases that often contain missing information (318,319). Consequently, manual curation using experimental data to fill reaction gaps and correct stoichiometry remains essential for obtaining high-quality reconstructions (303). The publication of open-access semi-curated reconstructions of human colonic microbes, along with their continuous improvement, may overcome this limitation (278,279).

4.6. Conclusions

Five mathematical models simulating the effect of dietary compounds on the human colonic microbiota were assessed. The ODE-based models, microPop and microPopGut, use default conditions that do not capture the diversity of dietary compounds in human diets or their impact on colonic commensals. Modifying these conditions requires knowledge of microbial kinetic parameters for different substrates, which is a current bottleneck. MGCMs were better suited for modelling large microbial communities and detailed dietary fluxes. Among them, MICOM stood out for its user-friendly workflows, integrating all steps from data processing to visualisation within a single package. It was used to simulate the effect of high-fibre and Western diets on the colonic microbiota of healthy adults, producing results that qualitatively align with experimental data. However, its accuracy under realistic dietary patterns remains unvalidated. In summary, MICOM shows promise in generating hypotheses on how dietary compounds influence colonic microbiota function, thereby aiding in the design of *in vitro* or

in vivo experiments. Experimental methods remain indispensable for a comprehensive understanding of the relationship between colonic microbes and the host diet.

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.	
Student name:	Vitor Geniselli da Silva
Name and title of main supervisor:	Professor Warren McNabb
In which chapter is the manuscript/published work?	Chapter 5

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Vitor Geniselli da Silva (student): Conceptualisation, Data curation, Formal Analysis, Investigation, Visualisation, Writing – original draft, Writing – review & editing.
Nick Smith (co-supervisor): Conceptualisation, Supervision, Writing – review & editing.
Jane Mullaney (co-supervisor): Conceptualisation, Supervision, Writing – review & editing.
Clare Wall (co-supervisor): Conceptualisation, Funding acquisition, Supervision, Writing – review & editing.
Nicole Roy (co-supervisor): Conceptualisation, Funding acquisition, Supervision, Writing – review & editing.
Warren McNabb (main supervisor): Conceptualisation, Project administration, Supervision, Writing – review & editing.

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  da Silva VG, Smith NW, Mullaney JA, Wall C, Roy NC, McNabb WC. Food-breastmilk combinations alter the colonic microbiome of weaning infants: an in silico study. mSystems (2024) 9:e00577-24. doi: 10.1128/msystems.00577-24

- ( ) **The manuscript is currently under review for publication**

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Chapter 5: *In silico* simulation of the effects of complementary foods on the colonic microbiota of weaning infants[3]

5.1. Abstract

The introduction of solid foods to infants, also known as weaning, is a critical point for the development of the complex microbial community inhabiting the human colon, impacting host physiology in infancy and later in life. This chapter investigated *in silico* the impact of food-breastmilk combinations on growth and metabolite production by colonic microbes of New Zealand weaning infants using the metagenome-scale community metabolic model MICOM. Eighty-nine foods were individually combined with breastmilk, and the twelve combinations with the strongest influence on the microbial production of SCFAs and BCFAs were identified. Fibre-rich and polyphenol-rich foods, like pumpkin and blackcurrant, resulted in the greatest increase in predicted fluxes of total SCFAs and individual fluxes of propionate and acetate when combined respectively with breastmilk. Identified foods were further combined with other foods and breastmilk, resulting in 66 multiple food-breastmilk combinations. These combinations altered the impact of individual foods on the microbial production of SCFAs and BCFAs *in silico*, suggesting that the interaction between the dietary compounds composing a meal is the key factor influencing colonic microbes. Blackcurrants combined with other foods and breastmilk promoted the greatest increase in the production of acetate and total SCFAs, while pork combined with other foods and breastmilk decreased the total production of BCFAs.

5.2. Introduction

The human GIT is colonised by a complex microbial community, with the greatest concentration and diversity being found in the large intestine, or colon (34). Diet is a well-known key factor shaping the composition and function of colonic microbes throughout human life (3,25,80). In turn, colonic microbes impact host physiology and are associated with health and disease biomarkers (45). One mechanism by which the colonic microbiota affects host health is the production of metabolites that are later absorbed by the host (320). Organic acids, such as SCFAs and BCFAs, are among the most studied

[3] This chapter has been published as da Silva VG, Smith NW, Mullaney JA, Wall C, Roy NC, McNabb WC. Food-breastmilk combinations alter the colonic microbiome of weaning infants: an in silico study. *mSystems* (2024) 9:e00577-24. doi: 10.1128/msystems.00577-24

microbial metabolites produced in the colon and decreases in their production have been associated with disease, particularly gastrointestinal and metabolic disorders (75,210).

One of the challenges of investigating the colonic microbiota is that the composition of this microbial community is unique to each individual, making it difficult to define an ideal microbiota in terms of composition (22,43). On the other hand, the functional capacity of the microbiota is similar among individuals with the same health status (4), and colonic microbes are functionally redundant, meaning that different taxa can perform the same metabolic functions (24). Thus, focusing on what the colonic microbiota produces, rather than which microbes compose it, may be more relevant to host health implications, also reducing the complexity of microbiome investigations.

Animal and human studies have demonstrated that imbalances in the colonic microbiota, or dysbiosis, during infancy are associated with an increased risk for several diseases later in life (29,321–323). Therefore, adequate nutrition from an early postnatal age is crucial to prevent or limit dysbiosis. The introduction of solid foods represents a window of opportunity for establishing long-term beneficial host-microbiota interactions that may influence host physiology later in life (56,324). However, among the studies that examined the impact of solid foods on the colonic microbiota of infants (81,83,88,219,220,253), only a few assessed the microbial production of organic acids (84,218), limiting our understanding of how complementary foods affect colonic microbes in this crucial life stage.

Studies conducted *in vitro* or using animal models have investigated the effect of foods on the colonic microbiome of infants in the absence of human milk (86,89,91,325), not accurately reflecting how complementary foods are introduced to weaning infants. Furthermore, there is no evidence *in vitro*, in animals (such as mice and piglets), or *in silico* of how food-breastmilk combinations affect the colonic microbiota in early life.

Mathematical models are a rapid and inexpensive complementary strategy to study microbial communities, being able to generate hypotheses that can be further tested by experimental approaches. Genome-scale metabolic models stand out for using genome information to infer the metabolism of microorganisms, having the major advantage of predicting microbial growth rates and fluxes of produced metabolites without prior definition of kinetic parameters, whose experimental determination is technically challenging (303).

Recently, the metagenome-scale community metabolic model MICOM was proposed to predict how diet shapes the composition and function of the human colonic microbiota (29). So far, *in silico* investigations of the colonic microbiome using metagenome-scale metabolic models have mainly focused on adults (31,204,282,283). This chapter used this modelling approach to identify foods with the strongest impact on the production of organic acids by colonic microbes of weaning infants when combined with breastmilk. SCFAs and BCFAs were chosen due to their association with host health. Insights generated by this research help the design of future experiments, advancing the understanding of the relationship between complementary foods and colonic microbes in a decisive stage of human life.

5.3. Materials and methods

5.3.1. Software

Simulations were performed in Python (version 3.9) using the package MICOM (31) (version 0.32.5) and the integrated development environment Spyder (version 5). The solver CPLEX Optimisation Studio (IBM ILOG, version 22.1) was employed under an academic license. The code and data used in the simulations described in this thesis are available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/Chapter%205.

5.3.2. Modelling workflow

Simulations used the relative abundance of the genera present in the faeces of weaning infants, metabolic reconstructions for each taxon (a list of the biochemical reactions performed by a microorganism), and the fluxes of dietary compounds of food-breastmilk combinations (a list of nutrients composing a diet, in which their respective quantities are expressed in units of time) as inputs. Outputs were the predicted individual microbial growth rates and the fluxes of metabolites produced by the microbiota, particularly SCFAs and BCFAs. A graphical representation of the modelling workflow is depicted in Figure 5.1.

The average faecal microbial relative abundance of 14 New Zealand infants aged between five and twelve months, obtained from a previous *in vitro* study (86), was used to represent the colonic microbiota of weaning infants (while minimising variations in microbiota composition between individuals). Raw 16S rRNA sequencing data (Illumina MiSeq, 2 x 250 bp paired end reads) for the fermentation control (water, at time = 0 hours) were imported from NCBI archives (BioProject PRJNA669972) using the plugin q2-fondue (326). A total of 241,611 paired-end demultiplexed reads (~80,000 sequences/sample)

were imported as ".qza" artefact in QIIME2 (222) (version 2023.2), resulting in a quality score of approximately 37. DADA2 (327) was used to denoise and filter the reads, which were trimmed at 250 bp. Taxonomy assignment was carried out using the q2-feature-classifier plugin and the Greengenes2 database (328), in which amplicon sequence variants were collapsed at the genus level (Supplementary Table 5.1).

To reduce the numerical instability (a failure in optimally solving a numerical problem, see Appendix 4 for sensitivity analyses) and the processing time of the simulations, taxa were filtered to include only genera with at least 1 % relative abundance. As proposed by MICOM's authors (31), low-abundance taxa can be discarded as they are unlikely to affect the overall production of microbial metabolites but increase the computational cost of the simulations. Eleven genera remained, accounting for 95 % of the relative abundance of the microbial community (*Bacillus, Bacteroides, Bifidobacterium, Collinsella, Lacticaseibacillus, Lactobacillus, Limosilactobacillus, Prevotella, Streptococcus, Veillonella,* and *Succinispira*).

The Assembly of Gut Organisms through Reconstruction and Analysis version 2 (AGORA2) (279) metabolic reconstructions were employed in the simulations. AGORA2 reconstructions were available for ten of these genera, representing 94 % of the relative abundance used as input in the simulations. The genus rank was chosen because it suited both the resolution of 16S rRNA sequencing (329) and pan models of AGORA2 metabolic reconstructions. Pan models of the AGORA2 metabolic reconstructions at the genus rank were built by pooling individual metabolic reconstructions for strains of colonic microbes into higher taxonomic ranks (available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/AGORA_models).

Fluxes of dietary compounds were generated according to a pre-defined workflow, which is available on the MICOM GitHub page. Foods included in the simulations were identified through a literature survey. Those corresponded to food items that are commonly introduced to infants at weaning (6 to 12 months old) in New Zealand (330,331). To account for cultural variations in dietary habits, complementary foods that are introduced to infants in other geographical locations, such as North America (332) and Europe (333), were also included. In alignment with current nutrition recommendations (111,228), included food items were from various food groups, such as vegetables, fruits, nuts, legumes, meats, cereals, and dairy products, while food groups not recommended to infants, such as sweets and fast-food products, were not included.

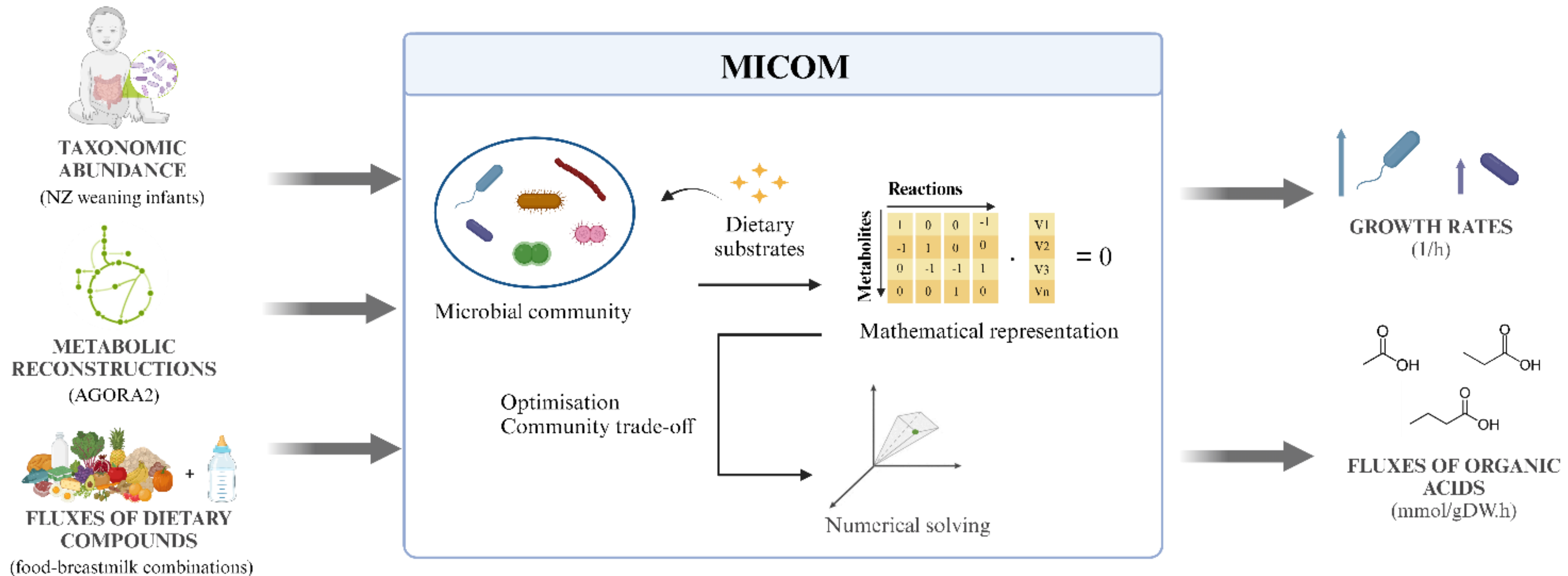

Figure 5.1. Workflow to predict the influence of food-breastmilk combinations on growth rates of colonic microbes of weaning infants and produced fluxes of organic acids using MICOM.

To represent how solid foods are consumed by weaning infants, identified food items were combined, individually or in pairs, with mature breastmilk using the "Design a diet" function of the VMH database (291) (see Supplementary Table 5.2 for the list of foods, including descriptions of breastmilk and infant formula used as controls). Food varieties were prioritised based on New Zealand production or local availability. When New Zealand varieties were not included in the VMH database (which is based on the USDA National Nutrient Database), they were replaced with similar available varieties.

Previous evidence estimated that at six months of age, the average weight of infants is 7.6 kg (334) and their energy requirement is 85 kcal/kg/day (335), of which 85 % is supplied by breastmilk (335). Therefore, it was assumed that diets for 6-month-old infants would consist of 85 % of breastmilk and 15 % of complementary foods, totalling 608 kcal/day. In total, dietary fluxes of 155 food-breastmilk combinations and two controls (infant formula only and breastmilk only) were imported from the VMH database and processed in their original unit, mmol/day, as this reduced the numerical instability of the simulations.

Host secreted mucin cores and the bile acids glycocholic acid and taurocholic acid were then added to the dietary fluxes (values of 1 mmol/h) to better represent the substrates available for microbes in the human colon. The next step of the MICOM workflow used the human metabolic network Recon3D (336) resource to identify the dietary compounds that are absorbed in the small intestine. The flux of these compounds was then multiplied by a factor of 0.2 to account for their intestinal absorption (dilution factor value followed the pre-defined workflow). Finally, AGORA2 (279) metabolic reconstructions were used to identify and add the missing nutrients necessary to allow growth rates of at least 0.01/h for all microbial species of colonic microbes comprised by these metabolic reconstructions. This step is necessary because the dietary fluxes provided by food databases often lack essential cofactors for microbial growth, such as vitamins and minerals. After supplementing the fluxes with the minimal additional substrates (which were added in mmol/hour), the final fluxes were in mmol/hour.

5.3.3. Modelling assumptions

This modelling approach was based on flux balance analysis under a mass steady state assumption, meaning that there is no accumulation of substances inside the microbial cells (276). A constrained linear programming problem (a mathematical problem) for the fluxes (v) was created by using a stoichiometric matrix (S), which is a matrix representing all biochemical reactions (as columns) and involved metabolites (as rows) performed by the microbial community. The objective of the flux balance analysis

was to maximise biomass reaction (v_{bm}) such that there is no accumulation of substrates in the system ($S*v=0$).

The steady state assumption represents the exponential phase of bacterial growth, in which growth rates can be assumed constant, and the linear problem describing fluxes of metabolites can be solved by a programming solver. The community growth rate (μ_c) is determined by the sum of growth rates of individual microbes (μ_i) weighted by their relative abundances (a_i), as described by the following equation:

$$\mu_c = \sum_i a_i \mu_i$$

MICOM also distributes growth across all members of the microbial community by limiting the maximal community growth with a cooperative trade-off (a value between 0 and 1). The optimal trade-off between maximal microbial community growth and maximal individual microbial growth was determined for each food-breastmilk combination. This allows most microbes present in the community to grow, rather than only the dominant members, thus better representing *in vivo* conditions in which multiple microbes are found in different abundances in the human colon (4). The cooperative trade-off (α) was calculated in two steps: initially determining the maximal community growth rate (μ_c^{max}) and then calculating the optimal individual growth rates (μ_i^{opt}), which are positively correlated with the relative abundance (a_i) of individual taxa (31):

$$\mu_i^{opt} = \frac{\alpha\ \mu_c^{max}}{a_i^T\ a_i}\ a_i$$

MICOM assumes that the relative abundance of microbes present in a sample corresponds to their relative biomass, in which the biomass reaction is normalised to produce one gram, predicting the production of microbial metabolites in mmol/g.h (of microbial biomass in dry weight) [see (31)]. The total flux of a given microbial metabolite (v_{tot}^m) is calculated as the sum of the fluxes of this metabolite produced by individual microbes (v_i^m) weighted by their relative abundances (a_i), as described by the equation below:

$$v_{tot}^m = \sum_i a_i\ v_i^m$$

5.3.4. Criteria for selecting food-breastmilk combinations

To identify food and food combinations having the strongest influence on the colonic microbiome of New Zealand weaning infants, this chapter focused on the production of microbial metabolites (microbial function) rather than changes in microbial composition. This is due to the potential of functional analyses to be more informative about how diets shape colonic microbes. Indeed, evidence linked variations in the genetic content of colonic commensals between individuals to their ability to metabolise dietary compounds and produce bioactive metabolites (337,338).

Predicted microbial fluxes of the major SCFAs (acetate, propionate, and butyrate) and BCFAs (isovalerate and isobutyrate) were chosen due to the importance of these organic acids on host physiology and because their concentration is known to be influenced by host dietary patterns during weaning (339,340). As criteria of choice, selected foods and food combinations were representative of different model outputs, corresponding to candidates that, when combined with breastmilk, resulted in the greatest increase, decrease, or did not change the fluxes of total SCFAs and BCFAs, in comparison to breastmilk alone. Foods commonly consumed by weaning infants in New Zealand and produced in the country were prioritised.

5.4. Results

5.4.1. Predicted fluxes of SCFAs and BCFAs

Combining foods with breastmilk altered the fluxes of major SCFAs and BCFAs compared to breastmilk alone (Supplementary Table 5.3). Twenty-nine food-breastmilk combinations increased the flux of total SCFAs (32 % of the evaluated combinations), and 64 food-breastmilk combinations reduced the flux of total BCFAs (72 % of the combinations). The twelve food-breastmilk combinations that, compared to breastmilk alone, resulted in the greatest increase, decrease, or similar values of total SCFA and total BCFA fluxes were identified (Figure 5.2).

The combination of pumpkin with breastmilk promoted the greatest increase in the flux of total SCFAs (11.7 %) and the second greatest decrease in the flux of total BCFAs (40.2 %), compared to breastmilk alone. Raspberries-breastmilk and blackcurrant-breastmilk increased the production of total SCFAs (6.4 and 6.2 %, respectively) with no change in the flux of total BCFAs. Similarly, soybean-breastmilk and sweet potato-breastmilk elevated fluxes of total SCFAs (4.0 and 3.0 %, respectively) without changing total BCFA production.

Figure 5.2. Heatmap of food-breastmilk combinations with the greatest influence on predicted fluxes of SCFAs and BCFAs. Fluxes of organic acids are expressed in relative variation in comparison to breastmilk alone. Cells are coloured according to intensity, with the highest values in green and the lowest values in red.

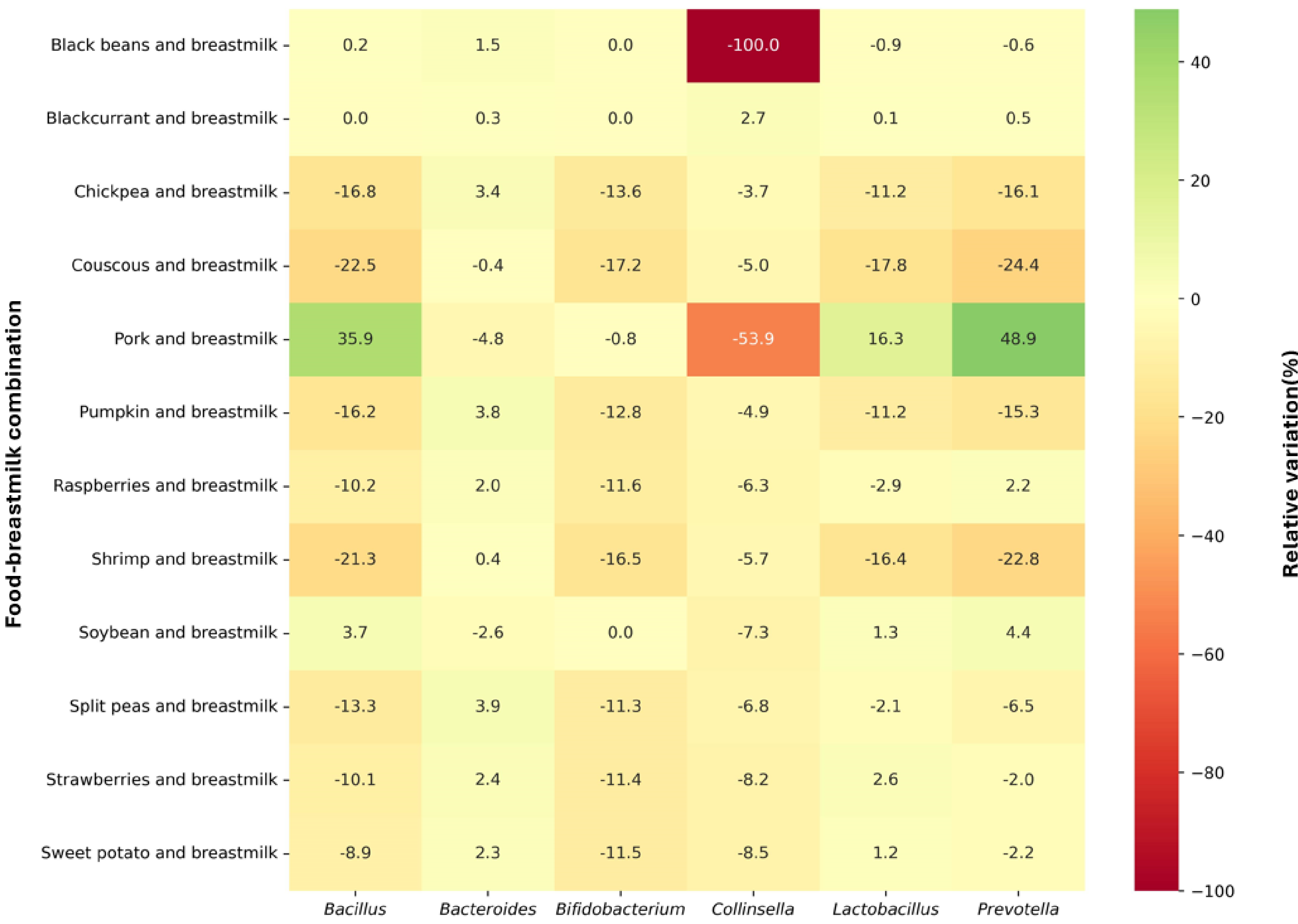

Figure 5.3. Heatmap of microbial growth rates for food-breastmilk combinations with the greatest influence on predicted fluxes of SCFAs and BCFAs. Microbial growth rates are expressed in relative variation in comparison to breastmilk alone. Cells are coloured according to intensity, with the highest values in green and the lowest values in red. Genera with predicted negligible growth are not presented.

The increase in the flux of total SCFAs obtained with the pumpkin-breastmilk combination was driven by heightened fluxes of propionate and butyrate. On the other hand, the other four food-breastmilk combinations, which increased total SCFA flux, mainly increased the flux of acetate. Indeed, combining pumpkin with breastmilk resulted in the largest increase in propionate (65.0 %), while breastmilk combined with blackcurrant resulted in the largest increase in acetate (13.3 %).

The couscous-breastmilk combination resulted in the highest increase in butyrate (104.8 %) but decreased acetate and propionate fluxes. Additionally, it caused the largest reduction in total BCFA flux (61.7 %). Split peas-breastmilk reduced the flux of total BCFAs by 26.9 % while increasing the production of acetate (12.8 %) and butyrate (14.8 %). On the other hand, the combination of strawberry with breastmilk resulted in the greatest reduction in the total SCFA flux (60.9 %), also reducing total BCFA flux (33.9 %), while shrimp-breastmilk had the greatest increase in the production of BCFAs (22.9 %). Conversely, black beans, pork, and chickpeas produced little to no change in the fluxes of SCFAs and BCFAs when individually combined with breastmilk.

5.4.2. Predicted microbial growth rates

The modelling approach assumes that the system is in a steady state, meaning that there is no accumulation of substrates. Additionally, MICOM employs a linearisation strategy that correlates the predicted growth rates of individual taxa to their relative abundance. As a result, the profile of faster-growing microbes was not expected to change much under different food-breastmilk combinations. High-abundance genera were expected to have the greatest growth rates, while low-abundance genera were expected to have slower growth rates.

As expected, the high-abundance genera *Bifidobacterium*, *Bacteroides*, and *Bacillus* had higher growth rates for most of the food-breastmilk combinations, while low-abundance genera, such as *Lacticaseibacillus* and *Streptococcus*, had slower growth rates (Supplementary Table 5.4). However, other low-abundance genera also had negligible growth (growth rates lower than 10^{-6} h^{-1}). This result suggests a failure to respect the trade-off between community growth and individual growth, resulting in a numerical problem that was not optimally solved (numerical instabilities).

Food-breastmilk combinations with the greatest influence on the fluxes of SCFAs and BCFAs impacted the microbial growth rates of high-abundance genera in different ways (Figure 5.3). Compared to breastmilk alone, little to no changes in the growth rates of the genus *Bacteroides* were observed for these breastmilk-food combinations. On the other hand, most of the food-breastmilk combinations

reduced the growth of the *Bifidobacterium* and *Lactobacillus* genera. The greatest reduction in the growth rates of these genera was observed for couscous-breastmilk (17 % reduction), followed by shrimp-breastmilk (16 %). In addition, a few food-breastmilk combinations altered the growth of *Prevotella*, *Collinsella*, and *Bacillus* genera. The pork-breastmilk combination had the strongest influence on the growth rates of these genera, increasing them by 48 % for the *Prevotella* genus and 35 % for the *Bacillus* genus, while decreasing growth rates by 53 % for the *Collinsella* genus.

5.4.3. Impact of multiple food-breastmilk combinations on colonic microbes of weaning infants

As complementary foods are normally introduced to infants in combination with other foods rather than consumed individually, the identified foods with the strongest impact on microbial SCFA and BCFA fluxes were combined with other identified foods and breastmilk for additional simulations. Sixty-six combinations were generated (multiple food-breastmilk combinations), composed of 7.5 % of a first food item, 7.5 % of a second food item, and 85 % of breastmilk by caloric intake. In comparison to breastmilk alone, 24 multiple food-breastmilk combinations increased the flux of total SCFAs (36 % of the combinations), while 47 reduced the flux of total BCFAs (70 % of the combinations, Supplementary Table 5.5).

The combination of blackcurrant with breastmilk and soybean, strawberries, or sweet potato, promoted the highest increases in acetate production, leading to the greatest increases in total SCFA flux by 11.7, 6.9, and 6.6 %, respectively, as compared to breastmilk (Figure 5.4). Pumpkin combined with breastmilk and couscous, or breastmilk and split peas, resulted in the greatest increase in propionate (9.4 and 8.4 %, respectively) but did not alter total SCFA flux. On the other hand, the combination black beans-pumpkin-breastmilk resulted in the greatest increase in butyrate flux (410.9 %). The combination also had the greatest reduction in isobutyrate, isovalerate, and total BCFA fluxes (84.5 %). The combination chickpea-split peas-breastmilk followed, with a total BCFA flux reduction of 75.6 %. Pork combined with breastmilk and other foods also decreased total flux of BCFAs; reductions were observed for the pork-couscous-breastmilk (26.4 %), pork-chickpea-breastmilk (36.5 %), and pork-blackcurrant-breastmilk combinations (47.7 %).

Combining foods with other foods and breastmilk shifted the way foods influenced the production of total SCFAs and total BCFAs when combined with breastmilk singly. Among the foods individually

combined with breastmilk, pumpkin promoted the greatest increase in the flux of total SCFAs, while combining pumpkin with other foods and breastmilk, such as pumpkin-split peas-breastmilk and couscous-pumpkin-breastmilk, gave little to no changes in the flux of total SCFAs (Figure 5.5). In turn, among the multiple food-breastmilk combinations, it was blackcurrant that stood out for promoting the greatest increases in total SCFA flux when combined with breastmilk and soybean, strawberries, or sweet potato. Most concerning, the combination strawberries-breastmilk promoted the smallest total SCFA flux, but when strawberries were combined with blackcurrant and breastmilk, it resulted in the second highest increase in total SCFA flux (Figure 5.5).

Nevertheless, the influences of pumpkin on the production of propionate and blackcurrant on the production of acetate were maintained when combining those foods with other foods and breastmilk. Although the combinations couscous-pumpkin-breastmilk and pumpkin-split peas-breastmilk did not change total SCFA flux, they resulted in the greatest increase in propionate. Similarly, the combinations blackcurrant-soybean-breastmilk, blackcurrant-strawberries-breastmilk, and blackcurrant-sweet potato-breastmilk had the highest increases in acetate flux.

As expected, high-abundance genera, like *Bifidobacterium, Bacteroides,* and *Bacillus*, grew faster, while low-abundance genera, such as *Lacticaseibacillus* and *Streptococcus*, grew slower under multiple food-breastmilk combinations (Supplementary Table 5.6). Negligible growth (growth rates near zero) for the low-abundance genera was also observed. For instance, 55 out of 89 multiple-food breastmilk combinations did not promote the growth of at least one genus included in the simulations, indicating that the trade-off between maximal community growth and individual growth was not respected, probably due to numerical issues.

Overall, multiple food-breastmilk combinations tended towards decreasing the growth of the *Bifidobacterium* and *Lactobacillus* genera. Among the combinations with the greatest influence on the production of total SCFAs and BCFAs, blackcurrant-soybean-breastmilk resulted in the greatest increase in the growth rates of the *Bacteroides* genus (13.8 %, Figure 5.6). Blackcurrant-sweet potato-breastmilk promoted the greatest relative increases in the growth rates of the genera *Prevotella* (36.4 %), *Bacillus* (28.5 %), and *Lactobacillus* (17.0 %), while black beans-blackcurrant-breastmilk promoted the greatest relative decreases for these same genera (78.9, 76.5, and 76.7 %, respectively). On the other hand, the combinations chickpea-split peas-breastmilk, black beans-blackcurrant-breastmilk, and black beans-pumpkin-breastmilk decreased the growth rates of all high-abundance genera.

Figure 5.4. Heatmap of multiple food-breastmilk combinations with the greatest influence on predicted fluxes of SCFAs and BCFAs. Fluxes of organic acids are expressed in relative variation in comparison to breastmilk alone. Cells are coloured according to intensity, with the highest values in green and the lowest values in red.

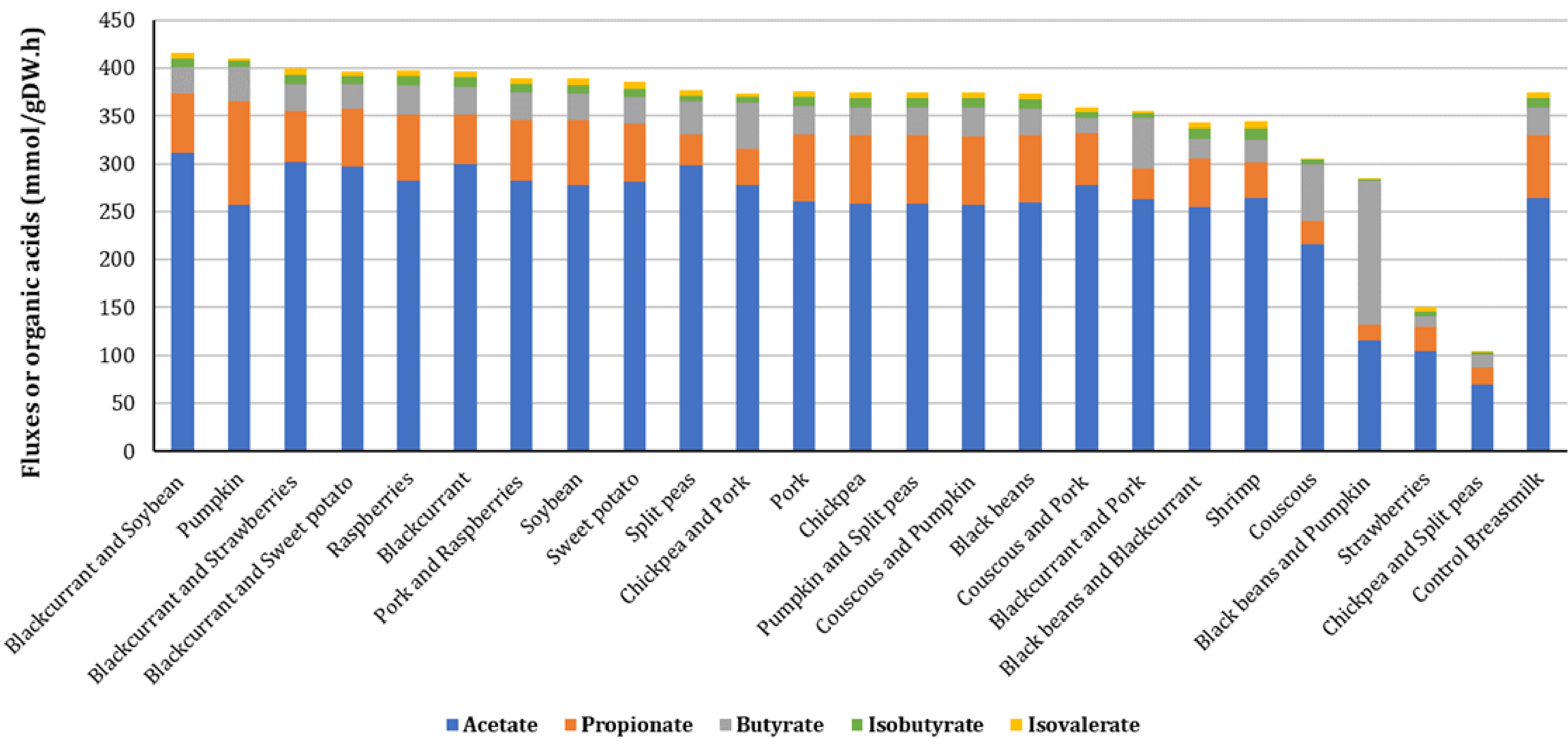

Figure 5.5. Comparison of SCFAs and BCFAs fluxes when foods are combined individually with breastmilk or with other foods and breastmilk. Food-breastmilk combinations are ordered according to their predicted total SCFA flux, in which combinations resulting in higher fluxes are shown on the left. Fluxes for breastmilk alone (control) are shown on the right.

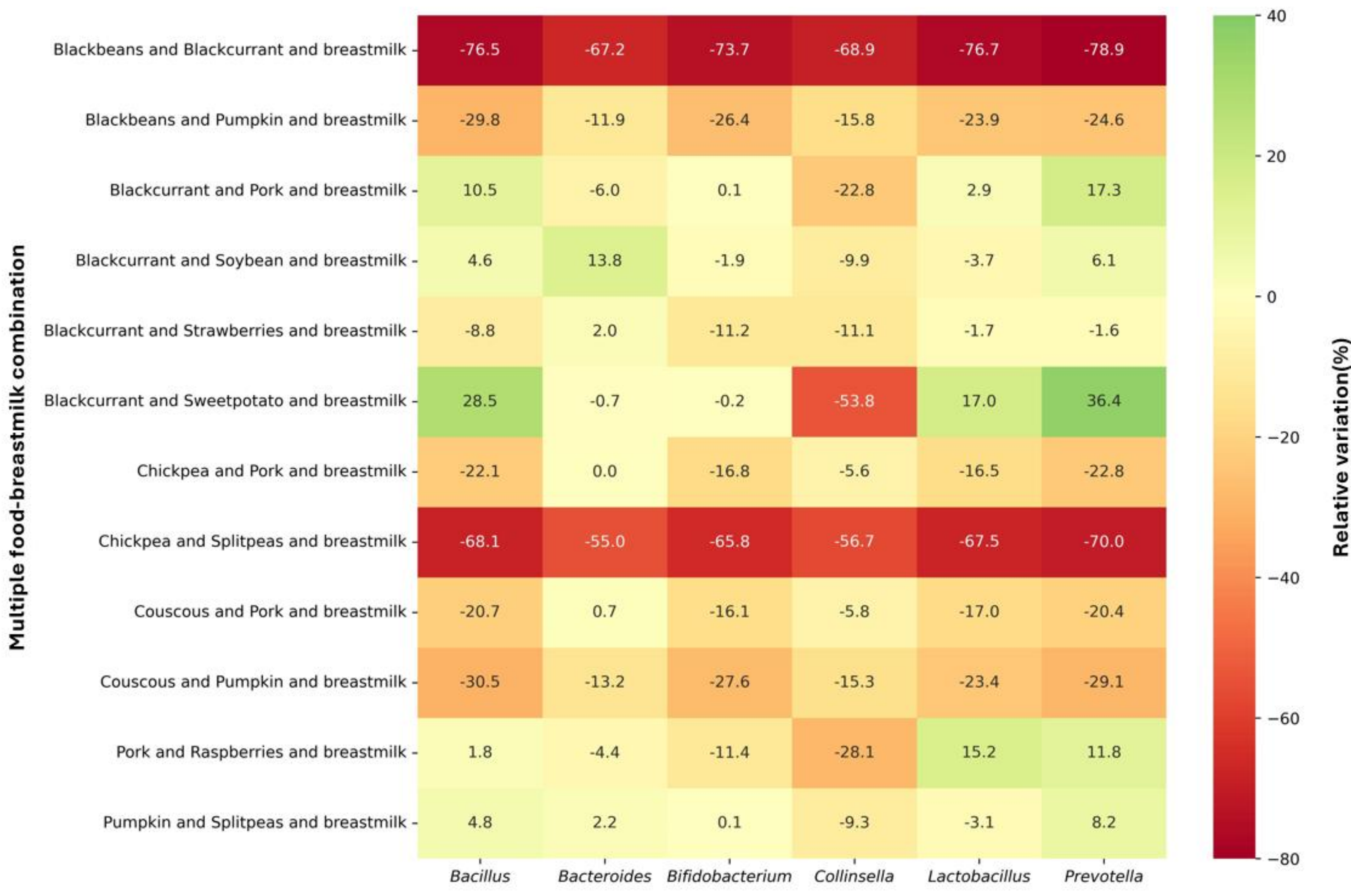

Figure 5.6. Heatmap of microbial growth rates for multiple food-breastmilk combinations with the greatest influence on predicted fluxes of SCFAs and BCFAs. Microbial growth rates are expressed in relative variation in comparison to breastmilk alone. Cells are coloured according to intensity, with the highest values in green and the lowest values in red. Genera with predicted negligible growth are not presented.

5.5. Discussion

In this chapter, a metagenome-scale metabolic modelling approach was employed to identify foods and food combinations with the greatest impact on total SCFA and BCFA production by the colonic microbiota of New Zealand weaning infants. This is the first *in silico* screening of the influence of complementary foods on the colonic microbes of weaning infants. Another originality of this chapter was the *in silico* evaluation of foods combined with breastmilk and with other foods, allowing a better representation of how complementary foods are introduced to weaning infants.

A total of 155 food-breastmilk combinations (89 single food-breastmilk and 66 multiple-food breastmilk combinations) were investigated, and the 12 individual foods and 12 food combinations with the strongest influence on predicted fluxes of total SCFAs and BCFAs, when combined with breastmilk, were identified. Consistent with nutritional recommendations for weaning infants (228), foods included in the simulations were from diverse food groups, including legumes, fruits, vegetables, and animal proteins, while sweets and fast-food products were excluded. For instance, legumes and meats are recommended for infants as sources of protein and iron, while fresh fruits and vegetables are sources of certain vitamins, other minerals, and dietary fibre (111,228).

MICOM assumes steady state and employs a two-step linearisation strategy that predicts growth rates based on the relative abundance of individual taxa (31). The food-breastmilk combinations tested consisted of 85 % breastmilk by caloric intake, resulting in a similar nutritional profile across all diets tested. As a result, the predicted profile of faster-growing microbes was expected to remain consistent across different food-breastmilk combinations, with high-abundance genera having higher growth rates than low-abundance genera, and this is what was observed. However, predicted microbial growth was not homogenous under different dietary conditions and growth rates near zero were also predicted for the less abundant genera *Limosilactobacillus*, *Lacticaseibacillus*, *Streptococcus*, and *Veillonella*. This indicates that the solver failed to optimally solve the linear problem, likely affecting outcomes for fluxes of organic acids.

Compared to breastmilk (control), most of the food-breastmilk combinations decreased the growth rate of the *Bifidobacterium* and *Collinsella* genera, agreeing with findings from longitudinal studies showing a decrease in the faecal relative abundance of these genera over the course of weaning (56,80,253). On the other hand, only small increases (up to 3.9 %) in the growth rate of the genus *Bacteroides*, which is associated with complex carbohydrates and protein degradation, were predicted *in*

silico. This contrasts with the observed increase in faecal relative abundance of the *Bacteroides* genus when solid foods are introduced to infants (3,341).

Nevertheless, our ability to compare *in silico* predictions of microbial growth rates with *in vitro* or *in vivo* observed microbial abundance is limited. It is a technical challenge to accurately measure the growth rates of individual colonic microbes experimentally *ex vivo* and nearly impossible *in vivo* (342), so available data are rare, particularly for under-investigated groups like weaning infants. Furthermore, changes in microbiota composition are not as informative as changes in microbiota function, considering that healthy individuals tend to have distinct microbiota composition but similar functionality (4,22). Thus, the focus of the study was on how food-breastmilk combinations affect the microbial production of health-relevant organic acids, particularly SCFAs and BCFAs, in the colonic microbiota of infants, rather than their impact on microbial composition.

Compared to breastmilk alone, pumpkin, raspberries, blackcurrants, soybeans, and sweet potato, individually combined with breastmilk, resulted in the greatest increases in the flux of total SCFAs. Among these foods, pumpkin and blackcurrants also stood out for promoting the greatest increase in the production of propionate and acetate, respectively. These findings suggest beneficial alterations to the colonic microbiome of infants at weaning, as SCFAs are associated with health benefits (343–346).

The *in silico* predicted increase in SCFA production can be attributed to the high content of dietary fibre and phytochemicals in these foods, as suggested by other microbiome investigations *in vitro*, in animals, and clinical trials. For instance, colonic microbes of adults rapidly fermented pumpkin skin *in vitro*, resulting in increased production of propionate and total SCFAs (347), while adding pumpkin polyphenols to the high-fat diet of type 2 diabetic rats increased the colonic concentration of butyrate and total SCFAs in digesta (348).

Soybeans, which contain insoluble dietary fibre, increased the faecal content of acetate, propionate, and butyrate in mice on a high-fat diet (349), while sweet potato, which contains non-starch polysaccharides, increased the faecal content of acetate, propionate, and isobutyrate in diarrhoeic mice (350). Faecal fermentations of sweet potatoes using inoculum from healthy adults increased the production of acetate and total SCFAs due to the presence of fibres (351,352), and also increased acetate production, likely due to anthocyanins found in the purple variety (353).

Berries contain bioactive polyphenols and flavonoids, which influence the composition and function of colonic microbes. For example, blackcurrant anthocyanins decreased the ratio Bacillota/Bacteroidota phyla in murine models (354) and increased caecal and serum concentrations of propionate and butyrate (355,356). The faecal fermentation of different raspberry compounds, including phenolic extract and total dietary fibre, showed that SCFA production was mainly driven by polyphenol content and, to a lesser extent, by fibre content (357).

When combined with breastmilk, couscous resulted in the greatest increase in butyrate production, although it decreased total SCFA flux. This observation may be justified by the presence of dietary fibres in durum wheat, in particular, arabinoxylan (358). Interventions on adults consuming wheat arabinoxylan reported increased faecal butyrate concentration (359,360). Similarly, the faecal fermentation of wheat cereal using inoculum from six healthy weaning infants increased the production of butyrate, although the extent of the increase varied among individuals (89).

Couscous, pumpkin, strawberries, and split peas also reduced total BCFA flux when combined with breastmilk. BCFAs, like isobutyrate and isovalerate, are produced in the colon through microbial fermentation of non-absorbed amino acids. Less is known about their influence on host health, but evidence suggests a link with metabolic functions (361). BCFAs are biomarkers for protein fermentation in the distal colon (11), which can generate potentially deleterious metabolites, such as ammonia and phenols (236). Colonic microbial production of BCFAs was negatively correlated with dietary insoluble fibre intake (362), which could partially explain the decreased BCFA production observed *in silico* when adding the above fibre-rich foods to breastmilk.

For instance, an intervention with yellow pea fibre decreased the faecal concentration of isovalerate in overweight adults (363). Nevertheless, an *in vitro* faecal fermentation of 22 plant sources of fibre, including whole cereals, seeds, and pulses, using inoculum from healthy adults, found no changes in the production of BCFAs (364). In this *in* silico study, other fibre-rich foods like sweet potato, black beans, and chickpeas also did not change the flux of BCFAs when combined with breastmilk, suggesting that other dietary compounds affect BCFA production. Indeed, a recent murine model study demonstrated that the protein source is a key factor affecting the faecal BCFA content (365).

