Metagenomic Analysis of The Effect of Formula versus Blended Food on Gut Microbiota of Children, using an *in vitro* gut model.

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Abstract

Background: Artificial nutrition is needed for many children with complex medical conditions who can no longer support their nutritional needs orally. Enteral nutrition in the home environment uses almost exclusively sterile commercial formulas, however, the use of blended tube feeds made from food liquidised at home is becoming more commonly used. Dietary patterns have been shown to impact the bacterial composition of the gut microbiota in real time. The gut microbiome affects virtually all aspects of human health and influences the development of chronic diseases. The aim of this study was to use a triple stage chemostat model to simulate the human gut and study the effects of blended tube feeds and commercial formula on the gut microbial population.

Methodology: Pre-digested commercial formula and blended tube feed was added to an *in vitro* gut model seeded by pooled faecal samples of healthy children. Microbiota community profiles were evaluated through bacterial culture and long read Nanopore sequencing, and CZ-ID. Construction of metagenome assembled genomes (MAGs) identified microbial community structure and provided prevalence data. Alpha and beta diversity metrics were used to quantify microbial diversity and species richness.

Results: Microbiota composition changed during the commercial formula feeding with increased prevalence of *Bacteroides, Bifidobacterium, Lactobacillus, Lacrimispora*, *Megasphaera* and *Hungatella* compared to the control model. Microbiota composition also changed during the blended tube feeding with increased prevalence of *Lachnospiraceae, Bacteroides, Lactobacillus, Bifidobacterium* and *Faecalibacterium* compared to the control model. Compositional changes within the gut microbial populations occurred with the introduction of both commercial formula and the blended recipe. More increases were seen in beneficial bacteria in response to the blended tube feed compared to the commercial formula. No increases in pathogenic bacteria were seen in response to the blended feed compared with the commercial formula.

Conclusion: The blended tube feed promoted a greater prevalence of bacterial species that are considered beneficial within the gut microbiota compared with commercial formula. These changes in gut microbial composition are associated with a healthier state.

1 Background

1.1.1 Enteral tube feeding

Artificial nutrition, including enteral (EN) nutrition is recommended when oral nutrition fails to adequately supply the necessary nutrient needs to the body. Considered a convenient and well tolerated form of feeding intervention, it can be used within a hospital and home setting (Reber et al., 2019). Children with complex medical needs, including neuro-disabilities and severe chronic illnesses, may be unable to consume sufficient food orally to meet their nutritional needs. As a result, children may be fed via an enteral feeding tube which may continue long-term in their own home. Conditions such as preterm low birthweight, cleft palate, cerebral palsy, autistic spectrum disorder, gastro-oesophageal reflux and cancer treatments are some of the reasons why children have difficulties eating and drinking orally. Consequences of these difficulties includes malnourishment which requires intervention strategies (Diamanti et al., 2013). One such strategy for individuals unable to independently support their own nutritional needs is the insertion of a feeding tube. Several types of enteral feeding solutions are available, and one of the most common is the nasogastric tube (Figure 1A). The tube is inserted through the nasal cavity through to the stomach and is primarily for individuals who require short-term feeding interventions. For individuals who require longer-term feeding interventions a percutaneous endoscopic gastrostomy (PEG) tube is inserted (Figure 1B). The insertion site for the tube is located around the navel, where the tube is inserted through the skin into the stomach, allowing for feeds to be directly administered and facilitating absorption and digestion of nutrients through lumen of the gastrointestinal (GI) tract (Page et al., 2019). In England, during 2014 /2015 the total number of children with life limiting conditions, aged 0-19 years who have ever had a gastrostomy was 10,154 (Taylor et al., 2019).

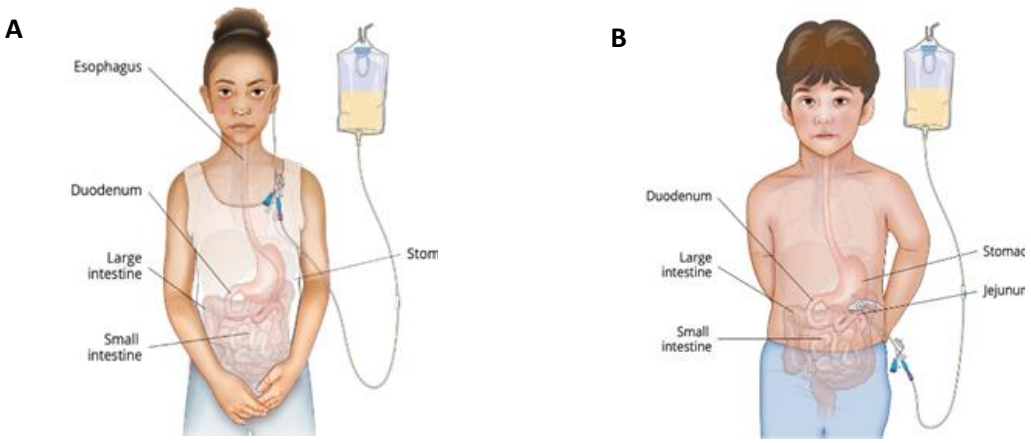


Figure 1. Enteral tube placements. A, nasogastric tube (NG tube) that is inserted through the nose past the oesophagus and into the stomach. **B**, gastrostomy long tube (G tube) which is inserted through the abdominal wall, a small internal balloon or mushroom shaped end keep it in place. Image modified from (together.stjude.org, 2018)

Children who require enteral nutrition (EN) may be cared for at home by parents or carers. The benefits of feeding children at home are shorter hospital stays, less malnutrition related complications (Rosen et al., 2016), and psychosocial benefits such as improved emotional wellbeing and social inclusion and participation (Hopwood et al., 2020). Home enteral nutrition (HEN) is typically a commercially produced complete nutritional formula (CF) which are sterile, ready-to-feed liquids available in pouches, cartons, or bottles. The use of these formulas have been normal practice since the early 1970s when Gormican and Catli published developmental procedures to test the results of a fortified sterile milk-based feed (Gormican & Catli, 1972). The results of this study led to the development of the Wisconsin formula, which contributed to the idea that patients needed to be fed a similar ratio of carbohydrate, fat and protein as found in the average diet of a healthy person. Additionally, this diet not only met nutritional needs better but to also appeared to decrease gastrointestinal symptoms often associated with the early milk and raw egg-based feeds (Harkness, 2002). Prior to Gormican and Catli's findings, in the 1950-60s European and North American hospitals routinely administered blended (liquidised) real food for enteral feeds as standard, however, due to the financial costs and the introduction of complete CF the practice fell out of favour (Parrish, 2014). Today, CF are prescribed as the standard enteral feeding practice, due to the fact they are sterile, have quantified nutritional compositions and are easy for the caregiver to administer (Gramlich et al., 2018). However, some children receiving long term enteral feeding suffer from gut dysbiosis which is a disruption in the composition of the microbiota, symptoms often associated with dysbiosis is the irritation of the GI tract, such as nausea, regurgitation, vomiting

and diarrhoea with 63% suffering with one or more symptoms throughout the duration of their feeding course (Blumenstein et al., 2014). Many dietitians and medical practitioners support the use of CF because nutrient and energy content are precise, feeds are sterile until opened and, they are convenient, portable and practical to administer (Novak et al., 2009). It has been hypothesised that sterile CF diets reduce the richness and diversity of the microbiome influencing the number of distinct bacterial species and the evenness of abundance of these species within the gut. Reductions in these bacterial communities, lead to dysbiosis of the gastrointestinal tract, and often resulting in GI symptoms, impaired systemic health and reduced quality of life (QoL) (Gallagher et al., 2018; Hron et al., 2019; Trollip et al., 2020).