On the other hand, when strawberries and shrimp were individually combined with breastmilk, they respectively showed the greatest decrease in flux of total SCFAs and the greatest increase in total BCFA flux *in silico*. These observations suggest a potential deleterious alteration in the function of colonic

microbes. Evidence demonstrated that reduced SCFA faecal concentrations in infancy are associated with an increased risk of diseases and allergies (29,366,367), while increased content of BCFAs in infants' faeces was linked to weight gain (368). These imbalances in organic acid production by the colonic microbiota during infancy may affect later life. In adults, decreased faecal concentration of SCFAs has been linked with colonic dysbiosis and diseases, such as encephalitis, Parkinson's and type 2 diabetes (263,344,369).

Currently, there is a lack of studies evaluating how the consumption of shrimp affects colonic microbes, while evidence found for strawberries contrasts with the *in silico* observations. Strawberry interventions did not change the faecal SCFA content in healthy adults (370) but increased the caecal production of acetate, propionate, and butyrate in mice with colitis (371).

Importantly, combining foods with other foods and with breastmilk shifted their individual influence on colonic microbes. The combination pumpkin-breastmilk promoted the highest flux of total SCFAs, but when pumpkin was combined with other foods and breastmilk, it resulted in little or no change in total SCFA fluxes. Among the multiple food-breastmilk combinations, blackcurrants stood out for promoting the greatest increases in SCFA production. This observation suggests that the interaction between dietary compounds composing a meal has a stronger influence on the metabolism of colonic microbes than individual foods. As foods are rarely consumed individually, dietary patterns rather than individual foods are more likely to promote alterations in colonic microbes that may affect host health (372,373).

A recent *in vitro* study evaluated how 32 foods are fermented by the colonic microbiota of New Zealand infants at weaning age, using faeces as a proxy. After 24 hours of fermentation, blackcurrants, pumpkin, and sweet potato increased total SCFA production, which is consistent with the *in silico* observations reported here (86). Similarly, pumpkin and blackcurrants increased the production of acetate after 24 hours of fermentation using faecal inoculum from New Zealand weaning infants (91). However, none of these *in vitro* studies combined foods with human milk or with other foods and human milk, providing limited information about how foods may influence the colonic microbiota of infants when added to their pre-existing dietary patterns.

In vivo approaches can better evaluate the relationship between dietary patterns and the colonic microbiota, measuring host health outcomes. For example, a series of studies investigated the colonic microbes of malnourished infants using animal models to identify food combinations that promoted the

growth of bacterial taxa associated with healthy colonic microbial development during weaning (325,374,375). Observational trials evaluating dietary patterns in weaning infants associated increased dietary diversity with increased microbial diversity and consequently the stabilisation of the colonic microbiome (109), and with increased SCFA production (3), highlighting the importance of introducing infants to meals composed of diverse foods.

Nevertheless, clinical trials evaluating the relationship between diet and the colonic microbiome are resource- and time-consuming, also facing other limitations, such as ethical concerns, low patient recruitment and adherence to the intervention, and poor accuracy of food questionnaires. In this scenario, computational modelling is a useful complementary tool to evaluate conditions that cannot be investigated with traditional *in vitro* and *in vivo* methods due to technical or logistical limitations.

However, this *in silico* approach has limitations. To simulate the impact of foods on the colonic microbiota of weaning infants, average faecal relative abundance data were used rather than data at the individual level. Repeating the simulations with the same dietary conditions produces identical outcomes. Consequently, it was impossible to conduct statistical analyses to assess potential differences in the effects of various food-breastmilk combinations on microbial growth and metabolite production. To reduce computational demands, a threshold of 1 % of relative abundance was used to select the microbial genera included in the simulations. This resulted in a small-scale microbial community that excluded genera usually found in low abundance in the colon of weaning infants but that have key metabolic functions, such as the butyrate producers *Clostridium* and *Faecalibacterium*. Thus, the findings presented are preliminary and require further experimental validation.

The design of food-breastmilk combinations was limited by the accuracy and availability of data in food composition databases, which predominantly focus on Western-type foods, lacking information on the diversity of food varieties and cooking/preparation methods [for a review on the limitations of food composition databases, see (376)]. This chapter prioritised foods produced or available in New Zealand. However, the Virtual Metabolic Human database (291) is based on the USDA National Nutrient Database for Standard Reference Release 28 (377) and does not cover all foods consumed by weaning infants in New Zealand. For example, traditional indigenous foods like kūmara (sweet potato variety) are not covered in the database and had to be replaced by the most similar food available in the database. Furthermore, data available in the VMH database do not account for individual variability in breastmilk composition.

Our study did not consider key factors influencing the composition of the colonic microbiota in infants during the first months of life, such as the mode of delivery, breastfeeding versus infant formula feeding, and the mother's diet (62,63). Since our simulations focused on the weaning period (typically between 5 and 12 months of age), we assumed that these early-life factors would have a lesser impact on the infant microbiome compared to dietary changes introduced during weaning (consumption of solids and reduced intake of breastmilk or infant formula). Nonetheless, metagenome-scale community metabolic models create personalised simulations based on input data. This methodology is adaptable and can be applied to other infant populations, potentially accounting for varying early-life influences on the microbiome in future investigations.

Another drawback is that the accuracy of the simulations presented here is dependent on the quality of the microbial metabolic reconstructions (a mathematical representation of the biochemical reactions that a microorganism can perform), which may contain missing information (303) and greatly affect the prediction capability of genome-scale metabolic models (281). Microbial relative abundance data were obtained from 16S rRNA sequencing of faecal samples. This is the most common method to assess colonic microbes of weaning infants, but 16S rRNA sequencing provides resolution suitable only to the genus rank (329), while the use of faeces does not accurately represent the ratios of microbial communities found in the colonic mucosa and the proximal colon (200). Relative abundances are not independent by nature, meaning that the relative abundance of one microbe is affected by the abundance of any other microbe in the community. As a result, without data on absolute quantities, it is not possible to determine whether the abundance of a specific taxon has truly increased or decreased, or whether changes in relative values are due to changes in the growth of other taxa.

Although the metabolic reconstructions of colonic microbes used in the simulations were validated using independent datasets (279), the simulations using MICOM described in this chapter were not validated experimentally (due to resource and ethical constraints) or using an external dataset (due to a lack of available data). Experimental validation of the MICOM model for predicting infant gut microbial responses will be presented later in the thesis. A recent study using MICOM reported agreement between fluxes of propionate and butyrate predicted *in silico* and those estimated *in vitro* (280). The package stood out among seven other metabolic modelling tools in a qualitative and quantitative assessment (305), but did not accurately correlate growth rates predicted *in silico* with those observed *in vitro* (281). Furthermore, numerical instabilities are common in MICOM when solving quadratic programming

problems in large community models (31). Near-zero growth rates were observed in our simulations for the less abundant genera, indicating instabilities that are likely to alter predicted fluxes of SCFAs and BCFAs.

The lack of research evaluating the effect of food-breastmilk combinations on the colonic microbiome of weaning infants and the differences between predicted *in silico* outcomes (microbial growth rates and fluxes of metabolites) and experimental outcomes (microbial relative abundance and concentration of metabolites) limits even a qualitative comparison between *in silico* observations reported here and published *in vitro* or *in vivo* results.

To calculate the fluxes of microbial metabolites through flux balance analysis, a mass steady state was assumed, implying that there is no accumulation of substrates in the intracellular space [see review (276)]. However, this assumption strongly differs from *in vitro* static conditions, where resources are depleted, and microbial products accumulate over time. Due to this assumption, parameters used in the simulation, such as substrate fluxes and microbial growth rates, are fixed and thus may not represent the rapid changes of colonic microbes in response to diet (25,46). Dynamic flux balance analysis may be a promising alternative to better represent *in vivo* conditions (313). However, this strategy is computationally intensive when dealing with complex microbial communities (315), hence limiting its use for screening the effect of a broad range of food combinations on colonic microbes.

Finally, *in silico* predictions may diverge from the behaviour of colonic microbes observed *in vitro* or *in vivo*. Both unabsorbed carbohydrates and amino acids contribute to colonic microbial production of SCFAs, but colonic microbes preferentially ferment carbohydrates (74,226). Acetate is produced in larger quantities by most colonic commensals, while propionate and butyrate are produced in lesser amounts by only a few genera, normally through cross-feeding interactions (378,379). Reduced dietary carbohydrate intake, even if replaced with protein, ultimately decreases the production of butyrate (380).

Thus, one could expect that fibre-rich plant foods would increase the *in silico* production of SCFAs when combined with breastmilk. However, that was not the case for all food combinations evaluated here. This result may be justified by the highly individualised and varied response of colonic microbes to dietary compounds (381), particularly dietary fibres like resistant starch (382,383), but also impacted by the limitations cited above. Therefore, further experimental investigation is essential to validate the foods and food combinations identified in this chapter. Nevertheless, the data generated by this research

provide a direction for future food-microbiome investigations and show the potential of modelling approaches to complement *in vitro* and *in vivo* techniques.

5.6. Conclusions

Currently, there is a lack of knowledge about how solid foods affect the colonic microbiome of weaning infants. This chapter evaluated for the first time how food-breastmilk combinations affect the colonic microbial production of SCFAs and BCFAs using a metagenome-scale community metabolic model. By quickly and inexpensively generating insights *in silico*, this study helps the design of future research *in vitro* and *in vivo*, contributing to filling a crucial knowledge gap in infant nutrition. Furthermore, our *in silico* observations suggest that the interaction of foods composing a meal has a key influence on the colonic microbial production of SCFAs and BCFAs. This encourages future microbiome investigations to focus on the combined effect of foods on colonic microbes instead of focusing on the effect of individual food items.

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Vitor Geniselli da Silva
Name and title of main supervisor:	Professor Warren McNabb
In which chapter is the manuscript/published work?	Chapter 6

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Vitor Geniselli da Silva (student): Conceptualisation, Data curation, Formal Analysis, Investigation, Visualisation, Writing – original draft, Writing – review & editing. Jane Mullaney (co-supervisor): Conceptualisation, Data curation, Formal Analysis, Supervision, Writing – review & editing. Nicole Roy (co-supervisor): Conceptualisation, Funding acquisition, Supervision, Writing – review & editing. Nick Smith (co-supervisor): Supervision, Writing – review & editing. Clare Wall (co-supervisor): Funding acquisition, Supervision, Writing – review & editing. Warren McNabb (main supervisor): Conceptualisation, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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1

Doctoral Research Committee
May 2023

Chapter 6: *In vitro* effects of complementary foods on the colonic microbiota of weaning infants[4]

6.1. Abstract

The transition from breastmilk to complementary foods is critical for maturing the colonic microbiota of infants. Dietary choices at weaning can lead to long-lasting microbial changes, potentially influencing health later in life. However, the weaning phase remains underexplored in colonic microbiome research, and the current understanding of how complementary foods impact the infant's colonic microbiota is limited. To address this knowledge gap, this chapter assessed the influence of 13 food ingredients on the *in vitro* microbial composition and production of organic acids by the colonic microbiota in New Zealand infants aged five to eleven months, using faecal samples as a proxy. To better represent real feeding practices, ingredients were combined with infant formula, other complementary foods, or both infant formula and other foods. Among the individual food ingredients, fermentation with peeled kūmara (sweet potato) increased the production of lactate and the relative abundance of the genus *Enterococcus*. Fermentation with blackcurrants, strawberries, or raspberries enhanced acetate and propionate production. Additionally, fermentation with blackcurrants increased the relative abundance of the genus *Parabacteroides*, while raspberry fermentation increased the relative abundance of the genera *Parabacteroides* and *Eubacterium*. When combined with infant formula or with blackcurrants, fermenting black beans increased butyrate production and stimulated the relative abundance of *Clostridium sensu stricto 1*. These foods are promising candidates for future clinical trials.

6.2. Introduction

The large intestine harbours a diverse microbial community that relies on dietary compounds unabsorbed by the host. Numerous studies have highlighted the crucial role of the colonic microbiota in digestion and have demonstrated the impact of microbial metabolites produced in the colon on host health and well-being (170,374,384). The relationship between colonic commensals and the host is dynamic and mutual, influenced by multiple factors, with diet playing a major role. Notably, disruptions in faecal

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[4] This chapter has been published as Silva VG da, Mullaney JA, Roy NC, Smith NW, Wall C, Tatton CJ, McNabb WC. Complementary foods in infants: an in vitro study of the faecal microbial composition and organic acid production. *Food Funct* (2025) 16:3465-3481. doi: 10.1039/D5FO00414D

microbial composition and concentration of organic acids are often observed in individuals experiencing negative health outcomes, ranging from gastrointestinal diseases to neurological disorders, compared to healthy controls (263,385,386).

Dysbiosis in the colonic microbiota (an imbalance in the microbiota) is frequently marked by reduced faecal concentration of SCFAs (263,344). SCFAs are organic acids produced by the microbial metabolism of complex carbohydrates and benefit the host by supporting intestinal barrier integrity, supplying energy, and regulating metabolic functions, among other benefits (364,387–389). Given the relationship between the colonic microbiota and host physiology, understanding how diet shapes colonic microbes to promote health has attracted great interest recently. However, research in this area often neglects a critical period for the development of the colonic microbiota: infancy.

In early life, breastmilk is the gold standard for nourishing beneficial colonic commensals (211,390). However, little is known about how complementary foods influence the colonic microbiota when infants start consuming solids (weaning). Longitudinal observations demonstrated, through the usage of faecal samples as a proxy, that the colonic microbiota is particularly adaptable during weaning, with diet-induced changes potentially lasting into later life and affecting long-term health (29,391). At this stage, the GIT is still developing, allowing macronutrients from complementary foods to reach the colon and promote the growth of new commensal microbes (56,212). Therefore, a deeper understanding of how foods impact the microbiota of weaning infants is essential for fostering the adequate development of the colonic microbiota from an early age.

Clinical trials allow for assessing dietary interventions on the colonic microbiota and tracking related health outcomes. However, trials involving vulnerable populations, such as infants, can be particularly time-consuming, costly, and ethically complex. *In vitro* experimental models, while unable to capture the full complexity of host-microbiota interactions, offer a cheaper and less invasive alternative that addresses some of the ethical and logistical challenges associated with clinical trials (392). Among these methods, static *in vitro* protocols for food digestion and subsequent faecal fermentation of food remnants provide a useful screening approach to evaluate how dietary compounds influence colonic microbes (265,393).

This chapter investigated the effects of complementary foods on the microbial composition and organic acid production by the colonic microbiota in weaning infants after 24 hours of fermentation, using faecal samples as a proxy. Uniquely, food ingredients were combined with infant formula, other

foods, or both to better replicate real-life infant feeding patterns. This research aimed to identify *in vitro* foods that support adequate development of the colonic microbiota in New Zealand weaning infants.

6.3. Materials and methods

6.3.1. Food ingredients

A total of 13 food ingredients were used in this chapter (Table 6.1). These included vegetables (pumpkin), legumes (black beans, chickpeas, soybeans, and yellow peas), starchy foods (kūmara and couscous), meat (pork), seafood (prawn), and berries (blackcurrants, raspberries, and strawberries). Due to ethical and practical considerations, a commercial infant formula powder (NAN SUPREMEpro 2, Nestlé, Auckland, New Zealand) was used as a substitute for human breastmilk (see Appendix 5 for composition). These foods were identified through an *in silico* analysis as candidates for promoting changes in the production of SCFAs by the colonic microbiota of New Zealand weaning infants (117). Foods were purchased from local stores and prepared under various conditions. Fruits and infant formula were obtained as dried powders and used as purchased, while the other ingredients were brought fresh, sous-vide cooked, freeze-dried, and ground using a standardised method (see Supplementary Table 6.1 for cooking conditions). Potato starch (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control due to its resistant starch content, which serves as a fermentable substrate for colonic microbes. The moisture content of food ingredient powders was determined by the weight difference before and after 48 hours of incubation at 105 °C. Additionally, compositional analyses of freeze-dried and ground food ingredients were performed at the Massey University Nutrition Laboratory. Analyses were conducted in duplicates for carbohydrates, sugar, total dietary fibre, protein, fat, saturated fat, and energy content (Supplementary Table 6.2).

6.3.2. Simulated infant digestion of the food ingredients

Food ingredients were digested *in vitro* either alone, combined with other foods (1:1 food-food ratio), combined with infant formula (1:4 food-formula ratio), or combined with other foods and infant formula (1:1:8 food-food-formula ratio). These ratios were selected to reflect the high intake of infant formula by formula-fed infants at six months of age, which accounts for approximately 80 % of their caloric intake (335). A total of 53 samples, each with three replicates, were randomised into batches and independently digested using a protocol adapted to mimic the digestion of a 6-month-old infant. Simulated digestive fluids were prepared as described in the adult INFOGEST protocol (393,394), with

enzyme concentrations modified according to a dynamic model for infant digestion (395) and a static model for newborn digestion (396).

Table 6.1. List of food ingredients.

Ingredient	Description	Source
Black beans	Dried grains of turtle black beans	Davis Food Ingredients, Palmerston North, New Zealand
Blackcurrants	Freeze-dried New Zealand-grown blackcurrants	Fresh As, Auckland, New Zealand
Chickpeas	Dried grains of chickpeas (garbanzo beans)	Davis Food Ingredients, Palmerston North, New Zealand
Couscous	Medium-sized grains of dried couscous (Durum wheat)	DARI, Salé, Morocco
Infant formula	Nestlé NAN SUPREMEpro 2	Nestlé New Zealand Limited, Auckland, New Zealand
Kūmara	Fresh red kūmara	Countdown, Palmerston North, New Zealand
Pork	Fresh lean pork fillet (tenderloin)	Online meats, Ōtāhuhu, New Zealand
Prawn	Fresh Australian prawn	Solander Seafood & Fishing, Nelson, New Zealand
Pumpkin	Fresh crown pumpkin	Countdown, Palmerston North, New Zealand
Raspberries	Freeze-dried New Zealand-grown raspberries	Fresh As, Auckland, New Zealand
Soybeans	Dehulled grains of soybeans	Jia Hua Asian Mart, Palmerston North, New Zealand
Strawberries	Freeze-dried New Zealand-grown strawberries	Fresh As, Auckland, New Zealand
Yellow peas	Dried grains of yellow peas	Davis Food Ingredients, Palmerston North, New Zealand

To simulate oral digestion, 1.5 g of food ingredients were homogenised with 5 mL of deionised water and 5 mL of simulated salivary fluids. No mastication was assumed due to the liquid nature of the resulting mixture. The mixture was incubated for 2 minutes at pH 7.0 and 37 °C with 75 U/mL of α-amylase under agitation at 150 rpm. The reaction was stopped with concentrated hydrochloric acid, and simulated gastric fluid was added to bring the volume to 20 mL. The mixture was then incubated for 2 hours at pH 3.0 and 37 °C with 500 U/mL of porcine pepsin under agitation at 150 rpm. The reaction was stopped with concentrated sodium hydroxide, and simulated intestinal fluid was added to bring the final volume to 40 mL. Intestinal digestion was simulated by incubating the mixture for 2 hours at pH 7.0 and 37 °C with 100 U/mL of protease activity of pancreatin, 200 U/mL of pancreatic lipase, 100 U/mL of amyloglucosidase, and 10 mmol/L of bile salts under agitation at 150 rpm. All chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Intestinal digestion was stopped by heat treatment (3 minutes at 95 °C). After digestion, nutrient absorption in the large intestine was simulated by placing digested samples into Spectra/Por® cellulose membrane dialysis tubing (Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours, with at least 2

changes of room-temperature deionised water. Post-dialysis samples were stored at -20 °C until fermentation.

6.3.3. Faecal fermentation of the digested food ingredients

This research was approved by the Massey University Human Ethics Committee Southern A (Application 22/48). A total of six healthy New Zealand infants at weaning age (5-11 months) were recruited for this chapter after written consent for their participation was obtained from their primary caregivers (see Appendix 6 for advertisement). The recruited infants were born at over 32 gestational weeks and weighed more than 2.5 kg (Supplementary Table 6.3). None had received antibiotics in the three weeks before sampling and were not consuming prebiotics or probiotics. All infants had already been exposed to complementary foods and had no known medical conditions.

Participants donated multiple faecal samples and were provided with scooping-lid plastic containers and written instructions on collecting and storing the stool samples (see Appendix 7 for information sheet). Samples were preferentially collected fresh after defaecation and transported refrigerated to the laboratory. Alternatively, samples could be stored in the participant's freezer until transportation. Upon arrival at the laboratory, samples were diluted with 50 mM potassium phosphate buffer pH 6.8 to a concentration of 32 % (w/v). The resulting faecal slurry was filtered using a filter bag and stored at -80 °C.

Faecal fermentations followed a standard batch protocol with slight modifications (265). Before fermentation, aliquots from different donors were defrosted and pooled in equal proportions to create an inoculum representative of the colonic microbiota of New Zealand weaning infants. Digested food samples were randomised into independent fermentation batches, and 6 mL of each sample was mixed with 2 mL of 0.15 M potassium phosphate buffer pH 7.4 in two 16 x 125 mm Hungate tubes. The potassium phosphate buffer was also used as a negative control. The mixture was degassed with nitrogen, and the headspace of the tubes was filled with carbon dioxide. To ensure the absence of oxygen, 100 µL of 3 % (w/v) L-cysteine was added to the tubes. Finally, 2 mL of faecal inoculum was added to the tubes, resulting in a total volume of 10.1 mL. Half of the tubes were immediately incubated on ice (time zero), while the remaining tubes were incubated for 24 hours at 37 °C.

6.3.4. Gas pressure and pH

After 24 hours of fermentation, the gas pressure of the Hungate tubes was measured (in kPa) using the Go Direct® Gas Pressure Sensor and the software Vernier Graphical Analysis (Vernier Science Education, Beaverton, OR, USA). The pH of samples at the start and end of fermentation was measured using the PL-700AL bench meter (Pacific Sensor Technologies, Rowville, VIC, Australia). Results were expressed as a decrease in pH after 24 hours. Additionally, 1 mL aliquots were collected and centrifuged at 13,000 × g for 1 min using the Minispin Plus mini centrifuge (Eppendorf, Hamburg, Germany). The supernatants and pellets were recovered and stored at -80 °C for subsequent analysis of organic acids and microbial composition, respectively.

6.3.5. Organic acids analysis

Organic acids were extracted and derivatised following a published protocol (397), with slight modifications. Extractions were performed by mixing 450 µL of fermentation supernatant with 50 µL of the internal standard 50 mM 2-ethyl butyric acid (Sigma-Aldrich, St. Louis, MO, USA). Then, 1250 µL of diethyl ether and 250 µL of hydrochloric acid (37 %) were added to the mixture. Samples were vortexed, and 100 µL of the diethyl ether phase was transferred to a glass vial containing 20 µL of the derivatising agent N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich, St. Louis, MO, USA). Derivatisation occurred by incubating the mixture for 20 minutes at 80 °C, followed by 48 hours at room temperature.

Standard solutions of the organic acids formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, hexanoate, heptanoate, lactate, and succinate, containing 5 mM 2-ethyl butyric acid were prepared alongside the samples. The standard solutions at varying concentrations (0.15, 0.25, 0.50, 1, 2.50, 5, 10, and 20 mM) were used to generate a calibration curve for determining the concentration of the organic acids in the samples. The supernatants from samples at the end of fermentation were diluted with 0.15 M potassium phosphate buffer pH 7.4 to ensure that the concentrations fell within the range of the calibration curve. Organic acid production was calculated as the difference between the concentrations at time zero and 24 hours, expressed in mmol/g (dry weight) to account for the theoretical dry mass of the fermented sample.

Organic acids were detected using the GC-2010 gas chromatograph system coupled with a flame ionisation detector (Shimadzu, Kyoto, Japan) and fitted with an HP-1 column (30 m × 0.25 mm ID ×

0.25 μm; Agilent Technologies, Santa Clara, CA, USA). Helium was used as carrier gas with a flow rate of 21.2 mL/min, a pressure of 131.2 kPa, and a split ratio of 5:1. The temperature programme began at 70 °C, increasing to 115 °C at a rate of 6 °C/min, followed by a final increase to 300 °C at 60 °C/min, holding for 3 minutes. The detector temperature was 310 °C. Data were acquired and processed using the LabSolutions software (version 5.98) (Shimadzu, Kyoto, Japan).

6.3.6. Microbial composition analysis

The DNA from fermentation pellets was extracted using the NucleoSpin DNA Soil kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. The quantity of extracted DNA was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its quality was assessed through gel electrophoresis using a 1 % agarose gel and the lambda HindIII DNA marker (Thermo Fisher Scientific, Waltham, MA, USA; **Supplementary Figure 6.1**). Extracted DNA was stored at -80 °C before sequencing. The V3-V4 regions of the 16S rRNA were amplified using the 341 forward (5′-CCTACGGGAGGCAGCAG-3′) and the 806 reverse (5′-GGACTACHVGGGTWTCTAAT-3′) primers with custom barcodes. All samples from the 24 hours of fermentation were sequenced, while only five randomly selected samples from time zero were sequenced due to resource limitations.

PCR amplification, amplicon quantification, purification, and sequencing using a MiSeq platform (Illumina, San Diego, CA, USA) with 2 x 250 bp paired-end reads were performed at Magigene Biotechnology Co. Ltd. (Guangzhou, China) (data deposited in the NCBI SRA repository, BioProject accession number PRJNA1327581). Raw data were processed using the New Zealand eScience Infrastructure (NeSI) high-performance computing facilities (code and data available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/Chapter%206). In short, primers were removed from raw demultiplexed reads using Cutadapt (398) (version 2.3), followed by Trimmomatic (399). The DADA2 pipeline (version 1.32) (327) was employed for denoising, truncating reads (to 214 bp for forward reads and 195 bp for reverse reads), chimera removal, and inferring amplicon sequence variants (ASVs) in R (version 4.4) (400). Taxonomy assignment was performed using the SILVA database (version 138.1) (223).

Inconsistencies and missing classifications in the ASV data were addressed using the microbiome package (version 1.26) (401) by collapsing taxa into higher taxonomic ranks. Microbial alpha diversity analyses were conducted on unfiltered and unrarefied ASVs using the package phyloseq (version 1.48)

(402) to measure the Chao1 richness estimator, Shannon index, and Simpson index. For microbial beta diversity analysis, samples were rarified to 49,433 reads, and dissimilarities in microbial abundances were assessed using the Bray-Curtis index. Data were ordinated using principal coordinate analysis (PCoA) based on the Bray-Curtis index employing phyloseq. Microbial relative abundance was visualised using the microViz package (version 0.12.4) (403) after filtering taxa that were present in at least 10 % of samples and had a relative abundance greater than 0.01 %.

6.3.7. Statistical analysis

All statistical analyses were performed individually for each subset of samples, which were grouped according to their composition: food ingredients alone, foods combined with infant formula, foods combined with other foods, and foods combined with both infant formula and other foods. A one-way analysis of variance (ANOVA) was used to assess the influence of the substrate (food ingredient or food combination) on pH, gas pressure, and organic acid production after 24 hours of fermentation. Differences in absolute pH changes, gas pressure, and organic acid production between samples were determined using the Tukey Honestly Significant Difference (HSD) test with a 95 % confidence level to account for multiple comparisons. Results were plotted using the ggplot2 package (version 3.5.1) (404).

The effect of the substrate on the microbial alpha diversity of samples was assessed using the Kruskal-Wallis test. For diversity indices with significant differences, subsequent pairwise comparisons were performed using Dunn's test via the FSA package (version 0.9.5) (405). The Benjamini-Hochberg adjustment was employed to control for false discovery rates. Differences in beta diversity between samples were evaluated through a pairwise permutational multivariate analysis of variance (PERMANOVA), with p-values adjusted using the Benjamini-Hochberg method. Analyses were conducted using the adonis2 function from the vegan package with 9,999 permutations (version 2.6-6) (406).

Differential abundance testing was performed for taxa present in more than 5 % of the samples using the ANCOM-BC2 package (version 2.6) (407). The ANCOM-BC2 global test served as a preliminary approach to identify taxa varying between at least two samples, while sensitivity analyses assessed the reliability of the results. For taxa identified through the global test, abundance log-fold changes (LFC) between samples were evaluated through multiple pairwise comparisons using a Dunnett-type test, with p-values adjusted using the Holm-Bonferroni method.

Two-sided Spearman's rank correlation tests were performed to assess the strength of the associations between the following pairs: the nutritional composition of food samples and organic acids produced after 24 hours of fermentation; the nutritional composition of food samples and the relative abundance of microbial genera after 24 hours of fermentation; and produced organic acids and the relative abundance of microbial genera at the end of the fermentation. Thresholds for the strength of the correlations were considered as follows: weak (0.2 - 0.39), moderate (0.4 - 0.59), and strong (> 0.6). Only genera with more than 0.05 % relative abundance were included in the analyses. The Benjamini-Hochberg method was used to control for false discovery rates. Significant correlations (at a false discovery rate-adjusted $p < 0.05$) were displayed as heatmaps using the corrplot package (version 0.95) (408).

6.4. Results

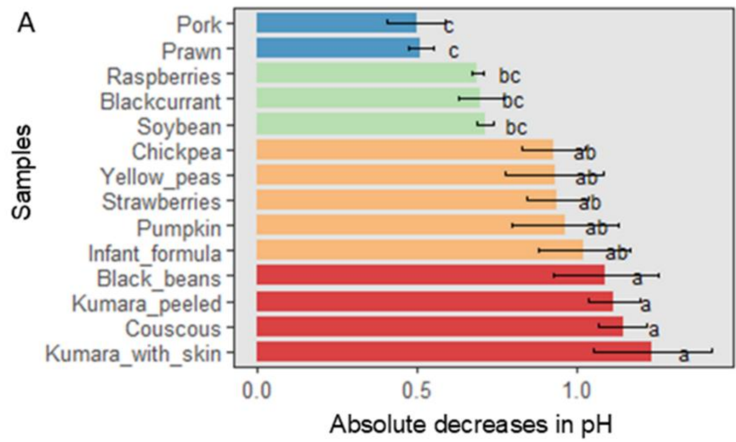
6.4.1. Changes in pH and gas pressure

Changes in pH and gas pressure between fermented substrates were only observed for fermentations with food ingredients alone (ANOVA, $p < 0.001$). Fermentations with kūmara with skin and couscous resulted in the greatest decreases in pH and increases in gas pressure. Fermentation with peeled kūmara exhibited one of the greatest decreases in pH but a moderate gas pressure change. In contrast, fermenting pork, prawn, raspberries, and blackcurrants resulted in the smallest decreases in pH and the lowest gas pressures (Figure 6.1). Fermentation with strawberries showed one of the lowest gas pressures but a moderate decrease in pH. Additionally, combining foods with infant formula resulted in greater pH decreases and increased gas pressures compared to food ingredients alone (Supplementary Figure 6.2 and Supplementary Figure 6.3).

6.4.2. Produced organic acids

After 24 hours of fermentation, fermented food samples produced formate, acetate, propionate, butyrate, isovalerate, lactate, and succinate. Isobutyrate, valerate, hexanoate, and heptanoate were undetected (see Supplementary Table 6.4 for detection limits). Individually, fermenting blackcurrants and strawberries resulted in the highest production of organic acids. The same was observed for the fermentation of these berries when combined with infant formula, with each other, or in combination with each other and formula (Supplementary Figure 6.4).

The type of food ingredient significantly influenced the production of formate, acetate, propionate, butyrate, isovalerate, lactate, and succinate, as well as total SCFAs (sum of acetate, propionate, and butyrate; ANOVA one-way, $p < 0.05$). Fermentations with blackcurrants, strawberries, and, to a lesser extent, raspberries increased the production of acetate, propionate, and total SCFAs compared to other foods (Tukey HSD, adjusted $p < 0.05$). The fermentation of kūmara, either peeled or with skin, primarily produced lactate (Figure 6.2; Supplementary Table 6.5).



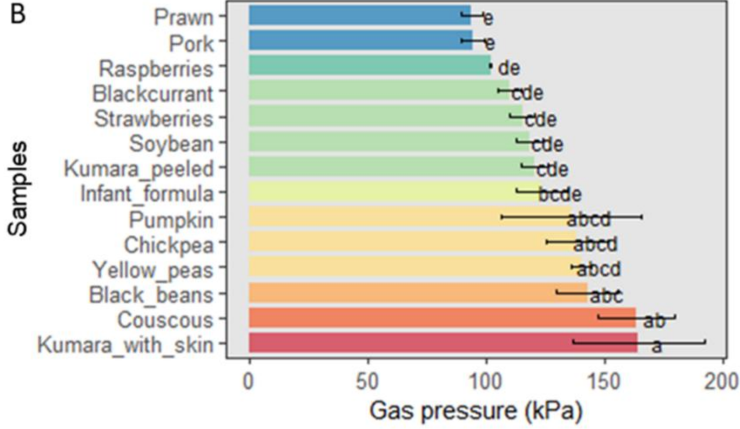


Figure 6.1. Absolute decreases in pH and final gas pressure after 24 hours of fermentation for food ingredients alone. Changes in pH are expressed as the difference between after 24 hours of fermentation and fermentation time zero. Bars are coloured according to intensity and statistical significance, with higher values in red and lower values in blue. Samples with the same colour and same letters belong to the same group according to the Tukey HSD test with a 95 % confidence interval. Decreases in pH (A) and gas pressure (B) are displayed.

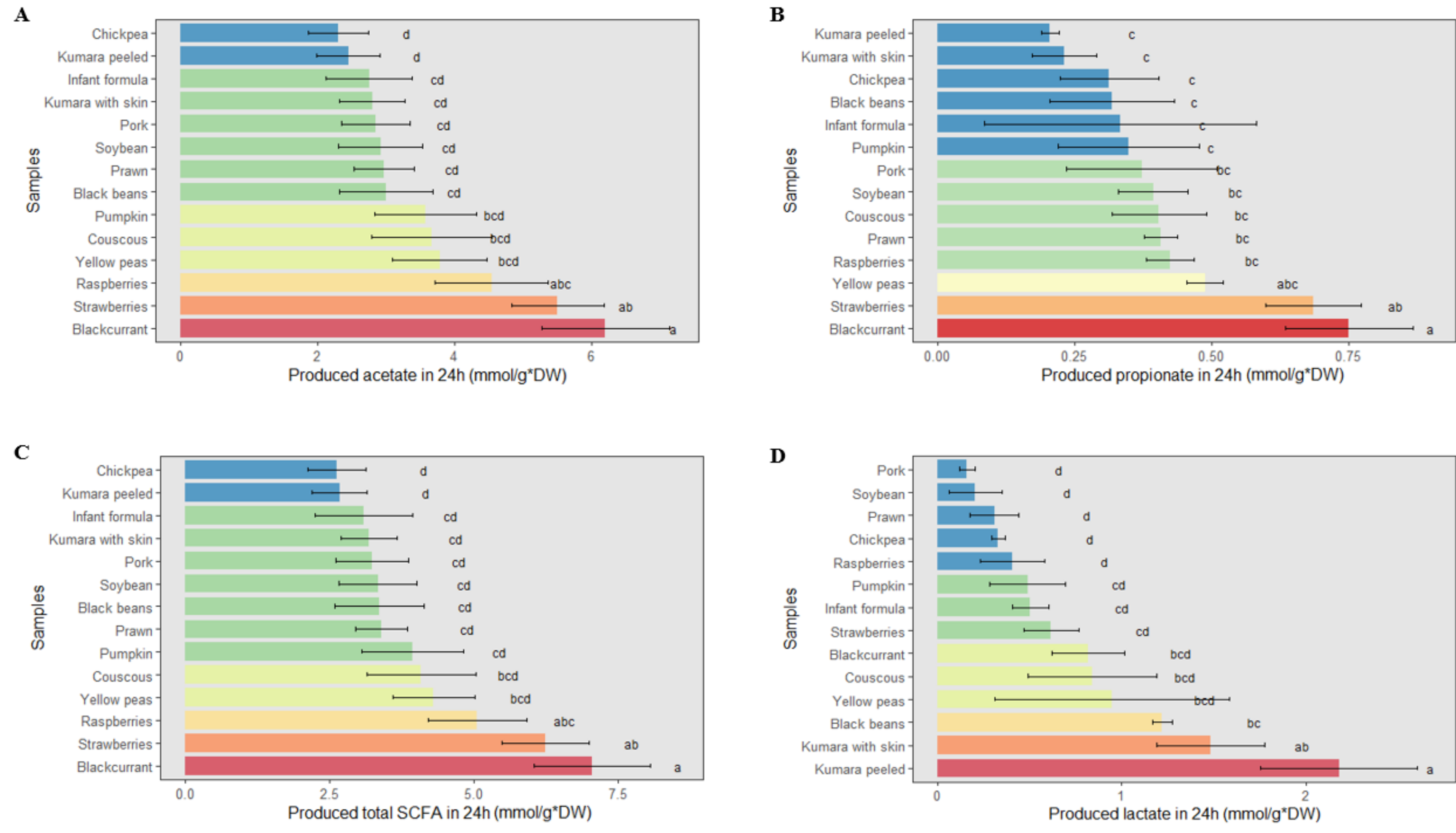


Figure 6.2. Production of organic acids after 24 hours of fermentation of individual food ingredients. Only key results are presented, as follows: acetate (A), propionate (B), total major SCFAs (C), and lactate (D). Bars are coloured according to intensity and statistical significance, with higher values in red and lower values in blue. Samples with the same colour and letters belong to the same group according to the Tukey HSD test with a 95 % confidence interval.

Fermenting food ingredients combined with infant formula or other foods resulted in fewer differences in organic acid production across samples (Supplementary Table 6.6 and Supplementary Table 6.7). The type of fermented food-formula combination only influenced butyrate production after adjusting for multiple comparisons, which was highest in the fermentation with black beans combined with infant formula (Tukey HSD, adjusted $p < 0.005$). Similarly, differences between fermented food-food combination samples were only noted for butyrate, with fermentation with black beans combined with blackcurrants yielding the highest production (Tukey HSD, adjusted $p < 0.005$). No differences in organic acid production were observed between fermentations of food-food-formula combinations after adjusting for multiple comparisons (Supplementary Table 6.8).

6.4.3. Microbial diversity

Samples at fermentation time zero exhibited higher alpha diversity indices (Chao1, Shannon, and Simpson) compared to those at the end (Kruskal test, $p < 0.001$; Supplementary Figure 6.5). There were no differences in microbial alpha diversity scores between samples at the end of the fermentation after adjusting for multiple comparisons (Dunn's test, adjusted $p > 0.05$). Samples at fermentation time zero had distinct beta diversity from samples at 24 hours of fermentation, as measured by the Bray-Curtis dissimilarity index (PERMANOVA, $p < 0.001$; Supplementary Figure 6.6). No differences in the Bray-Curtis dissimilarity index were observed between samples at the end of the fermentation after adjusting for multiple comparisons.

6.4.4. Microbial relative abundance

The major phyla present in samples at fermentation time zero were Actinobacteriota, Firmicutes (or Bacillota), Proteobacteria (or Pseudomonadota), and Bacteroidota, with respective relative abundances of 35 %, 32 %, 20 %, and 10 %. The most abundant families included *Bifidobacteriaceae*, *Enterobacteriaceae*, *Lachnospiraceae*, and *Bacteroidaceae*, while the predominant genera were *Bifidobacterium*, *Escherichia-Shigella*, *Bacteroides*, and *Veillonella*. After 24 hours of fermentation, a shift in the dominant microbial taxa was observed. Bacteroidota, followed by Proteobacteria, became the most abundant phyla. The predominant families were *Bacteroidaceae*, *Enterobacteriaceae*, *Bifidobacteriaceae*, and *Enterococcaceae*, while *Bacteroides*, *Escherichia-Shigella*, *Bifidobacterium*, and *Enterococcus* became the dominant genera (Supplementary Figure 6.7).

Differential abundance testing for fermentations with food ingredients alone revealed significant changes between samples at the phylum, family, and genus levels (ANCOM-BC2 global test, adjusted $p < 0.05$) (Supplementary Tables 6.9, 6.10, and 6.11 are available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/Chapter%206). The fermentation with pork, followed by the fermentation with raspberries, had the highest relative abundances of the phylum Bacteroidota, the family *Bacteroidaceae,* and the genus *Bacteroides* (41 % and 40 %, respectively, at the genus level). In contrast, the fermentation with kūmara with skin exhibited the lowest relative abundances of these taxa, with *Bacteroides* accounting for 32 %. The family *Tannerellaceae* and the genus *Parabacteroides* reached their highest abundances in fermentations with pork and blackcurrants (1 % and 0.8 %, respectively) and their lowest in fermentations with kūmara, both peeled and with skin (0.08 %).

The relative abundances of the phylum Actinobacteriota, the family *Bifidobacteriaceae*, and the genus *Bifidobacterium* were highest in fermentations with peeled kūmara and prawn (20 % at the genus level) and lowest in fermentations with blackcurrants and strawberries (15 %). Fermentations with peeled kūmara, blackcurrants, and raspberries promoted the highest abundances of the phylum Firmicutes, the family *Enterococcaceae*, and the genus *Enterococcus* (18 %, 17 %, and 17 %, respectively, at the genus level). In contrast, fermentations with soybeans and chickpeas had the lowest abundances of these taxa, with *Enterococcus* accounting for 5 % in each case. The family *Streptococcaceae* and the genus *Streptococcus* were least abundant in fermentations with blackcurrants and strawberries (0.9 % each at the genus level) but showed their highest abundances in fermentation with prawns (2.1 %). Additionally, fermentations with kūmara peeled and with skin had the highest abundances of the family *Lactobacillaceae* and the genus *Lacticaseibacillus*. Fermentations with raspberries and blackcurrants exhibited the highest abundances of the family *Eubacteriaceae* and the genus *Eubacterium*.

Multiple pairwise comparisons against a reference group (ANCOM-BC2 Dunnett-type test) demonstrated that fermentations with blackcurrants and raspberries significantly increased the log-fold change (LFC) in the abundance of the family *Tannerellaceae* and the genus *Parabacteroides* and, to a lesser extent, *Enterococcus*, compared to fermentations with other food ingredients (adjusted $p < 0.05$; **Figure 6.3**; Supplementary Figure 6.8). Importantly, LFC values represent differences in bias-correct abundances between groups and do not directly reflect the relative abundance of taxa. Fermentation with raspberries also exhibited higher LFC values for the phylum Firmicutes, the families *Eubacteriaceae* and

Enterococcaceae, and the genera *Sellimonas* and *Eubacterium* compared to fermentations with other foods (Supplementary Figure 6.9). In contrast, fermentations with kūmara peeled or with skin decreased the LFC in the abundance of the genus *Parabacteroides* compared to fermentations with other foods (**Figure 6.3**).

Significant differences in taxa relative abundance between fermentations with food-food combinations were at the phylum, family, and genus levels (ANCOM-BC2 global test, p adjusted < 0.05). Fermentation with the couscous-pork combination promoted the highest abundances of the phylum Bacteroidota, the family *Bacteroidaceae*, and the genus *Bacteroides* (43 % at the genus level), while fermentation with couscous-pumpkin exhibited the lowest abundance of these taxa (35 %). The family *Tannerellaceae* and the genus *Parabacteroides* showed the highest relative abundances in the fermentation with pork-raspberries (1.2 %) and the lowest in the fermentation with couscous-pork (0.2 %).

The phylum Proteobacteria and the family *Enterobacteriaceae* had the highest abundances in the fermentation with the blackcurrants-strawberries combination (30 % at the family level) and the lowest in the fermentation with blackcurrants-kūmara with skin (20 %). In contrast, the phylum Actinobacteria, the family *Bifidobacteriaceae*, and the genus *Bifidobacterium* exhibited the highest abundances in the fermentation with black beans-blackcurrants (19 %) and the lowest in the fermentation with blackcurrants-strawberries (14 % at the genus level). Fermentation with blackcurrants-pork had the highest relative abundances of the phylum Verrucomicrobiota, the family *Akkermansiaceae*, and the genus *Akkermansia* (1.4 % at the genus level).

The relative abundances of the phylum Firmicutes, the family *Enterococcaceae*, and the genus *Enterococcus* were highest in fermentations with blackcurrants combined with kūmara peeled or kūmara with skin (19 % at the genus level). In contrast, the fermentation of the combination blackcurrants-soybean exhibited the lowest abundance of these taxa, with *Enterococcus* accounting for 6 %. The families *Eubacteriaceae* and *Clostridiaceae* and the genera *Eubacterium* and *Clostridium sensu stricto 1* had their highest relative abundances in fermentation with black beans-blackcurrants (0.2 % for each genus).