1.1.2 Blended tube feeding

Alternatives to CF are blended tube feeds (BTF), also known as liquidized or a blenderised diet, which are blended foods and liquids that are usually prepared domestically and directly administered via a feeding tube (Oparaji et al., 2019). A blended feed is usually homemade and prepared by the caregiver in the home and may include the same foods that family members are eating at mealtimes. Preparation consists of using a range of whole foods including meats, vegetables, fruit, dairy and other liquids such as fruit juice. Ingredients are pureed using a domestic blender and administered via the enteral feeding tube either as a bolus using a syringe or as a continuous feed using a reservoir or pump. However, the term, blended feeds can also refer to CF when mixed with pureed baby food and other commercially available food products (Martin & Gardner, 2017). There is growing interest in BTF as an alternative to CF among patients, families and healthcare professionals (Bobo, 2016; Phillips & Coad, 2023). This stems from perceived health benefits including improved physical symptoms i.e. less regurgitation, diarrhoea and constipation and systemic improvements including reduced feelings of isolation, anxiety, and depression (Hurt et al., 2015; O'Connor et al., 2022; Trollip et al., 2020). This interest was focused on the positive aspects of BTF, such as eating real food and inclusion within the family unit by eating the same thing at mealtimes, whilst moving away from medicalised feeding regimes (Parrish, 2014). However, some concerns about BTF including microbial risks, nutritional adequacy, potential to block feeding tubes and additional burden for families considering BTF remain.

There is growing evidence of the reported benefits of children receiving blended feed diets; a retrospective cohort study by Batsis *et al* in 2020 demonstrated that three months after initiating a BTF 95% of patients experienced an improvement in gastrointestinal symptoms compared to 91% of a matched group that remained on the CF (Batsis et al., 2020). Additionally, a prospective cohort study by

Hron et al in 2019 reported that children receiving BTF had a 53% reduction in total hospital admissions compared to children receiving CF. Further, the study reported BTF fed children had less vomiting and reflux, with lower rates of healthcare utilization (Hron et al., 2019). A retrospective cohort study by Schmidt *et al*, 2019 and a prospective observation study by Fabiani *et al*, 2020 showed a significant reduction (p = 0.023) in probability of diarrhea in long term tube fed critical care patients (Fabiani et al., 2020; Schmidt et al., 2019). Further, a case study by Phillips in 2022, showed that a child with development delay and faltering growth even after food fortification and oral nutrient supplements, when introduced to a BTF showed growth velocity increase from 75% to 190%. They also exhibited a reduction in reflux allowing reflux medication to be stopped and an improvement in bowel movements and stool consistency (Phillips, 2022).

Additionally, a systematic review of intervention and observational studies of blended diet for enteral tube feeding in young people was undertaken by McCormack et al. And demonstrated through two questionnaires the Pediatric Gastroesophageal Reflux Disease Symptom and Quality of Life Questionnaire (PGSQ) and Pediatric Quality of life Inventory Gastrointestinal Symptoms Scale (GI-PedsQL), that although studies were heterogenous, BTF was associated with positive outcomes with an emphasis on tolerance of diet (p = 0.007), control of GI symptoms (p = 0.03), reduced reliance on medication for GI symptoms from 88% to 76% (p = 0.007) and improved weight for age scores (76% vs. 82%, p = 0.001) and fewer reports of mild adverse events (McCormack et al., 2023). These studies provide useful evidence, but their study designs limit the interpretation of the findings. Randomised controlled trials (RCT) are considered the gold standard for evaluating health interventions but, to date, no RCTs comparing the effects of BTF and CF have been published. These studies however support the use of BTF for children with complex medical needs, showing that diet can potentially reduce systemic symptoms of dysbiosis.

However, as well as potential benefits from BTF there are also concerns associated with risk of microbial contamination, potential for blocking feeding tubes, uncertainty and variability of nutrient composition with the increased time required to make and administer BTF. Until recently, switching to a blended diet has been met with caution due to concerns over foodborne infection, however, correct food training and refrigeration of feeds has a significant impact on bacterial loads (Milton et al., 2020; Phillips & Coad, 2023). The risk of microbial contamination of feeds was investigated by Madden et al (2019) comparing the microbial load of BTF compared to sterile CF in a laboratory-based setting. Although food blended in a domestic environment did not achieve levels of sterility compared to CF production, the BTF was

within guidelines for assessing the microbiological safety of ready-to-eat food on the market (Health Protection Agency, 2009). They concluded that BTF could be an option, from a microbial perspective, for individuals who were not immunocompromised or medically unstable (Madden et al., 2019). This was further supported by Johnson et al (2019) when making BTF from both whole foods and baby food and comparing them to CF within a hospital kitchen. Results from the study showed that all three feeds were categorised as acceptable for human consumption according to food safety standards expected of U.S. hospitals (FDA.gov, 2017). However, the BTF ingredients substituted fresh baked chicken and raw vegetables with chicken from a can and frozen vegetables which potentially reduced the risk of bacterial contamination (Johnson et al., 2019). Microbial load was assessed by Milton et al (2020) in an observational study who asked 50 participants to prepare homogeneous meal kits for BTF in a home setting with a set of standard procedures for minimising bacterial growth. Results showed that 88% of the feeds prepared met the US food code for safe food consumption (FDA.gov, 2017), 11% didn't meet US standards but were considered 'marginal' by standards from other countries (Swanson, 1992) and one sample exceeded acceptable microbial load by US food code standards (Johnson et al., 2019). Suggesting that if safe food handling and storage, and adequate hygiene procedures are in place, safe production of BTF in a domestic environment is attainable (Milton et al., 2020). Overall, these studies illustrate that producing completely sterile food in a domestic setting is not possible, however, with procedures in place to reduce bacterial contamination, such as cleaning and sanitizing kitchen surfaces and equipment such as blenders, correct refrigeration, and storage of BTF, microbial levels that are accepted for the general population can be achieved.

1.2 Gut Microbiota

1.2.1 Human Gut Microbiota Diversity

The human gastrointestinal tract is often referred to as the "gut" and contains an ecosystem of microbes, which are complex and dynamic, each person has a composition of microbial communities that is variable and unique, especially across healthy individuals (Johnson et al., 2019). In terms of broad composition of the adult gut microbiome, 93.6% of bacterial species isolated fall into four bacterial phyla; Firmicutes (31%); Proteobacteria (30%); Actinobacteria (26%) and Bacteroidetes (7%) (Hugon et al., 2015). Many influences shape the composition of the microbiome, such as genetics (Goodrich et al., 2014), mode by which the person was delivered at birth (Bäckhed et al., 2015), host immune response to pathogenic bacteria (inflammation) and symbiotic bacteria (tolerance) (Wang et al., 2021). Diet (David et al., 2014), antimicrobial regimes (Cho et al., 2012) and environmental factors also influence

microbiome composition (Fujimura et al., 2014) and underlying health conditions (Valdes et al., 2018). However, the composition of an individual's microbiome is highly dynamic and can experience large shifts in microbial composition over a short period of time in response to changes in diet, the environment and disease states. Consistent changes from any of these sources can alter diversity in microbial communities, this biodiversity can be measured using alpha and beta diversity metrics, alpha diversity observes the number of bacterial taxa (richness) and those relative abundances of those taxa (evenness) in a single sample, whereas beta diversity measures the similarity or dissimilarity between two microbial communities (LeBlanc et al., 2013; Lu et al., 2013; Walters & Martiny, 2020). To assess these changes in microbial populations, faecal samples are a good representation of those within the gut, a non-invasive method of sample collection from patients for examining community dynamics and explore changing bacterial populations within a microbiome. However, although faecal samples are pragmatic and convenient, they are limited in the accurate representation of microbial composition along the gastrointestinal tract. Niche populations of bacterial are more prolific in different regions of the gut which faecal samples are unable to fully capture.