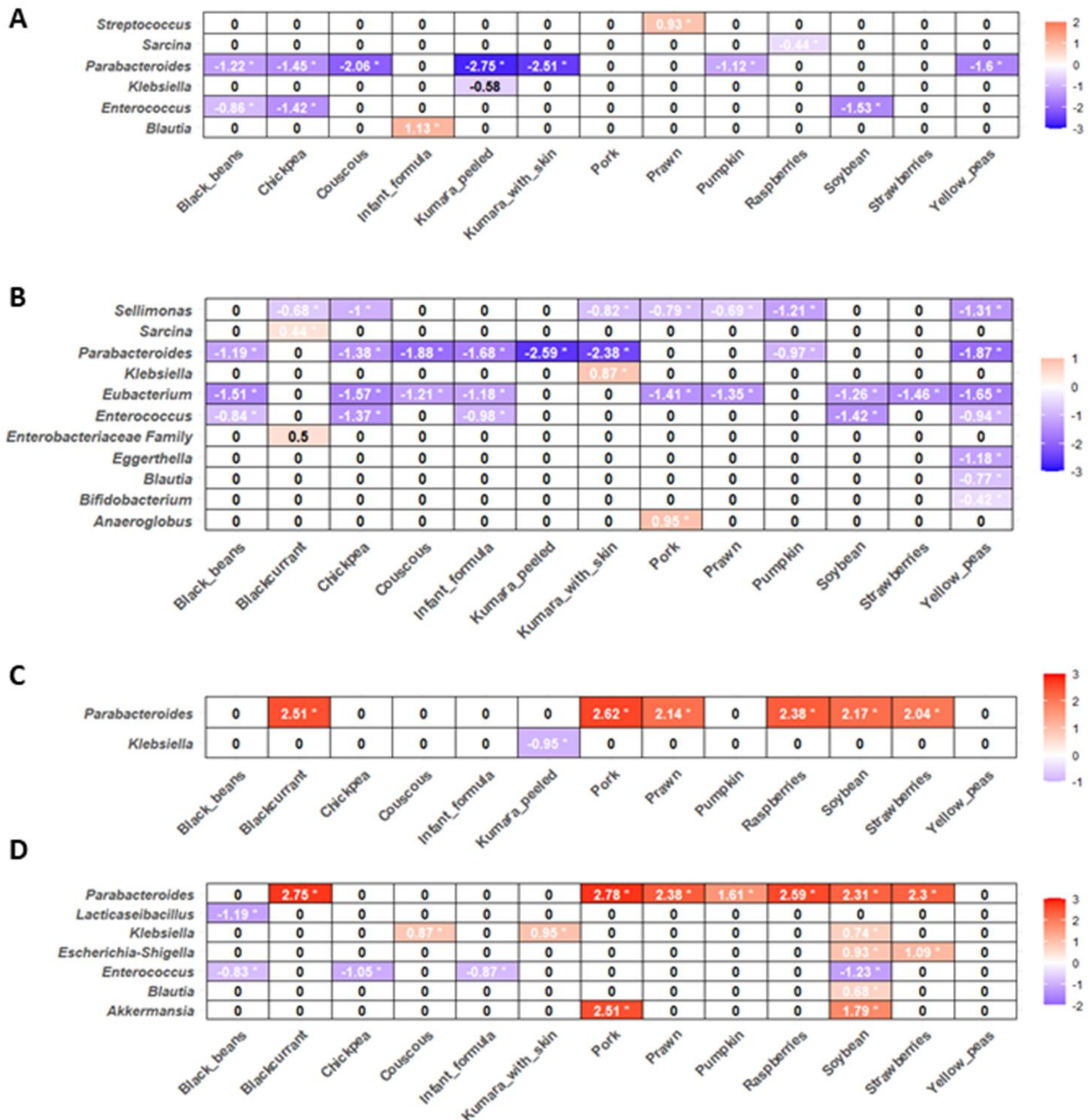


Figure 6.3. Heatmap of log-fold changes (LFC) in the abundance of bacterial genera after 24 hours of fermentation with selected food ingredients. LFC values are presented in comparison to other foods. Fermentation with blackcurrant, compared to fermentation with other foods, is at the top (A), followed by raspberries (B), kūmara with skin (C), and peeled kūmara (D), compared to other food ingredients. Cells are coloured according to intensity, with higher values in red and lower values in blue. Significant changes in LFC (adjusted $p < 0.05$) that passed sensitivity analyses are marked with an asterisk (*).

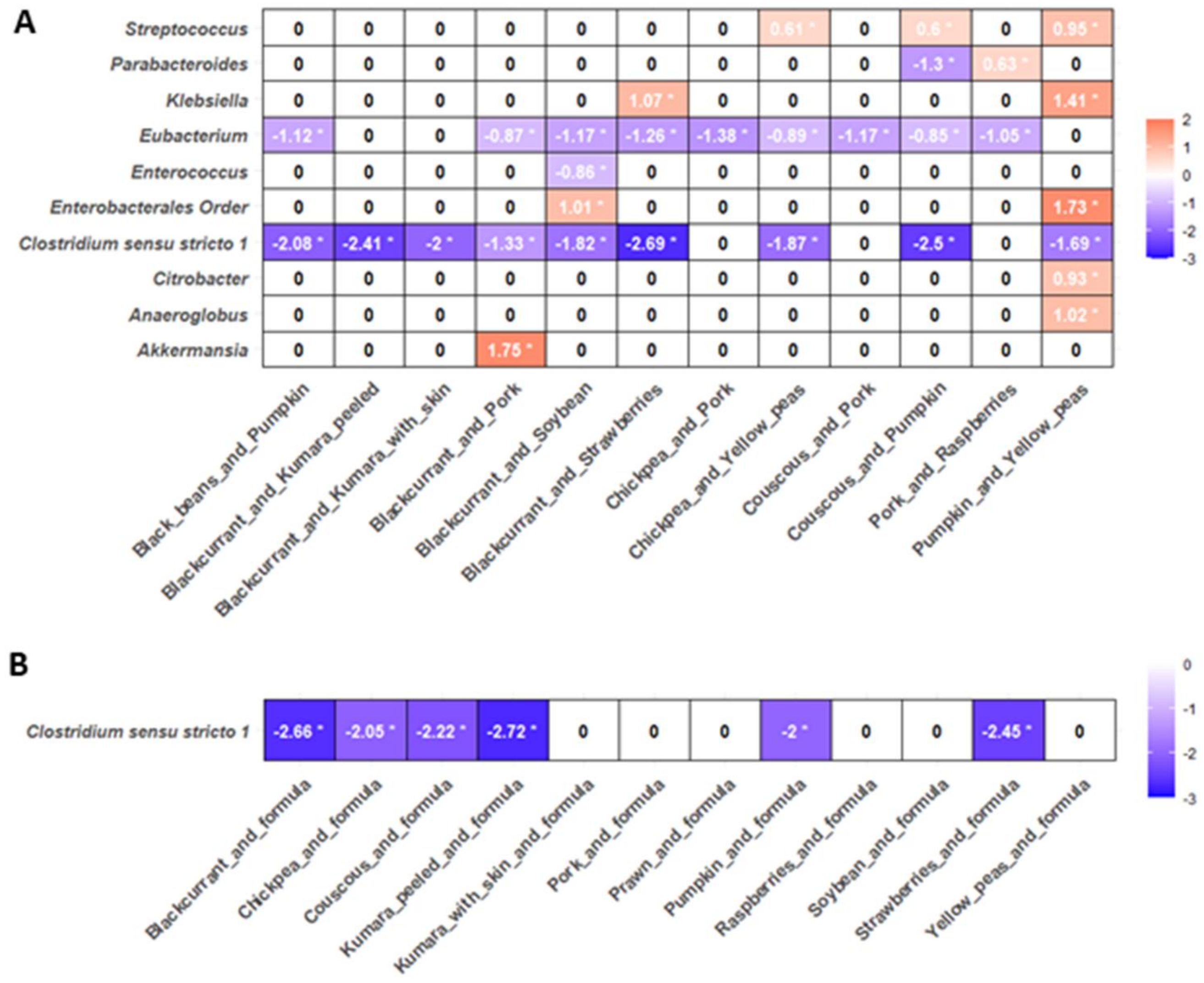


Figure 6.4. Heatmap of log-fold changes (LFC) in the abundance of bacterial genera after 24 hours of fermentation with food-formula and food-food combinations. LFC values are presented in comparison to other food combinations. Fermentation with black beans combined with blackcurrant, versus fermentation with other food-food combinations, is at the top (A), while black beans combined with infant formula, compared to other food-formula combinations, is at the bottom (B). Cells are coloured according to intensity, with higher values in red and lower values in blue. Significant changes in LFC (adjusted $p < 0.05$) that passed sensitivity analyses are marked with an asterisk (*).

Multiple pairwise comparisons of taxa LFC demonstrated that fermentations with the combination of black beans-blackcurrants had increased LFC values in the abundance of the genera *Eubacterium* and *Clostridium sensu stricto 1* compared to fermentations with other food-food combinations (Figure 6.4). Fermentation with black beans-blackcurrant also had higher LFC for the families *Eubacteriaceae* and *Clostridiaceae* (ANCOM-BC2 Dunnett's test, adjusted $p < 0.05$), while no significant differences were observed at the phylum level (Supplementary Figure 6.10).

Combining infant formula with food ingredients or food-food combinations reduced the observed differences in microbial relative abundance between fermented samples. No differences in the abundance of bacterial phyla, families, or genera detected between fermentations with food-formula combinations by the ANCOM-BC2 global test passed the sensitivity analyses. This suggests that the variations between fermented samples were likely due to model parameters or assumptions rather than biological differences. Multiple pairwise comparisons, using fermentation with black beans-formula as a reference group due to its increased butyrate production after 24 hours of fermentation, showed increased LFC in the abundance of the family *Clostridiaceae* and the genus *Clostridium sensu stricto 1* (adjusted $p < 0.05$), compared to fermentations with other food-formula combinations (Figure 6.4).

Significant differences in taxa abundance between fermentations with food-food-formula combinations were observed at the family and genus levels (ANCOM-BC2 global test, adjusted $p < 0.05$). The family Bacteroidaceae and the genus *Bacteroides* had the highest abundances in fermentation with chickpea-yellow peas-formula (43 % at the genus level) and the lowest in fermentation with blackcurrants-kūmara with skin-formula (32 %). Additionally, fermentation with chickpea-yellow peas-formula combination exhibited the lowest abundances of the families *Streptococcaceae* and *Eubacteriaceae* and the *genera Streptococcus* and *Eubacterium* (1.2 % and 0.06 %, respectively, at the genus level). In contrast, fermentation with couscous-pork-formula promoted the highest abundances of these taxa (1.7 % for *Streptococcus* and 0.1 % for *Eubacterium*). No significant changes in bacterial taxa LFC values between fermentations with food-food-formula combinations were observed after multiple pairwise comparisons using the fermentation with blackcurrants-strawberries-formula combination as a reference group.

6.4.5. Correlations between food composition, organic acids and microbiota composition

The produced major and total SCFAs exhibited weak positive correlations with the total dietary fibre content across all fermented food samples (Spearman's rank correlation, adjusted $p < 0.05$). In contrast, fat, energy, protein, and sugar content correlated negatively with the production of these organic acids (Figure 6.5). Notably, acetate production had a strong negative correlation with fat and energy content (Spearman's rank correlation coefficient r_s values of -0.66 and -0.72, respectively). Lactate production also showed negative correlations with energy, fat, and protein content, but had a weak positive correlation with carbohydrate content. Additionally, when analysing individual food ingredients and food-food combinations, lactate production had a strong positive correlation with carbohydrate content and a strong negative correlation with fat content (Supplementary Figure 6.11).

The relative abundances of the genera *Veillonella* and *Enterococcus* were positively correlated with the total fibre content in all samples. Trends indicating a weak positive correlation between fibre content and the relative abundance of the genera *Parabacteroides* and *Lacticaseibacillus* were also observed (Spearman's rank correlation, adjusted $p < 0.1$). Furthermore, the relative abundance of *Lacticaseibacillus* demonstrated a moderate positive correlation with carbohydrate content when analysing only food ingredients ($r_s = 0.52$). In contrast, energy, fat, and sugar content negatively correlated with the relative abundance of *Enterococcus* and *Lacticaseibacillus* (r_s ranging from -0.23 to -0.52), while they positively correlated with the relative abundance of the genera *Streptococcus* and *Blautia* (r_s ranging from 0.22 to 0.60). Protein content exhibited weak positive correlations with the relative abundance of the genera *Akkermansia*, *Anaeroglobus*, *Clostridium sensu stricto 1*, and *Streptococcus*, also showing a trend toward a positive correlation with the abundance of *Bacteroides* (Figure 6.5). A moderate positive correlation was also observed between *Clostridium sensu stricto 1* abundance and protein content in food-food combinations ($r_s = 0.49$; Supplementary Figure 6.11).

When considering the entire set of samples, the production of acetate, propionate, and total SCFAs positively correlated with the relative abundance of *Parabacteroides*, *Lacticaseibacillus*, and *Enterococcus*, among other genera (Figure 6.5). Notably, there were moderate correlations between the abundance of *Parabacteroides* and propionate ($r_s = 0.50$) and between *Enterococcus* and acetate ($r_s = 0.43$). *Enterococcus* abundance also showed positive correlations with lactate production, alongside *Lacticaseibacillus*, as well as with butyrate production in conjunction with *Clostridium sensu stricto 1*.

Additionally, the relative abundance of *Lacticaseibacillus* demonstrated a moderate positive correlation with lactate production from food ingredients, while *Clostridium sensu stricto 1* exhibited a similar correlation with butyrate production from food-formula combinations (Supplementary Figure 6.11). In contrast, the abundances of *Streptococcus* and *Blautia* exhibited negative correlations with the production of major and total SCFAs (r_s ranging from -0.57 to -0.24).

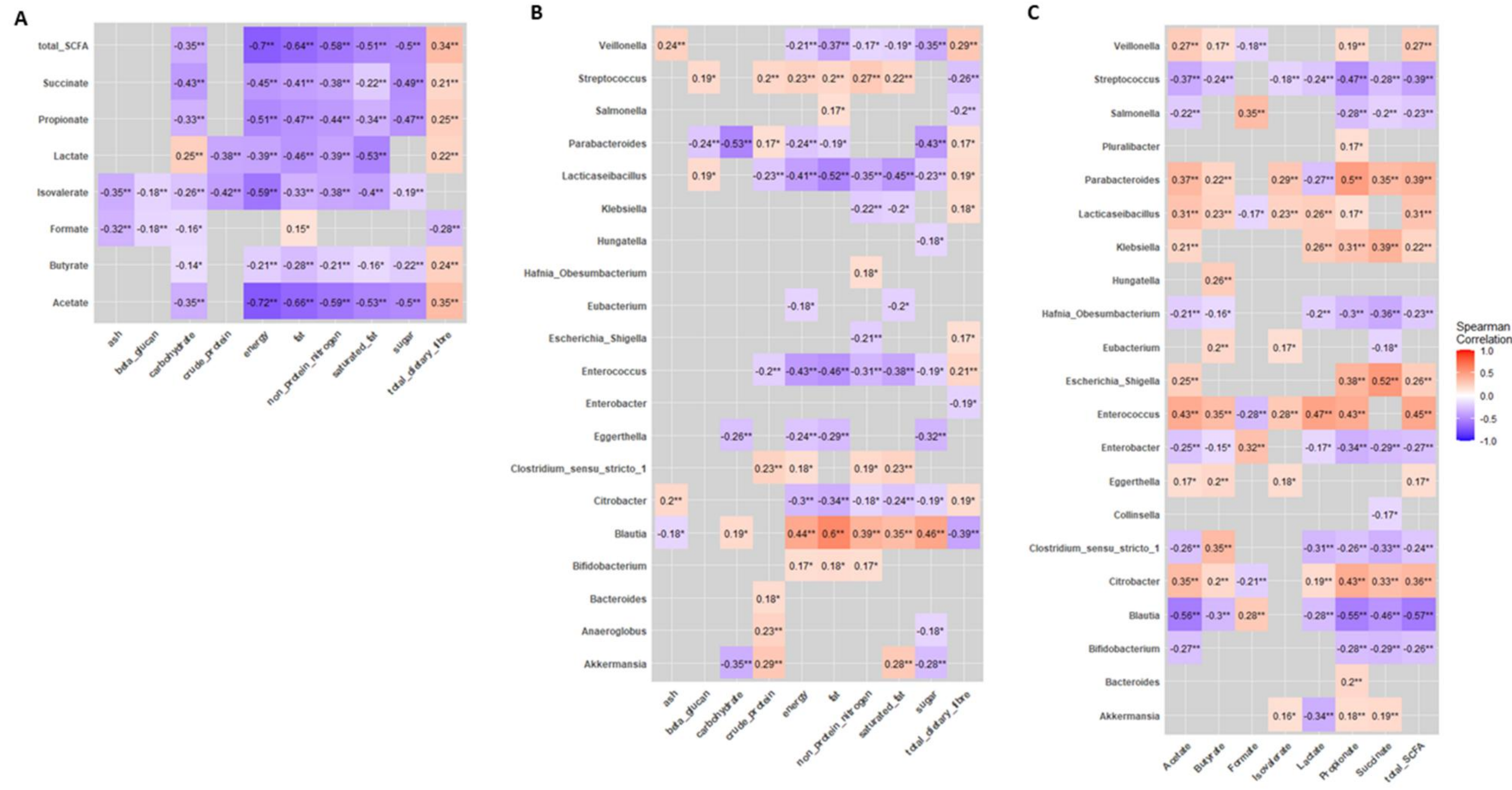


Figure 6.5. Spearman's rank correlation heatmaps. (A) Correlations between food composition and organic acid production. (B) Correlations between food composition and microbiota composition. (C) Correlations between organic acid production and microbiota composition. Only significant correlations are shown. Significant relationships (adjusted $p < 0.05$) are marked with a double asterisk (**), while trends (adjusted $p < 0.1$) are marked with a single asterisk (*). Positive correlations are shown in red and negative in blue.

6.5. Discussion

This chapter evaluated the effects of complementary foods from various sources, including meat, seafood, starchy foods, and fruits, on the *in vitro* composition and function of the colonic microbiota of New Zealand weaning infants, using faecal samples as a proxy. The transition from breastmilk to solid foods is a critical period for the development of the colonic microbiota of infants, yet it has traditionally been neglected in microbiome research (409). To address this knowledge gap in infant nutrition, this chapter assessed the fermentation of food ingredients combined with infant formula, other foods, or both infant formula and other foods. This unique aspect of our study aims to better replicate how complementary foods are introduced to infants in real life while also considering the impact of interactions between different dietary compounds on the relative abundance and organic acid production of colonic microbes. Another strength of our study was using standardised protocols and the latest methods for *in vitro* digestion, faecal fermentation, DNA sequencing, and bioinformatics.

Among the food ingredients, fermentation with kūmara (sweet potato) or couscous produced the most gas and promoted the greatest pH changes. In turn, fermentation with pork, followed by fermentation with prawn, blackcurrants, or raspberries, had the least impact on both measurements. Another *in vitro* study using faecal inoculum from weaning infants assessed the fermentation of plant-based foods, reporting that fermenting oats, sweetcorn, and carrot produced more gas than apple, blackcurrants, and kiwifruit (91). These findings indicate that the infant microbiota has adapted to fermenting complex carbohydrates rather than sugar or animal protein.

In particular, the soluble fibre content may be a major factor influencing pH and gas production during fermentation. Evidence from swine faecal fermentation of different ratios of soluble to insoluble fibre indicated that a higher proportion of soluble fibre increases total gas production while decreasing pH (410). Additionally, soluble fibre content was associated with higher production of lactate and acetate, whereas insoluble fibres were associated with propionate and butyrate yield (410). However, the study used simple substrates derived from mixes of inulin with non-starch polysaccharides, which did not reflect the complexity of foods. In addition to carbohydrates, foods contain various other components, such as fats and protein, which influence the digestion of other nutrients and impact colonic microbes [see reviews (411,412)]. Furthermore, phytochemicals found in plant-based foods can be metabolised by colonic microbes, generating more absorbable and bioactive molecules (413), or exhibit antimicrobial or prebiotic properties, selectively promoting the growth of certain microbes in the colon (414,415).

In our study, fermentation with kūmara produced more lactate than fermentations with other food ingredients. This result is likely an artefact of the *in vitro* static fermentation, as lactate accumulation is often observed in faecal fermentations due to an excess of fermentable substrates (416). Kūmara is rich in complex carbohydrates, primarily in the form of starch, and contains pectin as a soluble fibre and cellulose, hemicellulose, and lignin as insoluble fibres (417). Consistent with the increased lactate production, fermentation with peeled kūmara promoted the highest relative abundances of lactic acid bacteria from the genera *Bifidobacterium*, *Enterococcus*, and *Lacticaseibacillus*. Additionally, fermentation with peeled kūmara had higher LFC values for the abundance of the genus *Enterococcus* compared to other food ingredients. Correlation analyses demonstrated that the carbohydrate content in food ingredients was positively associated with lactate production and the relative abundance of *Lacticaseibacillus*. Furthermore, *Lacticaseibacillus* and *Enterococcus* abundances were positively linked with lactate production and total dietary fibre content.

These bacteria belong to a group of potentially beneficial microbes that produce lactate as their major fermentation product, ultimately contributing to SCFA production in the colon through cross-feeding with other microbes (418,419). For instance, the *Bifidobacterium* genus breaks down carbohydrates, particularly human milk oligosaccharides, through the fructose 6-phosphate pathway to produce acetate and lactate. Most members of the *Lacticaseibacillus* genus (previously classified under *Lactobacillus*) are homofermentative, mainly converting carbohydrates into lactate, although some strains are heterofermentative and produce acetate (420).

The highest lactate yield in the fermentation with kūmara also explains the greatest pH drop, as lactic acid is a stronger acid than the other major SCFAs. In the colon, lactate can be oxidised to pyruvate and subsequently converted into acetyl-CoA, contributing to the pool of acetate and butyrate (421,422), while propionate can be generated from lactate via the methylmalonyl-CoA or acrylyl-CoA pathways (423). Notably, the microbial conversion of lactate into other major SCFAs is sensitive to pH. Evidence *in vitro* demonstrated that lactate is efficiently transformed into propionate and butyrate at around pH 6.5, whereas at pH 5.5 or lower, its conversion is inhibited, resulting in lactate accumulation and overabundance of bifidobacteria (424,425). However, it is important to acknowledge that such low pH conditions do not accurately reflect the physiology of the human colon.

In line with those findings, we observed that the fermentation with kūmara peeled or with skin produced some of the lowest amounts of acetate and propionate, also resulting in a reduced abundance

of the genus *Parabacteroides*. This suggests that lactate was not efficiently converted into SCFAs and instead accumulated during fermentation. Our study used a static fermentation protocol, which does not reflect the dynamic inflow and outflow of substances in the human colon. Under more realistic conditions, lactate produced from kūmara fermentation may contribute to greater production of SCFAs, ultimately conferring host benefits.

Research in rats showed that dietary fibre from sweet potatoes stimulated the growth of *Bifidobacterium* and *Lactobacillus* during *in vitro* faecal fermentation (426). Additionally, it increased faecal propionate and butyrate levels in rats that received sweet potato fibre supplementation for four weeks (426). Similarly, faecal fermentation studies using adult inoculum reported that whole sweet potato and its extracted fibre promoted the production of major SCFAs and the abundance of bifidobacteria (352,427,428). Currently, there is no published research on the effect of kūmara on the colonic microbiota of weaning infants. However, two ongoing clinical trials are evaluating this topic, and their results could provide valuable insights into the field of infant nutrition (429,430).

Fermentations of blackcurrants, strawberries or raspberries led to the highest production of acetate and propionate. Consistently, fermentation with raspberries, followed by fermentation with blackcurrants, exhibited the highest abundances of the genus *Eubacterium.* Additionally, LFC values in the abundance of the genus *Parabacteroides* were greater in fermentations with raspberries or with blackcurrants compared to other food ingredients. These genera encode carbohydrate-active enzymes, allowing them to degrade complex carbohydrates to produce SCFAs (431,432). In contrast, genera colonising the colon in early life, such as *Streptococcus* and *Bifidobacterium*, had the lowest abundances in fermentations with blackcurrants or strawberries, suggesting a transition from infant to adult microbiota.

Blackcurrants, strawberries, and raspberries are sources of dietary fibre, particularly insoluble fibre (mostly cellulose), and contain high amounts of polyphenols, mainly anthocyanins, flavonols, ellagitannins, and ellagic acid (433–435). Evidence suggests that polyphenols and dietary fibre synergistically affect the colonic microbiota by changing the carbohydrate metabolism of colonic commensals. For instance, cranberry proanthocyanidins enhanced the fermentation of xyloglucans, a type of soluble fibre, by lactic acid bacteria *in vitro*, leading to increased acetate production (436). Whole-fruit cranberry powder, rather than its fibrous fraction alone, was more efficient in restoring colonic dysbiosis and reducing body weight in obese mice (437).

Consistently, the production of major and total SCFAs positively correlated with the dietary fibre content of fermented foods here. The productions of acetate, propionate, and total SCFAs were also positively associated with a higher relative abundance of *Parabacteroides* and lower abundances of *Streptococcus* and *Bifidobacterium* genera. In agreement with our findings, the faecal fermentation of raspberry using adult inoculum mainly produced acetate and propionate (357). Additionally, the same study demonstrated that polyphenols contributed more to the production of these SCFAs than dietary fibres (357). A four-week raspberry intervention in prediabetic adults reported no changes in faecal microbial alpha and beta diversity compared to baseline values, but an increase in the relative abundance of *Eubacterium eligens* and *Clostridium orbiscindens*, as well as reduced plasma total and low-density lipoprotein cholesterol (438).

In contrast to our results, another faecal fermentation study using inoculum from weaning infants found no changes in the production of acetate, propionate, and butyrate between blackcurrant and control fermentations (86). However, clinical studies assessing the effect of blackcurrant intervention observed an increase in the faecal abundance of the *Ruminococcus* genus in postmenopausal women after six months (439); as well as an increase in the faecal abundance of the genera *Lactobacillus* and *Bifidobacterium*, alongside a decrease in the abundance of *Clostridium* and *Bacteroides* genera after two weeks in healthy adults (440).

Little is known about the impact of strawberries on the human colonic microbiota. A study using mice with colitis reported that strawberry supplementation increased the faecal abundance of the genera *Bifidobacterium* and *Lactobacillus*, as well as the caecal content of SCFAs (371). Additionally, strawberry supplementation increased the colonic abundance of *Bifidobacterium* in diabetic mice (441). A four-week trial involving healthy adults who consumed strawberries observed increased faecal abundance of the genera *Akkermansia*, *Bacteroides*, and *Bifidobacterium,* but no changes in faecal SCFA levels (370). The evidence above suggests that blackcurrants, strawberries, and raspberries are promising complementary foods for increasing the abundance of SCFA-producing bacteria in the colonic microbiota of infants.

Unlike other major SCFAs, butyrate production did not vary between food ingredient fermentations. However, when black beans were fermented with infant formula or blackcurrants, there was an increase in butyrate production compared to other food-formula or food-food combinations. Similarly, combining black beans with infant formula or blackcurrants in fermentation led to the highest relative abundance of

Clostridium sensu stricto 1, a group of bacteria that metabolise carbohydrates and amino acids, producing butyrate via the butyryl-CoA and butyrate kinase pathways (442,443). Correlation analyses supported these findings, showing a relationship between higher protein content and increased relative abundance of *Clostridium sensu stricto 1*, whose abundance was also positively associated with butyrate production.

Black beans are a source of protein, dietary fibre, and polyphenols, notably containing high amounts of resistant starch (444). Additionally, soaking and cooking beans before consumption further increases their resistant starch content (445). Traditionally, the colonic fermentation of resistant starch produces butyrate through a cross-feeding mechanism involving key resistant starch degraders, such as *Ruminococcus bromii* and *Bifidobacterium adolescentis*, along with butyrate producers from the genera *Faecalibacterium*, *Roseburia*, *Eubacterium*, and *Anaerostipes* (446,447). However, recent evidence demonstrated that members of *Clostridium sensu stricto 1* can also produce butyrate from resistant starch (448).

Previous faecal fermentation studies evaluating the effect of black beans on colonic microbes have shown contrasting results. One reported that black beans exhibited a prebiotic effect by increasing the abundance of the *Bifidobacterium* and *Lactobacillus* genera during fermentation. This increase was associated with a rise in the production of acetate and propionate, but a decrease in butyrate levels compared to the fermentation control (449). On the other hand, another study observed that the fermentation of the insoluble indigestible fraction of black beans produced butyrate, as well as acetate and propionate (450). It is important to note that neither study specified the age of the faecal donors nor evaluated changes in the overall composition of the microbiota, limiting comparison with our findings.

Evidence in murine models suggests that consuming black beans benefits the microbiota by increasing the abundance of key taxa producing SCFAs and subsequently leading to greater production of SCFAs. For instance, healthy mice had increased faecal abundance of *Prevotella* and caecal contents of acetate, propionate, and butyrate after black bean intervention (451). Similarly, rats fed a high-fat and high-sugar diet supplemented with cooked beans exhibited increased faecal abundance of the *Clostridia* class and the genera *Ruminococcus*, *Coprococcus*, and *Prevotella*, as well as elevated faecal butyrate levels (452). In contrast, a navy bean intervention did not alter faecal SCFA content in overweight adults, while a common bean intervention in weaning infants showed no changes in faecal microbiota diversity or taxa abundance (219,453).

Combining foods with infant formula drastically reduced the variability in organic acid production, taxa abundance, and microbial diversity scores between samples. Since the food-formula combinations consisted of 80 % infant formula by mass, this high proportion of formula probably masked the effects of the individual food ingredients on colonic microbes. Similarly, we observed fewer changes in microbiota composition and SCFA production between fermentations with food-food and food-food-formula combinations, suggesting that the impact of specific foods on colonic microbes is less evident when considering the overall dietary pattern. Ultimately, long-term dietary patterns rather than spontaneous consumption of individual foods are more likely to promote notable and lasting changes in colonic commensals (131,454).

Nevertheless, our study has limitations. During the transition to solid foods, infants often continue to consume breastmilk (254). However, our study did not evaluate the effect of combining complementary foods with human milk. Instead, breastmilk was replaced with infant formula (due to practical and ethical reasons), which may have influenced the observed effects of complementary foods on the colonic microbiota of weaning infants. This limitation is particularly relevant during the first year of life, as breastfed infants have distinct faecal microbial composition and metabolite profiles compared to formula-fed infants (255).

Faeces were used due to the ease of collection and non-invasive procedure, which are essential when involving vulnerable participants. However, faecal samples mainly represent microbial communities from the distal colon and do not accurately reflect the microbes that adhere to the mucosa or those found in the proximal colon (200). Due to the screening nature of this chapter, static protocols were used to simulate infant digestion and subsequent colonic fermentation of foods. These static conditions do not capture the dynamic nature of the GIT of infants. Notably, in static faecal fermentations, microbial metabolites can accumulate, and substrates may become depleted, potentially distorting the microbial community compared to what would be found in a dynamic environment (455).

The microbial composition was characterised by 16S rRNA sequencing. While this method is accurate, it has limitations, particularly in resolution and typically cannot resolve taxonomy at the species level (329). The composition of the identified bacterial taxa was expressed as relative abundances, indicating the proportion of individual microbes within the entire community. Consequently, changes in relative values may reflect changes in the growth of other taxa, not necessarily reflecting a true increase or decrease in the absolute quantities of a given taxon.

Our study evaluated a higher number of plant-based foods compared to animal-based foods. This choice was justified by Chapter 5, which identified *in silico* complementary foods with the greatest impact on infant colonic microbial production of SCFAs (117). Although the food ingredients were prepared as similarly as possible to real-life conditions, the preparation likely altered their original structure, ultimately influencing their impact on colonic microbes (456). For instance, cooking and cooling plant-based foods can increase their resistant starch content (445). Finally, while correlation analyses could link changes in the production of SCFAs or microbial relative abundance to protein, fat, and fibre content in the evaluated foods, phytochemicals were not analysed, which is warranted in further research.

Finally, as our study focused on the colonic microbiota of New Zealand weaning infants, our findings may not be directly generalisable to infants from other geographic locations. Geographic location is known to influence the composition of the infant colonic microbiota (457). Furthermore, dietary patterns and eating habits may differ across countries and cultures (458). Consequently, the complementary foods evaluated in our *in vitro* study may not fully represent those consumed by weaning infants in other parts of the world.

6.6. Conclusions

This chapter investigated how various food ingredients and food combinations affect the *in vitro* composition and function of the colonic microbiota in New Zealand weaning infants, using faecal samples as a proxy. Foods promoting the most favourable changes in the infant microbiota were identified. Notably, fermentation with kūmara, a variety of sweet potatoes rich in complex carbohydrates, effectively promoted lactate production by stimulating the growth of the lactic acid bacteria from the genera *Enterococcus* and *Lacticaseibacillus*. Fermentation with blackcurrants, strawberries, and raspberries, notable sources of dietary fibre and polyphenols, increased acetate and propionate production. This increase was linked to a higher relative abundance of *Parabacteroides* and *Eubacterium* genera. Additionally, when black beans were fermented with infant formula or blackcurrants, they produced the highest yields of butyrate and increased the abundance of the group *Clostridium sensu stricto 1*. This is likely due to the high protein and resistant starch content in black beans. Overall, these findings contribute to an under-investigated topic of colonic microbiome research in infants. Kūmara, berries, and black beans are promising candidates for further clinical trials involving infants.

STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Vitor Geniselli da Silva
Name and title of main supervisor:	Professor Warren McNabb
In which chapter is the manuscript/published work?	Chapter 7

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Vitor Geniselli da Silva (student): Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Visualisation, Writing – original draft, Writing – review & editing.
Nick Smith (co-supervisor): Conceptualisation, Supervision, Writing – review & editing.
Jane Mullaney (co-supervisor): Formal analysis, Supervision, Writing – review & editing.
Nicole Roy (co-supervisor): Funding acquisition, Supervision, Writing – review & editing.
Clare Wall (co-supervisor): Funding acquisition, Supervision, Writing – review & editing.
Warren McNabb (main supervisor): Project administration, Supervision, Writing – review & editing.

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Chapter 7: Evaluating the accuracy of the computational model in predicting *in vitro* faecal fermentation data[5]

7.1. Abstract

Traditional approaches for studying diet-colonic microbiota interactions are time-consuming, resource-intensive, and often hindered by technical and ethical concerns. Metagenome-scale community metabolic models show promise as complementary tools that do not face these limitations. However, their experimental validation is challenging, and their accuracy in predicting colonic microbial function under realistic dietary conditions remains unclear. This chapter assessed the accuracy of the Microbial Community model (MICOM) in predicting major SCFA production by the colonic microbiota of weaning infants, using faecal samples as a proxy. Model predictions were compared with experimental SCFA production using *in vitro* faecal fermentation data at the genus level. The model exhibited overall poor accuracy, with only a weak, significant correlation between measured and predicted acetate production (r = 0.17, $p = 0.03$). However, agreement between predicted and measured SCFA production improved for samples primarily composed of plant-based foods: acetate exhibited a moderate positive correlation (r = 0.31, $p = 0.005$), and butyrate a trend toward a weak positive correlation (r = 0.21, $p = 0.06$). These findings suggest that the model is better suited for predicting the influence of complex carbohydrates on the infant colonic microbiota than for other dietary compounds. Our study demonstrates that, given current limitations, modelling approaches for diet-colonic microbiota interactions should complement rather than replace traditional experimental methods. Further refinement of computational models for microbial communities is essential to advance research on dietary compound-colonic microbiota interactions in weaning infants.

7.2. Introduction

The relationship between dietary compounds and the colonic microbiota has garnered intense scientific interest due to its impact on host health (1,128). From a health perspective, changes in colonic microbial function are more relevant than alterations in composition: imbalances in microbial metabolite

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production may distinguish individuals with disease from healthy controls, despite individual variations in microbial taxonomy (4,37,263,459). Numerous metabolites play crucial roles in the bidirectional communication between the colonic microbiota and the host, such as neurotransmitters, polyamines, vitamins, bile acids, and organic acids (460). Among them, the most abundant SCFAs, acetate, propionate, and butyrate, offer numerous benefits to the host, including maintaining colonic barrier integrity, serving as an energy source for colonocytes, and exerting neuroprotective effects (344,387–389).

Most of our current understanding of diet-colonic microbiota interactions centres on the effects of complex carbohydrates on the adult microbiota. In contrast, the impact of other dietary compounds, such as fatty acids and polyphenols, remains underexplored, particularly in underrepresented populations such as infants and older adults (461). A better understanding of how dietary compounds affect colonic microbial function across diverse human populations is crucial. Clinical trials are standard approaches for evaluating this impact and assessing potential health outcomes from diet-microbiota interactions. However, they are time- and resource-consuming, and often challenged by technical and ethical concerns (30). Consequently, new techniques have been developed to study diet-colonic microbiota interactions, including mathematical models (31,202). Models show promise as complementary tools that help overcome the limitations of traditional methods by using pre-existing data to conduct simulations, thereby reducing the cost and time required for microbiota investigations.

Various models have been proposed to investigate diet-colonic microbiota interactions, including ODE-based (268,271,274,275), agent-based (284,462,463), and genome-scale metabolic models (277,464–466). GEMs are distinguished from other models by their mechanistic approach. They use metabolic reconstructions, a mathematical representation of a microorganism's metabolism, and FBA to predict microbial metabolite production as fluxes (units of concentration per time) (276). MGCMs extend this concept to microbial communities (31,202,467). Among MGCMs, the MICOM model stands out for its user-friendly approach, extensive documentation, and pre-made workflows that range from data preparation to visualisation (31,305).

However, experimentally validating the predictions of MGCMs is a technical challenge (468). A recent study compared measured SCFA fluxes from *ex vivo* faecal incubations with predicted fluxes obtained from MICOM (280). An agreement between predictions and experimental measurements was reported for propionate and butyrate. Nevertheless, while the study partially validated *in silico*

predictions for the influence of isolated dietary fibres on the colonic microbiota of adults, the model's accuracy in predicting how whole foods shape colonic microbial function in other populations remains unexplored. Importantly, foods are matrices containing multiple dietary compounds that can interact during digestion and modulate their collective impact on colonic microbes (411,412).

This chapter aimed to evaluate MICOM's accuracy in predicting microbial SCFA production by the colonic microbiota of weaning infants under dietary conditions mimicking real infant feeding scenarios. Predicted acetate, propionate, and butyrate fluxes were compared with fluxes measured experimentally from the *in vitro* faecal fermentations described in Chapter 6 (118). The *in silico* simulations were designed to match the experimental setup as closely as possible, which examined how complementary foods, alone or combined with infant formula and/or other foods, affected major SCFA production by the colonic microbiota of weaning infants.

7.3. Materials and methods

7.3.1. Experimentally measured fluxes of SCFAs

Measured acetate, propionate, and butyrate fluxes were estimated using data from the faecal fermentations described in Chapter 6 (118). The chapter evaluated the effects of complementary foods on the composition and SCFA production of the colonic microbiota in weaning infants, using faecal samples as a proxy. Food ingredients were tested individually and in combination with other foods, infant formula, or both, resulting in 53 samples. Samples were digested *in vitro* using a static model adapted from the INFOGEST protocol (393) to mimic the gastrointestinal conditions of 6-month-old infants, then fermented for 24 hours at 37 ºC using a pooled faecal inoculum from six healthy weaning infants (aged 5-11 months).

Organic acids were acidified with hydrochloric acid, extracted with diethyl ether, derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide, and detected using gas chromatography with a flame ionisation detector (see full methods description in Chapter 6). A solution of 2-ethyl butyric acid was used as an internal standard to account for batch variations. Organic acids were quantified using standard solutions of acetate, propionate, and butyrate. The production of SCFAs was determined for each sample as the difference between measured SCFAs before and after fermentation, normalised by the dry weight of the fermented sample. Fluxes of acetate, propionate, and butyrate were calculated by dividing the concentration of each organic acid by the fermentation time (24 hours).

7.3.2. Software

Simulations were conducted in Python (version 3.9.10) within the Spyder integrated development environment (version 5.4.2) using MICOM (31) (version 0.37.0) and the CPLEX Optimization Studio solver (IBM ILOG, version 22.1), which was accessed under an academic license. Additionally, the AGORA (279) metabolic reconstructions (version 2) were employed to infer the metabolism of the infant faecal microbiota. Data and code are available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/Chapter%207.

7.3.3. In silico media design

Media for the simulations were designed following the workflow described in Chapter 5, which employed MICOM to assess the impact of complementary foods on the colonic microbiota of weaning infants, using faecal microbial relative abundances. In short, the "Design a diet" function of the VMH database was used to select the foods composing the *in silico* media (291). Foods were chosen to match the experimental samples as closely as possible (Supplementary Table 7.1). Additionally, the *in silico* media were designed to have the same dry mass of foods (150 g) to replicate the experimental conditions, which used 1.5 g of freeze-dried foods (a scalar increase was necessary to mitigate numerical instability in the simulations). The same approach was used to design media composed of food combinations, including foods with other foods, infant formula, or both, keeping the same ratios used in the *in vitro* experiments: 50 % $food_1$ with 50 % $food_2$; 20 % food with 80 % infant formula; and 10 % $food_1$ with 10 % $food_2$ and 80 % infant formula.

Imported data were processed through the MICOM workflow for media design (117,280), which added host-secreted compounds (mucin cores and bile acids), removed diluted compounds that are absorbed in the small intestine, and supplemented the media with minimal missing nutrients to ensure a community growth rate of 0.3/h. Finally, media compounds were diluted by a factor of 10 to match experimental conditions, where approximately 10 % of the volume of post-dialysis digested food samples was fermented with faecal inoculum (118). As MICOM's workflow does not directly account for digestion, the composition of the *in silico* media was assumed to reflect the chemical profile of the experimentally digested food samples.

7.3.4. In silico simulations

The relative abundance of the microbial community used in the simulations reflects baseline values from *in vitro* faecal fermentations described in Chapter 6 (118) (Supplementary Table 7.2). Simulations were performed at the genus level due to the use of 16S rRNA gene sequencing in the experimental work. Briefly, raw paired-end sequencing data were generated by amplifying the V3-V4 regions of the 16S rRNA gene using an Illumina MiSeq platform. Primers were removed using Cutadapt (version 2.3) (398) and Trimmomatic (399). The DADA2 (version 1.32) (327) pipeline was followed for denoising, read truncation, chimera removal, and inferring amplicon sequence variants. The SILVA database (version 138.1) (223) was used for taxonomy assignment, and amplicon sequence variants were collapsed at the genus level using the microbiome (version 1.26) (401) package. Only genera with at least 0.001 relative abundance were included in the simulations to reduce numerical instability and processing time. A total of 31 genera (out of 54) were included, representing 99.3 % of the relative abundance of the microbial community. Pan models of the AGORA2 metabolic reconstructions (279) for these genera were built by pooling microbial metabolic strains into higher taxonomic ranks.

Simulations followed published protocols (117,280). MICOM is based on FBA under a mass steady state assumption (31), representing the exponential phase of microbial growth, during which growth rates remain constant. Fluxes of microbial metabolites are calculated as the solution to a constrained linear programming problem, integrating the biochemical reactions performed by the microbial community, assuming no accumulation of substrates in the system, to maximise microbial community biomass. Notably, MICOM incorporates a trade-off between maximal community growth and maximal individual microbial growth (31). This strategy prevents the most abundant microbes from growing at the expense of low-abundance ones. The optimal cooperative trade-off was determined for each *in silico* medium (values ranged from 0.4 to 0.7) using MICOM's "tradeoff" function. Additionally, MICOM employs a linearization strategy that relates the growth rates of individual taxa to their relative abundance (31). Consequently, the microbial community was expected to exhibit consistent growth patterns across different *in silico* media, with high-abundance genera predicted to have higher growth rates than those of lower abundance. Importantly, microbial relative abundance was used as a proxy for microbial biomass in the simulations, and fluxes of microbial metabolites were normalised by the dry weight of microbial biomass (expressed in millimoles per gram per hour {mmol/gDW.h}).

7.3.5. Statistical analyses

To account for dissimilarities between the design of *in silico* and *in vitro* studies, standard scores (z-scores) were calculated for measured and predicted fluxes. The z-score describes the number of standard deviations a value differs from the mean. This strategy enables a comparison of results across studies with different designs (280). Pearson correlation coefficients (r) and two-tailed *p*-values (*p*) between measured and predicted z-scores for acetate, propionate, and butyrate production at the 95% confidence interval were calculated in Python (version 3.10.9) using pandas (version 2.2.3) (469) and SciPy (version 1.10.0) (470). Plotnine (version 0.14.5) was used to plot the correlations (471). To further assess the agreement between predicted and measured z-scores, the Bland-Altman analysis (472) was performed, and the 95 % limits of agreement (mean difference ± 1.96 standard deviations) were calculated using the packages NumPy (version 1.23.5) (473) and matplotlib (version 3.10.0) (474). The normal distribution of the data was verified through the Shapiro-Wilk test using SciPy (version 1.10.0) (470). Heatmaps and radar charts were generated using matplotlib (version 3.10.0) (474), seaborn (version 0.13.2) (475), and SciPy (version 1.10.0) (470).

7.4. Results

7.4.1. Pearson correlations between predicted and measured SCFA production

Correlation analyses demonstrated a weak agreement between predicted and measured acetate production ($r = 0.17$, $p = 0.03$). However, this was the only significant correlation ($p < 0.05$) observed when considering the entire dataset (Supplementary Figure 7.1). To investigate whether combining food ingredients with other dietary compounds would impact the accuracy of the model, subsequent analyses clustered samples into the following categories: food ingredients alone, foods combined with infant formula (food-formula combinations), foods combined with other foods (food-food combinations), and foods combined with both (food-food-formula combinations). A trend towards a weak correlation was observed between predicted and measured butyrate production for food ingredients alone ($r = 0.28$, $p = 0.07$). Additionally, a moderate correlation was observed between model predictions and experimental productions of acetate and total SCFAs for food-food combinations (r = 0.43 and 0.41, with $p = 0.006$ and 0.01, respectively), while propionate exhibited a trend towards a weak negative correlation ($r = -0.30$, $p = 0.06$) (Supplementary Figure 7.2). In contrast, no significant correlations were observed

between predicted and measured z-scores for food-formula and food-food-formula combinations (Supplementary Figure 7.2).

Given that combining infant formula with foods reduced the model's accuracy, an analysis was performed excluding food combinations that contained infant formula (i.e., food ingredients and food-food combinations only, corresponding to samples predominantly composed of plant-based foods). For this subset of samples, acetate exhibited a moderate agreement between predicted and measured z-scores ($r = 0.31$, $p = 0.005$), butyrate demonstrated a trend towards a weak positive correlation ($r = 0.21$, $p = 0.06$), and propionate showed a trend towards a weak negative correlation ($r = -0.19$, $p = 0.08$; Figure 7.1). Similar results were observed when animal-based food samples were completely excluded from the dataset (Supplementary Figure 7.3).

7.4.2. Bland-Altman analysis

The normality of the dataset, comprising food ingredients and food-food combinations, was verified by the Shapiro-Wilk test. While the differences between predicted and measured z-scores for individual SCFAs suggested a normal distribution ($p > 0.05$), their sum did not ($p = 0.01$; Supplementary Table 7.3). Consequently, Bland-Altman plots were generated only for acetate, propionate, and butyrate individually (Figure 7.2). Since fluxes were standardised into z-scores, all plots' mean differences between z-scores were zero. Among the SCFAs, acetate exhibited the lowest limits of agreement, indicating better concordance between predicted and measured z-scores. Overall, most samples fell within the 95 % limits of agreement for the major SCFAs. However, some samples exceeded these limits: 5 out of 81 for acetate, 2 out of 81 for propionate, and 7 out of 81 for butyrate. This suggests that the model had limitations in accurately predicting experimental outcomes across the entire dataset.

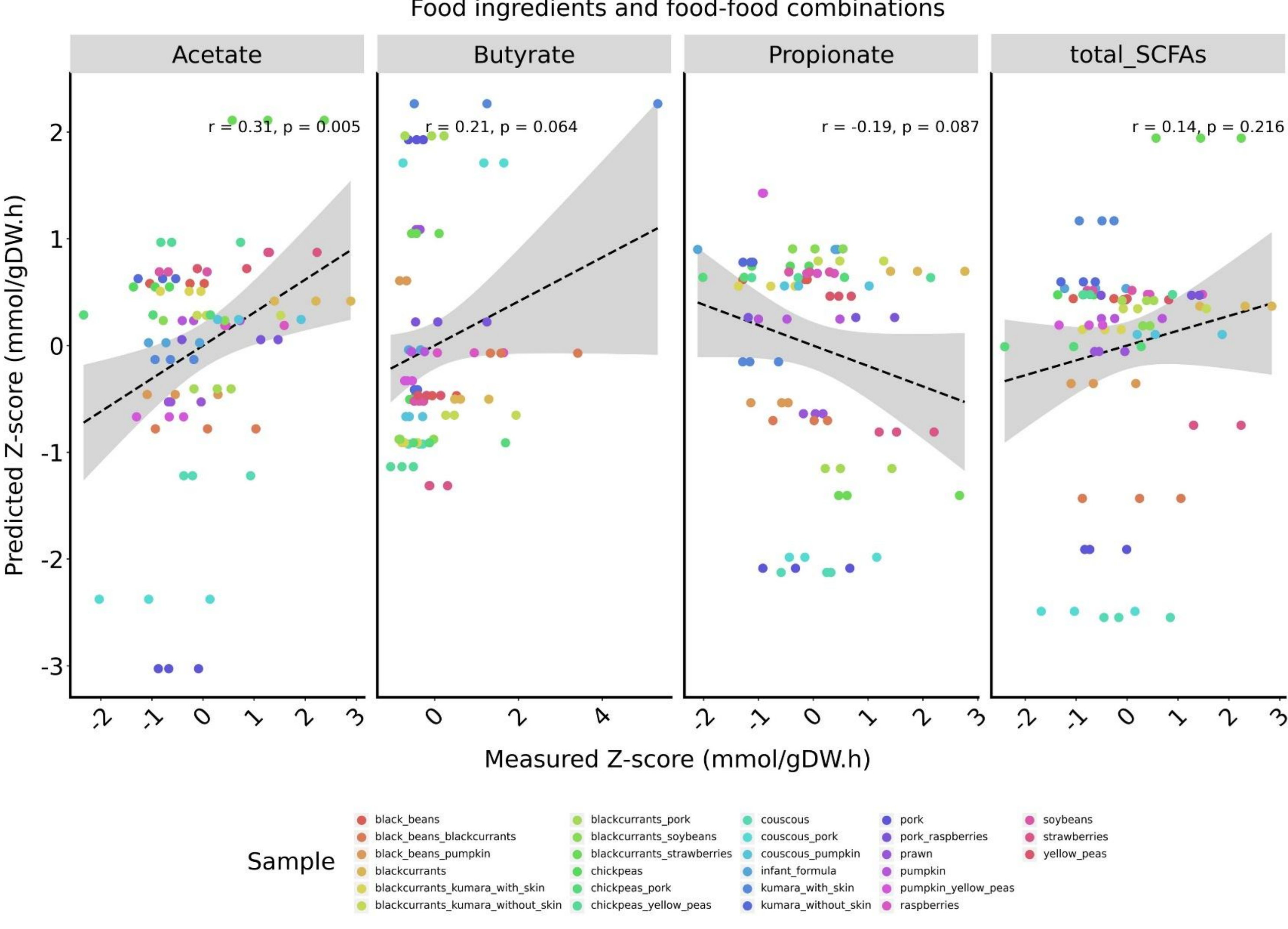

Figure 7.1. Pearson correlations between measured and predicted z-scores of major SCFAs for food ingredients and food-food combinations. SCFAs are displayed from left to right: acetate, butyrate, and propionate. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Pearson correlation coefficients (r) and two-tailed *p*-values are calculated for each plot individually. A regression line is shown in black, with the corresponding 95 % confidence interval in grey.

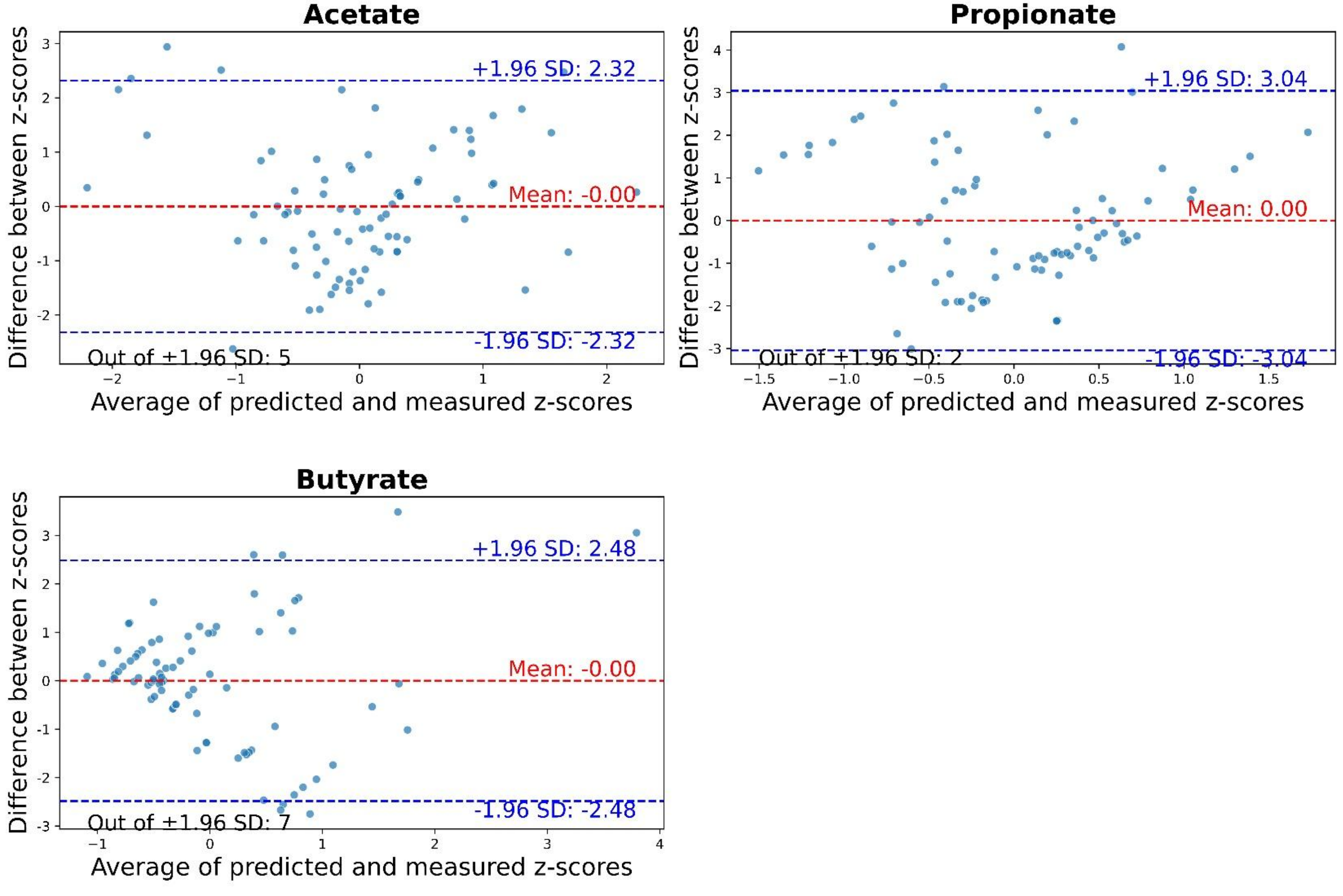

Figure 7.2. Bland-Altman plots comparing predicted and measured z-scores of major SCFAs for food ingredients and food-food combinations. The red line represents the mean difference between z-scores, while the blue lines indicate the upper and lower 95 % limits of agreement (mean difference ± 1.96 standard deviation). Each sample is depicted as a dot.

7.4.3. Comparison between predicted and measured z-scores

To better visualise the comparison between *in silico* and *in vitro* outcomes, a heatmap (Figure 7.3) and a radar chart (Supplementary Figure 7.4) were plotted. Only food ingredients and food-food combinations were included, as they demonstrated better agreement between predicted and measured values. Results showed several disagreements between *in silico* predictions and experimentally measured z-scores for these samples. For instance, black beans combined with blackcurrants had the greatest experimental production of butyrate among food-food combinations. In contrast, the model predicted the greatest butyrate production for pork combined with blackcurrants or couscous. For food ingredients, notable discrepancies were observed in the z-scores for chickpeas, couscous, soybeans, and kūmara samples, with only the butyrate prediction matching the experimental outcomes. Furthermore, the model did not accurately predict the ability of strawberries to increase propionate and total SCFA production *in vitro*. On the other hand, the model satisfactorily predicted the greatest production of butyrate for kūmara with skin among the food ingredients and the greatest production of acetate and total SCFAs for blackcurrants combined with strawberries among the food-food combinations. Additionally, the model captured the impact of blackcurrants and raspberries in increasing the total SCFA production compared to other foods.

7.4.4. Sensitivity analyses

To assess whether prediction quality depends on input microbial composition, additional simulations were conducted using post-fermentation relative abundances for each sample. Consistent with our initial results, the model exhibited poor overall agreement with experimental outcomes, with no significant correlations observed between predicted and measured z-scores for all samples. However, acetate production showed a moderate positive correlation ($r = 0.42$, $p = 0.008$) and total SCFA production trended positively ($r = 0.28$, $p = 0.08$) when analyses were restricted to food-formula combinations (Supplementary Figure 7.5). Similar to the initial results, food-formula combinations involving protein-rich foods, such as pork-formula and prawn-formula, showed the greatest discrepancies between predicted and measured outcomes (Supplementary Figure 7.6 and Supplementary Figure 7.7).

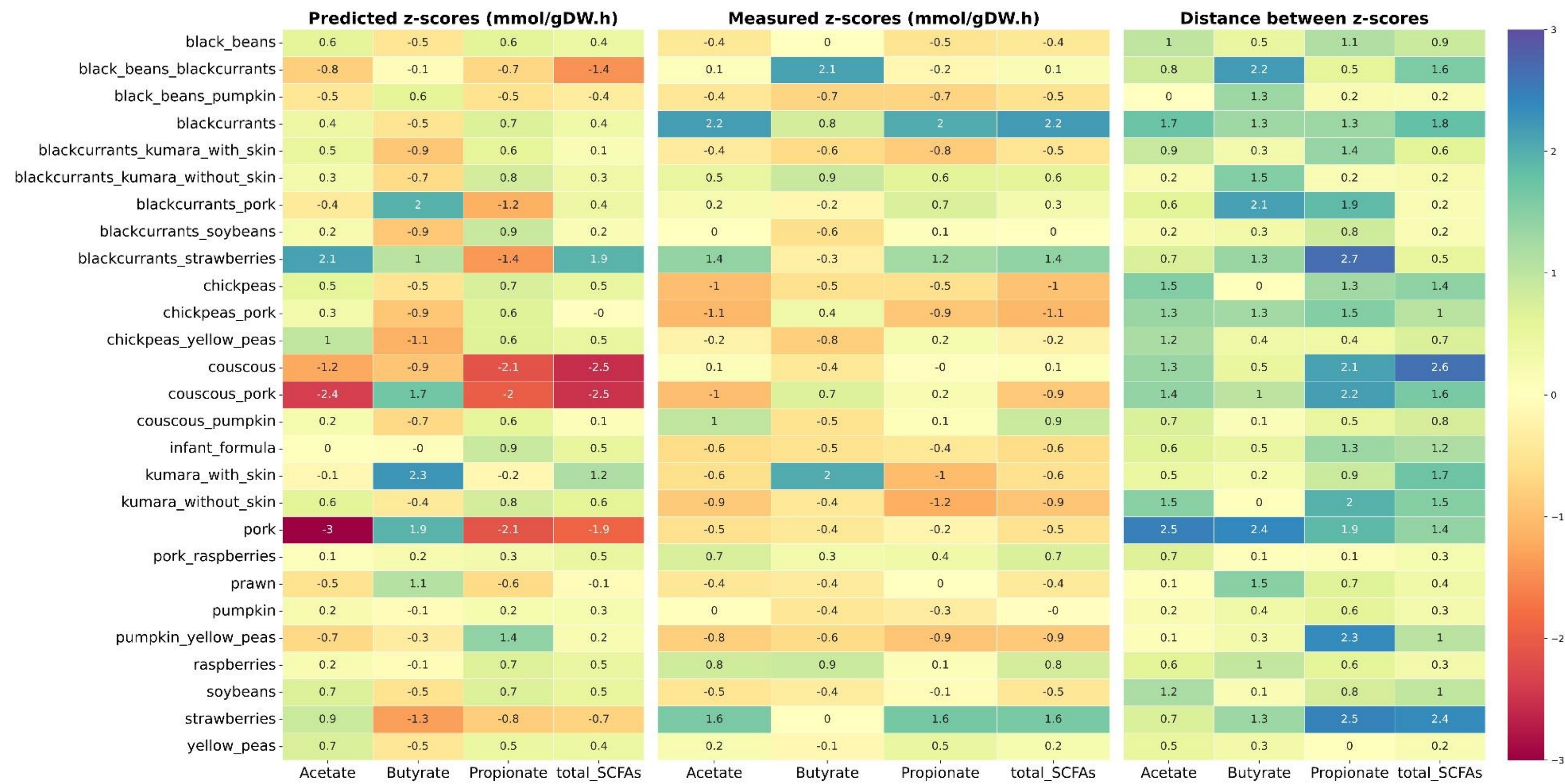

Figure 7.3. Heatmap of measured and predicted z-scores of major SCFAs for food ingredients and food-food combinations. *In silico* predicted z-scores are displayed on the left and *in vitro* measured z-scores on the middle. The absolute difference between *in silico* and *in vitro* z-scores is displayed on the right. Cells are coloured by intensity, with the lowest values in red and the highest values in blue.

7.5. Discussion

This chapter compared the predicted production of the major SCFAs (acetate, propionate, and butyrate) *in silico* using MICOM (31) with experimental values obtained from the faecal fermentations described in Chapter 6 (118). Our focus on assessing the model's accuracy in predicting the production of health-relevant metabolites, rather than microbial growth, was driven by the lack of available data on *in vitro* individual microbial growth rates. Additionally, from a health perspective, microbial function is more informative than composition, as colonic microbes are functionally redundant, and the colonic microbiota of healthy individuals maintains similar functionality despite taxonomic differences (4,37). The workflows for *in silico* simulations and statistical comparison between *in silico* and *in vitro* outcomes followed published protocols (117,280). Another strength was the assessment of the model's accuracy in predicting SCFA production by the colonic microbiota of weaning infants using faecal samples under realistic infant feeding patterns, including whole foods, foods combined with other foods, infant formula, or a combination of both.

When evaluating all samples, the model demonstrated poor accuracy, with only acetate showing a weak correlation between predicted and measured outcomes. Interestingly, we observed increased accuracy when analysing samples predominantly composed of plant-based foods. Acetate exhibited a moderate positive correlation, while propionate and butyrate showed a trend towards weak positive and weak negative correlations, respectively. These findings suggest that MICOM's accuracy in predicting the function of the infant microbiota increases for media enriched in complex carbohydrates. This result is expected, considering that the influence of complex carbohydrates on colonic microbes is far better understood than that of other dietary compounds. Consequently, metabolic reconstructions of colonic microbes are often not curated for biochemical reactions involving amino acid and lipid utilisation due to limited available data (278). Additionally, this result is likely driven by the functional capacity of the colonic microbiota of weaning infants. Observational studies have demonstrated that the weaning infant microbiota transitions from primarily degrading human milk oligosaccharides to metabolising complex carbohydrates, while amino acid fermentation increases but remains less prominent compared to that of the adult colonic microbiota (56,61).

Given that correlation analyses assess the strength of the relationship between two variables but do not indicate how these variables differ from each other, a Bland-Altman analysis was conducted to determine the limits of agreement (95 % confidence interval) between predicted and measured SCFA

production. Standard scores were used to account for differences in magnitude and unit between methods (476). Acetate had lower limits of agreement than propionate and butyrate, suggesting stronger concordance between predicted and measured z-scores, which aligns with the correlation results. Overall, most food and food-food combination samples fell within the 95 % limits of agreement. However, some exceptions highlighted the model's limited ability to accurately predict SCFA experimental production across the entire dataset.

Among food ingredients and food-food combinations, disagreements were observed between *in vitro* and *in silico* outcomes. For example, strawberries and the combination black beans-blackcurrants had positive z-scores for total SCFA production *in vitro* but negative z-scores *in silico*. On the other hand, some samples that drove the greatest total SCFA production *in vitro*, such as blackcurrants, raspberries, and the blackcurrants-strawberries combination, also resulted in high positive z-scores *in silico*. Sensitivity analyses assessing the impact of microbial composition on prediction accuracy indicated that community composition strongly influences simulation outcomes. However, the model consistently showed limited predictive performance across SCFAs and samples, with acetate showing better agreement in carbohydrate-rich samples. As a take-home message, this chapter demonstrates that emerging modelling approaches for diet-colonic microbiota interactions are imperfect and should not replace experimental methods. Instead, given their cost- and time-efficiency, and ability to leverage existing data, these models offer a valuable starting point for generating insights that can guide the design of *in vitro* and *in vivo* studies. Their further refinement and use as complementary tools represent a promising opportunity to advance diet-colonic microbiota research.

Future directions for the development of MGCMs include expanding the number of high-quality microbial metabolic reconstructions, which should be built using sequencing data that meet quality standards and subsequently curated with experimental data (303,477,478). Shotgun metagenomic sequencing data is preferable to 16S rRNA sequencing data, as it more accurately captures the metabolic potential of colonic microbes (479). Importantly, experimental data remain crucial for model curation and validation (480), with a notable need for more research into the behaviour of colonic microorganisms in response to various dietary compounds. The accuracy of MGCMs could also benefit from incorporating omics data, such as metatranscriptomics, to better personalise the model's conditions (481). Additionally, integrating dynamic FBA could improve the representation of changes in the colonic

environment over time (315,465). Finally, databases used for designing media in *in silico* simulations require further refinement to better account for the heterogeneity of dietary patterns across individuals.

Our results partially contrast with a previous study that reported agreement between MICOM's prediction for propionate and butyrate and experimental outcomes from *ex vivo* faecal incubations with isolated dietary fibres using adult inoculum (280). The modest performance observed here is likely due to the different study designs. For instance, the colonic microbiota of weaning infants has distinct functionality compared to the adult colonic microbiota, characterised by a higher proportional production of acetate and lower production of propionate and butyrate (339,482). Furthermore, assessing the effects of individual fibres on SCFA production is not as informative as using whole foods. Foods are complex matrices containing other dietary compounds, such as protein, fat, and phytochemicals, all of which impact the function of colonic microbes (112,123,297). Finally, the study mentioned above used metagenomic data to build models at the species level and normalised SCFA predictions based on microbial biomass, using metagenomic reads mapping to the human genome as a proxy (280). In contrast, our simulations were limited to the genus rank and did not incorporate biomass normalisation due to using 16S rRNA sequencing data, thereby reducing metabolic specificity. These contrasting results highlight the need for further investigations of the model accuracy across different study conditions and host populations.

A major limitation of our study is the difference in outcomes obtained from distinct methods, as fluxes predicted *in silico* are not equivalent to the concentration of metabolites measured *in vitro*. To address this limitation, we followed a published protocol that used standard scores to compare results from different methodologies and validate MICOM's predictions of propionate and butyrate production (280). However, estimating SCFA fluxes using an artificial system like static *in vitro* fermentation is only an approximation of dynamic conditions, as metabolites accumulate and substrates are depleted (455). Over long fermentation periods, these conditions diverge from the steady state assumption inherent in FBA-based models, which assumes no accumulation of substrates in the system (276).

Additionally, the accuracy of models based on FBA strongly depends on the quality of the metabolic reconstructions used in the simulations. A recent systematic evaluation of FBA-based tools, including MICOM, reported low prediction accuracy of microbial growth rates compared to experimental data when using the AGORA metabolic reconstructions (281). The authors highlighted that semi-curated metabolic reconstructions were not sufficiently accurate for predicting the behaviour of microorganisms

(281). Our simulations were performed using the second version of AGORA, which was generated via a semi-automated pipeline and manually refined (279). However, it is important to recognise that these reconstructions may contain gaps or inaccurately assigned biochemical reactions (278).

Another limitation was the use of pooled faecal data, justified by the absence of individual-level data in the faecal fermentations. Although pooling faecal samples is a common practice in faecal fermentation studies, it inevitably alters the original microbial community structure, reducing inter-individual variability and functional resolution. These changes are likely to influence microbial functional dynamics in ways that are not captured by the taxonomic profile of the pooled sample (483,484). Using amplicon 16S sequencing data limited model construction to the genus level, potentially masking metabolic differences between microbial species (280,485). Additionally, this approach reduced the number of taxa included in the simulations and hindered the normalisation of SCFA predictions by bacterial biomass. Notably, bacterial species that are present in low abundance yet biologically meaningful (keystone species) may be underrepresented when using 16S rRNA sequencing data (486,487). Moreover, the simulations used microbial relative abundance as a proxy for absolute quantities, which are inherently interdependent and cannot determine whether the abundance of a specific taxon has truly increased or decreased, or whether an observed relative change simply reflects shifts in the growth of other taxa (488).

Furthermore, the *in silico* media were designed using the VMH database (291), which lacks information on diverse cooking methods and food ingredients. Notably, indigenous foods available in New Zealand and used in the experimental work (118), such as kūmara (sweet potato variety), were not included in the database and had to be substituted with the most similar available food. Finally, while the *in vitro* study used a static protocol to mimic the digestion and absorption of dietary compounds (118), MICOM, like other diet-microbiota GEM-based models, currently does not account for digestion. Instead, it represents absorption by diluting the dietary compounds identified to be absorbed by the human intestine using a scalar (31). Not accounting for digestion is a potential major source of variation between *in silico* and *in vitro* outcomes, as digestion influences food structure and, consequently, the nutrient profile accessible for microbial fermentation (489). However, this limitation is intrinsic to the modelling framework, as computationally simulating food digestion is challenging. Although promising tools are emerging (490), they have not yet been integrated into MGCMs.

7.6. Conclusions

This chapter evaluated the accuracy of the metagenome-scale community metabolic model MICOM in predicting SCFA production by the colonic microbiota of weaning infants under realistic complementary infant feeding patterns, using static *in vitro* faecal fermentation data as a comparator. A weak positive correlation was observed between predicted and measured acetate production. The agreement between predicted and measured SCFA fluxes improved when analysing samples predominantly composed of plant-based foods. These findings suggest that the model more accurately replicates experimental results when simulating media rich in complex carbohydrates. Despite disagreements between experimental and simulated outcomes for specific SCFAs, the model identified samples with the highest total SCFA production *in vitro*. This exemplifies the model's limitations as a replacement for traditional experimental methods but supports its potential as a complementary tool. Further model development is essential to improve its accuracy, particularly for media-rich in fat and protein. Refined versions of the model would contribute to advancing research on the relationship between diet and colonic microbiota.

Chapter 8: General discussion

8.1. Summary of thesis findings

The interaction between dietary compounds and the colonic microbiota strongly impacts host physiology, influencing both disease risk and the promotion of health and well-being (45,135,374). This thesis aimed to identify foods that support the composition and function of the infant colonic microbiota. To achieve this, a novel approach combining *in silico* and *in vitro* methodologies was employed. This strategy enabled a time- and resource-efficient evaluation of the effects of a wide variety of complementary foods and food combinations on the colonic microbiota of New Zealand infants transitioning from breastmilk to solid foods, using faecal samples as a proxy. Foods were identified that positively impacted the infant colonic microbiota by increasing the abundance of saccharolytic bacterial taxa and SCFA production, including blackcurrants, raspberries, strawberries, and black beans. These findings support the introduction of these foods during weaning to support colonic microbiota development. Importantly, they are promising candidates for further clinical trials in weaning infants, which could assess potential health outcomes driven by microbiota-diet interactions. Ultimately, the knowledge generated from this thesis (Figure 8.1) can help design future research on interactions between dietary compounds and the colonic microbiota, contributing to improved dietary recommendations and/or complementary food formulations for infants.

Chapter 2 presented a narrative review of current literature on how dietary patterns influence the colonic microbiota across the human lifespan. By prioritising evidence from intervention trials, systematic reviews, and meta-analyses, the review aimed to provide a state-of-the-art synthesis of optimal dietary choices to support colonic microbes in different life stages: early infancy, post-weaning infancy, adulthood, older adulthood, and centenarian age. Dietary patterns characterised by high food diversity, predominantly plant-based, and including daily consumption of fermented foods (e.g., dairy products), and moderate intake of lean meats (two to four times a week) were associated with greater microbial diversity, increased abundance of saccharolytic bacterial taxa, and enhanced production of SCFAs. Additionally, these dietary patterns were linked to a reduced risk of developing non-communicable diseases, including obesity, type 2 diabetes, and cardiovascular disorders.

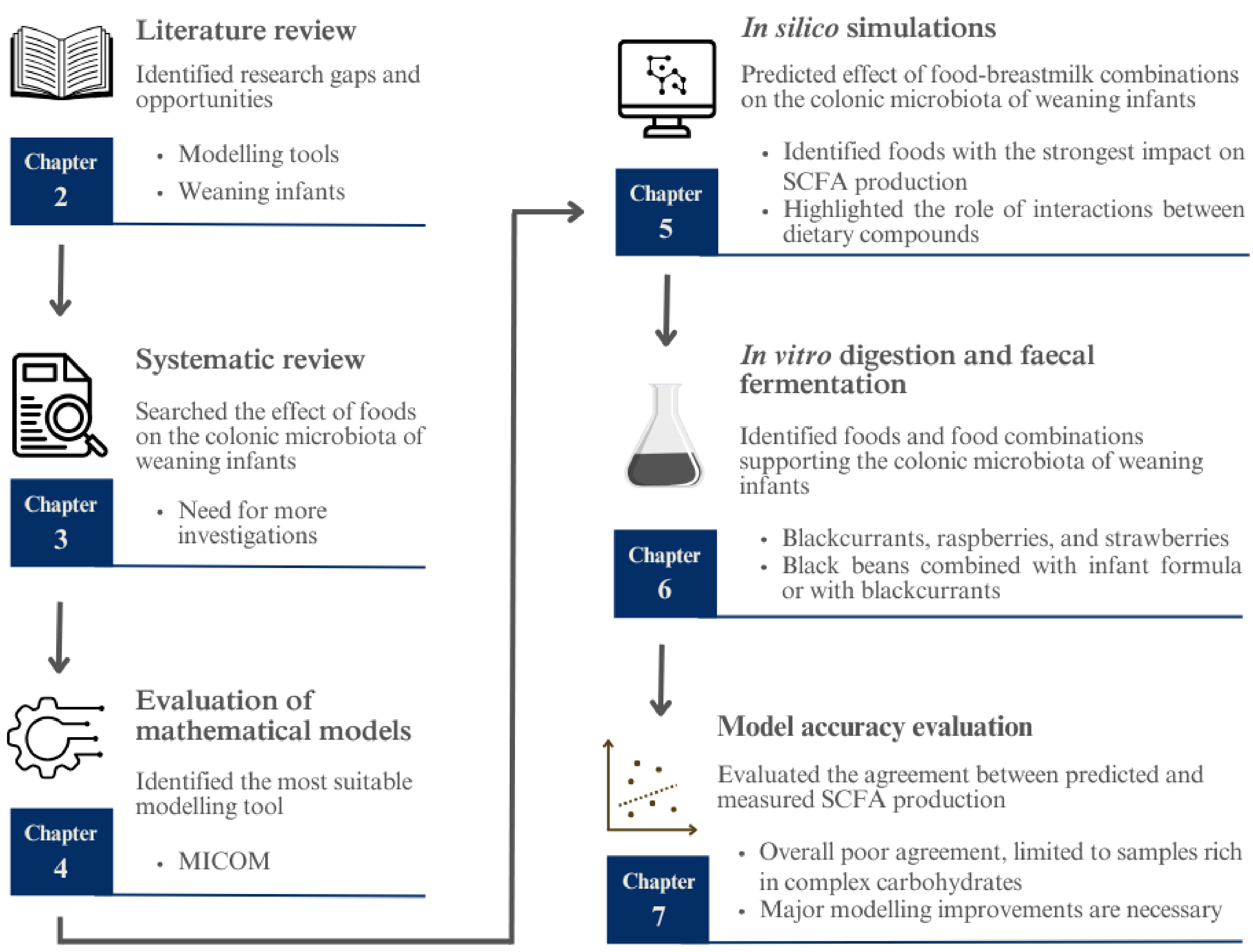

Figure 8.1. Summary of the main thesis findings.

Chapter 2 also identified knowledge gaps and research opportunities in the study of interactions between dietary compounds and the colonic microbiota. These insights set the foundations for the subsequent experimental chapters of this thesis. For instance, modelling strategies predicting how dietary compounds impact gut microbial communities showed promise as complementary tools to address some of the technical and ethical limitations of traditional *in vitro* and *in vivo* methods. Moreover, advancing our understanding of therapies that target the colonic microbiota through diet requires further research into how dietary choices influence colonic microbes in population groups typically underrepresented in microbiota research, such as weaning infants. The weaning period is a critical window for the maturation of the colonic microbiota, during which microbial communities established can persist over time and potentially influence disease susceptibility later in life (26–29). Therefore, weaning represents a valuable opportunity to modulate the colonic microbiota through diet, fostering beneficial host-microbe interactions that may support long-term health and well-being.

To further explore this often-overlooked topic in diet-colonic microbiota research, Chapter 3 presented a systematic review of intervention trials assessing the effects of complementary foods on the composition and function of the infant colonic microbiota. The systematic review aimed to identify dietary choices that support the development of the infant colonic microbiota. It included seven food intervention studies conducted in healthy infants aged 4 to 12 months, encompassing a total of 983 participants. Findings indicated that increases in the abundance of microbes metabolising complex carbohydrates and amino acids in the faeces of infants were more likely attributable to age rather than the consumption of a specific complementary food. Among the evaluated foods, wholegrain cereal and pureed beef were associated with increased faecal abundance of bacterial taxa producing SCFAs. The conclusions of the systematic review were limited by the small number of included studies and their varied methodologies and reported outcomes, highlighting the need for more investigations on how complementary foods affect the colonic microbiota during weaning.

The limited clinical evidence motivated the use of modelling methodologies in this thesis to rapidly and inexpensively generate preliminary insights into the influence of dietary choices on the colonic microbiota of weaning infants. Chapter 4 described a qualitative assessment of computational tools designed to simulate the effects of dietary compounds on gut microbial communities, intending to identify the most suitable model for predicting the impact of foods on the human colonic microbiota. Five tools were evaluated, encompassing two main modelling strategies: ODE-based models and GEM-based models, the latter including MGCMs. ODE-based models offered easier customisation, lower data

requirements, and rapid runtimes, but their reductionistic design oversimplified the role of individual nutrients and failed to capture the diversity of the human colonic microbiota. In contrast, MGCMs were better suited to generating personalised representations of the colonic microbiota and offered deeper dietary resolution. Among the evaluated tools, the MICOM model was selected as the most promising candidate due to its integrated workflows for data integration, simulation, and result visualisation. Given that experimental validation of the model is still in progress, this offered a timely opportunity for this thesis to assess MICOM's accuracy under realistic infant feeding scenarios, thereby contributing to addressing an important knowledge gap in gut microbiota modelling tools.

In Chapter 5, MICOM was employed to screen *in silico* the effects of 89 food ingredients, commonly introduced to infants in New Zealand and globally, on SCFA and BCFA production by the colonic microbiota of weaning infants, using data from faecal samples from New Zealand infants as a proxy. To mimic realistic weaning dietary patterns, food ingredients were combined with breastmilk. The twelve food-breastmilk combinations with the strongest influence on the *in silico* microbial organic acid production were identified. These included fibre- and polyphenol-rich foods like raspberries and blackcurrants. These food ingredients were further combined with other foods and breastmilk to assess how combining multiple ingredients affected predicted colonic microbial function. Findings revealed that food combinations could alter the individual effects of food ingredients on SCFA and BCFA production, suggesting that the interaction between dietary compounds is a key factor influencing the metabolism of colonic microbes. Consequently, the effect of combining different food ingredients was incorporated into the experimental work of this thesis.

Subsequently, Chapter 6 examined the impact of the previously identified foods and food combinations on the *in vitro* microbial composition and organic acid production by the colonic microbiota of New Zealand weaning infants, using faecal samples as a proxy. These foods and food combinations were evaluated individually or in combination with infant formula, which replaced breastmilk due to practical and ethical considerations. Samples were digested using a static INFOGEST protocol adapted to mimic the gastrointestinal conditions of weaning infants (393). Following digestion and dialysis, samples were fermented for 24 hours using a pooled faecal inoculum from six healthy New Zealand infants aged five to eleven months. Microbial composition post-fermentation was characterised by 16S rRNA amplicon sequencing, while microbial function was assessed by quantifying organic acids via gas chromatography.

Foods and food combinations that increased the *in vitro* SCFA production and the relative abundance of saccharolytic bacterial taxa were identified. Among individual food ingredients, blackcurrants, strawberries, and raspberries enhanced acetate and propionate production. Supporting these findings, these berries promoted the growth of SCFA-producing bacterial genera: blackcurrants increased the abundance of *Parabacteroides*, while raspberries enriched both *Parabacteroides* and *Eubacterium*. Regarding food combinations, black beans increased butyrate production and the relative abundance of the group *Clostridium sensu stricto 1* when combined with either infant formula or blackcurrants. These foods are promising candidates for future clinical trials involving weaning infants.

Finally, Chapter 7 compared *in silico* predictions with *in vitro* outcomes to evaluate the accuracy of the mathematical modelling strategy in predicting SCFA production by the colonic microbiota of New Zealand weaning infants under realistic feeding patterns. Pearson correlation analyses were used to determine the strength of the relationship between predicted and measured outcomes. Overall, the model exhibited limited predictive accuracy, with only a weak positive correlation observed for acetate production across all samples. However, the agreement between predicted and measured SCFA production increased when focusing on samples predominantly composed of plant foods: acetate exhibited a moderate positive correlation and butyrate a trend towards weak positive correlation. This suggests that the model is more effective at predicting the effect of media rich in complex carbohydrates on the function of the infant colonic microbiota, compared to media rich in other dietary compounds, such as protein or fat.

Importantly, our ability to compare *in silico* and *in vitro* outcomes was limited by their different study designs: while the *in silico* model assumes a steady state, representing a system in which conditions remain constant over time, the static *in vitro* faecal fermentation reflects dynamic conditions, where substrates are gradually depleted and metabolites accumulate. To improve model accuracy, major improvements are needed in both the modelling framework and simulation inputs, particularly regarding the quality of the microbial metabolic reconstructions and the availability of data in food databases. Despite its intrinsic limitations, the model effectively identified *in silico* candidates, such as berries, that supported the colonic microbiota of weaning infants *in vitro*. In conclusion, when combined with traditional *in vitro* experiments, the MICOM model demonstrated potential as a complementary tool for accelerating future research on the interaction between dietary compounds and the colonic microbiota.

8.2. Thesis strengths and limitations

An original aspect of this thesis was the use of mathematical models to predict the impact of complementary foods on the colonic microbiota of weaning infants. This was particularly timely, given the recent rise in *in silico* studies simulating dietary effects on the adult colonic microbiota, while the weaning period has received little attention (204,282,283). Another novelty was the evaluation of common foods combined with breastmilk in the *in silico* simulations and with infant formula in the *in vitro* experiments, as opposed to evaluating foods singly. This design more accurately reflected feeding patterns during weaning, when breastmilk or infant formula remains a significant part of the infant's diet (85,335). Additionally, food ingredients were combined with other foods to account for interactions between dietary compounds, an aspect often overlooked in investigations assessing the influence of dietary choices on the infant colonic microbiota. As highlighted in a literature review, such interactions can influence host digestion and, consequently, the availability of nutrients for colonic microbial fermentation (412).

A major strength of this research was that *in silico* predictions guided the design of *in vitro* experiments. This approach reduced the cost and time required for experimental work, allowing the assessment of the effects of a wide range of foods and food combinations on the infant microbiota within the timeframe and budget of a PhD project. Over 150 samples were analysed *in silico*, and more than 40 were evaluated *in vitro*. Moreover, comparing *in silico* and *in vitro* outcomes enabled an evaluation of the modelling strategy's accuracy under realistic infant feeding patterns, contributing to addressing a knowledge gap in diet-gut microbiota modelling approaches. Another strength was the use of standardised protocols and the latest methods to evaluate interactions between dietary compounds and colonic microbes. Notably, *in silico* simulations were conducted using a reference modelling tool along with an expanded repository of semi-curated metabolic reconstructions of colonic microbes (31,279). The *in vitro* digestion and faecal fermentation experiments followed the reference INFOGEST protocol, which was adapted to mimic the gastrointestinal conditions of weaning infants (265,393). Bioinformatics and data analysis adhered to current best practices, using relevant pipelines and updated versions of reference databases (223,327,407).

On the other hand, this research has several limitations. *In silico* simulations were performed using food compositional data from the VMH database, which lacks diverse preparation methods and food ingredients typically consumed in New Zealand, such as indigenous foods (291). Additionally, using

microbial relative abundance data obtained from 16S rRNA sequencing for building *in silico* models of the infant colonic microbiota limited their resolution to the genus level, which is less accurate than models built at the species level (280). Moreover, the simulations relied on relative abundances as a proxy for absolute quantities. Relative abundances are interdependent by nature, and without data on absolute microbial quantities, changes in their values cannot capture whether the absolute abundance of a specific taxon has increased or decreased, or whether an observed relative change simply reflects shifts in the growth of other taxa (488). Static protocols were used for *in vitro* digestion and faecal fermentation. While these methods enabled the simultaneous evaluation of multiple samples, suiting the screening scope of this project well, they do not accurately reflect the dynamic conditions of the infant GIT (265,393). Furthermore, metabolites accumulate and substrates are depleted over time in static fermentation systems (455). This diverges from the steady state assumption inherent to the *in silico* modelling approach, thereby limiting the comparability between *in silico* and *in vitro* outcomes (276).

Additionally, faecal samples were used as a proxy for the colonic microbiota due to the ease and non-invasive nature of collection. Nonetheless, faeces mainly represent microbial communities from the distal colon, providing limited representation of the microbes attached to the colonic mucosa or residing in other parts of the colon (200). Faecal samples were collected from six healthy New Zealand weaning infants aged 5-11 months, offering an adequate coverage of the weaning period. Nevertheless, the small sample size limited the generalisability of our findings to the broader population of New Zealand weaning infants. The pooling of samples prevented insights into specific weaning stages, and the focus on this group further restricted the generalisation of results beyond the studied population. It is important to acknowledge that, despite efforts in minimising intraindividual and interindividual variations, by collecting multiple samples from the same individual and pooling them to create a representative inoculum, the colonic microbiota remains highly variable (21,22). Consequently, colonic microbes from different individuals may respond differently to the foods identified in this thesis. Finally, while this thesis identified promising foods for supporting the infant colonic microbiota and potentially contributing to health and well-being, it did not evaluate host health outcomes related to microbiota modifications. Such evaluations would require clinical trials.

8.3. Future perspectives

This thesis provided a comprehensive *in silico* and *in vitro* characterisation of the effects of common complementary foods on the colonic microbiota of weaning infants. Foods identified as supportive of

infant colonic microbiota development, such as kūmara, blackcurrants, and black beans, are promising candidates for future infant intervention trials. To date, few clinical studies have assessed how complementary foods influence the colonic microbiota during weaning. Conducting such clinical trials would deepen our understanding of the role these foods play in shaping the development of the infant microbiota, offering insights into potential health outcomes (e.g., growth indicators like weight and length-for-age z-scores, diarrhoea frequency, or biomarkers of inflammation like faecal calprotectin). Ultimately, the evidence generated could help address an important knowledge gap in infant nutrition and inform improved dietary recommendations and/or the development of food formulations for early life.

Although this thesis used pooled faeces from New Zealand infants for both *in silico* simulations and *in vitro* fermentations, the combined *in silico* and *in vitro* approach outlined here is adaptable and has potential for advancing personalised nutrition. For instance, it can be employed to assess individual-specific responses in the colonic microbiota. Additionally, this combined approach could be applied to populations traditionally underrepresented in microbiota research. One of these groups is older adults, in whom studying the relationship between dietary choices and the colonic microbiota represents an opportunity to contribute to healthy ageing (187). Another promising direction is studying dietary strategies to modulate the colonic microbiota as therapeutic interventions for individuals with gastrointestinal disorders [see review (491)].

Future research using *in silico* and *in vitro* combined methods can be improved by incorporating dynamic models of food digestion and faecal fermentation, which more accurately reflect the conditions of the human GIT (492). The use of dynamic *in vitro* systems also allows for the experimental measurement of microbial metabolite fluxes and growth rates, enabling more effective comparisons between *in vitro* and *in silico* outcomes. Sequencing methods with higher resolution, such as shotgun metagenomics, are preferred to 16S rRNA amplicon sequencing as they provide a more accurate representation of the metabolic potential of colonic microbes and enable the construction of *in silico* models at the species level (280,479). Additionally, there is potential to refine colonic microbiota computational modelling tools by integrating simulations of host-microbe interactions and food digestion (490,493,494). Finally, these tools could be further improved by integrating multi-omics data and by incorporating dynamic FBA to represent temporal changes in the colonic environment (465,481).