The microbiota in healthy children consuming a varied diet, contain two phyla that dominate bacterial composition, *Firmicutes* and *Bacteroidetes*, with smaller amounts of *Proteobacteria, Actinobacteria* and *Verrucomicrobia*. Additionally, the gut microbiome also contains a rich virome, with eukaryotes that consist of mainly yeasts and methanogenic archaea (Eckburg et al., 2005; Reyes et al., 2010). The gut microbiome of children differs from that of adults, suggesting a slower rate of maturity, and allowing additional opportunities for interventions (Derrien et al., 2019). Generally, the profile of an adolescent's microbiome is predominated by phyla such as Bacteroidetes which exhibit the most stability, followed by Proteobacteria. To assess the microbiota of children de Meij et al (2016) collected faecal samples from 61 healthy children aged 2-18 years in the Netherlands; samples (weekly) were sequenced to show diversity and stability of the microbiome. The study duration was six weeks with a follow up sample after 18 months, with no dietary interventions. The stability of some phyla declined rapidly for short intervals, stabilised, then naturally declined further at a gradual pace. This suggested phylum-specific temporal shifts following variation in diet. Overall, around 70% of identified bacterial strains showed general stability within the microbiome over the 18-month study period (de Meij et al., 2016). These studies show that natural temporal shifts occur within the microbiota of children as they mature, however, these changes take place over a long period of time.

1.2.2 Gut Microbiota and metabolite production

Bacteria within the gut microbiota produce metabolites that can benefit the host or contribute to disease pathogenesis. Bacterial taxa within the gut microbiota are essential for the fermentation of dietary fibre and endogenous intestinal mucus. Fermentation of polysaccharides, oligosaccharides, proteins, glycoproteins, and peptides result in the production of metabolites such as short chain fatty acids (SCFA). SCFA are bacterial derived metabolites that modulate key functions for the host especially intestinal homeostasis maintenance. Carbohydrates fermented by saccharolytic bacteria producing linear SCFA, proteins and amino acids are fermented by proteolytic bacteria producing branched SCFA, phenols and amines in the colon. This fermentation process not only supports growth of bacteria within the gut but impacts gut integrity, metabolism and host immunity (Martin-Gallausiaux et al., 2021; Wong et al., 2006). The predominate SCFA are acetate, butyrate and propionate which are essential for the maintenance of gut integrity, neuronal health and immune system regulation. Butyrate is an important metabolite for cell regulation and as the predominant energy source for colonocytes that induce apoptosis of colon cancer cells. Additionally, butyrate facilitates the activation of intestinal gluconeogenesis which has a beneficial effect on energy and glucose homeostasis and prevents leaky gut. Acetate aids in the regulation of gut pH, appetite and protects against pathogens (Valdes et al., 2018).

Gut microbes have been shown to regulate systemic inflammation through the production of anti-inflammatory metabolites, SCFA can cross the blood brain barrier (BBB) and interact with brain cells. Additionally, SCFA are also essential in the maintenance of the BBB. Braniste et al demonstrated in mice models the important role of the microbiota in the production of SCFA's, by upregulating the expressions of tight junction proteins occludin and claudin-5 that regulate barrier function in endothelial tissues and by decreasing BBB permeability. They also reported that when germ free mice received either a faecal transfer from a pathogen free mouse or a treatment containing bacteria that produced SCFA, a decrease in the permeability and a reinforcement of integrity of the BBB was observed in mice (Braniste et al., 2014). The use of animal model studies are able to demonstrate the impact of SCFA *in vivo,* with findings being comparable to the understanding of SCFA synthesis and utilization in humans (Braniste et al., 2014; Devkota et al., 2012; Park et al., 2013).