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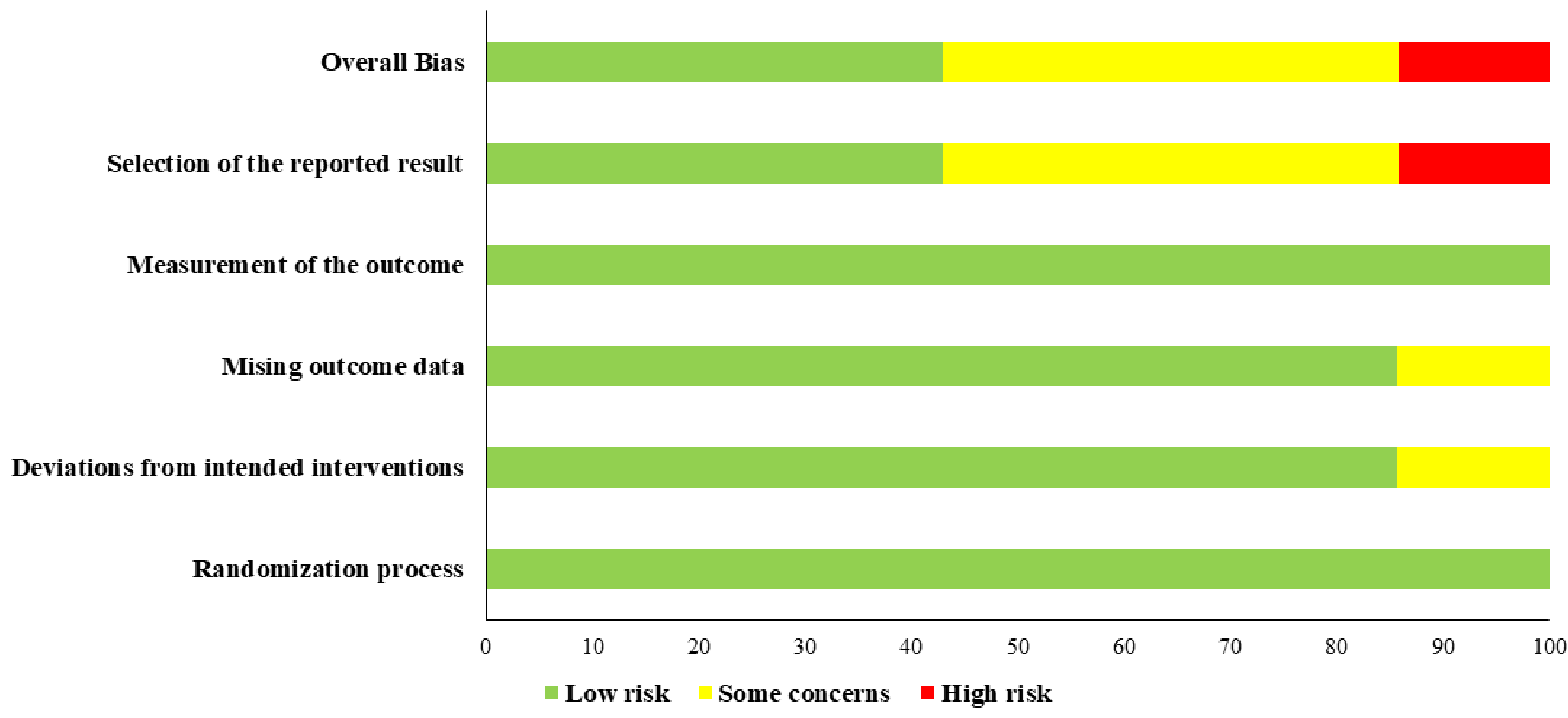
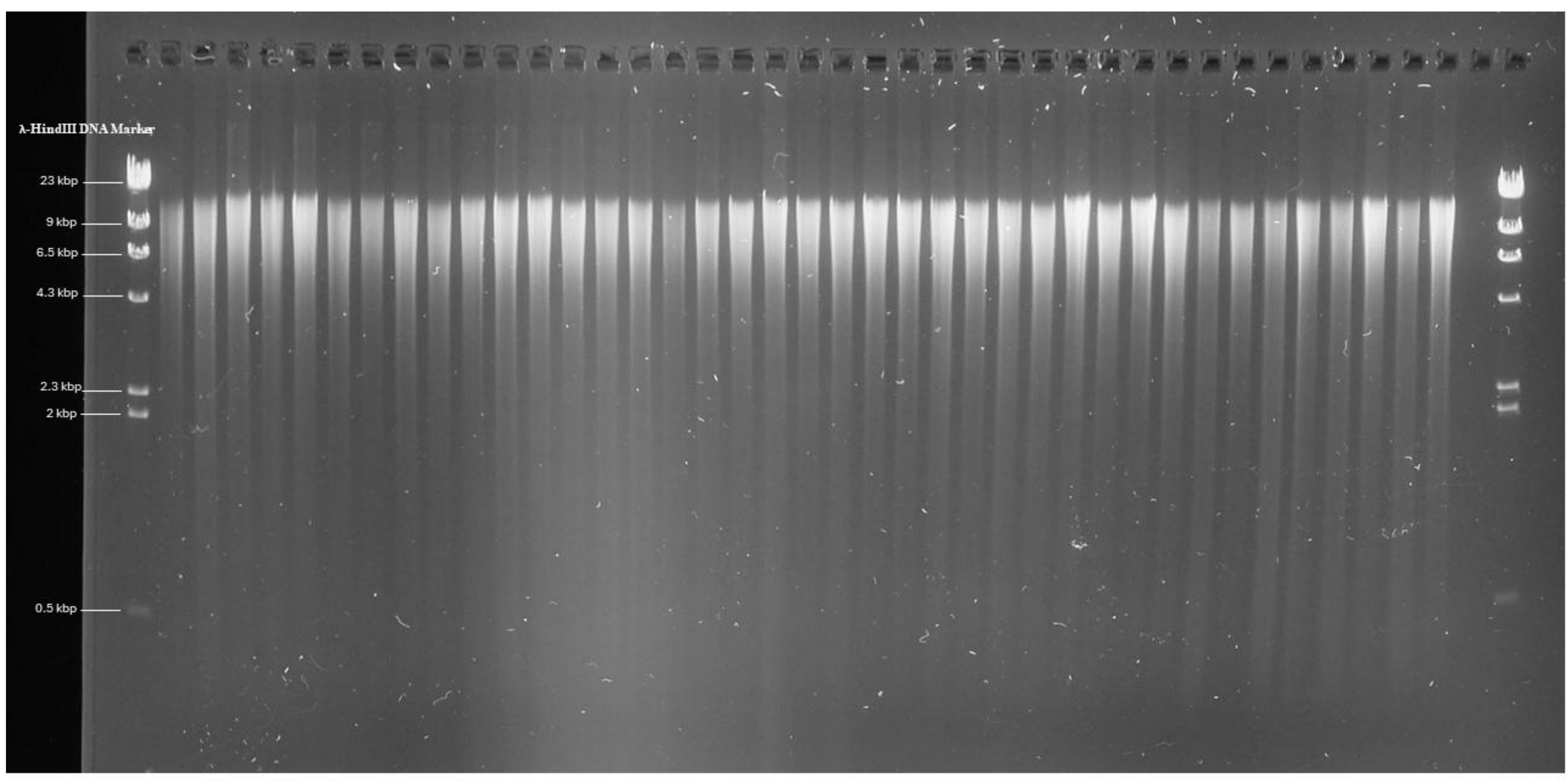
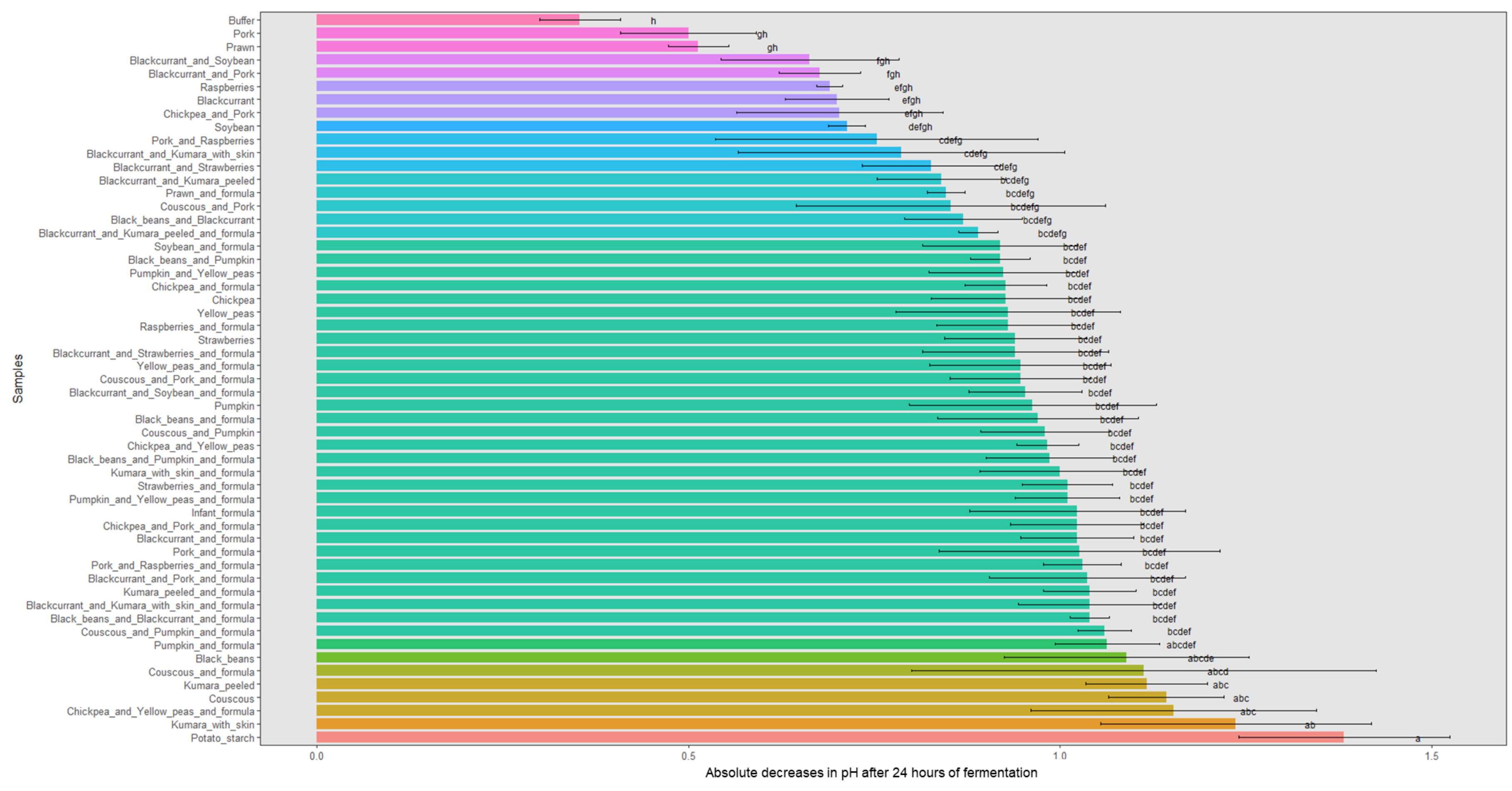
Appendix 1: Supplementary Figures



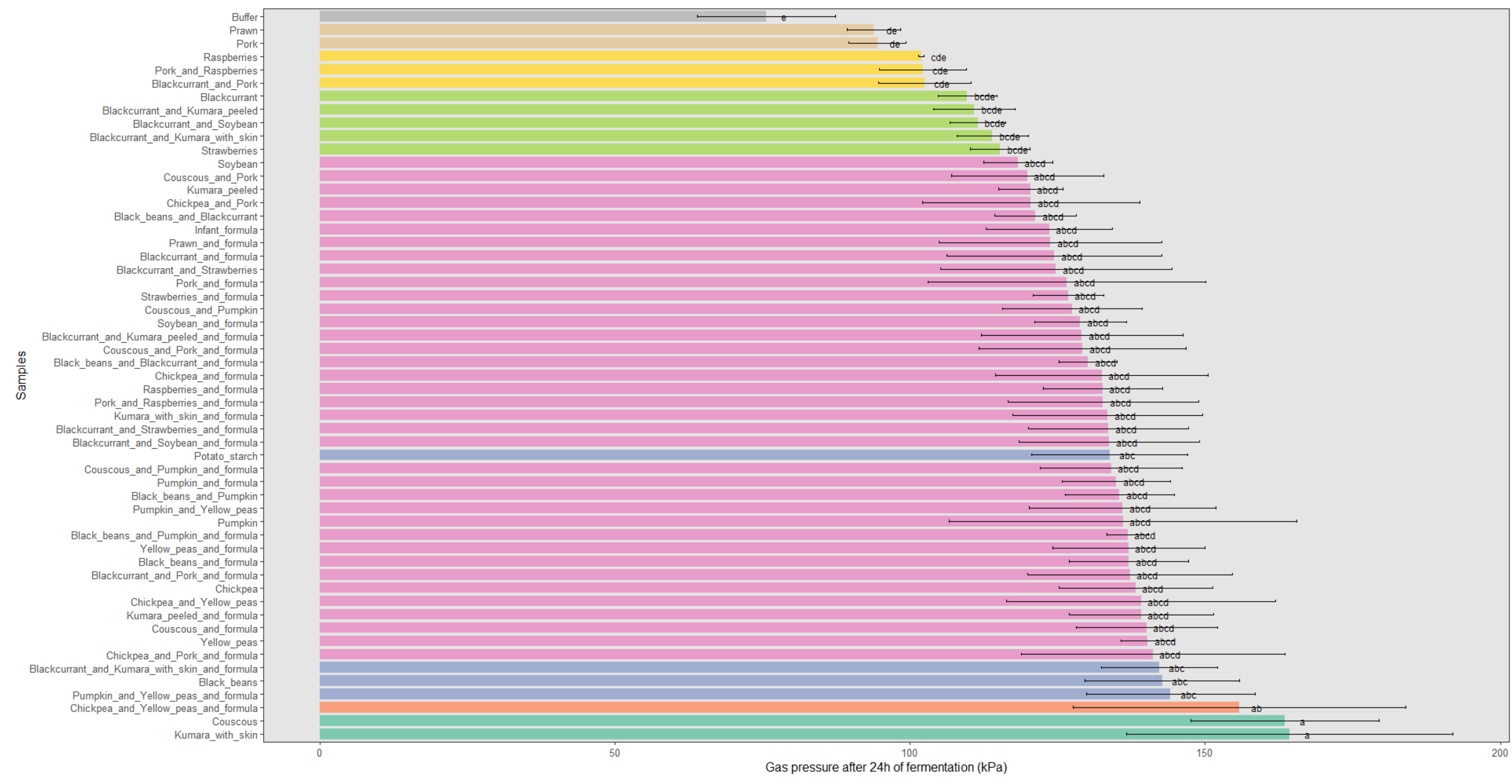
Supplementary Figure 3.1. Percentage of scores for risk of bias.



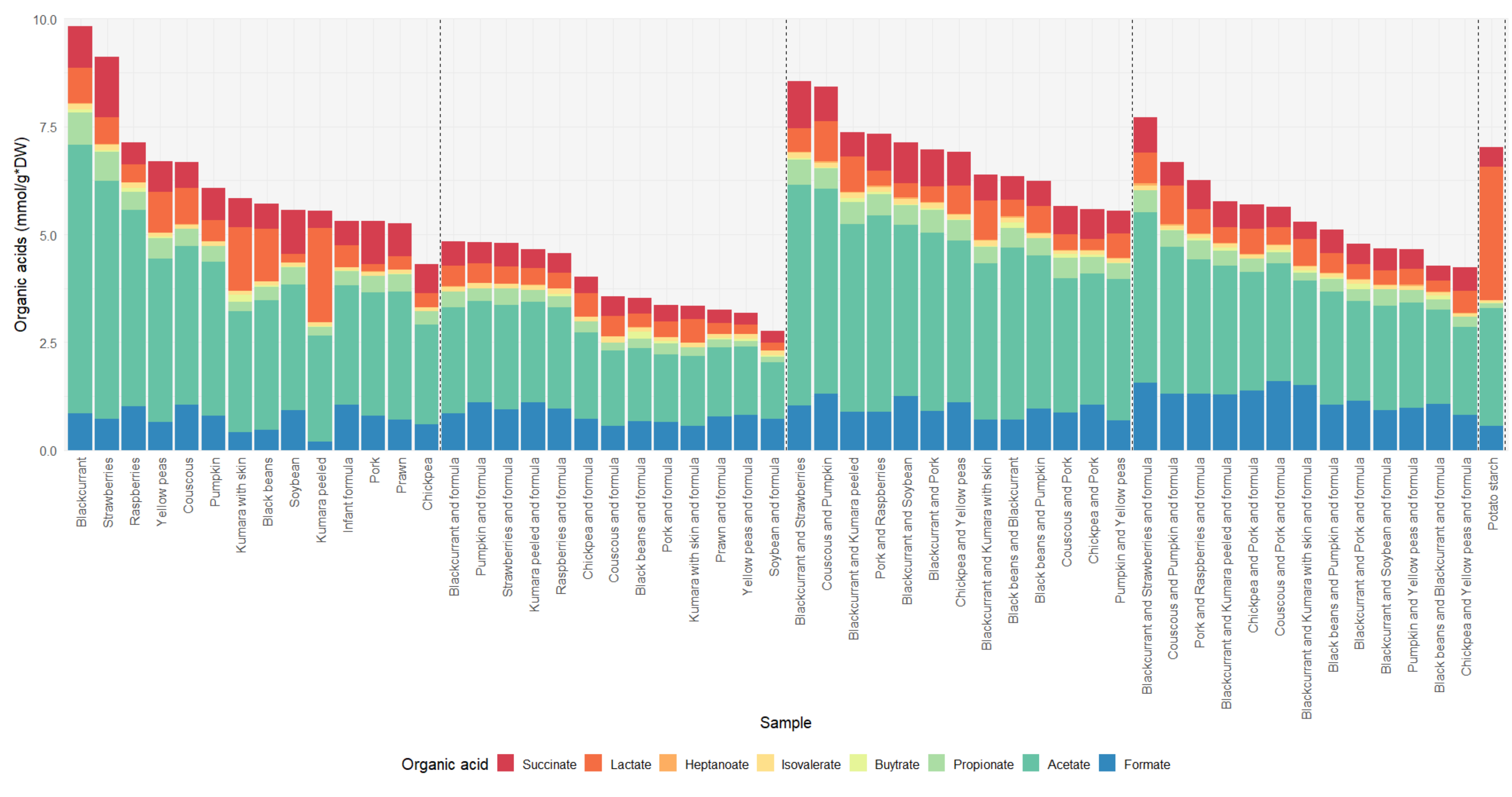
Supplementary Figure 6.1. DNA electrophoresis in agarose gel. Gels were loaded with 2 µL of extracted DNA and 5 µL of the lambda-HindIII DNA marker. The figure displays the electrophoresis results for the first 39 extracted DNA samples. The remaining samples were processed under identical conditions in subsequent runs.



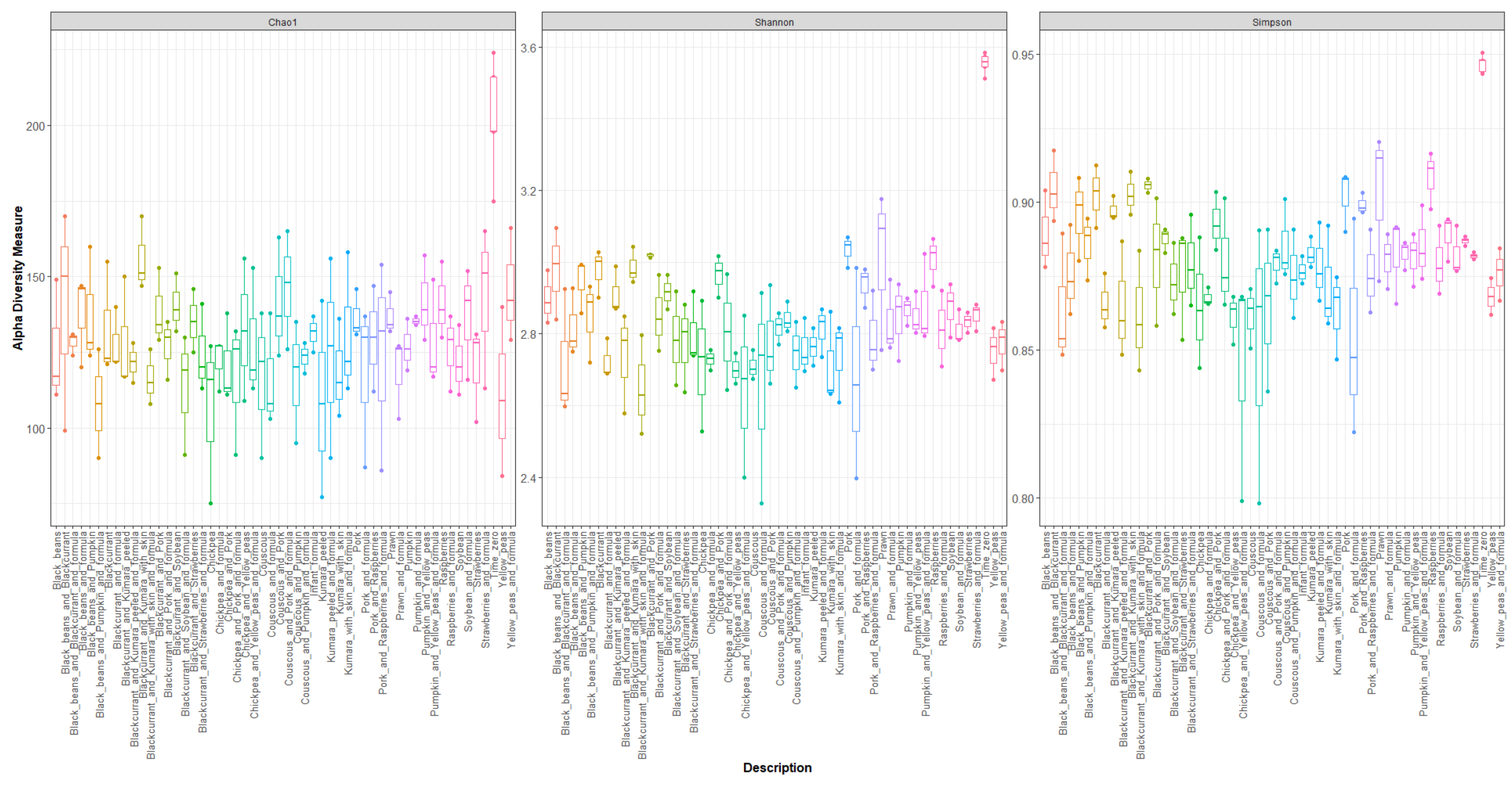
Supplementary Figure 6.2. Absolute decreases in pH after 24 hours of fermentation. Changes in pH are expressed as the difference between after 24 hours of fermentation and fermentation time zero. Bars are coloured according to intensity and statistical significance, with higher values represented in red and lower values in blue. Samples with the same colour and same letters belong to the same group according to the Tukey HSD test with a 95 % confidence interval.



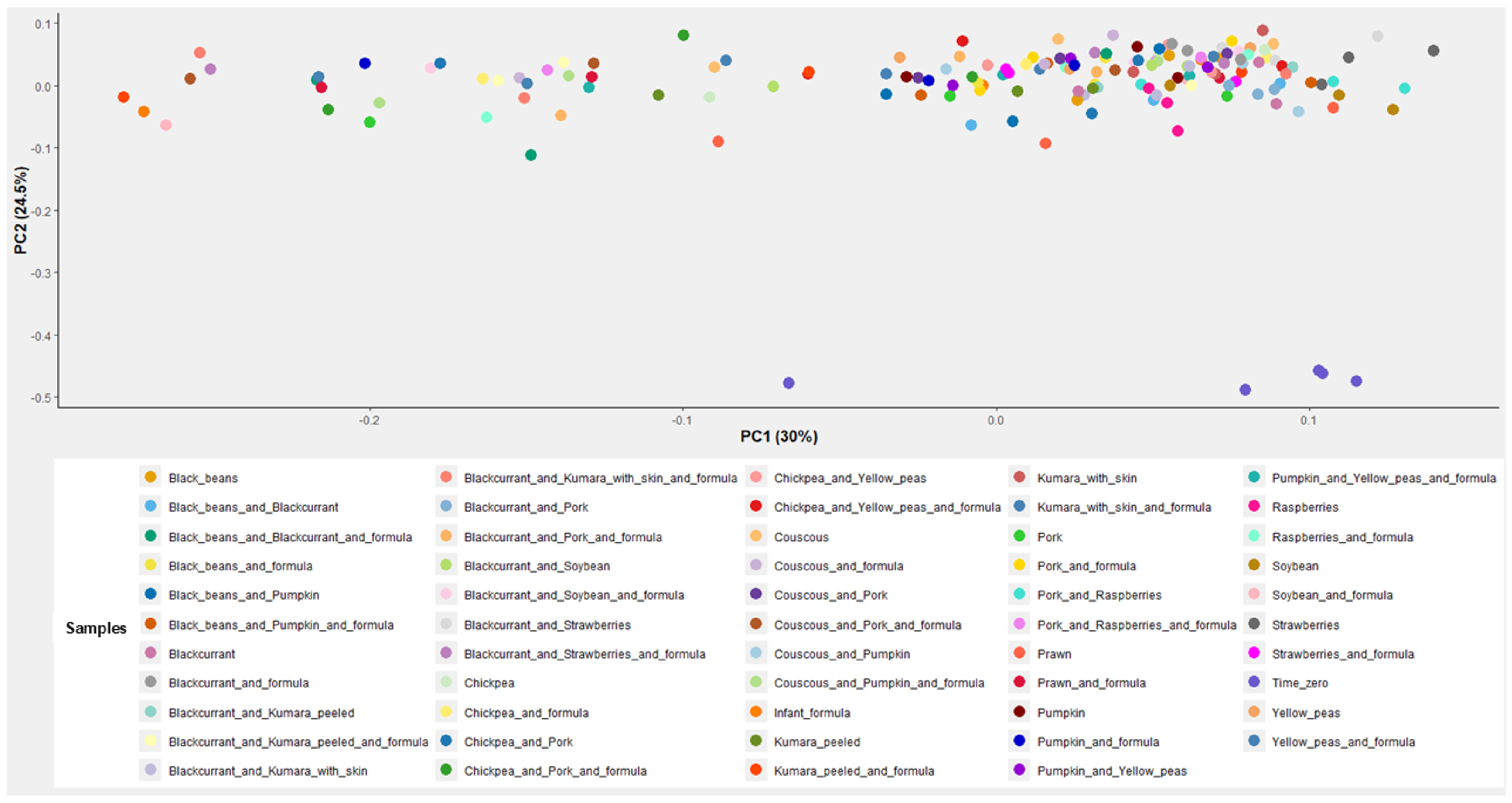
Supplementary Figure 6.3. Gas pressure after 24 hours of fermentation. Bars are coloured according to intensity and statistical significance, with higher values represented in red and lower values in blue. Samples with the same colour and same letters belong to the same group according to the Tukey HSD test with a 95 % confidence interval.



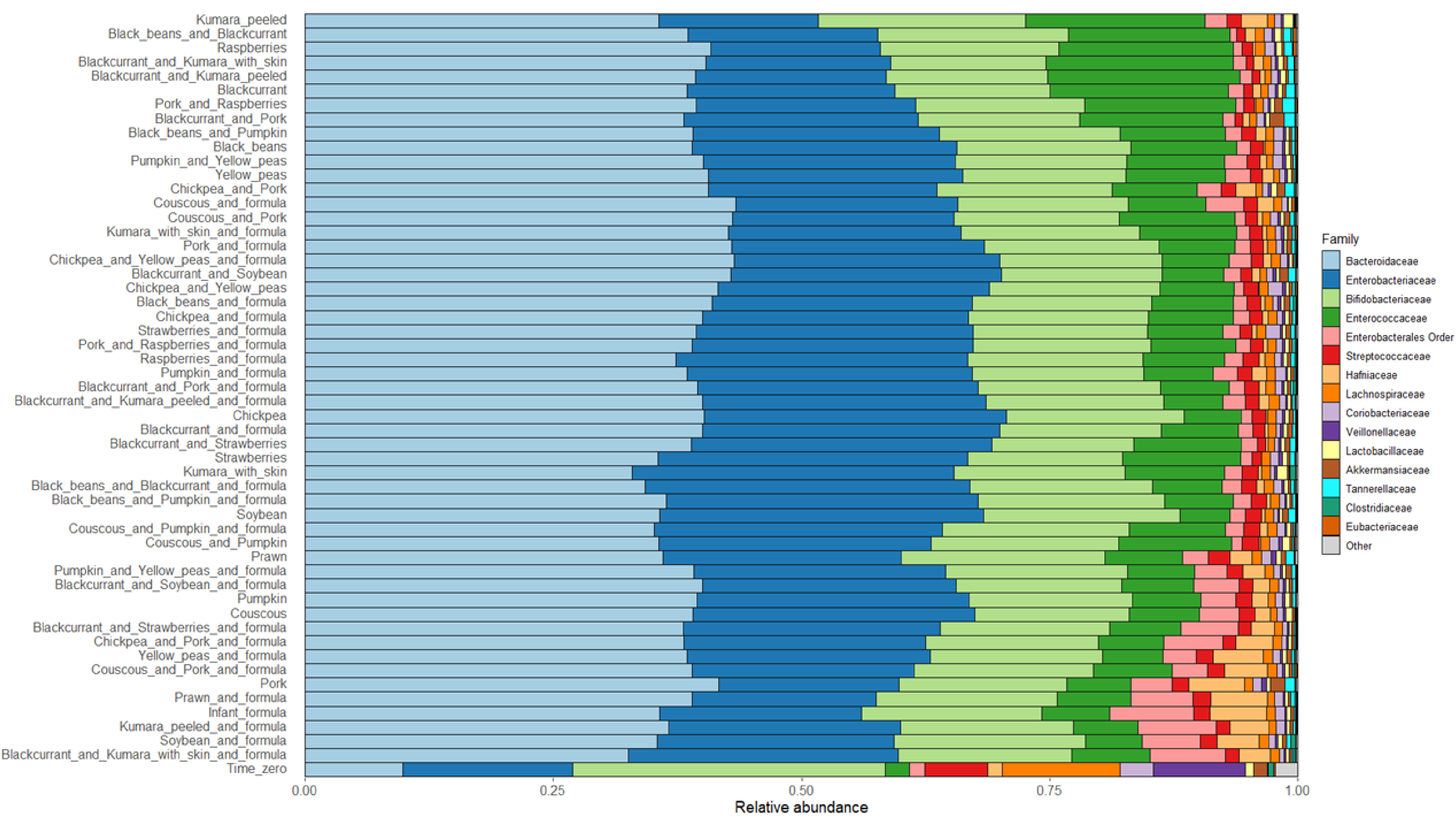
Supplementary Figure 6.4. Organic acids produced after 24 hours of fermentation with food ingredients individually, combined with infant formula, combined with other foods, or combined with infant formula and other foods. Samples are grouped by composition, with food ingredients alone displayed on the left and food-food-formula combinations on the right. Potato starch was used as a positive fermentation control.



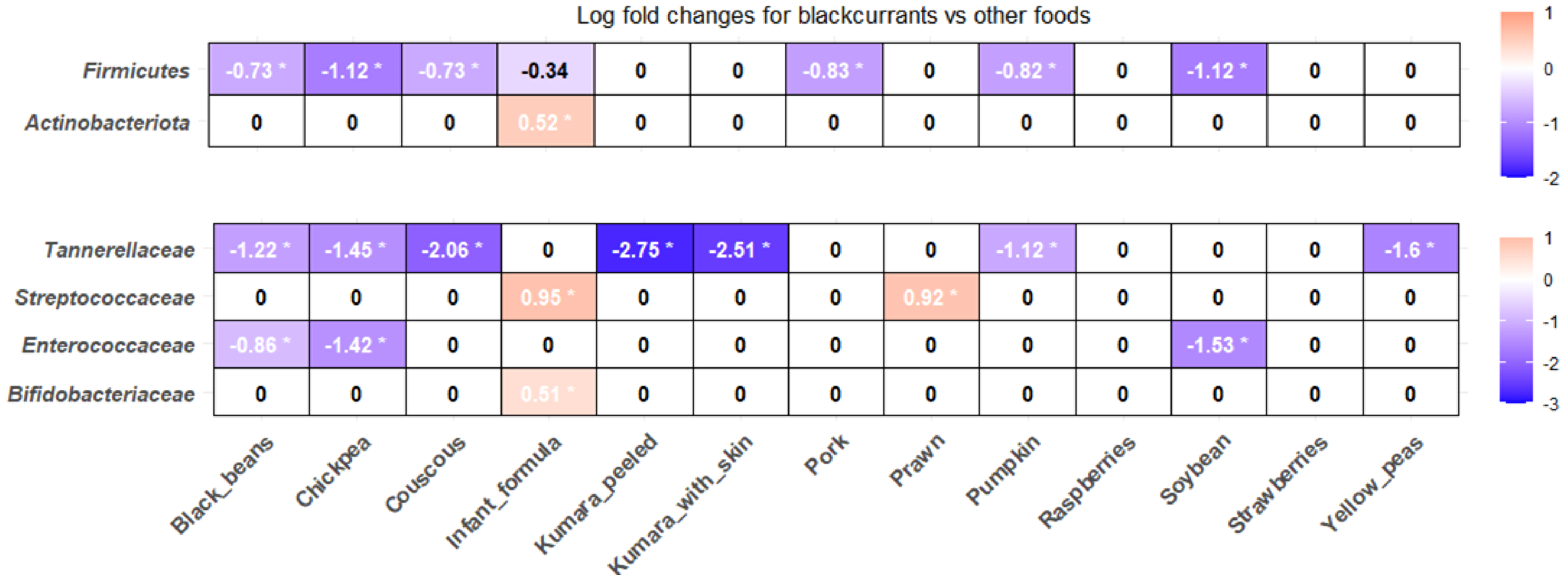
Supplementary Figure 6.5. Influence of food ingredients and food combinations on microbial alpha diversity. Food ingredients and food combination samples were analysed after 24 hours of fermentation. Five randomly selected samples at time zero were included to represent the microbial baseline conditions.



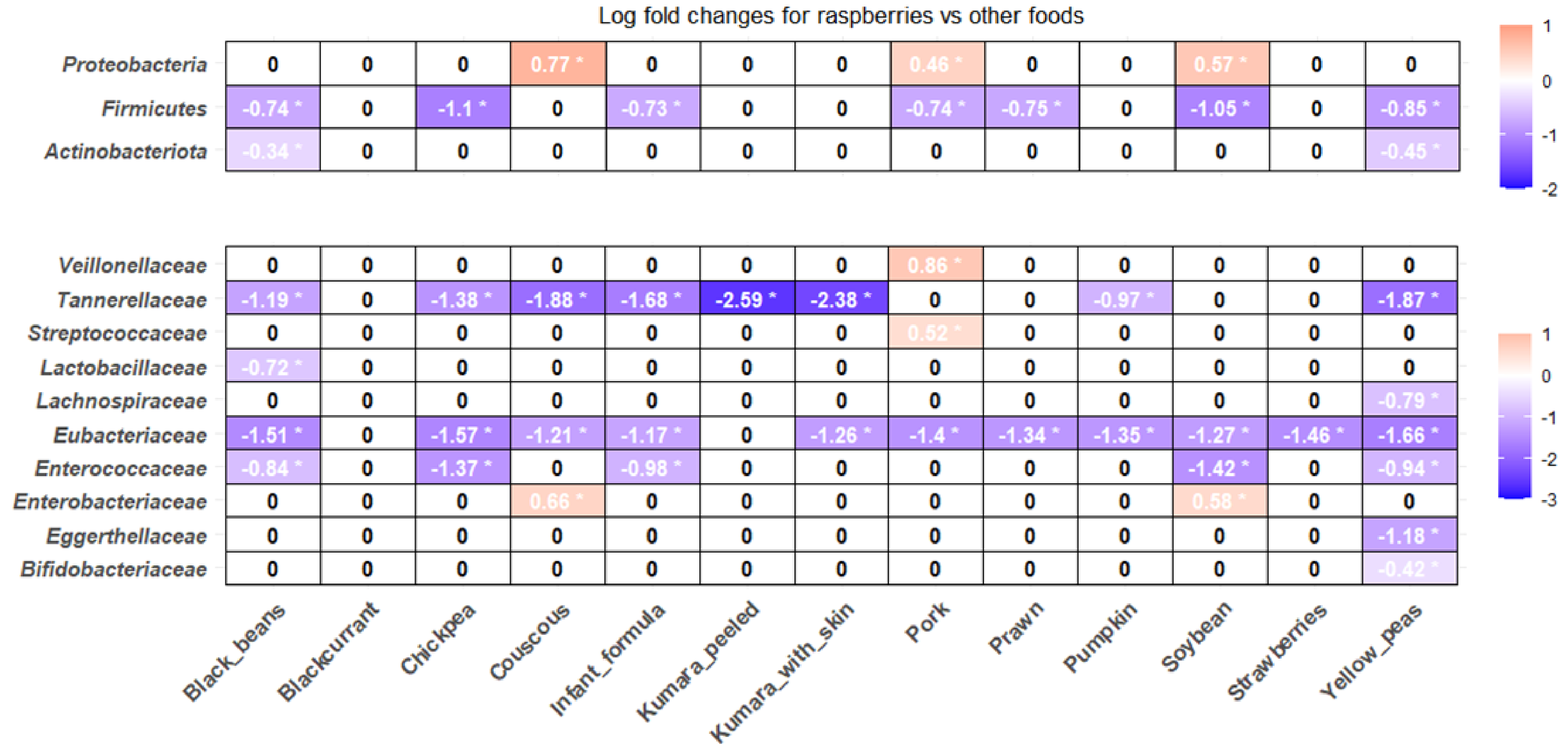
Supplementary Figure 6.6. Influence of food ingredients and food combinations on microbial beta diversity. Food ingredients and food combination samples were analysed after 24 hours of fermentation. Five randomly selected samples at time zero were included to represent the microbial baseline conditions.



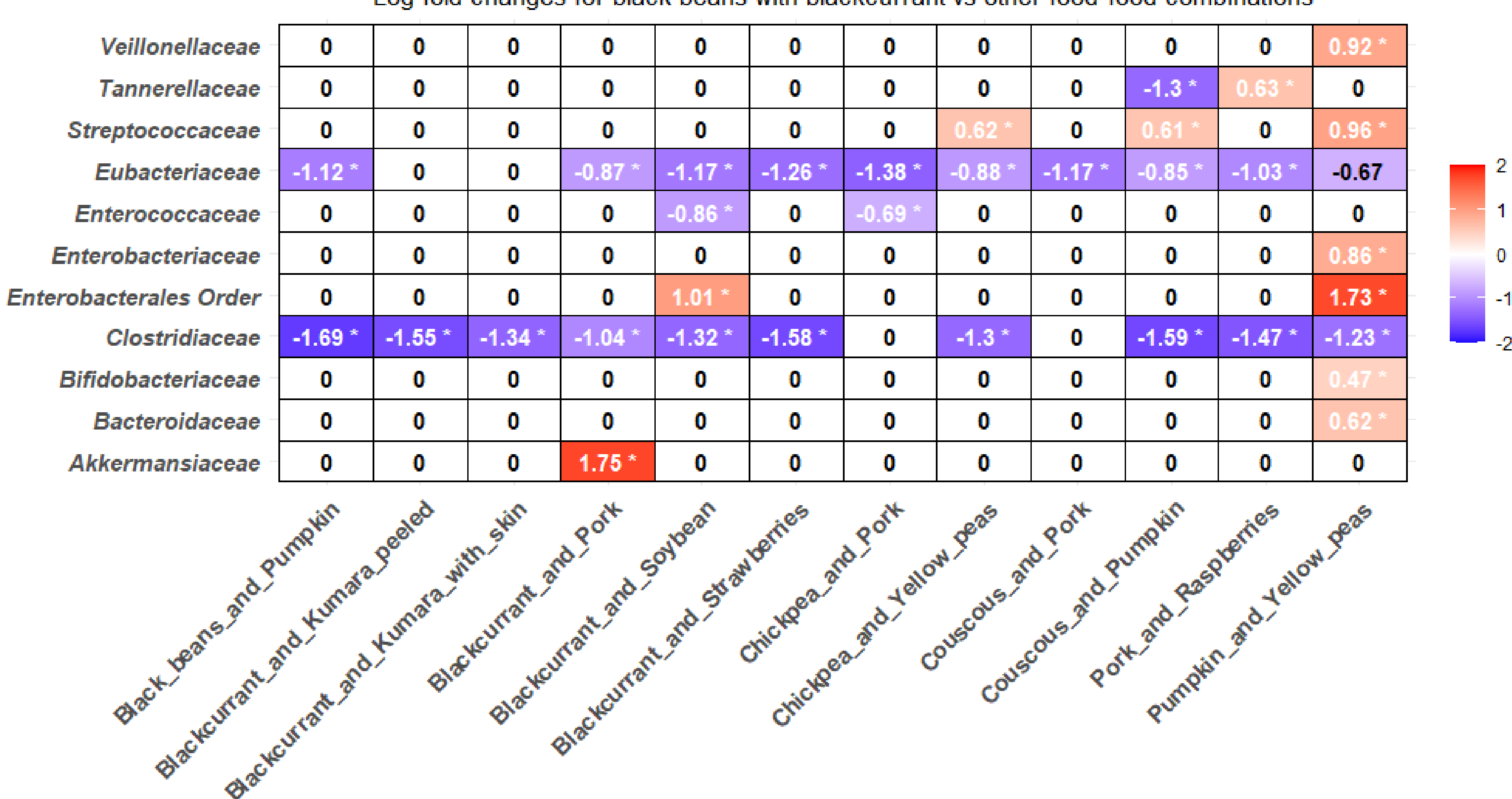
Supplementary Figure 6.7. Influence of food ingredients and food combinations on the relative abundance of bacterial families after 24 hours of fermentation. Only the 15 most abundant families are presented. Taxa with ambiguous or missing classifications were collapsed into higher taxonomic ranks. Five randomly selected samples at time zero were included to represent the microbial baseline conditions.



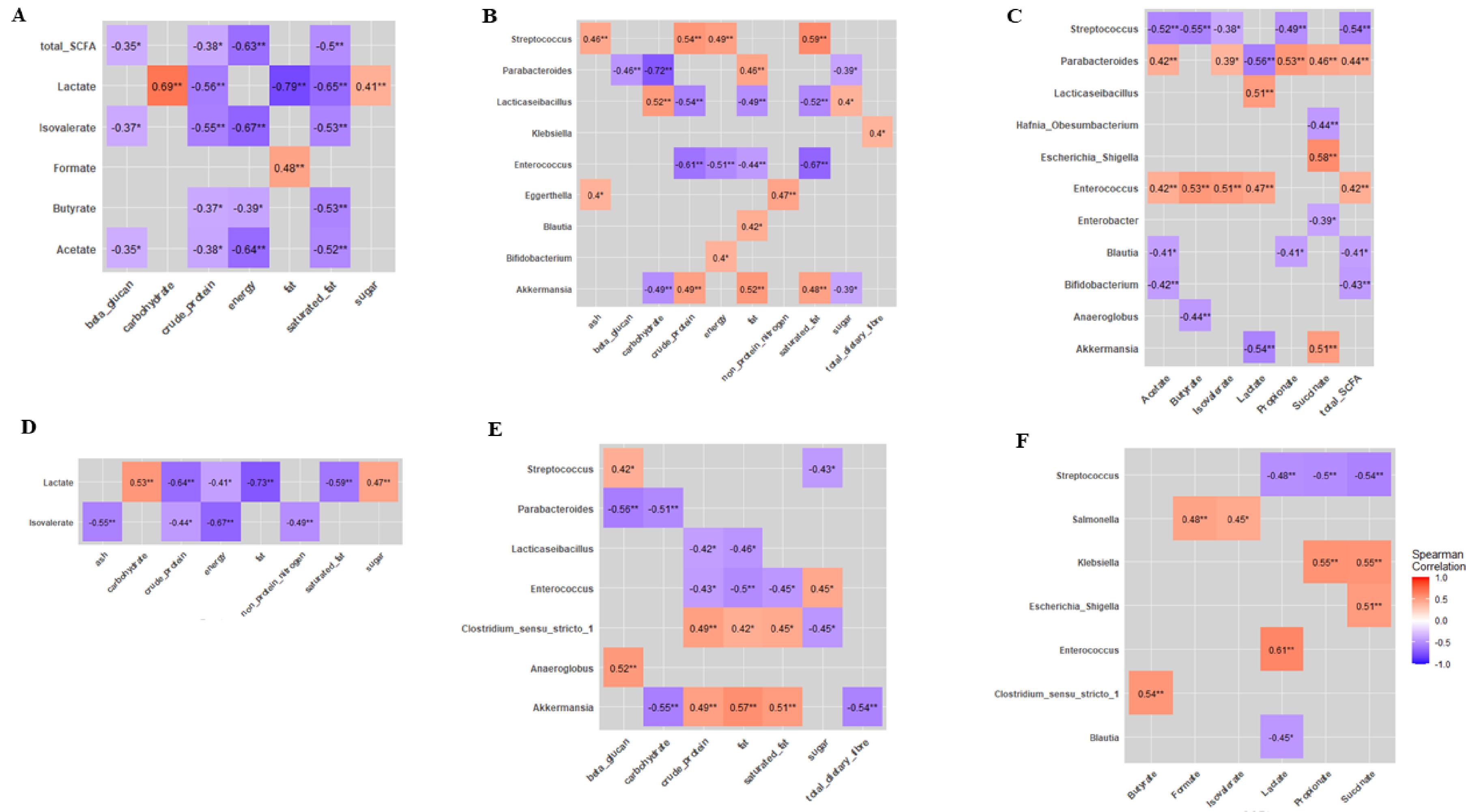
Supplementary Figure 6.8. Heatmap of log-fold changes for food ingredients compared to blackcurrants, showing the abundance of bacterial phyla and families after 24 hours of fermentation. Cells are coloured according to intensity, with higher values in red and lower values in blue. Significant changes in log-fold-changes (adjusted $p < 0.05$) that passed sensitivity analyses are marked with an asterisk (*).



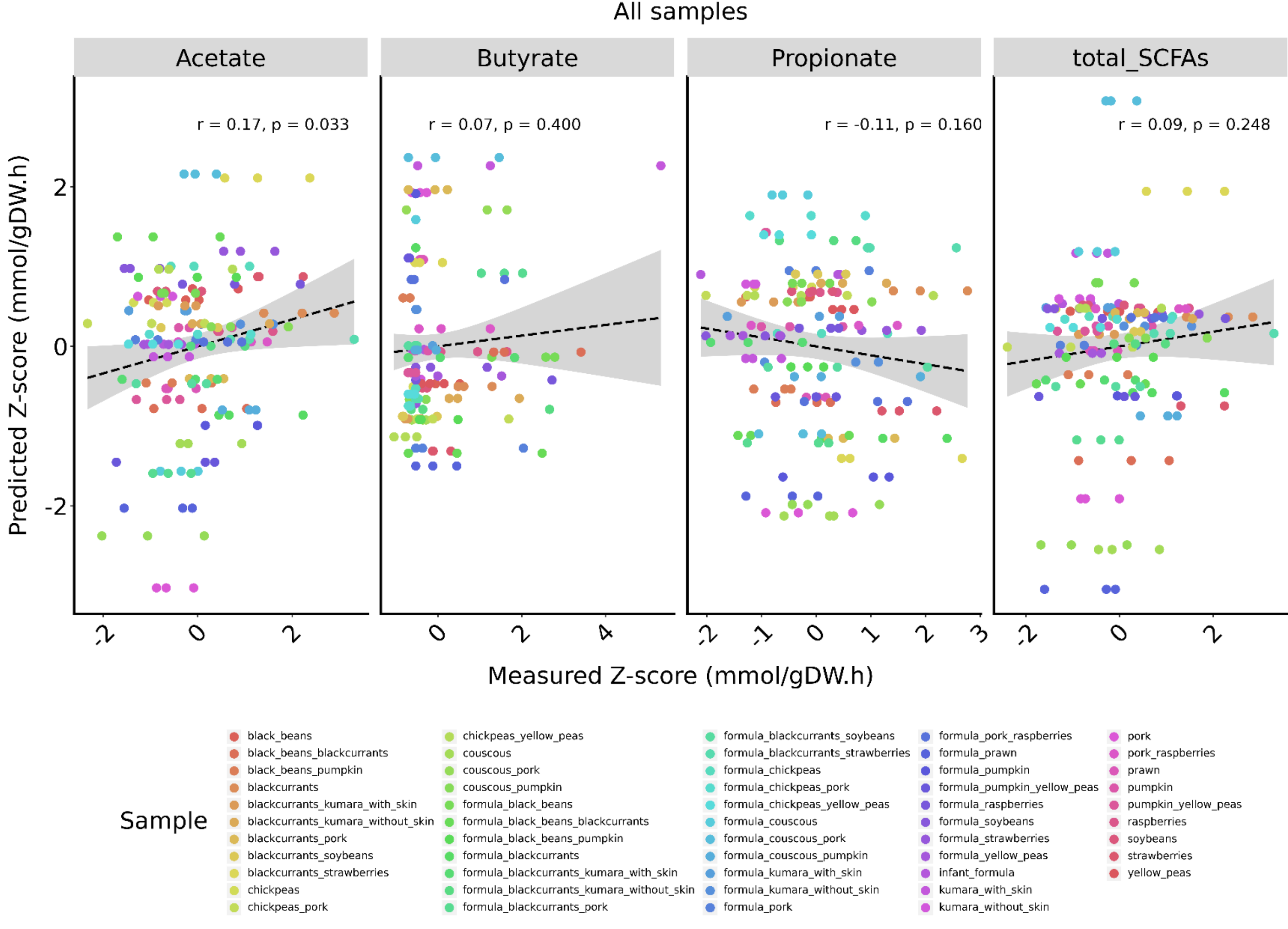
Supplementary Figure 6.9. Heatmap of log-fold changes for food ingredients compared to raspberries, showing the abundance of bacterial phyla and families after 24 hours of fermentation. Cells are coloured according to intensity, with higher values in red and lower values in blue. Significant changes in log-fold changes (adjusted $p < 0.05$) that passed sensitivity analyses are marked with an asterisk (*).



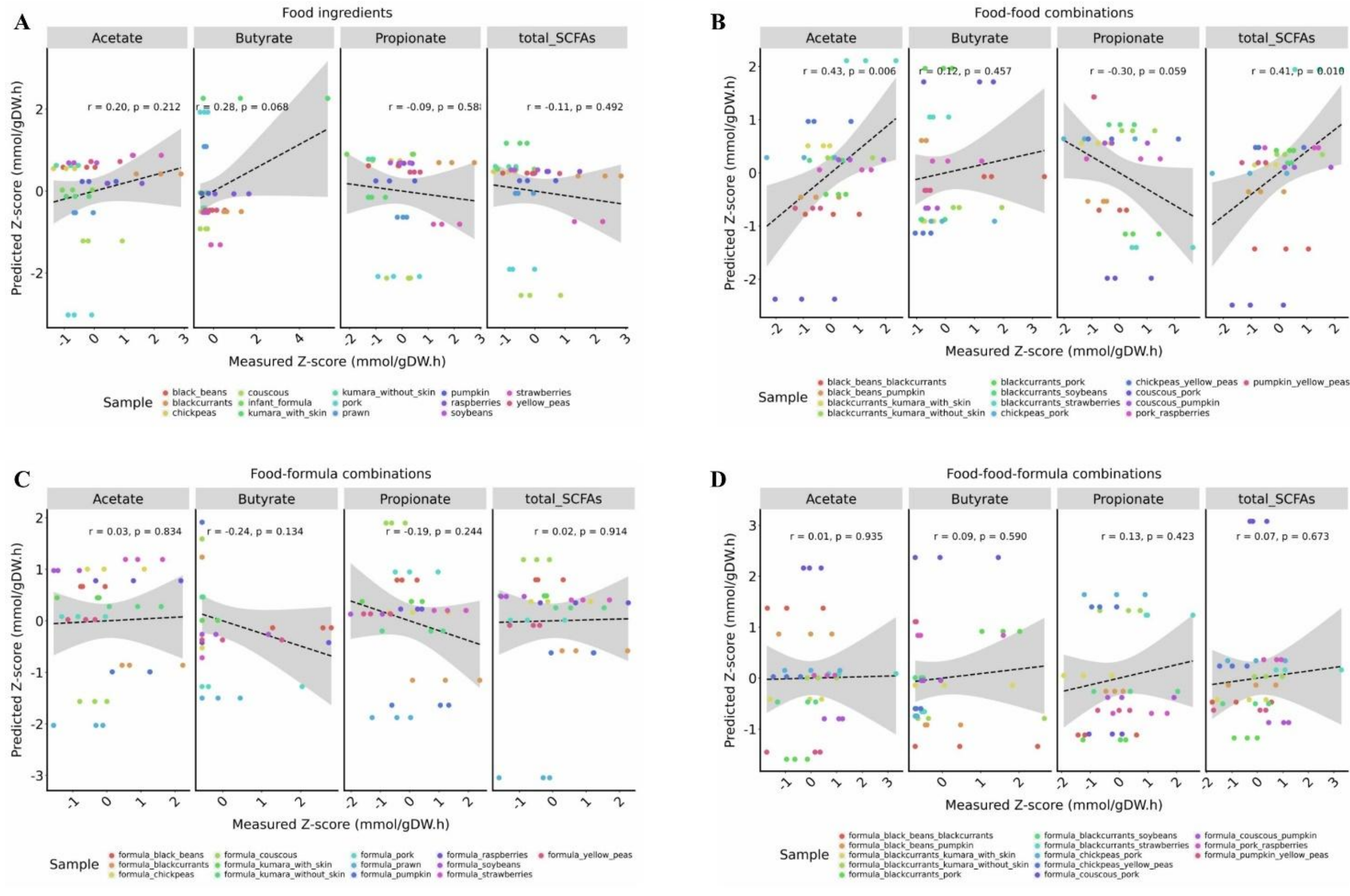
Supplementary Figure 6.10. Heatmap of log-fold changes for food-food combinations compared to black beans-blackcurrants, showing the abundance of bacterial families after 24 hours of fermentation. Cells are coloured according to intensity, with higher values in red and lower values in blue. Significant changes in log-fold changes (adjusted $p < 0.05$) that passed sensitivity analyses are marked with an asterisk (*).



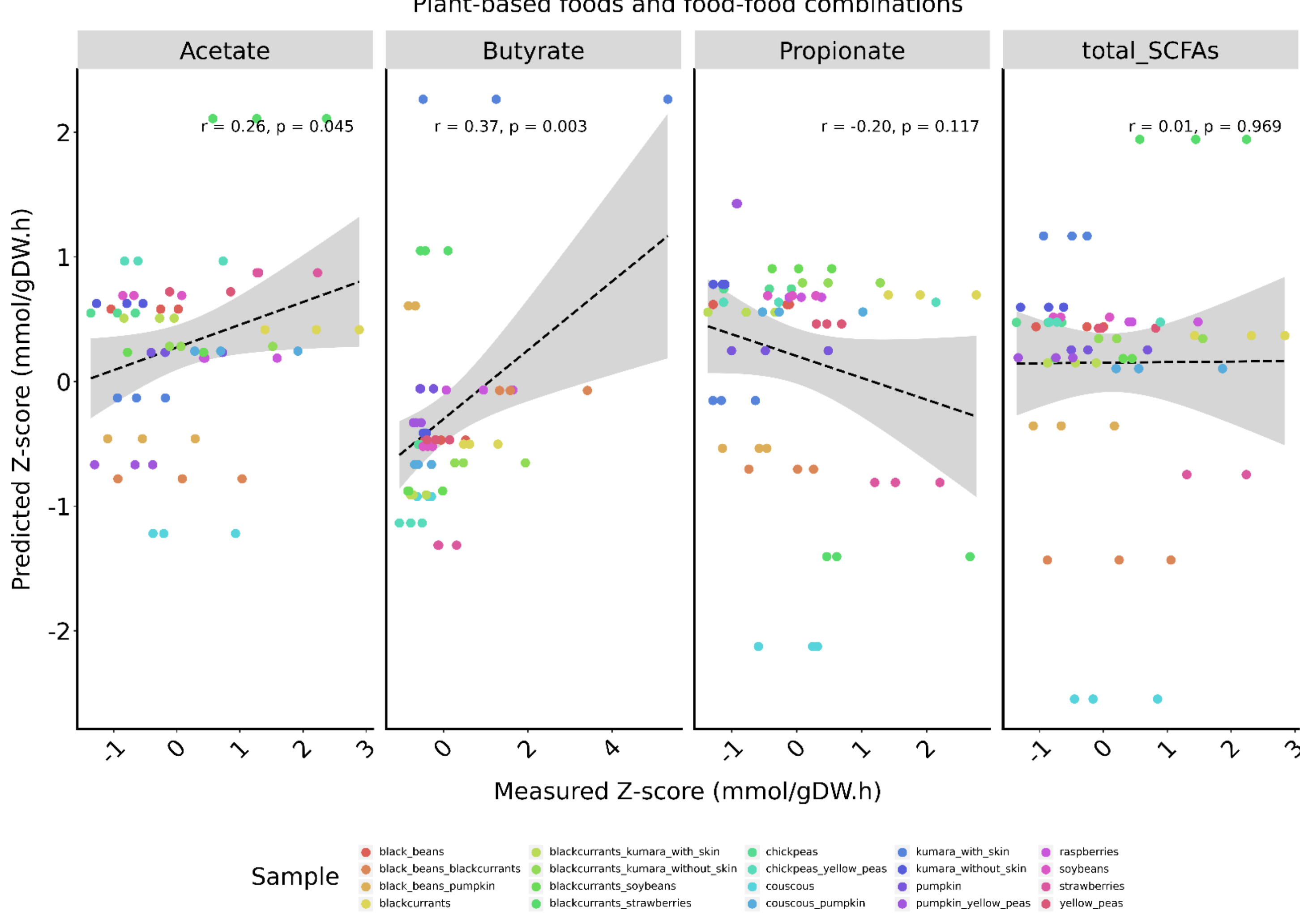
Supplementary Figure 6.11. Spearman's rank correlation heatmaps. Correlations between food composition and organic acids for food ingredients (A) and food-food combinations (D); food composition and microbiota for food ingredients (B) and food-food combinations (E); and microbiota and organic acids for food ingredients (C) and food-formula combinations (F). Significant correlations (adjusted $p < 0.05$) are marked with an asterisk (**), while trends (adjusted $p < 0.1$) are marked with a single asterisk (*). Positive correlations are shown in red and negative in blue.



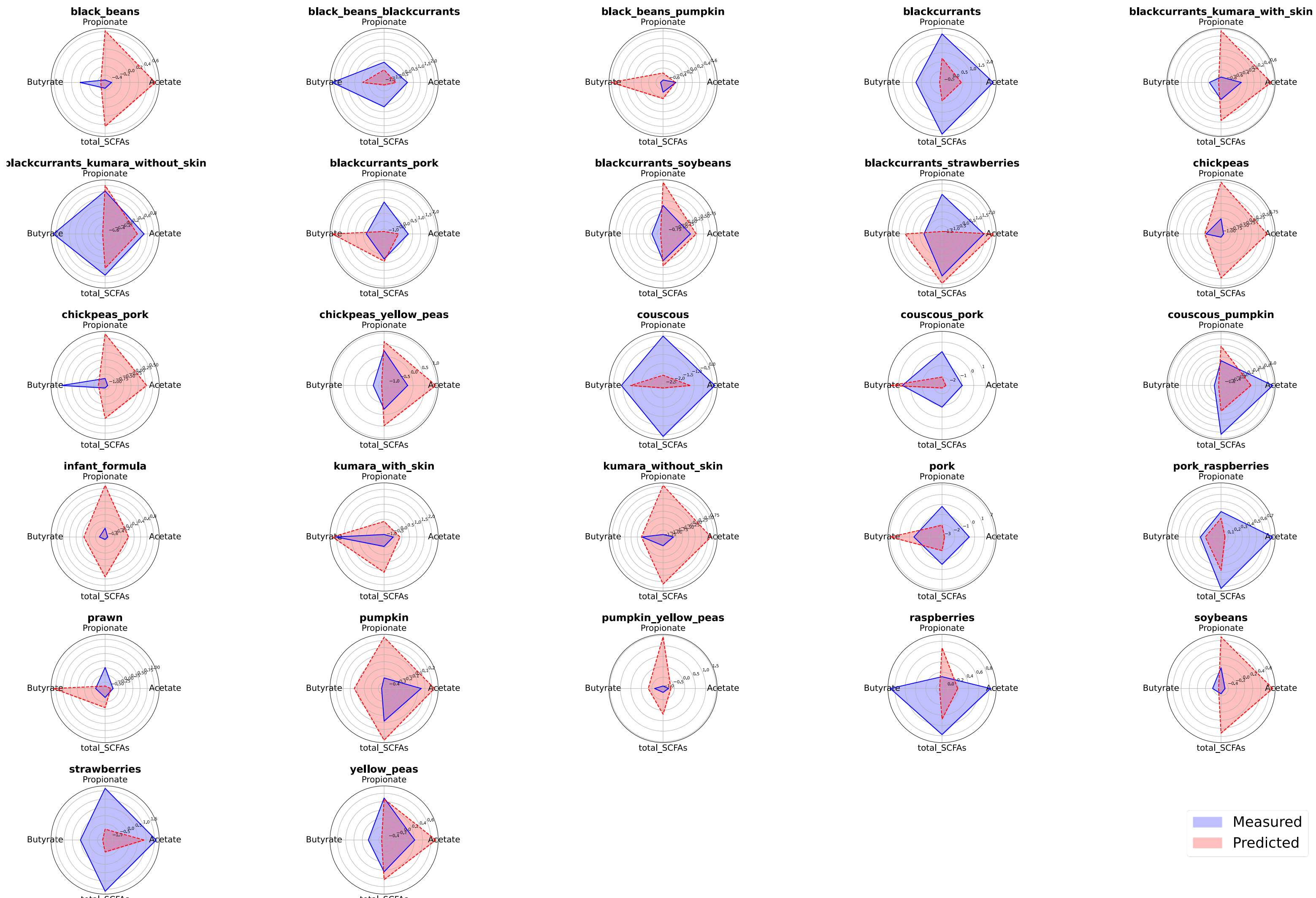
Supplementary Figure 7.1. Pearson correlations between measured and predicted z-scores. SCFAs are displayed from left to right as follows: acetate, butyrate, and propionate. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Pearson correlation coefficients (r) and two-tailed p-values are calculated for each plot individually. A regression line is shown in black, with the corresponding 95 % confidence interval in grey.



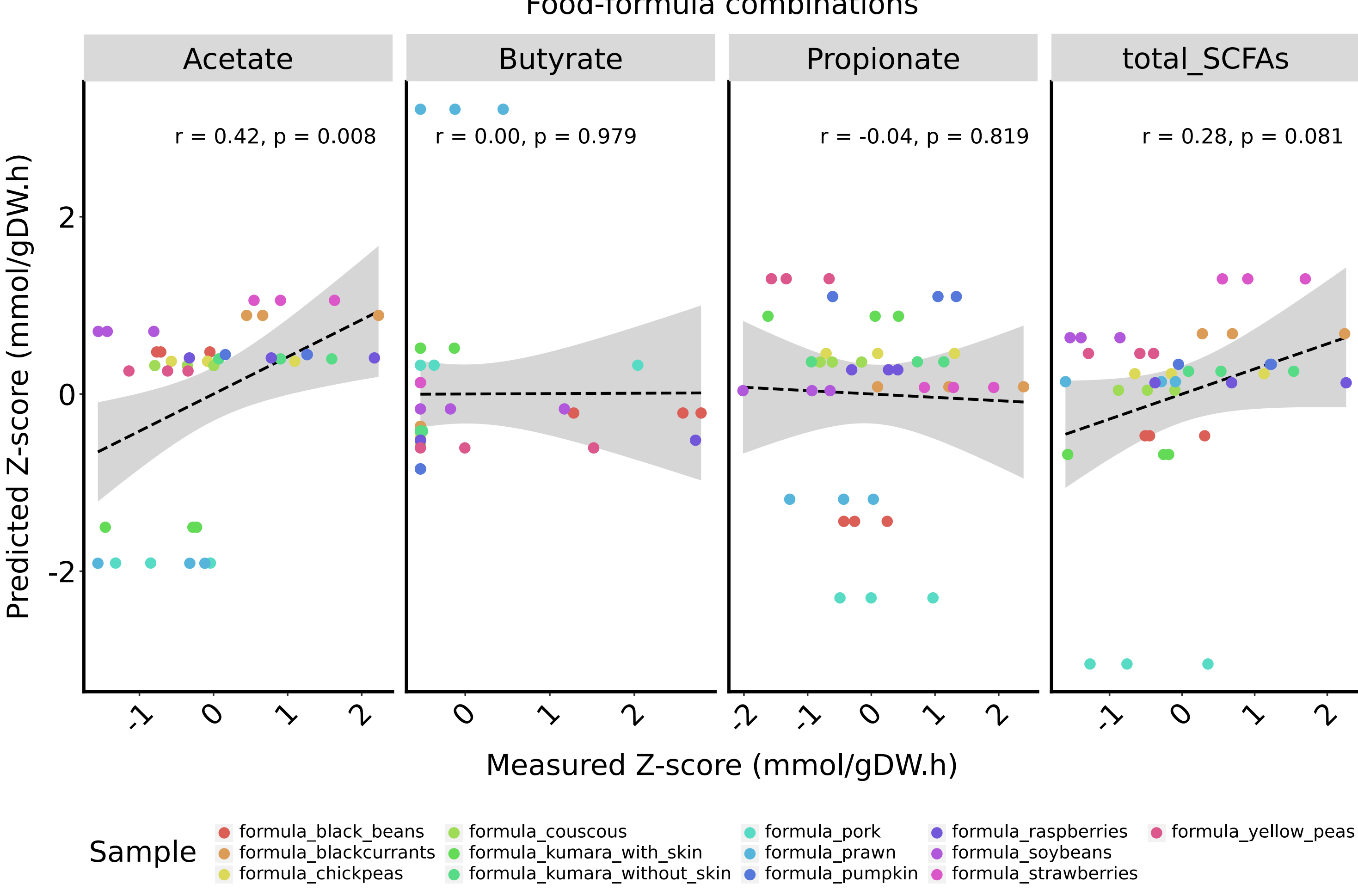
Supplementary Figure 7.2. Pearson correlations between measured and predicted z-scores of major SCFAs. Food ingredients (A), food-food combinations (B), food-formula combinations (C), and food-food-formula combinations (D). SCFAs are displayed from left to right as follows: acetate, butyrate, and propionate. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Pearson correlation coefficients (r) and two-tailed *p*-values are calculated for each plot individually. A regression line is shown in black, with the corresponding 95 % confidence interval in grey.



Supplementary Figure 7.3. Pearson correlations between measured and predicted z-scores of major SCFAs for plant-based food samples. SCFAs are displayed from left to right as follows: acetate, butyrate, and propionate. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Pearson correlation coefficients (r) and two-tailed *p*-values are calculated for each plot individually. A regression line is shown in black, with the corresponding 95 % confidence interval in grey.



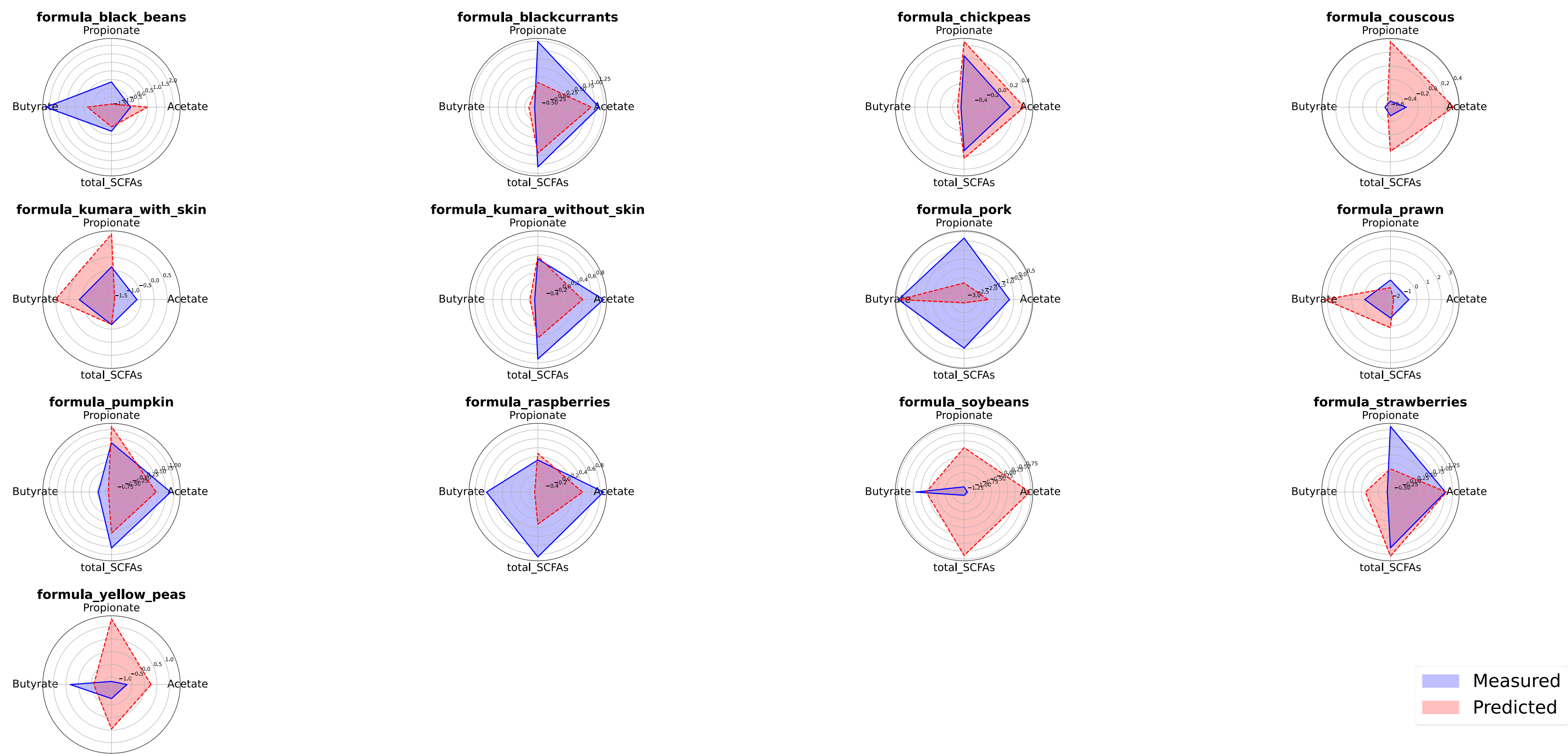
Supplementary Figure 7.4. Radar charts comparing measured and predicted z-scores for major SCFAs in food ingredients and food-food combinations. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Predicted values are displayed in red, while measured z-scores are shown in blue.



Supplementary Figure 7.5. Pearson correlations between measured and predicted z-scores of major SCFAs for food-formula samples. Simulations used post-fermentation microbial relative abundances for each sample. SCFAs are displayed from left to right as follows: acetate, butyrate, and propionate. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Pearson correlation coefficients (r) and two-tailed *p*-values are calculated for each plot individually. A regression line is shown in black, with the corresponding 95 % confidence interval in grey.

	Predicted z-scores (mmol/gDW.h)				Measured z-scores (mmol/gDW.h)				Distance between z-scores			
	Acetate	Butyrate	Propionate	total_SCFAs	Acetate	Butyrate	Propionate	total_SCFAs	Acetate	Butyrate	Propionate	total_SCFAs
formula_black_beans	0.5	-0.2	-1.4	-0.5	-0.5	2.2	-0.1	-0.2	1	2.4	1.3	0.3
formula_blackcurrants	0.9	-0.4	0.1	0.7	1.1	-0.5	1.2	1.1	0.2	0.2	1.2	0.4
formula_chickpeas	0.4	-0.5	0.5	0.2	0.1	-0.5	0.2	0.1	0.2	0.1	0.2	0.1
formula_couscous	0.3	-0.6	0.4	0	-0.4	-0.5	-0.5	-0.5	0.7	0	0.9	0.5
formula_kumara_with_skin	-1.5	0.5	0.9	-0.7	-0.7	-0.4	-0.4	-0.7	0.8	0.9	1.3	0
formula_kumara_without_skin	0.4	-0.4	0.4	0.3	0.9	-0.5	0.3	0.7	0.5	0.1	0.1	0.5
formula_pork	-1.9	0.3	-2.3	-3	-0.7	0.4	0.2	-0.6	1.2	0.1	2.5	2.5
formula_prawn	-1.9	3.2	-1.2	0.1	-0.7	-0.1	-0.6	-0.7	1.2	3.3	0.6	0.8
formula_pumpkin	0.4	-0.8	1.1	0.3	0.9	-0.5	0.6	0.8	0.5	0.3	0.5	0.5
formula_raspberries	0.4	-0.5	0.3	0.1	0.9	0.6	0.1	0.9	0.5	1.1	0.1	0.7
formula_soybeans	0.7	-0.2	0	0.6	-1.3	0.2	-1.2	-1.3	2	0.3	1.2	1.9
formula_strawberries	1.1	0.1	0.1	1.3	1	-0.5	1.3	1.1	0	0.7	1.3	0.2
formula_yellow_peas	0.3	-0.6	1.3	0.5	-0.7	0.3	-1.2	-0.8	1	0.9	2.5	1.2

Supplementary Figure 7.6. Heatmap of measured and predicted z-scores of major SCFAs for food-formula combinations. Simulations used post-fermentation microbial relative abundances for each sample. Predicted z-scores are displayed on the left and measured z-scores on the middle. The absolute difference between predicted and measured z-scores is displayed on the right. Cells are coloured by intensity, with the lowest values in red and the highest values in blue.



Supplementary Figure 7.7. Radar charts comparing measured and predicted z-scores for major SCFAs in food-formula combinations. Simulations used post-fermentation microbial relative abundances for each sample. Total SCFAs correspond to the sum of acetate, propionate, and butyrate. Predicted values are displayed in red, while measured z-scores are shown in blue.

Appendix 2: Supplementary Tables

Supplementary Table 2.1. Principal bacteria, archaea, and fungi composing the human colonic microbiota.

Phylum	Genus	Characteristics	Colonic abundance and observed effect on host health			Reference
			Infants	Adults	Older adults	
Bacillota	*Streptococcus*	Saccharolytic and proteolytic, produces lactate and acetate. Includes both beneficial species (e.g., *S. thermophilus*) and pathogens (e.g., *S. bovis*)	Infants delivered by C-section and formula-fed had an increased abundance of *Streptococcus* compared to other infants (n = 27)	*Streptococcus* abundance was positively associated with coronary atherosclerosis (n = 8973)	A higher abundance of *Streptococcus* was associated with unhealthy ageing (n = 32)	(209,495,496)
	Lactobacillus	Mainly saccharolytic, produces lactate and bacteriocins. Species are typically considered beneficial (e.g., *L. rhamnosus*)	Vaginally delivered infants had a higher abundance of *Lactobacillus* compared to those delivered via C-section during the first days of life (n = 37)	A higher abundance of *Lactobacillus* was associated with type 2 diabetes (n = 18) and obesity (n = 20) in adults	Long-living older adults (aged 97-100) had increased *Lactobacillus* abundance compared to healthy older adults (aged 60-76; n = 20)	(497–500)
	Clostridium	Saccharolytic and proteolytic, produces acetate, propionate, and butyrate. Contains both beneficial species (e.g., *C. butyricum*) and pathogens (e.g., *C. difficile*)	Higher abundance of *Clostridium sensu stricto* in infants with food allergies (n = 34)	A higher abundance of *Clostridium* was associated with obesity in adults (n = 307)	Older adults with Parkinson's disease had a decreased abundance of *Clostridium* compared to healthy controls (n = 45)	(6,501,502)
	Ruminococcus	Mainly saccharolytic, produces acetate. Degrades resistant starch, contributing to butyrate production	Lower abundance of *Ruminococcus* in children and adolescents with Crohn's disease (n = 64)	Lower abundance of *Ruminococcus* in adults with Crohn's disease compared to healthy controls (n = 10)	Higher abundance of *Ruminococcus* in older adults with frailty and sarcopenia (n = 18)	(503–505)

		via cross-feeding (e.g., *R. bromii*)				
	Faecalibacterium	Mainly saccharolytic, produces SCFAs, including butyrate. Typically considered beneficial, some species produce anti-inflammatory molecules (e.g., *F. prausnitzii*)	Lower abundance of *F. prausnitzii* in children with allergic asthma (n = 92)	Lower abundance of *F. prausnitzii* in adults with Crohn's disease (n = 68)	Lower abundance of *F. prausnitzii* in older adults with mild cognitive impairment (n = 15)	(506–508)
	Enterococcus	Saccharolytic and proteolytic, produces lactate and acetate. Some species raise concern due to the capacity to acquire antibiotic resistance (e.g., *E. faecalis*)	Higher abundance of *Enterococcus* in infants with food allergies (n = 34)	Higher abundance of *E. faecalis* in adults with colorectal cancer (n = 25)	Higher abundance of *Enterococcus* in older adults with Parkinson's disease (n = 24)	(502,509,510)
	Eubacterium	Chemoheterotroph, produces SCFAs, including butyrate. Typically associated with the promotion of colonic homeostasis (e.g., *E. rectale*)	Lower abundance of *E. rectale* in children with ulcerative colitis (n = 6) and in children with neurodevelopmental disorders (n = 36)	Lower abundance of *Eubacterium* in adults with Crohn's disease compared to healthy controls (n = 10)	Lower abundance of *Eubacterium* in older adults with frailty and sarcopenia (n = 18)	(504,505,511,512)
Bacteroidota	*Bacteroides*	Saccharolytic and proteolytic, produces acetate, propionate, and succinate. Contains both beneficial species (e.g., *B. thetaiotaomicron*) and pathogens (e.g., *B. fragilis*)	Decreased abundance of *Bacteroides* in infants delivered via C-section compared to infants vaginally delivered (n = 9)	A meta-analysis reported a lower abundance of *Bacteroides* in adults and adolescents with Crohn's disease and ulcerative colitis (n = 706)	Higher abundance of *Bacteroides* in older adults was positively associated with increased risk of all-cause mortality (n = 706)	(160,513,514)
	Prevotella	Saccharolytic and proteolytic, produces acetate and	Higher abundance of *Prevotella* in infants	Higher abundance of *Prevotella* in adults with hypertension (n =	A systematic review reported a lower abundance	(290,502,515,516)

		propionate. Contains species with potential role in promoting (e.g., *P. copri*) or suppressing inflammation (e.g., *P. histicola*)	with food allergies (n = 34)	99). Increased abundance of *Prevotella* was associated with improved glucose metabolism in healthy adults (n = 10)	of *Prevotella* in frail older adults (n = 912)	
	Alistipes	Saccharolytic and proteolytic, produces acetate and propionate. Contains pathogenic species that produce pro-inflammatory toxins (e.g., *A. finegoldii*)	Lower abundance of *A. putredinis* in infancy was associated with neurodevelopmental disorders later in life (n = 1748)	Lower abundance of *Alistipes* in adults with atrial fibrillation (n = 50) and higher abundance in adults with chronic fatigue syndrome (n = 25)	Higher abundance of *Alistipes* in older adults with frailty compared to healthy controls (n = 47)	(264,517–519)
Actinomycetota	*Bifidobacterium*	Saccharolytic, produces acetate and lactate. Predominant in the infant colon. Typically considered beneficial (e.g., *B. breve* and *B. longum*)	Vaginally delivered and breastfed infants have higher *Bifidobacterium* abundance compared to those delivered via C-section and formula-fed (n = 8)	Lower abundance of *Bifidobacterium* in obese women (n = 15)	Higher abundance of *Bifidobacterium* in older adults with frailty compared to healthy controls (n = 47)	(496,518,520)
Pseudomonadota	*Escherichia*	Saccharolytic and proteolytic. Contains pathogenic species that produce pro-inflammatory toxins (e.g., *E. coli*)	Higher abundance of *Escherichia* in children with non-alcoholic steatohepatitis (n = 22)	A meta-analysis reported a higher abundance of *E. coli* in adults with irritable bowel syndrome compared to healthy controls (n = 1340)	Higher abundance of *Escherichia-Shigella* in critically ill older adults (n = 72)	(385,521,522)
	Desulfovibrio	Reduces sulphate, producing hydrogen sulphide (excessive production is deleterious)	Higher abundance of *Desulfovibrio* in infants with stunting compared to healthy controls (n = 10)	Higher abundance of *Desulfovibrio* in adults with systemic sclerosis (n = 59)	Higher abundance of *Desulfovibrio* in older adults with Parkinson's disease (n = 20)	(459,523,524)
Verrucomicrobiota	*Akkermansia*	Mucin degrader. Produces acetate, propionate, and butyrate. Assumed to	Lower abundance of *A. muciniphila* in overweight children (n = 20)	Lower abundance of *A. muciniphila* in pregnant women (n = 16) and patients with	Higher abundance of *Akkermansia* in older adults with frailty compared to	(518,525–527)

		promote colonic barrier integrity (e.g., *A. muciniphila*)		inflammatory bowel disease (n = 46)	healthy controls (n = 47)	
Euryarchaeota	*Methanobrevibacter*	Archaea. Consumes hydrogen to produce methane, facilitating fermentation by anaerobic saccharolytic bacteria (e.g., *M. smithii*)	Lower abundance of *Methanobrevibacter* in children with severe acute malnutrition (n = 143)	Higher abundance of *Methanobrevibacter* in anorexic adults (n = 20)	Higher abundance of *M. smithii* in older adults was positively associated with the severity of cognitive impairment (n = 159)	(497,528,529)
Ascomycota	*Candida*	Fungi. Converts simple carbohydrates into ethanol and acetate. Contains opportunistic pathogens (e.g., *C. albicans*)	Higher abundance of *Candida* in children with autism compared to neurotypical controls (n = 40)	Higher abundance of *Candida* in adults with inflammatory bowel disease (n = 235)	Higher abundance of *C. tropicalis* in older adults with Alzheimer's disease (n = 88)	(530–532)
	Saccharomyces	Fungi. Converts simple carbohydrates into ethanol and carbon dioxide. Typically considered commensals (e.g., *S. cerevisiae*)	Higher abundance of *S. cerevisiae* in children with autism compared to neurotypical controls (n = 29)	Higher abundance of *Saccharomyces* in adults with colorectal cancer (n = 71). Lower abundance of *Saccharomyces* in adults with inflammatory bowel disease (n = 235)	*Saccharomyces* abundance was positively associated with higher levels of circulating plasma triglycerides and very low-density lipoprotein in older adults (n = 99)	(164,530,533,534)

Supplementary Table 2.2. Principal metabolites produced by the human colonic microbiota.

Category	Compound	Substrate or precursor	Major producing microbial taxa	Observed effect on host health			Reference
				Infants	Adults	Older adults	
Short-chain fatty acids (SCFAs)	Acetate	Primarily dietary fibre and resistant starch, but also amino acids	*Bifidobacterium, Lactobacillus, Prevotella, Ruminococcus, A. muciniphila*	Infants exclusively breastfed had a lower concentration of acetate, propionate, and butyrate (n = 48)	A meta-analysis found an increased faecal concentration of acetate, propionate, and butyrate in obese adults (n = 221)	Lower concentration of faecal acetate, propionate, and butyrate in older adults with Alzheimer's disease compared to healthy controls (n = 27)	(535–537)
	Propionate		*Prevotella, Bacteroides, Propionibacterium*				
	Butyrate		*Clostridium, Roseburia, F. prausnitzii, E. rectale, B. fragilis, R. bromii, A. muciniphila*				
Branched-chain fatty acids (BCFAs)	Isobutyrate and isovalerate	Amino acids (valine, leucine, and isoleucine)	*Bacteroides, Clostridium*	Increased faecal levels of isobutyrate and isovalerate in formula-fed infants compared to breastfed infants (n = 33)	Increased isobutyrate faecal levels in adults with non-alcoholic fatty liver disease (n = 24) and increased isovalerate in adults with depression (n = 34)	Increased faecal levels of isobutyrate and isovalerate in older adults with colorectal cancer (n = 50)	(538–541)
Conjugated fatty acids	Conjugated linoleic acid	Linoleic acid	*Bifidobacterium, Enterobacter, Lactobacillus, Clostridium*	Conjugated linoleic acid supplementation in obese children decreased body fat and high-density lipoprotein compared to placebo (n = 28)	Conjugated linoleic acid supplementation in adults decreased T lymphocyte activation (n = 39)	Conjugated linoleic acid supplementation in adults older with type 2 diabetes reduced insulin sensitivity (n = 16)	(542–544)

Vitamins	Complex B vitamins	Carbohydrates and amino acids	*Bifidobacterium, Lactobacillus, Bacteroides*	Deficiency of complex B vitamins in infants was linked with compromised brain development (n = 6)	Decreased serum levels of vitamin B7 in adults were associated with obesity and type 2 diabetes (n = 24)	Deficiency of complex B vitamins in older adults was linked with increased risk of dementia (n = 228)	(545–547)
	Vitamin K family		*Bacteroides, Prevotella*	Vitamin K family deficiency in infants was linked with convulsions, haemorrhage, and death (n = 30)	Serum vitamin K_1 levels were negatively associated with circulating inflammatory biomarkers in adults (n = 1381)	Serum vitamin K_1 levels were negatively associated with circulating inflammatory biomarkers in older adults (n = 662)	(548–550)
Gases	H_2	Carbohydrates	*Clostridium, Enterobacteriaceae*	Excessive H_2 production has been associated with the development of colic symptoms in infants (n = 8)	Higher H_2 production from *in vitro* starch fermentation using faecal inoculum from adults with irritable bowel syndrome compared to healthy controls (n = 14)	No differences in H_2 production from *in vitro* fermentation of different carbohydrates using faecal inoculum from older adults with pre-frailty compared to young controls (n = 6)	(551–553)
	CO_2		*Clostridium*	No changes in CO_2 production were observed during *in vitro* incubation of faecal inoculum from infants fed soy-based infant formula, milk-based formula, or breastmilk (n = 18)	CO_2 insufflation during colonoscopy in adults reduced the faecal abundance of colonic pathogens compared to air insufflation (n = 38)	CO_2 insufflation during colonoscopy in older adults was associated with less pain compared to air insufflation (n = 66)	(554–556)

	CH_4	H_2, CO_2	*Methanobrevibacter smithii*	Higher breath methane in children with chronic constipation compared to healthy controls (n = 75)	Higher breath methane in adults with multiple sclerosis compared to healthy controls (n = 60)	Higher CH_4 production from *in vitro* fermentation of different carbohydrates using faecal inoculum from older adults with pre-frailty compared to young controls (n = 6)	(553,557,558)
	H_2S	Sulphate	*Desulfovibrio*	Higher H_2S production was observed from *in vitro* incubation of faecal inoculum from infants fed soy-based infant formula compared to breastfed infants (n = 5)	Higher H_2S production from *in vitro* starch fermentation using faecal inoculum from adults with irritable bowel syndrome compared to healthy controls (n = 14)	A meta-analysis reported lower levels of circulating H_2S in older adults with chronic and degenerative diseases compared to healthy controls (n = 1721)	(552,554,559)
Secondary bile acids	Deoxycholic acid, lithocholic acid	Primary bile acids	*Clostridium, Bifidobacterium, Lactobacillus, Bacteroides, M. smithii*	Higher faecal levels of secondary bile acid in critically ill children compared to healthy controls (n = 39)	Higher levels of circulating lithocholic acid in adults with severe obstructive coronary heart disease compared to angiographically normal controls (n = 150)	Higher levels of circulating unconjugated secondary bile acids were associated with a higher risk for cardiovascular diseases in older adults with type 2 diabetes (n = 1234)	(560,561)
	Ursodeoxycholic acid	Primary bile acids	*Ruminococcus, Clostridium*	Higher levels of circulating ursodeoxycholate in formula-fed infants compared	Ursodeoxycholic acid use was associated with a reduced risk of colorectal cancer	Ursodeoxycholic acid use was associated with a reduced risk of colorectal cancer	(562–564)

				to breastfed infants (n = 48)	in adults (n = 2557)	in older adults (n = 1911)	
Neurotransmitters	Dopamine	Tyrosine, 3,4-dihydroxy-L-phenylalanine	*Bacillus, E. coli, Staphylococcus*	Higher circulating serotonin and serotonin transporter levels in children with autism compared to healthy controls (n = 60)	Higher plasma levels of dopamine and gamma-aminobutyric acid in adults with major depressive disorder compared to healthy controls (n = 49)	Alterations in the dopamine system were associated with the progression of Alzheimer's disease in older adults (n = 144)	(565–567)
	Norepinephrine	Tyrosine	*Bacillus*				
	Serotonin	Tryptophan, 5-hydroxytryptophan	*Lactobacillus, Streptococcus, Clostridium*				
	Gamma-aminobutyric acid	Acetate, glutamate	*Bifidobacterium, Lactobacillus, Eubacterium, Bacteroides*				
Nitrogen-derivatives	Ammonia	Amino acids and peptides	*Clostridium, Fusobacterium, Bacteroides*	Hyperammonaemia in infants was associated with liver failure and urea cycle defects (n = 90)	Higher circulating levels of ammonia were associated with hepatic steatosis in adults (n = 25)	Higher blood ammonia levels in patients with Alzheimer's disease compared to controls (n = 3)	(568–570)
	p-cresol	Tyrosine	*Fusobacterium, Enterobacter, Clostridium*	Higher urinary levels of p-cresol in children with autism compared to healthy controls (n = 33)	Higher circulating levels of p-cresol in adults undergoing haemodialysis were associated with increased risk for infection-related hospitalisations (n = 464)	Higher circulating levels of p-cresol in older adults undergoing haemodialysis compared to non-haemodialysis controls (n = 4)	(571–573)
	Indole	Tryptophan	*Peptostreptococcus, Akkermansia, Clostridium*	Faecal levels of indole-3-lactic acid correlated positively with increased faecal abundance of *Bifidobacterium*	Lower serum levels of indole-3-pyruvic acid in adults with ulcerative colitis compared to healthy controls (n = 15)	Lower faecal levels of indole-3-pyruvic acid in older adults with Alzheimer's disease compared to healthy controls (n = 27)	(537,574,575)

				infants in breastfed infants (n = 18)			
Endotoxins	Lipopolysaccharide	Lipid A, oligosaccharide, O antigen	*Enterobacteriaceae*, Bacteroidales	Increased exposure to lipopolysaccharides in early infancy was associated with the development of autoimmune diseases (n = 168)	Lipopolysaccharide exposure increased intestinal permeability in healthy adults (n = 14)	Trend towards higher circulating levels of lipopolysaccharides in older adults with Alzheimer's disease compared to healthy controls (n = 27)	(537,576,577)

Supplementary Table 3.1. Search terms and filters used in the different databases.

Database	Terms	Filters
PUBMED	(food OR diet OR feeding OR nutrition) AND (bowel OR gut OR colon* OR feces OR faeces OR fecal OR faecal OR gastrointestinal OR intestinal OR stool) AND (microb* OR bacteria OR microorganism* OR micro-organism*) AND (infancy OR "early life" OR "early-life" OR infant* OR baby OR babies OR toddler* OR child*) AND (weaning OR complementary OR supplementary)	No filters
CENTRAL	(food OR diet OR feeding OR nutrition) AND (bowel OR gut OR colon* OR feces OR faeces OR fecal OR faecal OR gastrointestinal OR intestinal OR stool) AND (microb* OR bacteria OR microorganism* OR micro-organism*) AND (infancy OR "early life" OR "early-life" OR infant* OR baby OR babies OR toddler* OR child*) AND (weaning OR complementary OR supplementary)	Only clinical trials
Scopus	(food OR diet OR feeding OR nutrition) AND (bowel OR gut OR colon* OR feces OR faeces OR fecal OR faecal OR gastrointestinal OR intestinal OR stool) AND (microb* OR bacteria OR microorganism* OR micro-organism*) AND (infancy OR "early life" OR "early-life" OR infant* OR baby OR babies OR toddler* OR child*) AND (weaning OR complementary OR supplementary)	Search only by titles, abstracts, and keywords. Reviews and book chapters were excluded. Only records in English
Web of Science	(food OR diet OR feeding OR nutrition) AND (bowel OR gut OR colon* OR feces OR faeces OR fecal OR faecal OR gastrointestinal OR intestinal OR stool) AND (microb* OR bacteria OR microorganism* OR micro-organism*) AND (infancy OR "early life" OR "early-life" OR infant* OR baby OR babies OR toddler* OR child*) AND (weaning OR complementary OR supplementary)	Reviews and book chapters were excluded. Only records in English
ScienceDirect	food OR diet) (gut OR colon OR fecal OR intestinal) (microbiota OR microbiome) (infant OR child OR baby) (weaning OR complementary)	Research articles only

Supplementary Table 5.1. Average relative abundance of microbes present in the faeces of 14 New Zealand weaning infants used in the simulations.

Genus	Relative abundance
Bifidobacterium	0.173518
Bacteroides	0.143229
Veillonella	0.131564
Bacillus	0.092016
Lacticaseibacillus	0.028031
Prevotella	0.024361
Collinsella	0.019328
Streptococcus	0.01494
Succinispira	0.008924
Limosilactobacillus	0.007138
Lactobacillus	0.006921
Erysipelatoclostridium	0.005582
Paramuribaculum	0.003222
Clostridium	0.003161
Anaeroglobus	0.002221
Cupriavidus	0.001675
Asaccharospora	0.001577
Faecalibacillus	0.001304
Coprenecus	0.001217
Eggerthella	0.001098
Holdemanella	0.000859
Niameybacter	0.000859
Flavonifractor	0.000773
Pseudomonas	0.000708
Bombilactobacillus	0.000707
Lactococcus	0.000671
Actinomyces	0.000588
Pantoea	0.000583
Cellulosilyticum	0.000569
Ligilactobacillus	0.000471
Gilliamella	0.000414
Clostridium	0.000405
Lactiplantibacillus	0.000295
Varibaculum	0.000277

Eubacterium	0.0002
Leuconostoc	0.000191
Fusobacterium	0.000188
Lactococcus	0.000171
Desulfovibrio	0.000161
Cryptobacteroides	0.000112
Ellagibacter	8.03248×10^{-5}
Frischella	7.55278×10^{-5}
Eubacterium	6.02868×10^{-5}
Propionispira	5.86995×10^{-5}
Bilophila	5.8418×10^{-5}
Agathobacter	5.21008×10^{-5}
Apilactobacillus	5.21008×10^{-5}
Mesorhizobium	4.46578×10^{-5}
Weissella	4.2721×10^{-5}
Christensenella	3.72148×10^{-5}
Gemella	3.6618×10^{-5}
Fimenecus	3.65113×10^{-5}
Duncaniella	3.65113×10^{-5}
Luxibacter	3.65113×10^{-5}
Peptoniphilus	2.97719×10^{-5}
Lawsonibacter	2.9209×10^{-5}
Sporobacter	2.9209×10^{-5}
Barnesiella	2.9209×10^{-5}
Bartonella	2.23289×10^{-5}
Gemmiger	2.19068×10^{-5}
Alistipes	2.19068×10^{-5}
Dysosmobacter	1.48859×10^{-5}
Massilioclostridium	1.46045×10^{-5}
Neisseria	1.46045×10^{-5}
Finegoldia	1.2206×10^{-5}

Supplementary Table 5.2. Foods used in the simulations and their respective description from the Virtual Metabolic Human database.