1.2.3 Gut Microbiota and diet

Diet and nutrition play a key role in the symbiotic relationship between the gut microbiota and the host, influencing health and metabolic homeostasis through metabolite production. The gut microbiome can

be modified by diet volume and composition which can have a systemic effect on health (Wilson et al., 2020). Some children with disabilities rely on enteral nutrition as the primary source of nutrition, especially if nutritional needs cannot be met orally. Diet has a high impact not only on nutritional requirements but on systemic health (Katagiri et al., 2023). The impact of high fat, high sugar content and low fibre diet on health has been widely researched (Clemente-Suárez et al., 2023; De Filippo et al., 2010; Graf et al., 2015). Including effect on gut microbial communities, metabolites and enzymes (Leeming et al., 2019) associated with chronic illness such as obesity and immune related disorders such as inflammatory bowel disease (IBD) (Devkota et al., 2012; Ley et al., 2006). Diet-induced depletion of commensal gut bacteria such as *Faecalibacterium* and *Ruminococcus,* and enrichment of pathogenic bacteria such as *Staphylococcus* and *Enterococcus*, cause dysbiosis and adverse outcomes for individuals with underlying medical needs.

Figure 2. High fibre vs Western diet. The image shows the consequences of diet on the gut and gut microbiota; Western diet vs a Healthy High Fibre diet and the consequences of each. Image created in BioRender.com modified from (Mills et al., 2019).

Research has highlighted the effect of a western diet (Figure 2) on the microbiome composition. Differences were found between the gut microbiota of individuals who consumed vegetarian diets

compared to omnivores (Glick-Bauer & Yeh, 2014). Demonstrating that individuals who consumed vegetable, fruit and fibre from a plant-based diet long term, had a greater richness and diversity within their microbiome compared to those who consumed a western based omnivore diet (Klimenko et al., 2018). A study conducted by De Filippo et al in 2010, showed that diet of children in different geographical populations also impacted the gut microbiota. Comparisons of faecal microbiota of children aged 1-6 years old in Europe (Italy) consuming a western diet compared with children from rural Africa consuming a high fibre vegetarian-based diet (Figure 2). The study showed significant differences ($P < 0.001$) in bacterial populations within the gut microbiota between the two groups, with a reduction of SCFA from European children compared to African children. Significant differences in richness and biodiversity were found between the two microbiota ($P < 0.01$), highlighting a richer and more diverse microbiota of African children, compared to European children (De Filippo et al., 2010).

Consequences of consuming a high fat, low fibre diet, is not just an imbalance in gut microbiota, gut dysbiosis and but also dysregulation leading to inflammation and loss of gut integrity. Gut dysbiosis has been seen in many children who rely on EN, exhibiting symptoms such as bloating and diarrhoea (Rogers et al., 2016). The gut microbiota interacts with bile acids (BA) and plays an important role in carbohydrate and lipid metabolism. Deconjugation of bile acids, and the production of enzymes synthesized to alter the composition of secondary BA, affected glucose and lipid metabolism. One of the consequences of dysregulation of BA metabolism is the reduction in bacterial genus such as *Bacteroides*, *Lactobacillus* and *Bifidobacterium* that express bile salt hydrolase (BSH). This causes bile toxicity and impedes normal excretion of BA (Odermatt et al., 2011) whilst also allowing for colonisation of bile resistant bacteria (M. Yang et al., 2021). This demonstrates the importance of diet on systemic health, especially children relying on enteral nutrition who often also have underlying medical conditions.

1.2.4 Gut Microbiota and systemic health of children

A diverse and healthy microbiome is key to systemic health within children. The microbiome is acquired from birth, other factors play a key role such as genetics and environmental factors account for susceptibility to metabolic disease (Bäckhed et al., 2015; Fujimura et al., 2014). A complex interplay of these factors shape a unique phenotype, however, as the microbiome can be highly dynamic it can be modified, so therefore act as a therapeutic target (Menni et al., 2017). The overall systemic health of gastrostomy fed children is complex to quantify, most children receiving enteral feeds have chronic illnesses and secondary conditions such as diabetes , however, this highlights the importance of good enteral nutrition on the health outcomes of these children (Bakewell et al., 2021).