Food group	Food	VMH Description
Vegetables	Broccoli	Broccoli, cooked, boiled, drained, without salt
	Brussel	Brussels sprouts, cooked, boiled, drained, without salt
	Cabbage	Cabbage, red, cooked, boiled, drained, without salt
	Carrot	Carrots, cooked, boiled, drained, without salt
	Cauliflower	Cauliflower, cooked, boiled, drained, without salt
	Celery	Celery, cooked, boiled, drained, without salt
	Cucumber	Cucumber, with peel, raw
	Eggplant	Eggplant, cooked, boiled, drained, without salt
	Green beans	Beans, snap, green, cooked, boiled, drained, without salt
	Green capsicum	Peppers, sweet, green, cooked, boiled, drained, without salt
	Lettuce	Lettuce, butterhead (includes boston and bibb types), raw
	Mushroom	Mushrooms, white, cooked, boiled, drained, without salt
	Onion	Onions, cooked, boiled, drained, without salt
	Pak choi	Cabbage, chinese (pak-choi), cooked, boiled, drained, without salt
	Potato	Potatoes, boiled, cooked in skin, flesh, without salt
	Pumpkin	Pumpkin, cooked, boiled, drained, without salt
	Sweetcorn	Corn, sweet, yellow, cooked, boiled, drained, without salt
	Spinach	Spinach, cooked, boiled, drained, without salt
	Squash	Squash, winter, butternut, cooked, baked, without salt
	Sweet potato	Sweet potato, cooked, boiled, without skin
	Tomato	Tomatoes, red, ripe, raw, year round average
	Yam	Yam, cooked, boiled, drained, or baked, without salt
	Zucchini	Squash, summer, zucchini, includes skin, cooked, boiled, drained, without salt
Fruits	Apple	Apples, raw, gala, with skin
	Banana	Bananas, raw
	Blackcurrant	Currants, european black, raw
	Blueberries	Blueberries, raw
	Cherry	Cherries, sweet, raw
	Feijoa	Feijoa, raw
	Gold kiwifruit	Kiwifruit, ZESPRI SunGold, raw
	Grape	Grapes, red or green (European type, such as Thompson seedless), raw
	Grapefruit	Grapefruit, raw, pink and red, all areas
	Green kiwifruit	Kiwifruit, green, raw
	Mandarin	Tangerines, (mandarin oranges), raw

	Mango	Mangos, raw
	Melon	Melons, cantaloupe, raw
	Nectarine	Nectarines, raw
	Orange	Oranges, raw, navels
	Peache	Peaches, yellow, raw
	Pear	Pears, raw, bartlett
	Pineapple	Pineapple, raw, traditional varieties
	Plum	Plums, raw
	Raspberries	Raspberries, raw
	Strawberries	Strawberries, raw
Cereals and starches	Barley	Barley, pearled, cooked
	Barley cereal	Babyfood, cereal, barley, prepared with whole milk
	Couscous	Couscous, cooked
	Noodles	Noodles, egg, unenriched, cooked, without added salt
	Oat cereal	Babyfood, cereal, oatmeal, prepared with whole milk
	Pasta	Pasta, cooked, unenriched, without added salt
	Rice	Rice, white, long-grain, regular, enriched, cooked
	Rice cereal	Babyfood, cereal, rice, prepared with whole milk
	Tapioca pudding	Puddings, tapioca, dry mix, prepared with whole milk
	White bread	Bread, white, commercially prepared (includes soft bread crumbs)
	Wholegrain bread	Bread, whole-wheat, commercially prepared
Dairy, eggs, and plant-based alternatives	Cottage cheese	Cheese, cottage, lowfat, 2 % milkfat
	Eggs	Egg, whole, cooked, hard-boiled
	Mozzarella cheese	Cheese, mozzarella, whole milk
	Soymilk	Soymilk, original and vanilla, unfortified
	Tofu	Tofu, raw, regular, prepared with calcium sulfate
	Whole milk	Milk, whole, 3.25 % milkfat, with added vitamin D
	Yoghurt	Yogurt, plain, whole milk, 8 grams protein per 8 ounce
Meats	Beef	Babyfood, meat, beef, strained
	Chicken	Babyfood, meat, chicken, strained
	Codfish	Fish, lingcod, cooked, dry heat
	Lamb	Babyfood, meat, lamb, strained
	Mackerel	Fish, mackerel, Atlantic, cooked, dry heat
	Mussels	Mollusks, mussel, blue, cooked, moist heat
	Pork	Babyfood, meat, pork, strained
	Salmon	Fish, salmon, Atlantic, farmed, cooked, dry heat
	Shrimp	Crustaceans, shrimp, cooked (not previously frozen)

	Tuna	Fish, tuna, white, canned in water, without salt, drained solids
	Turkey	Babyfood, meat, turkey, strained
Legumes, nuts, and seeds	Almond	Nuts, almonds, dry roasted, without salt add
	Black beans	Beans, black, mature seeds, cooked, boiled, without salt
	Cashew	Nuts, cashew nuts, dry roasted, without salt added
	Chia	Seeds, chia seeds, dried
	Chickpea	Chickpeas (garbanzo beans, bengal gram), mature seeds, cooked, boiled, without salt
	Green peas	Peas, green, cooked, boiled, drained, without salt
	Hazelnut	Nuts, hazelnuts or filberts, dry roasted, without salt added
	Lentils	Lentils, mature seeds, cooked, boiled, without salt
	Peanut	Peanuts, all types, dry-roasted, without salt
	Pecans	Nuts, pecans, dry roasted, without salt added
	Pumpkin seed	Seeds, pumpkin and squash seeds, whole, roasted, without salt
	Red beans	Beans, kidney, red, mature seeds, cooked, boiled, without salt
	Soybean	Soybeans, mature cooked, boiled, without salt
	Split peas	Peas, split, mature seeds, cooked, boiled, without salt
	Sunflower seed	Seeds, sunflower seed kernels, dry roasted, without salt
	White beans	Beans, white, mature seeds, cooked, boiled, without salt
Controls	Breastmilk	Milk, human, mature, fluid
	Infant formula	Infant formula, NESTLE, GOOD START SUPREME, with iron, ready-to-feed

Supplementary Table 5.3. Predicted fluxes of SCFAs and BCFAs according to different food-breastmilk combinations.

Food-breastmilk combination	Fluxes of organic acids (mmol/gDW.h)						
	Acetate	Propionate	Butyrate	SCFAs	Isobutyrate	Isovalerate	BCFAs
Control breastmilk	264.1	65.5	29.3	358.9	9.2	6.4	15.6
Control infant formula	256.0	69.0	30.9	355.9	9.1	6.3	15.4
Broccoli	243.1	77.9	28.2	349.2	9.1	5.9	15.1
Brussel	272.1	61.7	33.0	366.7	8.9	6.4	15.4
Cabbage	265.6	68.9	29.9	364.4	9.1	6.5	15.6
Carrot	264.3	37.0	8.7	310.0	8.3	5.5	13.8
Cauliflower	254.4	74.3	29.0	357.6	8.9	6.1	15.0
Celery	257.3	66.1	24.7	348.1	6.6	5.9	12.6
Cucumber	242.3	43.9	45.3	331.5	7.7	5.4	13.0
Eggplant	258.4	69.8	27.6	355.8	9.2	6.4	15.6
Green beans	243.4	79.2	28.5	351.1	9.2	6.2	15.4
Green capsicum	254.4	55.6	28.7	338.7	8.6	5.7	14.3
Lettuce	254.2	67.5	27.1	348.8	9.3	6.5	15.8
Mushroom	256.0	70.3	27.1	353.4	9.3	6.6	15.8
Onion	255.1	69.5	28.9	353.6	9.4	6.3	15.7
Pak choi	248.7	75.7	28.2	352.6	9.5	6.5	15.9
Potato	288.9	54.7	24.3	367.9	8.5	5.9	14.4
Pumpkin	257.0	108.1	35.8	400.8	6.1	3.2	9.3
Sweetcorn	288.3	54.9	22.5	365.8	7.6	5.9	13.5
Spinach	177.1	120.6	18.9	316.6	5.3	6.9	12.2
Squash	268.2	64.9	26.9	360.1	8.1	6.6	14.7
Sweet potato	281.9	60.4	27.3	369.6	9.2	6.3	15.5
Tomato	261.9	66.8	27.7	356.4	9.4	6.5	15.9
Yam	264.8	73.7	32.8	371.3	9.8	6.6	16.4
Zucchini	255.9	61.2	24.2	341.3	8.2	5.9	14.1
Apple	266.5	65.2	28.5	360.2	8.9	6.3	15.2
Banana	254.0	71.4	28.5	353.9	9.1	6.4	15.5
Blackcurrant	299.3	52.2	29.6	381.1	8.8	6.7	15.6
Blueberries	252.1	70.8	30.0	352.9	9.0	6.1	15.0
Cherry	257.9	70.2	29.2	357.3	9.3	6.4	15.7
Feijoa	264.2	60.2	30.4	354.7	9.2	6.4	15.6
Gold kiwifruit	292.0	37.2	25.1	354.3	8.6	6.7	15.3
Grape	261.8	68.4	28.1	358.3	8.8	6.3	15.0

Grapefruit	240.4	85.3	27.9	353.6	8.7	6.0	14.7
Green kiwifruit	263.4	63.3	27.4	354.2	8.8	6.4	15.2
Mandarin	238.6	66.0	24.8	329.4	8.6	6.0	14.6
Mango	259.8	66.7	29.0	355.5	9.7	6.5	16.1
Melon	261.5	64.2	28.6	354.3	9.3	6.3	15.6
Nectarine	237.4	79.9	30.2	347.5	9.0	6.2	15.3
Orange	244.9	69.1	27.5	341.5	9.2	6.4	15.5
Peach	259.0	70.9	30.1	360.1	9.1	6.5	15.6
Pear	255.1	19.4	15.6	290.0	5.7	8.2	13.9
Pineapple	273.1	60.0	27.4	360.4	9.5	6.3	15.7
Plum	250.4	49.3	20.2	319.9	8.3	6.7	15.0
Raspberries	282.7	69.5	29.6	381.8	9.3	6.2	15.5
Strawberries	105.1	24.4	10.9	140.3	5.8	4.5	10.3
Barley	250.8	74.0	27.7	352.5	8.7	6.4	15.1
Barley cereal	282.6	61.4	25.1	369.2	8.8	5.9	14.8
Couscous	215.8	23.9	60.1	299.8	4.1	1.9	6.0
Noodles	252.8	70.7	29.4	352.9	9.3	6.4	15.6
Oat cereal	253.1	74.5	27.0	354.5	9.0	6.4	15.5
Pasta	239.6	78.8	29.8	348.3	9.3	6.4	15.7
Rice	233.7	56.5	16.5	306.7	7.5	5.4	13.0
Rice cereal	263.6	74.4	35.9	373.9	9.9	6.7	16.6
Tapioca pudding	263.2	69.0	30.1	362.4	9.1	6.3	15.4
White bread	250.8	70.4	30.5	351.6	9.1	6.3	15.4
Wholegrain bread	171.6	36.0	17.9	225.4	7.7	6.4	14.1
Cottage cheese	259.6	69.6	31.5	360.7	9.6	6.4	16.0
Eggs	251.3	73.8	29.6	354.7	9.2	6.3	15.5
Mozzarella cheese	265.8	69.3	30.5	365.6	9.3	6.3	15.6
Soymilk	261.3	70.1	27.3	358.6	8.6	6.2	14.8
Tofu	257.4	70.1	29.2	356.7	9.3	6.5	15.8
Whole milk	264.9	57.1	27.1	349.1	8.6	6.4	15.0
Yoghurt	248.1	53.9	24.8	326.7	8.0	5.5	13.5
Beef	261.3	65.9	30.0	357.2	9.0	6.3	15.3
Chicken	264.3	71.6	31.8	367.6	9.8	6.7	16.4
Codfish	248.3	68.4	23.0	339.7	8.5	6.0	14.5
Lamb	262.6	66.5	35.8	364.8	9.3	6.5	15.8
Mackerel	274.5	55.1	27.8	357.4	9.0	6.3	15.4
Mussels	265.6	70.0	29.2	364.8	9.0	6.4	15.4

Pork	261.1	70.3	28.8	360.2	9.3	6.4	15.6
Salmon	251.3	73.5	31.8	356.7	9.2	6.3	15.5
Shrimp	264.3	38.1	23.1	325.6	11.7	7.5	19.2
Tuna	258.9	69.8	28.7	357.4	9.1	6.3	15.4
Turkey	267.2	64.0	28.3	359.4	9.2	6.2	15.3
Almond	223.4	73.1	29.8	326.3	9.2	6.1	15.3
Black beans	259.2	70.7	28.1	358.0	9.2	6.4	15.6
Cashew	257.7	62.9	28.5	349.1	8.5	6.4	14.9
Chia	260.7	67.0	28.7	356.4	8.9	6.4	15.3
Chickpea	258.1	71.6	29.7	359.4	9.3	6.4	15.7
Green peas	256.7	67.8	27.0	351.5	8.9	6.2	15.0
Hazelnut	165.4	27.9	21.8	215.0	7.0	7.0	14.1
Lentils	252.5	61.9	26.6	341.0	8.7	5.9	14.6
Peanut	294.1	46.0	28.0	368.1	8.3	6.1	14.4
Pecans	221.1	33.4	21.6	276.1	5.2	4.2	9.4
Pumpkin seed	244.3	71.2	28.2	343.7	9.0	6.2	15.2
Red beans	267.5	65.8	27.5	360.8	9.3	6.4	15.7
Soybean	277.7	67.5	28.1	373.3	9.0	6.5	15.4
Split peas	297.9	33.7	33.7	365.3	5.2	6.2	11.4
Sunflower seed	237.4	54.7	21.3	313.4	8.4	7.7	16.1
White beans	265.7	67.5	30.9	364.1	9.2	6.5	15.7

Values are coloured according to intensity for each organic acid, with the highest values in green and the lowest values in red.

Supplementary Table 5.4. Predicted microbial growth rates according to different food-breastmilk combinations.

Food-breastmilk combination	Growth rate (1/h)									
	Bacillus	*Bacteroides*	*Bifidobacterium*	*Collinsella*	*Lacticaseibacillus*	*Lactobacillus*	*Limosilactobacillus*	*Prevotella*	*Streptococcus*	*Veillonella*
Control breastmilk	0.064	0.068	0.103	0.009		0.005		0.019		
Control infant formula	0.045	0.065	0.080	0.008	0.006	0.004	0.003	0.013	0.003	0.028
Broccoli	0.057	0.070	0.091	0.008	0.002	0.004	0.003	0.019		
Brussel	0.051	0.069	0.087	0.009	0.007	0.004	0.003	0.015	0.001 <	0.009
Cabbage	0.058	0.070	0.091	0.009	0.001	0.005	0.003	0.018		
Carrot	0.050	0.067	0.085	0.009	0.009	0.004	0.003	0.015	0.001	0.015
Cauliflower	0.049	0.068	0.085	0.008	0.006	0.004	0.003	0.015	0.001	0.015
Celery	0.059	0.068	0.091	0.008	0.002	0.005	0.003	0.019		
Cucumber	0.057	0.068	0.092	0.008	0.005	0.005	0.003	0.018		
Eggplant	0.050	0.068	0.086	0.008	0.006	0.004	0.003	0.015	0.001	0.014
Green beans	0.054	0.071	0.091	0.009	0.004	0.004	0.003	0.017	0.001 <	0.001 <
Green capsicum	0.052	0.068	0.087	0.008	0.011	0.004	0.003	0.016		0.010
Lettuce	0.050	0.068	0.086	0.009	0.006	0.004	0.003	0.015	0.001	0.013
Mushroom	0.054	0.071	0.090	0.009	0.004	0.004	0.003	0.016		0.003
Onion	0.055	0.071	0.091	0.009	0.001 <	0.004	0.003	0.017		0.001 <
Pak choi	0.073	0.063	0.103	0.006		0.005	0.001	0.022		
Potato	0.067	0.066	0.103	0.008		0.005	0.002	0.020		
Pumpkin	0.053	0.070	0.090	0.009	0.002	0.004	0.003	0.016		0.004
Sweetcorn	0.057	0.069	0.091	0.008	0.003	0.005	0.003	0.020		
Spinach	0.055	0.071	0.091	0.009	0.003	0.004	0.003	0.016		
Squash	0.062	0.080	0.103	0.010	0.002	0.005	0.003	0.018	0.001 <	0.001 <
Sweet potato	0.058	0.069	0.091	0.008	0.001	0.005	0.003	0.018		
Tomato	0.051	0.068	0.086	0.009	0.010	0.004	0.003	0.016	0.001	0.011
Yam	0.060	0.068	0.091	0.009		0.005	0.002	0.018		
Zucchini	0.030	0.043	0.053	0.006	0.008	0.002	0.002	0.009	0.002	0.038
Apple	0.060	0.067	0.091	0.008		0.004	0.002	0.020		
Banana	0.050	0.068	0.086	0.008	0.005	0.004	0.003	0.015	0.001	0.014
Blackcurrant	0.063	0.068	0.103	0.009		0.005		0.019		
Blueberries	0.046	0.065	0.081	0.008	0.007	0.004	0.003	0.013	0.003	0.027
Cherry	0.057	0.078	0.098	0.010	0.003	0.004	0.003	0.016	0.001	0.004

Feijoa	0.050	0.068	0.086	0.008	0.004	0.004	0.003	0.015	0.001	0.012
Gold kiwifruit	0.050	0.067	0.085	0.008	0.009	0.004	0.003	0.015	0.001	0.014
Grape	0.084	0.066	0.103	0.003		0.005	0.001 <	0.027		
Grapefruit	0.059	0.068	0.091	0.008		0.005	0.003	0.019		
Green kiwifruit	0.050	0.068	0.086	0.008	0.004	0.004	0.003	0.014	0.001	0.014
Mandarin	0.052	0.069	0.088	0.009	0.007	0.004	0.003	0.016	0.001 <	0.007
Mango	0.052	0.069	0.088	0.009	0.006	0.004	0.003	0.015	0.001	0.009
Melon	0.049	0.068	0.085	0.008	0.006	0.004	0.003	0.015	0.001	0.015
Nectarine	0.057	0.078	0.098	0.009	0.005	0.004	0.003	0.016	0.001	0.001
Orange	0.072	0.064	0.102	0.007		0.005		0.023		
Peach	0.050	0.068	0.085	0.009	0.009	0.004	0.003	0.015	0.001	0.013
Pear	0.052	0.068	0.087	0.009	0.003	0.004	0.003	0.016	0.001 <	0.010
Pineapple	0.051	0.069	0.088	0.009	0.008	0.004	0.003	0.016	0.001	0.008
Plum	0.044	0.064	0.079	0.008	0.007	0.003	0.003	0.012	0.003	0.028
Raspberries	0.057	0.069	0.091	0.008	0.004	0.005	0.003	0.019		
Strawberries	0.057	0.070	0.091	0.008	0.002	0.005	0.003	0.018		
Barley	0.051	0.069	0.087	0.009	0.006	0.004	0.003	0.015	0.001	0.009
Barley cereal	0.065	0.067	0.103	0.009		0.005	0.003	0.019		
Couscous	0.049	0.068	0.085	0.009	0.007	0.004	0.003	0.014	0.001	0.015
Noodles	0.049	0.067	0.085	0.008	0.006	0.004	0.003	0.014	0.002	0.016
Oat cereal	0.037	0.054	0.067	0.007	0.009	0.003	0.003	0.011	0.003	0.032
Pasta	0.064	0.071	0.103	0.009		0.005		0.019		
Rice	0.037	0.050	0.062	0.006	0.006	0.003	0.002	0.011	0.001	0.015
Rice cereal	0.049	0.067	0.085	0.008	0.005	0.004	0.003	0.014	0.001	0.017
Tapioca pudding	0.052	0.070	0.088	0.009	0.004	0.004	0.003	0.016	0.001 <	0.007
White bread	0.050	0.068	0.086	0.009	0.006	0.004	0.003	0.014	0.001	0.014
Wholegrain bread	0.059	0.068	0.091	0.008		0.005	0.002	0.018		
Cottage cheese	0.050	0.068	0.085	0.009	0.007	0.004	0.003	0.015	0.001	0.014
Eggs	0.053	0.071	0.090	0.009	0.003	0.004	0.003	0.016	0.001 <	0.003
Mozzarella cheese	0.068	0.063	0.091	0.006		0.005	0.001	0.022		
Soymilk	0.050	0.068	0.086	0.008	0.007	0.004	0.003	0.015	0.001	0.014
Tofu	0.038	0.055	0.068	0.007	0.009	0.003	0.003	0.011	0.003	0.030
Whole milk	0.049	0.068	0.085	0.008	0.007	0.004	0.003	0.014	0.001	0.015
Yoghurt	0.051	0.068	0.086	0.009	0.007	0.004	0.003	0.015	0.001	0.012
Beef	0.051	0.069	0.087	0.008	0.003	0.004	0.003	0.014	0.001	0.012
Chicken	0.049	0.068	0.085	0.008	0.007	0.004	0.003	0.014	0.001	0.015
Codfish	0.054	0.069	0.089	0.009	0.006	0.004	0.003	0.017		0.005

Lamb	0.042	0.057	0.072	0.007	0.008	0.003	0.003	0.012	0.002	0.019
Mackerel	0.054	0.071	0.090	0.009	0.006	0.004	0.003	0.016		0.001
Mussels	0.067	0.067	0.103	0.008		0.005		0.020		
Pork	0.086	0.065	0.102	0.004	0.001 <	0.005	0.001	0.028		
Salmon	0.051	0.069	0.087	0.009	0.006	0.004	0.003	0.015	0.001	0.011
Shrimp	0.050	0.068	0.086	0.008	0.007	0.004	0.003	0.014	0.001	0.013
Tuna	0.054	0.071	0.090	0.009	0.004	0.004	0.003	0.016		0.002
Turkey	0.051	0.069	0.087	0.008	0.005	0.004	0.003	0.015	0.001	0.011
Almond	0.066	0.064	0.092	0.006		0.005	0.001	0.021		
Black beans	0.064	0.069	0.103			0.005		0.019		
Cashew	0.042	0.057	0.072	0.007	0.007	0.003	0.003	0.013	0.002	0.018
Chia	0.052	0.070	0.088	0.009	0.007	0.004	0.003	0.016	0.001 <	0.007
Chickpea	0.053	0.070	0.089	0.009	0.004	0.004	0.003	0.016	0.001 <	0.005
Green beans	0.054	0.071	0.091	0.009	0.004	0.004	0.003	0.017	0.001 <	0.001 <
Hazelnut	0.063	0.066	0.091	0.007		0.005	0.002	0.021		
Lentils	0.050	0.069	0.087	0.009	0.004	0.004	0.003	0.015	0.001	0.011
Peanut	0.062	0.080	0.103	0.009	0.001 <	0.004	0.003	0.018		
Pecans	0.065	0.065	0.091	0.006		0.005	0.001	0.022		
Pumpkin seed	0.062	0.071	0.103		0.001	0.004	0.003	0.018		
Red beans	0.050	0.068	0.086	0.009	0.006	0.004	0.003	0.014	0.001	0.012
Soybean	0.066	0.066	0.103	0.008		0.005		0.020		
Split peas	0.055	0.071	0.091	0.008	0.004	0.005	0.003	0.018		
Sunflower seed	0.025	0.036	0.044	0.005	0.007	0.002	0.002	0.007	0.003	0.032
White beans	0.050	0.068	0.085	0.009	0.006	0.004	0.003	0.015	0.001	0.014

Values are coloured according to intensity for each genus, with the highest values in green and the lowest values in red. Blank cells correspond to absent growth.

Supplementary Table 5.5. Predicted fluxes of SCFAs and BCFAs for multiple food-breastmilk combinations.

Multiple food-breastmilk combination	Fluxes of organic acids (mmol/gDW.h)						
	Acetate	Propionate	Butyrate	SCFAs	Isobutyrate	Isovalerate	BCFAs
Control breastmilk	264.1	65.5	29.3	358.9	9.2	6.4	15.6
Control infant formula	256.0	69.0	30.9	355.9	9.1	6.3	15.4
Black beans and blackcurrant	254.5	51.0	20.6	326.1	10.6	6.5	17.1
Black beans and chickpea	242.9	32.7	14.0	289.6	6.5	6.9	13.4
Black beans and couscous	254.6	72.2	31.2	358.0	9.2	6.3	15.4
Black beans and pork	246.8	71.9	28.0	346.7	9.2	6.3	15.5
Black beans and pumpkin	115.4	17.0	149.8	282.3	1.9	0.5	2.4
Black beans and raspberries	275.2	62.3	27.6	365.1	8.4	6.2	14.6
Black beans and shrimp	255.5	74.4	28.7	358.6	9.5	6.4	15.9
Black beans and soybean	239.5	67.5	33.0	340.0	9.5	6.5	16.0
Black beans and split peas	258.5	70.8	28.2	357.5	9.3	6.4	15.6
Black beans and strawberries	258.3	70.1	31.7	360.1	9.3	6.4	15.7
Black beans and sweet potato	269.7	55.0	24.9	349.6	7.4	5.5	12.9
Blackcurrant and chickpea	250.2	69.8	27.4	347.4	9.2	6.0	15.1
Blackcurrant and couscous	261.4	60.9	29.6	351.9	9.1	6.5	15.6
Blackcurrant and pork	263.7	31.2	52.7	347.6	5.2	3.0	8.2
Blackcurrant and pumpkin	253.1	75.5	30.3	358.9	9.3	6.4	15.7
Blackcurrant and raspberries	260.0	62.8	29.0	351.8	8.7	6.1	14.8
Blackcurrant and shrimp	254.4	70.4	27.2	351.9	8.7	6.2	14.9
Blackcurrant and soybean	311.9	61.6	27.5	401.0	8.4	6.5	14.9
Blackcurrant and split peas	257.8	68.7	26.9	353.4	8.6	6.3	14.9
Blackcurrant and strawberries	301.7	53.6	28.3	383.6	9.4	6.5	15.9
Blackcurrant and sweet potato	297.1	60.4	25.2	382.7	8.3	6.0	14.3
Chickpea and couscous	253.0	76.4	26.5	355.9	7.7	5.6	13.2
Chickpea and pork	277.7	37.0	49.2	363.9	6.1	3.8	9.9
Chickpea and pumpkin	257.6	74.5	31.6	363.7	9.2	6.2	15.4
Chickpea and raspberries	255.2	69.8	30.5	355.5	9.3	6.3	15.6
Chickpea and shrimp	260.3	70.6	30.0	360.9	9.3	6.4	15.7
Chickpea and Soybean	260.6	72.4	30.8	363.8	9.3	6.4	15.8
Chickpea and split peas	68.9	19.2	12.5	100.6	2.5	1.3	3.8
Chickpea and strawberries	258.3	70.2	30.0	358.6	9.5	6.4	15.9
Chickpea and sweet potato	256.2	73.1	28.3	357.6	9.1	6.4	15.5
Couscous and pork	277.7	54.7	15.2	347.6	6.7	4.8	11.5

Couscous and Pumpkin	257.2	71.6	30.4	359.2	9.1	6.4	15.5
Couscous and Raspberries	258.0	67.7	26.5	352.2	9.1	6.1	15.2
Couscous and Shrimp	279.5	58.9	26.9	365.3	9.0	6.3	15.3
Couscous and Soybean	261.5	70.7	31.1	363.3	9.1	6.4	15.5
Couscous and Split peas	255.8	67.9	33.8	357.6	8.8	6.6	15.3
Couscous and Strawberries	247.6	67.9	27.7	343.2	8.9	5.9	14.9
Couscous and Sweet potato	238.7	63.0	7.4	309.1	7.4	5.6	13.1
Pork and Pumpkin	258.6	71.0	32.0	361.6	9.2	6.3	15.6
Pork and Raspberries	282.0	64.1	28.0	374.1	9.0	6.3	15.2
Pork and Shrimp	259.6	67.3	30.7	357.6	9.3	6.4	15.7
Pork and Soybean	260.1	68.3	30.0	358.4	9.3	6.4	15.7
Pork and Split peas	269.1	75.7	33.0	377.9	9.8	6.5	16.3
Pork and Strawberries	290.2	34.6	25.6	350.4	7.6	6.1	13.7
Pork and Sweet potato	278.2	59.3	27.8	365.3	9.2	6.5	15.7
Pumpkin and Raspberries	260.1	77.7	30.3	368.1	9.2	6.6	15.8
Pumpkin and Shrimp	290.1	43.7	28.5	362.3	8.6	7.3	15.8
Pumpkin and Soybean	250.0	74.7	30.7	355.3	9.4	6.5	15.9
Pumpkin and Split peas	258.9	71.0	29.4	359.3	9.2	6.4	15.6
Pumpkin and Strawberries	249.3	76.2	29.3	354.8	9.2	6.4	15.6
Pumpkin and Sweet potato	270.7	61.2	28.7	360.5	8.9	6.2	15.2
Raspberries and Shrimp	268.2	55.8	24.1	348.1	8.9	6.5	15.4
Raspberries and Soybean	255.2	44.5	20.7	320.3	8.9	6.1	14.9
Raspberries and Split peas	256.7	59.2	27.2	343.0	8.4	6.0	14.4
Raspberries and Strawberries	203.8	25.1	19.6	248.4	7.3	6.6	13.9
Raspberries and Sweet potato	229.2	91.1	28.4	348.7	9.3	6.6	15.9
Shrimp and Soybean	272.0	54.8	27.9	354.7	9.1	6.8	15.9
Shrimp and Split peas	260.3	70.9	26.9	358.1	9.1	6.3	15.4
Shrimp and Strawberries	252.1	71.9	30.1	354.0	9.2	6.4	15.6
Shrimp and Sweet potato	247.0	70.5	29.2	346.7	9.2	6.3	15.5
Soybean and Split peas	272.0	72.6	27.8	372.3	8.7	6.2	14.9
Soybean and Strawberries	260.0	72.5	30.5	362.9	9.5	6.6	16.0
Soybean and Sweet potato	260.1	52.0	25.2	337.3	8.3	6.0	14.3
Split peas and Strawberries	255.3	67.9	29.3	352.5	9.2	6.3	15.4
Split peas and Sweet potato	265.0	69.4	26.4	360.8	9.0	6.4	15.4
Strawberries and Sweet potato	251.8	68.3	28.6	348.7	8.9	6.3	15.3

Values are coloured according to intensity for each organic acid, with the highest values in green and the lowest values in red.

Supplementary Table 5.6. Predicted microbial growth rates for multiple food-breastmilk combinations and controls.

Multiple food-breastmilk combination	Growth rate (1/h)									
	Bacillus	***Bacteroides***	***Bifidobacterium***	***Collinsella***	***Lacticaseibacillus***	***Lactobacillus***	***Limosilactobacillus***	***Prevotella***	***Streptococcus***	***Veillonella***
Control breastmilk	0.064	0.068	0.103	0.009		0.005		0.019		
Control infant formula	0.045	0.065	0.080	0.008	0.006	0.004	0.003	0.013	0.003	0.028
Black beans and blackcurrant	0.015	0.022	0.027	0.003	0.004	0.001	0.001	0.004	0.002	0.018
Black beans and chickpea	0.05	0.068	0.086	0.009	0.006	0.004	0.003	0.015	0.001	0.013
Black beans and couscous	0.031	0.045	0.056	0.006	0.008	0.002	0.002	0.009	0.003	0.031
Black beans and pork	0.067	0.067	0.103	0.008		0.004		0.020		
Black beans and pumpkin	0.045	0.060	0.076	0.008	0.005	0.004	0.003	0.014	0.001	0.010
Black beans and raspberries	0.052	0.069	0.087	0.009	0.008	0.004	0.003	0.015	0.001	0.009
Black beans and shrimp	0.056	0.070	0.091	0.008	0.003	0.004	0.003	0.018		
Black beans and soybean	0.072	0.063	0.103	0.007		0.005		0.022		
Black beans and split peas	0.065	0.069	0.103			0.005	0.003	0.020		
Black beans and strawberries	0.148	0.226	0.276	0.030	0.043	0.011	0.011	0.040	0.021	0.184
Black beans and sweet potato	0.055	0.068	0.089	0.009	0.005	0.004	0.003	0.018		0.006
Blackcurrant and chickpea	0.051	0.069	0.087	0.009	0.006	0.004	0.003	0.015	0.001	0.011
Blackcurrant and couscous	0.052	0.070	0.089	0.009	0.005	0.004	0.003	0.015	0.001 <	0.006
Blackcurrant and pork	0.070	0.064	0.103	0.007		0.005	0.002	0.022		
Blackcurrant and pumpkin	0.051	0.069	0.087	0.008	0.004	0.004	0.003	0.015	0.001	0.011
Blackcurrant and raspberries	0.053	0.070	0.089	0.009	0.006	0.004	0.003	0.017		0.005
Blackcurrant and shrimp	0.056	0.070	0.091	0.008	0.006	0.005	0.003	0.017		

Blackcurrant and soybean	0.066	0.077	0.101	0.008		0.005		0.020		
Blackcurrant and split peas	0.043	0.058	0.073	0.008	0.007	0.004	0.003	0.013	0.002	0.015
Blackcurrant and strawberries	0.058	0.069	0.091	0.008		0.005	0.003	0.018		
Blackcurrant and sweet potato	0.082	0.067	0.103	0.004		0.006	0.001	0.026		
Chickpea and couscous	0.076	0.061	0.103	0.006		0.004	0.001	0.023		
Chickpea and pork	0.049	0.068	0.085	0.008	0.007	0.004	0.003	0.015	0.002	0.014
Chickpea and pumpkin	0.051	0.068	0.087	0.009	0.006	0.004	0.003	0.015	0.001	0.011
Chickpea and raspberries	0.044	0.058	0.074	0.008	0.009	0.004	0.003	0.014	0.001 <	0.015
Chickpea and shrimp	0.143	0.197	0.248	0.024	0.014	0.010	0.008	0.040	0.002	0.037
Chickpea and soybean	0.050	0.068	0.086	0.008	0.005	0.004	0.003	0.015	0.001	0.013
Chickpea and split peas	0.020	0.031	0.035	0.004	0.006	0.002	0.001	0.006	0.002	0.024
Chickpea and strawberries	0.214	0.297	0.372	0.036	0.019	0.015	0.012	0.059	0.004	0.065
Chickpea and sweet potato	0.058	0.069	0.091	0.008	0.001	0.005	0.003	0.019		
Couscous and pork	0.050	0.068	0.086	0.008	0.006	0.004	0.003	0.015	0.001	0.012
Couscous and pumpkin	0.044	0.059	0.074	0.008	0.006	0.004	0.003	0.013	0.001	0.013
Couscous and raspberries	0.049	0.067	0.084	0.008	0.006	0.004	0.003	0.014	0.002	0.018
Couscous and shrimp	0.098	0.098	0.152	0.012		0.006	0.003	0.029		
Couscous and soybean	0.063	0.070	0.103			0.005	0.003	0.018		
Couscous and split peas	0.039	0.055	0.069	0.007	0.007	0.003	0.003	0.011	0.003	0.028
Couscous and strawberries	0.057	0.070	0.091	0.008	0.001	0.005	0.003	0.017		
Couscous and sweet potato	0.053	0.071	0.090	0.009	0.003	0.004	0.003	0.015	0.001 <	0.004
Pork and pumpkin	0.053	0.070	0.089	0.009	0.005	0.004	0.003	0.016		0.004
Pork and raspberries	0.065	0.065	0.091	0.006		0.005	0.003	0.021		
Pork and shrimp	0.033	0.047	0.059	0.006	0.009	0.003	0.002	0.010	0.003	0.023
Pork and soybean	0.051	0.069	0.087	0.009	0.006	0.004	0.003	0.015	0.001	0.011
Pork and split peas	0.05	0.068	0.085	0.009	0.007	0.004	0.003	0.015	0.001	0.015
Pork and strawberries	0.057	0.07	0.091	0.008	0.003	0.005	0.003	0.018		
Pork and sweet potato	0.055	0.071	0.091	0.009	0.004	0.004	0.003	0.017		

Pumpkin and raspberries	0.063	0.066	0.091	0.007		0.005	0.002	0.022		
Pumpkin and shrimp	0.032	0.045	0.057	0.006	0.009	0.002	0.002	0.009	0.003	0.029
Pumpkin and soybean	0.053	0.070	0.089	0.009	0.006	0.004	0.003	0.016	0.001 <	0.005
Pumpkin and split peas	0.067	0.069	0.103	0.008		0.005	0.002	0.020		
Pumpkin and strawberries	0.079	0.069	0.100	0.005		0.005		0.026		
Pumpkin and sweet potato	0.086	0.056	0.102	0.003		0.005		0.028		
Raspberries and shrimp	0.060	0.068	0.091	0.008		0.005	0.002	0.019		
Raspberries and soybean	0.052	0.070	0.089	0.009	0.008	0.004	0.003	0.016	0.001 <	0.006
Raspberries and split peas	0.055	0.070	0.090	0.009	0.005	0.004	0.003	0.017		0.001
Raspberries and strawberries	0.052	0.069	0.088	0.009	0.008	0.004	0.003	0.016	0.001 <	0.008
Raspberries and sweet potato	0.058	0.070	0.091	0.008		0.004	0.003	0.018		
Shrimp and soybean	0.038	0.050	0.064	0.007	0.007	0.003	0.002	0.012	0.001	0.010
Shrimp and split peas	0.050	0.068	0.085	0.009	0.007	0.004	0.003	0.015	0.001 <	0.015
Shrimp and strawberries	0.056	0.070	0.091	0.009	0.003	0.004	0.003	0.017		
Shrimp and sweet potato	0.055	0.071	0.091	0.009	0.002	0.004	0.003	0.017		
Soybean and split peas	0.055	0.071	0.091	0.009	0.003	0.004	0.003	0.017		
Soybean and strawberries	0.172	0.238	0.298	0.029	0.013	0.012	0.009	0.048	0.003	0.050
Soybean and sweet potato	0.058	0.069	0.091	0.008	0.001 <	0.005	0.003	0.018		
Split peas and strawberries	0.228	0.317	0.398	0.039	0.013	0.016	0.012	0.062	0.006	0.081
Split peas and sweet potato	0.094	0.051	0.102	0.002		0.004	0.001 <	0.030		
Strawberries and sweet potato	0.059	0.068	0.092	0.008		0.005	0.003	0.019		

Values are coloured according to intensity for each genus, with the highest values in green and the lowest values in red. Blank cells correspond to absent growth.

Supplementary Table 6.1. Preparation of food ingredients.

Ingredient	Preparation	Sous-vide cooking conditions
Black beans	Soak overnight before cooking. Drained and freeze-dried after cooking	7 h, 80 °C
Blackcurrant	No preparation (bought as powder)	No cooking
Chickpeas	Soak overnight before cooking. Drained and freeze-dried after cooking	7 h, 80 °C
Couscous	Soak in water (ratio 1:1). Freeze-dried after cooking	5 min, 80 °C
Infant formula	No preparation (bought as powder)	No cooking
Kūmara	Peeled and not peeled before cooking. Freeze-dried after cooking	4 h, 80 °C
Pork	Minced before cooking. Freeze-dried after cooking	3 h, 60 °C
Prawn	Peeled and tail removed before cooking. Freeze-dried after cooking	3 h, 60 °C
Pumpkin	Peeled before cooking. Freeze-dried after cooking	4 h, 80 °C
Raspberries	No preparation (bought as powder)	No cooking
Soybeans	Soak overnight before cooking. Drained and freeze-dried after cooking	7 h, 80 °C
Strawberries	No preparation (bought as powder)	No cooking
Yellow peas	Soak overnight before cooking. Drained and freeze-dried after cooking	7 h, 80 °C

Supplementary Table 6.2. Compositional analyses of food powder ingredients.

Sample Name	DM %	Ash %	Crude Protein %	Fat %	Carbohydrate %	TDF %	Sugars g/100g	NPN %	Energy kJ/100g	B-Glucan %	Saturated Fat g/100g
Pumpkin	88.1	6.4	9.2	5.4	45.1	22.1	48.5	0.9	1299	ND	0.59
	88.1	6.4	9.8	5.4	44.6	21.9	48.8	0.9	1299	ND	0.60
Kūmara, with skin	95.7	3.6	3.7	1.9	77.2	9.3	52.6	0.2	1520	0.1	0.20
	95.7	3.6	3.8	2.0	76.7	9.6	50.2	0.3	1518	0.1	0.20
Kūmara, without skin	95.3	3.2	3.7	1.2	79.0	8.2	52.1	0.3	1516	< 0.1	0.16
	95.3	3.2	3.8	1.2	79.1	8.0	52.1	0.3	1518	< 0.1	0.16
Black beans	98.3	2.5	28.4	3.4	35.6	28.3	1.4	0.2	1441	ND	0.54
	98.3	2.6	28.9	3.4	33.0	30.4	1.4	0.2	1422	ND	0.53
Chickpeas	98.3	2.4	19.9	9.0	43.6	23.4	1.7	0.1	1602	< 0.1	1.01
	98.3	2.3	20.5	9.0	43.7	22.9	1.6	0.1	1605	< 0.1	1.16
Soybeans	98.3	3.7	39.7	24.0	7.2	23.7	4.4	0.2	1876	0.1	3.76
	98.2	3.8	40.1	24.1	8.4	21.9	4.4	0.2	1890	0.1	3.94
Yellow peas	98.3	1.3	21.1	3.2	52.6	20.1	1.7	0.1	1533	< 0.1	0.40
	98.3	1.3	21.0	3.1	52.1	20.8	1.7	0.1	1524	< 0.1	0.40
Pork	98.7	4.2	83.1	12.4	ND	2.4	< 0.5	1.4	1892	ND	4.29
	98.9	4.2	84.3	12.2	ND	1.6	< 0.5	1.5	1897	ND	4.32
Prawn	96.3	4.9	90.8	4.5	ND	1.1	< 0.1	3.2	1718	< 0.1	0.81
	96.2	4.9	90.9	4.6	ND	1.3	< 0.1	3.3	1724	< 0.1	0.81
Couscous	97.9	0.9	15.2	2.6	74.1	5.1	1.9	0.1	1655	0.2	0.49
	97.9	0.9	15.1	2.6	73.7	5.5	1.9	0.1	1652	0.2	0.49
Blackcurrants	97.7	3.5	3.0	6.0	67.2	18.0	46.1	0.2	1560	ND	0.20
	97.8	3.5	3.4	5.9	67.1	17.9	46.7	0.2	1558	ND	0.20
Raspberries	97.3	3.2	6.4	7.3	66.8	13.6	49.0	0.4	1624	ND	0.32
	97.5	3.2	6.2	7.5	67.3	13.5	49.0	0.4	1632	ND	0.32
Strawberries	97.7	4.9	6.4	4.9	69.4	12.1	54.6	0.6	1567	ND	0.14
	97.4	4.9	6.3	4.9	68.8	12.6	52.0	0.6	1556	ND	0.15

Infant formula	97.7	2.7	8.8	23.3	59.9	2.9	61.4	1.1	2054	ND	1.99
	97.7	2.8	8.6	23.3	60.4	2.7	60.3	1.1	2055	ND	1.91

ND: non-detected

Supplementary Table 6.3. Characteristics of participants donating faecal samples.

Donor ID	Age (months)	Gender
1	11	Female
2	8.5	Female
3	5	Male
4	7	Male
5	6	Male
6	6	Male

Supplementary Table 6.4. Limits of detection of organic acids.

Organic acid	Limit of detection (mmol/L)
Formate	0.8
Acetate	1.9
Propionate	0.3
Isobutyrate	0.1
Butyrate	0.1
Isovalerate	0.2
Valerate	0.1
Hexanoate	0.1
Heptanoate	0.5
Lactate	1.9
Succinate	0.2

Supplementary Table 6.5. Produced organic acids after 24 hours of fermentation of different food ingredients.

Food ingredient	Formate	Acetate	Propionate	Butyrate	Total SCFAs	Isovalerate	Lactate	Succinate
Black beans	$(0.46 \pm 0.24)^{ab}$	$(3.00 \pm 0.68)^{cd}$	$(0.32 \pm 0.11)^{c}$	$(0.04 \pm 0.03)^{ab}$	$(3.36 \pm 0.77)^{cd}$	$(0.08 \pm 0.00)^{d}$	$(1.22 \pm 0.05)^{bc}$	$(0.6 \pm 0.15)^{b}$
Blackcurrant	$(0.86 \pm 0.24)^{ab}$	$(6.21 \pm 0.93)^{a}$	$(0.75 \pm 0.12)^{a}$	$(0.08 \pm 0.02)^{ab}$	$(7.04 \pm 1.00)^{a}$	$(0.13 \pm 0.02)^{ab}$	$(0.82 \pm 0.20)^{bcd}$	$(0.96 \pm 0.36)^{ab}$
Chickpea	$(0.6 \pm 0.09)^{ab}$	$(2.31 \pm 0.44)^{d}$	$(0.31 \pm 0.09)^{c}$	$(0.00 \pm 0.00)^{b}$	$(2.63 \pm 0.51)^{d}$	$(0.08 \pm 0.01)^{d}$	$(0.33 \pm 0.04)^{d}$	$(0.67 \pm 0.21)^{ab}$
Couscous	$(1.05 \pm 0.64)^{a}$	$(3.67 \pm 0.88)^{bcd}$	$(0.40 \pm 0.09)^{bc}$	$(0.01 \pm 0.01)^{ab}$	$(4.09 \pm 0.94)^{bcd}$	$(0.10 \pm 0.02)^{bcd}$	$(0.84 \pm 0.35)^{bcd}$	$(0.6 \pm 0.44)^{ab}$
Infant formula	$(1.06 \pm 0.03)^{a}$	$(2.76 \pm 0.63)^{cd}$	$(0.33 \pm 0.25)^{c}$	$(0.01 \pm 0.01)^{ab}$	$(3.1 \pm 0.84)^{cd}$	$(0.09 \pm 0.01)^{d}$	$(0.51 \pm 0.10)^{cd}$	$(0.56 \pm 0.29)^{b}$
Kūmara peeled	$(0.2 \pm 0.1)^{b}$	$(2.46 \pm 0.46)^{d}$	$(0.21 \pm 0.02)^{c}$	$(0.01 \pm 0.00)^{ab}$	$(2.67 \pm 0.48)^{d}$	$(0.09 \pm 0.01)^{cd}$	$(2.18 \pm 0.43)^{a}$	$(0.40 \pm 0.27)^{b}$
Kūmara with skin	$(0.41 \pm 0.12)^{ab}$	$(2.80 \pm 0.47)^{cd}$	$(0.23 \pm 0.06)^{c}$	$(0.15 \pm 0.17)^{a}$	$(3.18 \pm 0.48)^{cd}$	$(0.09 \pm 0.01)^{cd}$	$(1.48 \pm 0.29)^{ab}$	$(0.66 \pm 0.25)^{ab}$
Pork	$(0.8 \pm 0.28)^{ab}$	$(2.85 \pm 0.51)^{cd}$	$(0.37 \pm 0.14)^{bc}$	$(0.01 \pm 0.01)^{ab}$	$(3.24 \pm 0.63)^{cd}$	$(0.10 \pm 0.01)^{cd}$	$(0.16 \pm 0.04)^{d}$	$(1.01 \pm 0.31)^{ab}$
Prawn	$(0.7 \pm 0.07)^{ab}$	$(2.97 \pm 0.44)^{cd}$	$(0.41 \pm 0.03)^{bc}$	$(0.01 \pm 0.00)^{ab}$	$(3.39 \pm 0.45)^{cd}$	$(0.09 \pm 0.00)^{cd}$	$(0.31 \pm 0.13)^{d}$	$(0.77 \pm 0.06)^{ab}$
Pumpkin	$(0.79 \pm 0.12)^{ab}$	$(3.58 \pm 0.74)^{bcd}$	$(0.35 \pm 0.13)^{c}$	$(0.01 \pm 0.01)^{ab}$	$(3.94 \pm 0.88)^{cd}$	$(0.10 \pm 0.01)^{abcd}$	$(0.49 \pm 0.21)^{cd}$	$(0.74 \pm 0.09)^{ab}$
Raspberries	$(1.01 \pm 0.09)^{a}$	$(4.55 \pm 0.82)^{abc}$	$(0.42 \pm 0.04)^{bc}$	$(0.08 \pm 0.04)^{ab}$	$(5.05 \pm 0.86)^{abc}$	$(0.14 \pm 0.02)^{a}$	$(0.41 \pm 0.17)^{d}$	$(0.52 \pm 0.07)^{b}$
Soybean	$(0.91 \pm 0.15)^{a}$	$(2.93 \pm 0.61)^{cd}$	$(0.39 \pm 0.06)^{bc}$	$(0.01 \pm 0.01)^{ab}$	$(3.34 \pm 0.67)^{cd}$	$(0.09 \pm 0.00)^{cd}$	$(0.21 \pm 0.14)^{d}$	$(1.01 \pm 0.21)^{ab}$
Strawberries	$(0.73 \pm 0.16)^{ab}$	$(5.51 \pm 0.68)^{ab}$	$(0.69 \pm 0.09)^{ab}$	$(0.04 \pm 0.01)^{ab}$	$(6.23 \pm 0.75)^{ab}$	$(0.12 \pm 0.02)^{abc}$	$(0.62 \pm 0.15)^{cd}$	$(1.41 \pm 0.29)^{a}$
Yellow peas	$(0.64 \pm 0.1)^{ab}$	$(3.79 \pm 0.69)^{bcd}$	$(0.49 \pm 0.03)^{abc}$	$(0.03 \pm 0.02)^{ab}$	$(4.3 \pm 0.70)^{bcd}$	$(0.09 \pm 0.01)^{d}$	$(0.95 \pm 0.64)^{bcd}$	$(0.70 \pm 0.42)^{ab}$

Results are expressed as mmol/g (dry weight) of the theoretical fermented mass of each sample. Values correspond to mean and standard deviation, respectively. Superscript letters indicate significant differences between samples determined by Tukey's HSD test with a 95 % confidence level. Total SCFAs corresponds to acetate, propionate, and butyrate altogether.

Supplementary Table 6.6. Produced organic acids after 24 hours of fermentation of different food ingredients combined with infant formula.

Food-formula combination	Formate	Acetate	Propionate	Butyrate	Total SCFAs	Isovalerate	Lactate	Succinate
Black beans and formula	(0.67 ± 0.1)^{a}	(1.68 ± 0.19)^{ab}	(0.23 ± 0.03)^{ab}	(0.16 ± 0.05)^{a}	(2.07 ± 0.25)^{ab}	(0.10 ± 0.00)^{a}	(0.32 ± 0.10)^{a}	(0.36 ± 0.05)^{a}
Blackcurrant and formula	(0.85 ± 0.14)^{a}	(2.46 ± 0.47)^{a}	(0.35 ± 0.11)^{a}	(0.00 ± 0.00)^{b}	(2.82 ± 0.57)^{a}	(0.12 ± 0.01)^{a}	(0.48 ± 0.18)^{a}	(0.57 ± 0.13)^{a}
Chickpea and formula	(0.73 ± 0.14)^{a}	(2.00 ± 0.41)^{ab}	(0.26 ± 0.09)^{ab}	(0.00 ± 0.00)^{b}	(2.26 ± 0.51)^{ab}	(0.10 ± 0.01)^{a}	(0.54 ± 0.08)^{a}	(0.38 ± 0.12)^{a}
Couscous and formula	(0.56 ± 0.22)^{a}	(1.74 ± 0.19)^{ab}	(0.19 ± 0.03)^{ab}	(0.00 ± 0.00)^{b}	(1.93 ± 0.21)^{ab}	(0.14 ± 0.07)^{a}	(0.47 ± 0.32)^{a}	(0.46 ± 0.17)^{a}
Kūmara peeled and formula	(1.1 ± 0.24)^{a}	(2.34 ± 0.37)^{ab}	(0.27 ± 0.10)^{ab}	(0.00 ± 0.00)^{b}	(2.61 ± 0.40)^{ab}	(0.12 ± 0.01)^{a}	(0.40 ± 0.06)^{a}	(0.42 ± 0.08)^{a}
Kūmara with skin and formula	(0.57 ± 0.24)^{a}	(1.61 ± 0.34)^{ab}	(0.21 ± 0.10)^{ab}	(0.01 ± 0.01)^{b}	(1.82 ± 0.44)^{ab}	(0.11 ± 0.03)^{a}	(0.55 ± 0.23)^{a}	(0.31 ± 0.07)^{a}
Pork and formula	(0.65 ± 0.08)^{a}	(1.57 ± 0.31)^{ab}	(0.26 ± 0.07)^{ab}	(0.05 ± 0.08)^{ab}	(1.88 ± 0.46)^{ab}	(0.08 ± 0.02)^{a}	(0.36 ± 0.14)^{a}	(0.40 ± 0.14)^{a}
Prawn and formula	(0.77 ± 0.08)^{a}	(1.60 ± 0.38)^{ab}	(0.19 ± 0.06)^{ab}	(0.03 ± 0.03)^{ab}	(1.82 ± 0.46)^{ab}	(0.10 ± 0.01)^{a}	(0.24 ± 0.08)^{a}	(0.32 ± 0.12)^{a}
Pumpkin and formula	(1.1 ± 0.12)^{a}	(2.36 ± 0.31)^{a}	(0.30 ± 0.10)^{ab}	(0.00 ± 0.00)^{b}	(2.65 ± 0.40)^{ab}	(0.12 ± 0.01)^{a}	(0.46 ± 0.06)^{a}	(0.48 ± 0.14)^{a}
Raspberries and formula	(0.96 ± 0.53)^{a}	(2.35 ± 0.60)^{a}	(0.25 ± 0.04)^{ab}	(0.06 ± 0.11)^{ab}	(2.66 ± 0.74)^{ab}	(0.12 ± 0.02)^{a}	(0.35 ± 0.12)^{a}	(0.46 ± 0.10)^{a}
Soybean and formula	(0.72 ± 0.22)^{a}	(1.31 ± 0.19)^{b}	(0.13 ± 0.07)^{b}	(0.04 ± 0.05)^{ab}	(1.49 ± 0.20)^{b}	(0.10 ± 0.00)^{a}	(0.18 ± 0.08)^{a}	(0.28 ± 0.06)^{a}
Strawberries and formula	(0.95 ± 0.08)^{a}	(2.42 ± 0.27)^{a}	(0.37 ± 0.05)^{a}	(0.00 ± 0.00)^{b}	(2.79 ± 0.32)^{a}	(0.12 ± 0.00)^{a}	(0.40 ± 0.05)^{a}	(0.54 ± 0.06)^{a}
Yellow peas and formula	(0.81 ± 0.05)^{a}	(1.59 ± 0.2)^{ab}	(0.13 ± 0.04)^{b}	(0.05 ± 0.06)^{ab}	(1.77 ± 0.26)^{ab}	(0.11 ± 0.00)^{a}	(0.21 ± 0.05)^{a}	(0.28 ± 0.08)^{a}

Results are expressed as mmol/g (dry weight) of the theoretical fermented mass of each sample. Values correspond to mean and standard deviation, respectively. Superscript letters indicate significant differences between samples determined by Tukey's HSD test with a 95 % confidence level. Total SCFAs corresponds to acetate, propionate, and butyrate altogether.

Supplementary Table 6.7. Produced organic acids after 24 hours of fermentation of different food ingredients combined with other foods.

Food-food combination	Formate	Acetate	Propionate	Butyrate	Total SCFAs	Isovalerate	Lactate	Succinate
Black beans and blackcurrant	(0.71 ± 0.07)^{a}	(3.99 ± 0.82)^{a}	(0.44 ± 0.05)^{a}	(0.14 ± 0.05)^{a}	(4.57 ± 0.88)^{a}	(0.11 ± 0.01)^{a}	(0.38 ± 0.35)^{abc}	(0.55 ± 0.17)^{ab}
Black beans and pumpkin	(0.96 ± 0.24)^{a}	(3.56 ± 0.58)^{a}	(0.39 ± 0.04)^{a}	(0.01 ± 0.00)^{b}	(3.96 ± 0.59)^{a}	(0.11 ± 0.01)^{a}	(0.62 ± 0.07)^{abc}	(0.59 ± 0.22)^{ab}
Blackcurrant and kūmara peeled	(0.88 ± 0.12)^{a}	(4.35 ± 0.75)^{a}	(0.52 ± 0.06)^{a}	(0.08 ± 0.04)^{ab}	(4.96 ± 0.79)^{a}	(0.13 ± 0.02)^{a}	(0.82 ± 0.30)^{abc}	(0.56 ± 0.16)^{ab}
Blackcurrant and kūmara with skin	(0.71 ± 0.06)^{a}	(3.62 ± 0.34)^{a}	(0.38 ± 0.05)^{a}	(0.02 ± 0.01)^{b}	(4.01 ± 0.35)^{a}	(0.13 ± 0.01)^{a}	(0.91 ± 0.25)^{ab}	(0.59 ± 0.09)^{ab}
Blackcurrant and pork	(0.91 ± 0.1)^{a}	(4.12 ± 0.31)^{a}	(0.53 ± 0.07)^{a}	(0.04 ± 0.02)^{b}	(4.69 ± 0.30)^{a}	(0.12 ± 0.02)^{a}	(0.37 ± 0.07)^{abc}	(0.86 ± 0.16)^{ab}
Blackcurrant and soybean	(1.26 ± 0.35)^{a}	(3.96 ± 0.58)^{a}	(0.47 ± 0.05)^{a}	(0.02 ± 0.02)^{b}	(4.44 ± 0.60)^{a}	(0.13 ± 0.01)^{a}	(0.34 ± 0.09)^{bc}	(0.94 ± 0.23)^{ab}
Blackcurrant and strawberries	(1.03 ± 0.42)^{a}	(5.11 ± 0.76)^{a}	(0.59 ± 0.13)^{a}	(0.03 ± 0.02)^{b}	(5.73 ± 0.76)^{a}	(0.12 ± 0.04)^{a}	(0.54 ± 0.15)^{abc}	(1.10 ± 0.30)^{a}
Chickpea and pork	(1.05 ± 0.35)^{a}	(3.05 ± 1.04)^{a}	(0.37 ± 0.14)^{a}	(0.06 ± 0.05)^{ab}	(3.48 ± 1.22)^{a}	(0.10 ± 0.02)^{a}	(0.25 ± 0.20)^{c}	(0.70 ± 0.27)^{ab}
Chickpea and yellow peas	(1.11 ± 0.14)^{a}	(3.74 ± 0.71)^{a}	(0.49 ± 0.17)^{a}	(0.01 ± 0.01)^{b}	(4.24 ± 0.88)^{a}	(0.11 ± 0.01)^{a}	(0.66 ± 0.19)^{abc}	(0.77 ± 0.07)^{ab}
Couscous and pork	(0.87 ± 0.41)^{a}	(3.11 ± 0.91)^{a}	(0.48 ± 0.09)^{a}	(0.08 ± 0.06)^{ab}	(3.67 ± 0.85)^{a}	(0.09 ± 0.02)^{a}	(0.37 ± 0.10)^{abc}	(0.65 ± 0.17)^{ab}
Couscous and pumpkin	(1.31 ± 0.23)^{a}	(4.75 ± 0.71)^{a}	(0.47 ± 0.08)^{a}	(0.02 ± 0.01)^{b}	(5.24 ± 0.80)^{a}	(0.12 ± 0.01)^{a}	(0.93 ± 0.16)^{a}	(0.80 ± 0.08)^{ab}
Pork and raspberries	(0.88 ± 0.24)^{a}	(4.55 ± 0.84)^{a}	(0.50 ± 0.14)^{a}	(0.06 ± 0.04)^{ab}	(5.10 ± 0.98)^{a}	(0.11 ± 0.04)^{a}	(0.35 ± 0.11)^{abc}	(0.85 ± 0.25)^{ab}
Pumpkin and yellow peas	(0.68 ± 0.14)^{a}	(3.28 ± 0.40)^{a}	(0.37 ± 0.00)^{a}	(0.02 ± 0.00)^{b}	(3.67 ± 0.40)^{a}	(0.09 ± 0.01)^{a}	(0.57 ± 0.24)^{abc}	(0.53 ± 0.12)^{b}

Results are expressed as mmol/g (dry weight) of the theoretical fermented mass of each sample. Values correspond to mean and standard deviation, respectively. Superscript letters indicate significant differences between samples determined by Tukey's HSD test with a 95 % confidence level. Total SCFAs corresponds to acetate, propionate, and butyrate altogether.

Supplementary Table 6.8. Produced organic acids after 24 hours of fermentation of different food ingredients combined with other foods and infant formula

Food-food-formula combination	Formate	Acetate	Propionate	Butyrate	Total SCFAs	Isovalerate	Lactate	Succinate
Black beans and blackcurrant and formula	(1.06 ± 0.27)^{ab}	(2.20 ± 0.80)^{ab}	(0.24 ± 0.14)^{a}	(0.08 ± 0.09)^{a}	(2.52 ± 0.95)^{ab}	(0.08 ± 0.01)^{ab}	(0.27 ± 0.15)^{a}	(0.34 ± 0.18)^{b}
Black beans and pumpkin and formula	(1.06 ± 0.13)^{ab}	(2.61 ± 0.75)^{ab}	(0.30 ± 0.04)^{a}	(0.03 ± 0.03)^{a}	(2.94 ± 0.76)^{ab}	(0.09 ± 0.01)^{ab}	(0.47 ± 0.20)^{a}	(0.53 ± 0.15)^{ab}
Blackcurrant and Kūmara peeled and formula	(1.29 ± 0.2)^{ab}	(2.99 ± 0.42)^{ab}	(0.34 ± 0.09)^{a}	(0.07 ± 0.11)^{a}	(3.40 ± 0.45)^{ab}	(0.09 ± 0.00)^{ab}	(0.37 ± 0.24)^{a}	(0.59 ± 0.13)^{ab}
Blackcurrant and kūmara with skin and formula	(1.51 ± 0.19)^{a}	(2.42 ± 0.76)^{ab}	(0.18 ± 0.11)^{a}	(0.06 ± 0.08)^{a}	(2.66 ± 0.80)^{ab}	(0.09 ± 0.01)^{ab}	(0.62 ± 0.23)^{a}	(0.40 ± 0.17)^{ab}
Blackcurrant and pork and formula	(1.14 ± 0.19)^{ab}	(2.31 ± 0.29)^{ab}	(0.28 ± 0.10)^{a}	(0.13 ± 0.03)^{a}	(2.71 ± 0.37)^{ab}	(0.09 ± 0.01)^{ab}	(0.35 ± 0.19)^{a}	(0.47 ± 0.09)^{ab}
Blackcurrant and soybean and formula	(0.93 ± 0.15)^{ab}	(2.42 ± 0.57)^{ab}	(0.38 ± 0.18)^{a}	(0.01 ± 0.01)^{a}	(2.81 ± 0.74)^{ab}	(0.09 ± 0.01)^{ab}	(0.34 ± 0.01)^{a}	(0.51 ± 0.21)^{ab}
Blackcurrant and strawberries and formula	(1.56 ± 0.25)^{a}	(3.95 ± 1.02)^{a}	(0.51 ± 0.12)^{a}	(0.01 ± 0.00)^{a}	(4.47 ± 1.14)^{a}	(0.11 ± 0.04)^{a}	(0.72 ± 0.45)^{a}	(0.83 ± 0.19)a
Chickpea and pork and formula	(1.38 ± 0.27)^{ab}	(2.74 ± 0.78)^{ab}	(0.30 ± 0.13)^{a}	(0.00 ± 0.00)^{a}	(3.05 ± 0.91)^{ab}	(0.10 ± 0.01)^{ab}	(0.59 ± 0.35)^{a}	(0.57 ± 0.24)^{ab}
Chickpea and yellow peas and formula	(0.81 ± 0.31)^{b}	(2.04 ± 0.38)^{b}	(0.25 ± 0.06)^{a}	(0.00 ± 0.00)^{a}	(2.29 ± 0.44)^{b}	(0.07 ± 0.01)^{b}	(0.52 ± 0.21)^{a}	(0.54 ± 0.09)^{ab}
Couscous and pork and formula	(1.59 ± 0.23)^{a}	(2.73 ± 0.25)^{ab}	(0.27 ± 0.07)^{a}	(0.05 ± 0.06)^{a}	(3.06 ± 0.28)^{ab}	(0.11 ± 0.01)^{ab}	(0.41 ± 0.12)^{a}	(0.48 ± 0.04)^{ab}
Couscous and pumpkin and formula	(1.3 ± 0.21)^{ab}	(3.40 ± 0.27)^{ab}	(0.39 ± 0.15)^{a}	(0.02 ± 0.02)^{a}	(3.81 ± 0.33)^{ab}	(0.10 ± 0.01)^{ab}	(0.89 ± 0.33)^{a}	(0.54 ± 0.12)^{ab}
Pork and raspberries and formula	(1.3 ± 0.27)^{ab}	(3.13 ± 0.30)^{ab}	(0.43 ± 0.12)^{a}	(0.05 ± 0.07)^{a}	(3.60 ± 0.29)^{ab}	(0.10 ± 0.01)^{ab}	(0.56 ± 0.26)^{a}	(0.69 ± 0.11)^{ab}
Pumpkin and yellow peas and formula	(0.98 ± 0.3)^{ab}	(2.43 ± 0.83)^{ab}	(0.30 ± 0.07)^{a}	(0.00 ± 0.00)^{a}	(2.73 ± 0.90)^{ab}	(0.09 ± 0.01)^{ab}	(0.36 ± 0.18)^{a}	(0.46 ± 0.10)^{ab}

Results are expressed as mmol/g (dry weight) of the theoretical fermented mass of each sample. Values correspond to mean and standard deviation, respectively. Superscript letters indicate significant differences between samples determined by Tukey's HSD test with a 95 % confidence level. Total SCFAs corresponds to acetate, propionate, and butyrate altogether.

Supplementary Table 7.1. Overview of food ingredients used in the experimental study and *in silico* simulations.

Food ingredient	Description *in vitro*	Description *in silico*
Black beans	Dried grains of turtle black beans. Soaked in water, sous vide-cooked, drained, and freeze-dried	Beans, black turtle, mature seeds, cooked, boiled, without salt
Blackcurrants	Freeze-dried New Zealand-grown blackcurrants	Currants, european black, raw
Chickpeas	Dried grains of chickpeas (garbanzo beans). Soaked in water, sous vide-cooked, drained, and freeze-dried	Chickpeas (garbanzo beans, bengal gram), mature seeds, cooked, boiled, without salt
Couscous	Medium-sized grains of dried couscous (Durum wheat). Soaked and freeze-dried	Couscous, cooked
Infant formula	Nestlé NAN SUPREMEpro 2	Infant formula, NESTLE, GOOD START SUPREME, with iron, powder
Kūmara with skin	Fresh red kūmara. Sous-vide cooked and freeze-dried	Sweet potato, cooked, baked in skin, flesh, without salt
Kūmara without skin	Fresh red kūmara. Peeled, sous-vide cooked, and freeze-dried	Sweet potato, cooked, boiled, without skin
Pork	Fresh lean pork fillet (tenderloin). Minced, sous-vide cooked, and freeze-dried	Pork, fresh, loin, tenderloin, separable lean only, cooked, roasted
Prawn	Fresh Australian prawn. Peeled and tail removed, sous-vide cooked, and freeze-dried	Crustaceans, shrimp, mixed species, cooked, moist heat (may have been previously frozen)
Pumpkin	Fresh crown pumpkin. Peeled, sous-vide cooked, and freeze-dried	Pumpkin, cooked, boiled, drained, without salt
Raspberries	Freeze-dried New Zealand-grown raspberries	Raspberries, raw
Soybeans	Dehulled grains of soybeans. Soaked in water, sous vide-cooked, drained, and freeze-dried	Soybeans, mature cooked, boiled, without salt
Strawberries	Freeze-dried New Zealand-grown strawberries	Strawberries, raw
Yellow peas	Dried grains of yellow peas. Soaked in water, sous vide-cooked, drained, and freeze-dried	Peas, split, mature seeds, cooked, boiled, without salt

Supplementary Table 7.2. Relative abundance of microbial genera in *in silico* simulations.

Genus	Relative Abundance
Bacteroides	0.098840904
Escherichia	0.126670202
Bifidobacterium	0.314637515
Enterococcus	0.02414566
Klebsiella	0.016808687
Streptococcus	0.062634146
Salmonella	0.00198801
Hafnia	0.014599439
Collinsella	0.033465417
Enterobacter	0.001040013
Hungatella	0.024423848
Lacticaseibacillus	0.007332542
Akkermansia	0.013579309
Blautia	0.021549269
Parabacteroides	0.001054581
Ruminococcus	0.058628858
Anaeroglobus	0.020301747
Veillonella	0.065238355
Eubacterium	0.001421595
Clostridium	0.004486061
Pluralibacter	0.001778481
Eggerthella	0.000657763
Citrobacter	0.001246069
Megasphaera	0.006877301
Sellimonas	0.004216371
Raoultella	0.001199521
Sarcina	0.000658116
Kosakonia	0.002237159
Lachnoclostridium	0.002976097
Fusobacterium	0.013201995
Lactococcus	0.000294217
Lactobacillus	0.001008204
Intestinibacter	0.00086026
Anaerostipes	0.000876635
Erysipelatoclostridium	0.000609896
Faecalibacterium	0.001644026
Pseudomonas	0.001840693
Morganella	0.000472596
Bilophila	0.00019372
Varibaculum	9.38×10^{-5}

Actinomyces	0.000141696
Phascolarctobacterium	0.000716992
Flavonifractor	0.000125719
Caproiciproducens	0.000289309
Dysgonomonas	0.000244747
Lactiplantibacillus	1.40×10^{-5}
Turicibacter	4.17×10^{-5}
Staphylococcus	2.02×10^{-5}
Incertae Sedis	0.000219354
Arcanobacterium	7.07×10^{-6}
Campylobacter	3.00×10^{-5}
Rothia	1.69×10^{-5}
Haemophilus	3.08×10^{-5}
Monoglobus	3.40×10^{-5}
Buttiauxella	7.75×10^{-6}

Supplementary Table 7.3. Shapiro-Wilk normality test for food ingredients and food-food samples.

Analyte	Shapiro-Wilk test statistic (difference between z-scores)	*p*-value
Acetate	0.9828	0.3493
Propionate	0.9756	0.1249
Butyrate	0.9729	0.0836
Total SCFAs	0.9589	0.0109

Appendix 3. GitHub pages for modelling tools

microPop

Model repository: https://github.com/HelenKettle/microPop

Tutorial: https://cran.r-project.org/web/packages/microPop/vignettes/microPopTutorial.html

microPopGut

Model repository: https://github.com/HelenKettle/microPopGut

Tutorial: https://github.com/HelenKettle/microPopGut/blob/main/getStartedWithMicroPopGut.pdf

BacArena

Model repository: https://github.com/euba/BacArena

Tutorial: https://cran.r-project.org/web/packages/BacArena/vignettes/BacArena-Introduction.pdf

Website: https://bacarena.github.io/

The COBRA Toolbox

Model repository: https://github.com/opencobra/cobratoolbox

Tutorials: https://opencobra.github.io/cobratoolbox/latest/tutorials/index.html

Community forum: https://groups.google.com/g/cobra-toolbox

Website: https://opencobra.github.io/cobratoolbox/latest/index.html

MICOM

Model repository: https://github.com/micom-dev/micom

Tutorials: https://micom-dev.github.io/micom/high_level.html

Community forum: https://github.com/micom-dev/micom/discussions

Website: https://micom-dev.github.io/micom/index.html

Appendix 4. Modelling sensitivity analyses

To evaluate the numerical stability of the simulations, two sensitivity analyses were performed. Initially, simulations were conducted under identical conditions using three different solvers: OSQP (default open-source solver included in MICOM), and the commercial solvers CPLEX (IBM ILOG, version 22.1) and GUROBI (Gurobi Optimization, version 10.0.1), which have superior accuracy and speed. The influence of a breastmilk diet on the colonic microbiota of weaning infants was simulated using a trade-off parameter of 0.7. Dietary fluxes were designed using the VMH database with a total caloric intake of 608 kcal/day. The AGORA2 metabolic reconstructions were employed along with faecal microbial abundance from weaning infants, filtered to include only genera with at least 0.01 relative abundance. The simulation code is available at: https://github.com/vgenisel/Foods-to-optimise-the-colonic-microbiome-for-our-lifelong-health-and-wellbeing-PhD-thesis/tree/main/Chapter%205.

Microbial growth rate profiles obtained with CPLEX and OSQP were characterised by a more homogeneous distribution of growth across taxa. In turn, simulations using GUROBI resulted in the overgrowth of the *Bacillus* genus at the expense of the other bacterial genera (Figure 1). More concerningly, growth rates varied by several orders of magnitude across the tested solvers. For instance, the genera *Lacticaseibacillus* and *Streptococcus* had negligible growth under the OSQP solver but active growth under CPLEX and GUROBI, suggesting numerical instabilities in the simulations.

To evaluate the influence of resource limitation and further assess numerical stability, the medium composition was perturbed by multiplying all uptake bounds by two scalar values: 0.95 (reduced medium) and 1.05 (increased medium). Simulations were conducted as described above, but only with the CPLEX solver. In a numerically stable system, microbial growth rates are expected to scale proportionally with the 5 % change in dietary lower bounds. However, this was not observed (Figure 2). For instance, the genera *Bacillus*, *Lactobacillus*, and *Prevotella* had negligible increased growth under the increased medium. On the other hand, *Streptococcus* and *Veillonella* showed minimal response to the reduced medium but showed increased growth of almost 15 % (3 times the expected) in the increased medium, confirming the presence of numerical instabilities in the simulations.

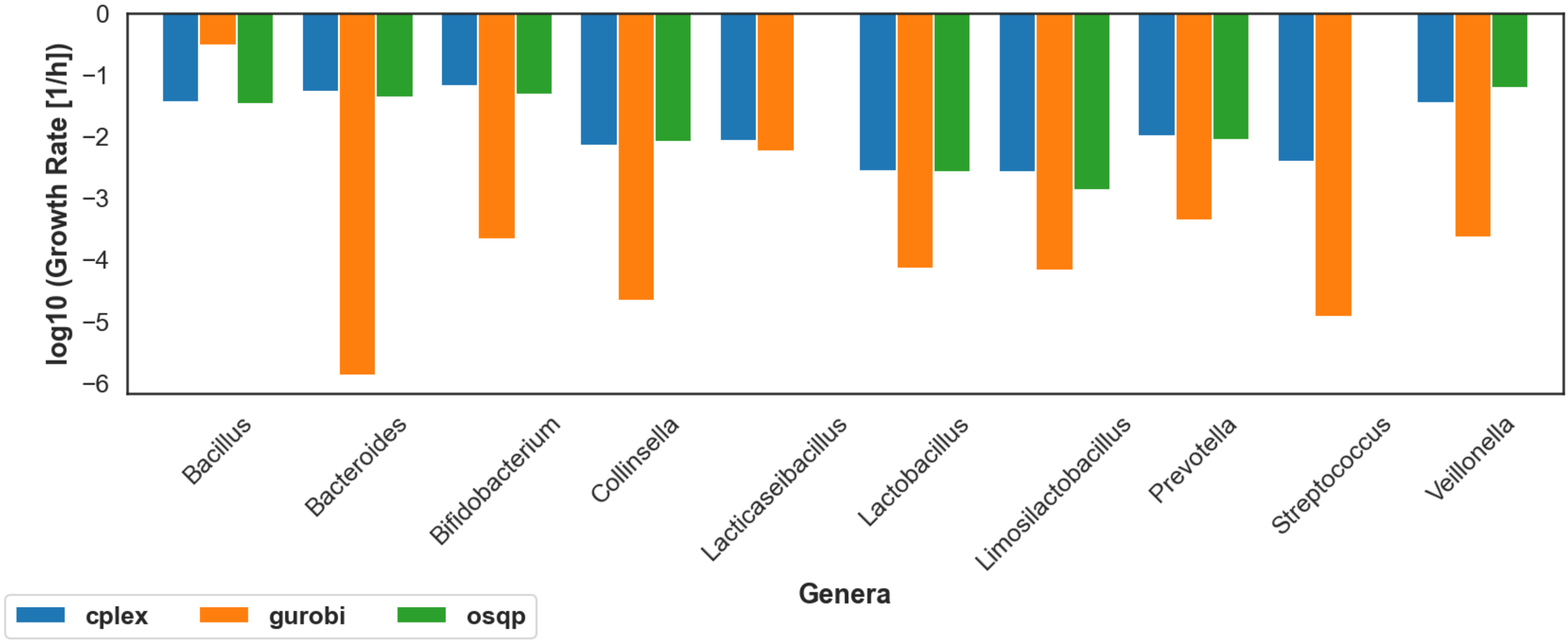


Figure 1. Comparison of bacterial genera growth rates under three different solvers. Microbial growth rates were predicted using the MICOM tool under the influence of a breastmilk diet on the colonic microbiota of weaning infants. Dietary fluxes were designed using the VMH database with a total caloric intake of 608 kcal/day. AGORA2 metabolic reconstructions, faecal microbial abundance from weaning infants (at the genus level), and the solvers OSQP (default version), GUROBI (version 10.0.1), and CPLEX (version 22.1), were used.

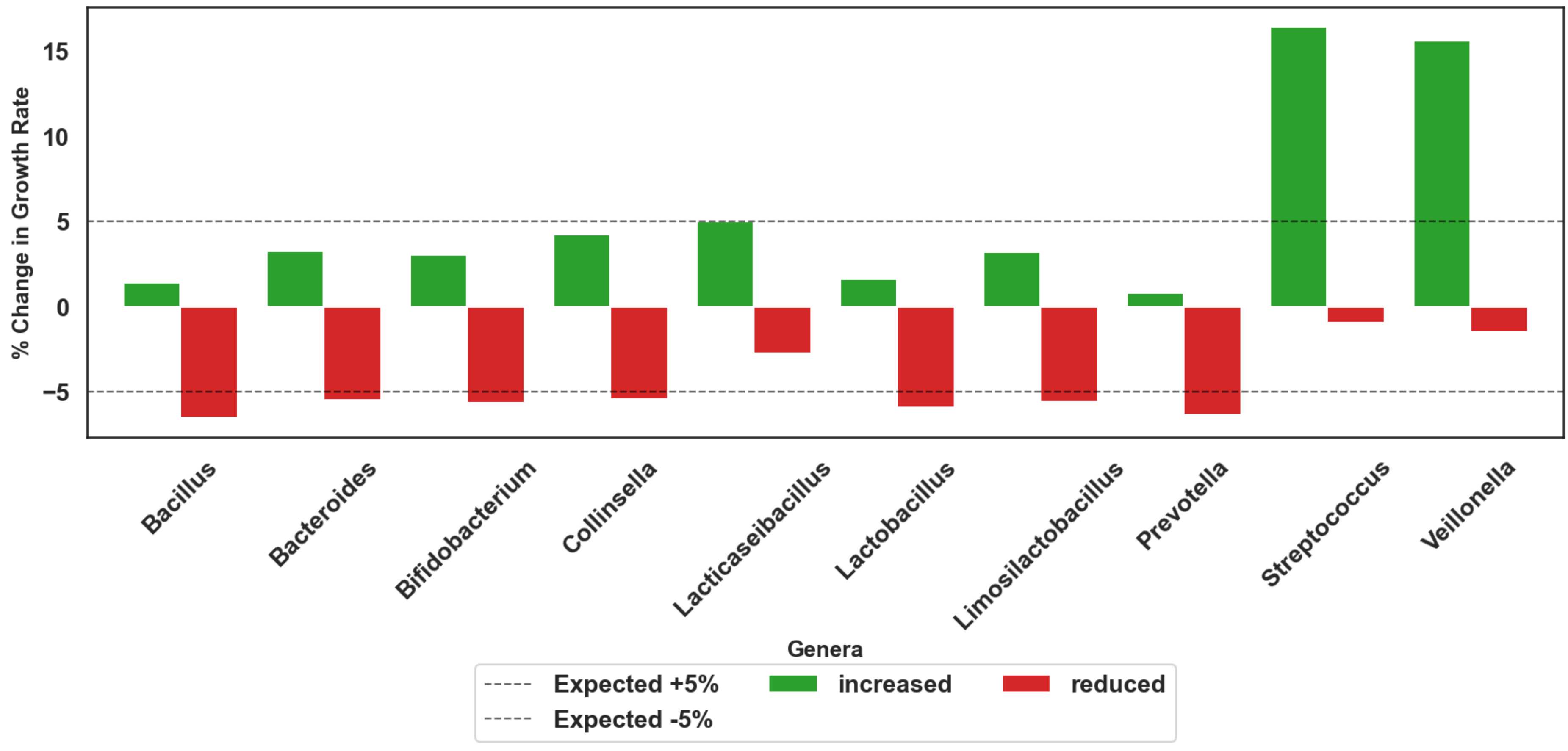


Figure 2. Comparison of bacterial genera growth rates under perturbed uptake bounds. Microbial growth rates were predicted using the MICOM tool under the influence of a breastmilk diet on the colonic microbiota of weaning infants. Dietary fluxes were designed using the VMH database with a total caloric intake of 608 kcal/day and then multiplied by scalar values of 0.95 and 1.05 to represent 5 % changes in the simulation lower bounds. AGORA2 metabolic reconstructions, faecal microbial abundance from weaning infants (at the genus level), and the solver CPLEX (version 22.1) were used.

Appendix 5. Infant formula composition

The information below corresponds to the infant formula Nestlé NAN SUPREMEpro 2, which was extracted from https://www.nestlefamilynes.com.au/nan-infant-formulas/nan-supremepro-2 [Accessed September 17, 2025].

Ingredients: Lactose (Milk), Vegetable Oils, Enzymatically Hydrolysed Whey Protein (Milk), Minerals (Calcium Glycerophosphate, Calcium Phosphate, Potassium Citrate, Magnesium Chloride, Calcium Chloride, Sodium Chloride, Ferrous Sulphate, Zinc Sulphate, Copper Sulphate, Manganese Sulphate, Potassium Iodide, Sodium Selenate), Oligosaccharides (2'0-Fucosyllactose (2'-FL), Lacto-N-Neotetraose (LNnT) (Milk)), Omega LCPUFAs (DHA from Fish Oil, AA), Acidity Regulator (Citric Acid), L-Phenylalanine, Vitamins (Sodium Ascorbate (Vit C), Calcium Pantothenate (Vit B5), Niacin, dl-Alpha-Tocopheryl Acetate (Vit E), Riboflavin (Vit B2), Retinyl Acetate (Vit A), Thiamine Mononitrate (Vit B1), Pyridoxine Hydrochloride (Vit B6), Folic Acid, Phylloquinone (Vit K1), Biotin, Cholecalciferol (Vit D3), Cyanocobalamin (Vit B12)), L-Histidine, L-Tyrosine, L-valine, Antioxidants (Mixed Tocopherols Concentrate, Ascorbyl Palmitate), Culture (*Bifidobacterium Lactis*)

Nutritional information:

Nutrient	**Amount per 100 mL of prepared formula***
Energy	281 kJ
Calories	67 kcal
Protein (100 % Whey (partially hydrolysed))	1.3 g
Fat	3.1 g
DHA (Omega 3)	5.7 mg
AA (Omega 6)	5.7 mg
Carbohydrate	8.4 g
Vitamins	
Vitamin A	68 µg
Vitamin B6	49 µg
Vitamin B12	0.2 µg
Vitamin C	9.6 mg
Vitamin D	0.8 µg
Vitamin E	1.2 mg
Vitamin K	4.2 µg
Biotin	1.4 µg
Niacin	0.7 mg
Folate	11 µg
Pantothenic Acid	711 µg
Riboflavin	137 µg
Thiamin	66 µg
Minerals	
Calcium	69 mg
Copper	53 µg
Iodine	8.9 µg

Iron	1 mg
Magnesium	7.5 mg
Manganese	12 mg
Phosphorus	41 µg
Selenium	2.2 mg
Zinc	0.6 µg
Chloride	53 mg
Potassium	72 mg
Sodium	23 mg
Oligosaccharides	
Oligosaccharide 2' -FL	26 mg
Oligosaccharide LNnT	13 mg

[*]One litre of preparation corresponds to 137 g of infant formula powder diluted in 900 mL of water.

Appendix 6. Advertisement for recruiting participants

LOOKING FOR INFANT PARTICIPANTS

Riddet Institute researchers are seeking participants in a study to evaluate foods for infant gut health

We are undertaking research on infant digestive health to support lifelong wellbeing and need samples of infant faeces.

Your infant may be able to participate if they:

- are currently healthy (and not receiving antibiotics or have conditions that affect feeding/nutrition)
- are between 4-7 months of age
- are about to be weaned or is starting solid food
- were at least 32 weeks gestation at birth and were heavier than 2.5kg
- are not receiving probiotic supplements
- have parents/legal guardians give informed written consent

If you would like to participate in this study or have any questions please email Callum Tatton on C.Tatton@massey.ac.nz.

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 22/48. If you have any concerns about the conduct of this research, please contact Dr Negar Partow, Chair, Massey University Human Ethics Committee: Southern A, telephone 04 801 5799 x 63363, email humanethicsoutha@massey.ac.nz.

Appendix 7. Information sheet

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Infant Stools Study

Collection of stools (faecal material) for evaluation of foods to support infant gut health

PARENT/CAREGIVER INFORMATION SHEET

You are invited to participate in a research study that will evaluate the effect of a complementary foods on infant gut bacteria and fermentation.

This Participant Information Sheet tells you about the study. It explains the tests and research involved, any benefits and risks, and what will happen after the study ends. Knowing what is involved will help you decide if you would like to take part. Please take the time to read this information sheet carefully. You may wish to talk about this study with other people such as family, whānau, friends, or healthcare providers. You will have the opportunity to discuss the information presented here with the study team, who will answer any questions you may have.

If you agree to take part in this study, you will be asked to sign the Consent Form. You will be given a copy of this information sheet and the signed consent form for your records. Please make sure you have read and understood all the pages.

If you have any cultural considerations or questions that relate to your potential participation in this study, please ask the study team before signing the consent form. It is the role of the investigators to ensure that you understand all procedures and risks.

What is the purpose of the study?

The human gut microbiota (gut bacteria) plays an important role in infant health. The introduction of solid foods and the changes in milk consumption are accompanied by significant gastrointestinal tract (GI) and developmental adaptations.

The purpose of this study is to evaluate how dietary nutrients in protein-rich, carbohydrate-rich or milk-based products used as complementary food is used by the infant's large intestinal microbiota. This is important for understanding the contribution of different nutrients and complementary foods to infant gut bacteria and fermentation.

This work will be conducted within the Riddet Institute, which is funded by through the Ministry of Business, Innovation and Employment (MBIE), Centre of Research Excellence funding, and by food industry partners. Work on infant digestive health may also be funded through High-Value Nutrition (HVN), Ko Ngā Kai Whai Painga, a National Science Challenge and their infant health priority research programme.

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Researchers

This study will be designed and performed by Professor Warren McNabb (Riddet Institute - Massey University), Dr. Jane Mullaney (AgResearch - Palmerston North), Ms Asher Brooke (Riddet Institute - Massey University), Mr Vitor Geniselli Da Silva (Riddet Institute - Massey University), and Mr. Callum Tatton (Riddet Institute - Massey University).

Who can participate in this study?

<u>You can participate in this study if your baby:</u>

- is healthy and free of illness for the past week
- is between 4-7 months of age
- was at least 32 weeks gestation at birth
- are either recently weaned or transitioning to solids
- has parents or legal guardians that can give written informed consent to participate in this study

<u>Babies are not eligible for participation if:</u>

- born <32 weeks gestation
- were small for gestational age (less than 2.5kg at birth, as they may have special dietary requirements)
- have any feeding difficulties
- have any digestive illnesses
- is receiving or has received antibiotic treatment in the last 3 weeks
- is currently taking a supplement with pre and/or probiotics

Participation in the study involves:

At the start of the recruitment process, volunteer parents/caregivers will be asked to complete a Screening Questionnaire to determine if your baby meets the study criteria. If they meet the study criteria and consent is given, they will be included in the pool of donors.

Prior to collection, Parents/caregivers will be provided with a health checklist (in case of changes in you infant's heath since recruitment) and a study collection kit (disposable gloves, hand sanitizer etc.) with instructions for the successful self-collection, storage, and transport of your infant's stool sample to the Riddet Institute Laboratory. We may ask you to provide further samples if your sample is not large enough or contaminated. You are under no obligation to provide any number of samples and may withdraw at any point.

Completion of the Screening Questionnaire and Consent Form will only take 10 minutes. Instructions on stool collection and provision of collection kits will take 10 minutes. Stool collection will take about 30 minutes per collection. Transportation of the sample to the Riddet Institute Laboratory will be arranged prior to sample collection.

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Will I benefit from the study?

Taking part in this study will provide you with useful information on the role of gut bacteria in early life and the fermentation of foods in the large intestine.

Researchers will gain knowledge of the fermentation behaviour of foods we believe to be helpful in supporting the healthy processes of infant gut bacteria. This knowledge will contribute to appropriate recommendations on the consumption of tested complementary food to develop good infant health, which may be of great interest to food manufacturers, health organizations and consumers. Your involvement in this study is of great value to the researchers; thank you for considering taking part.

Risks to you and your baby?

There are no major risks associated with taking part in this study. The method for sample collection will have as minimal impact as possible and researchers will have no contact with your baby. As with any research, there may be risks that are presently unknown or unforeseeable.

How will your confidentiality be protected?

We treat confidentiality and protection of your personal information as a matter of high priority during your participation in this study. The researchers will de-identify (remove) all personal information provided by you and there is no risk that you will be able to be identified should the results of this study be published.

On entering the study, your baby will be given a unique study identification number, which will be used for identification on all documents, samples, and data. This ensures that your baby cannot be identified in any way. All identifying participants information will be stored in a locked filling cabinet accessible by the research team only.

What will happen to your baby's stool samples?

Your baby's stool sample will be pooled with other infant stool samples. This will be used in the *in vitro* fermentation experiment to analyse how the nutrients in complementary foods are fermented in large intestine of infants. We will assess these fermentations through the variety of gut microbiota (through identifying and classifying the types of gut bacteria present), the production of short chain fatty acids and the production of gases (such as methane).

All samples will be labelled with the study ID number (and have no identifying personal names) and will be stored at the Riddet Institute at Te Ohu Ranaghau Kai. Your samples will not be sent overseas. We aim to use your entire stool sample in our study. Due to faecal matter being a biohazard risk, and our work taking place within a physical containment (PC2) environment, any utilized or excess sample(s) will need to be sterilized and appropriately disposed of as per biosafety requirements. If this is of concern to you for cultural, personal or religious reasons, please feel free to discuss

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this confidentially with Callum Tatton or any of the other researchers involved. These discussions will remain confidential and may help shape future study design.
We will seek your prior approval should any further unspecified research be made on your samples outside of the original study questions. It is important to know that neither your baby nor whānau/family will be identified in this data.

What happens with the results?

If you give us your permission by signing the Consent Form, findings from the study will be used in internal reports, conference presentations, and research publications. No personal information provided by you will be shared.

Following the completion of the study, a summary of the results will be available to each participant. To protect the development of high value kiwi products, The Riddet Institute often has to enter into non-disclosure agreements (NDAs) which may prevent us from initially identifying the precise complimentary food types we are investigating.

Will taking part in the study cost me anything, and will I be paid?

Taking part in this study should not cost you anything apart from your time, for which we thank you. Participants will receive a koha (gift) in the form of a $30 voucher for each collection they take part in as an expression of our thanks for dedicating time to this research.

Participant's Rights

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question.
- Withdraw from the study at any time without having to explain why. The information collected up to the point of withdrawal may continue to be used.
- Ask any questions about the study at any time during participation.
- Provide information on the understanding that your name will not be used unless you give permission to the researcher.
- Be given access to a summary of the project findings when it is concluded.
    - Due to NDAs we may be able to identify complimentary foods investigated immediately but can initially provide results without naming the foods investigated.
    - After relevant NDAs elapse, we can provide you with the omitted information and copies of any relevant publications if you desire.

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Project Contacts

If you have any questions or concerns about the study, please do not hesitate to contact Mr. Callum Tatton (Research Technician) directly at C.Tatton@massey.ac.nz.

Thank you for considering participation in this study!

Important work like this is not possible without volunteers like you.

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