An in-depth study by Murri et al, examined the compositional differences in microbial communities within the gut microbiota of sixteen children with type 1 diabetes compared to sixteen healthy children (without diabetes). The healthy children were matched to children with diabetes type 1 for age, gender, race, mode of delivery and duration of breastfeeding as an infant. Additional matching information was provided by parents on dietary habits, health status and lifestyle aspects. Microbiota composition was analysed from faecal samples and showed significant differences in bacterial composition between healthy children and children with diabetes type 1. The results showed a decrease in *Lactobacillus* and *Bifidobacterium* species from children with diabetes compared with controls. Additionally, analysis showed that compared to healthy children, children with diabetes had significant decreases in bacteria associated with production of lactic acid and butyrate, along with mucin degrading bacteria. These bacteria are essential for maintaining gut integrity and health, reduction in abundance could be responsible for altered gut permeability found in patients with type 1 diabetes (Murri et al., 2013).

Although the majority of research into the importance of maintaining a healthy microbiota is seen in murine models. Studies examining long term weight gain in humans supports the correlation of low microbial diversity and low dietary fibre intake with obesity, gut dysbiosis and systemic inflammation (Menni et al., 2017). Enterically fed children with neurodisabilities or long-term illnesses, such as chronic kidney disease, can be either overweight or obese. With excess calories being stored as adipose tissue rather than stimulating further growth improvements (Sienna et al., 2010). As enteral feeds administered contain more calories than is utilized this could lead to excessive weight gain especially with long term use, and should remain a consideration when choosing feeding regimes (Ramage et al., 1999). Reducing energy or changing diet to a BTF, is a possible consideration as BTF contains less calories from saturated fats and include high fibre ingredients increasing benefits such as enhanced microbial diversity. BTF could aid in reducing weight gain and reduce the symptoms of gut dysbiosis associated with obesity (Tanchoco et al., 2001).

Research into how diet is a contributing factor in shaping a gut microbiota that influences systemic health of the host, is invaluable in the understanding and progression of interventions associated with short- and long-term feeding tube requirements and systemic long-term diseases within the tube fed community.

1.2.5 Antimicrobials and the Gut Microbiome

Oral antimicrobials are one of the most common therapies administered to paediatric populations within European countries (Sturkenboom et al., 2008). A contributing factor to dysbiosis of the gut is

multiple antimicrobial exposures, resulting in a reduction in microbial diversity. Antimicrobials such as broad spectrum antimicrobials can cause a reduction of dominant communities of commensal bacteria allowing pathogenic bacteria to proliferate (Cho et al., 2012). Jernberg et al examined the long-term impact of a single 7-day course of clindamycin on the gut microbiota of adults over a two-year period. Results showed that a single treatment with clindamycin reduced *Bacteroides* permanently with no tendencies to return to pre-administration levels (Jernberg et al., 2007). Other studies also indicate permanent change to gut microbiota post antimicrobial exposure (Bajinka et al., 2020). However, the impact of antimicrobials within the gut microbiota is variable and is determined by intervention period, type of antimicrobial administered and concentration (Ghoshal et al., 2016). This was further demonstrated in a comparative study by Imhann et al who found bacterial genus such as *Collinsella*, *Lactobacillus* and *Anaerostipes* were reduced in the gut of children on antimicrobial therapy compared to an untreated group for up to two years (Imhann et al., 2016). It was shown that long-term antimicrobial use reduces the richness and diversity of the gut microbiota, impacting the immune system, including pro-inflammatory effects, increased permeability of the gut wall and a reduction in protective mucus layer (L. Yang et al., 2021)

Children that are tube fed often need antimicrobials due to their susceptibility to infections. It is essential to understand the broad impact of antimicrobials on the structure and abundance of bacterial species within the gut microbiome, as these changes can impact growth, drug metabolism and progression of chronic diseases.

1.3 Gut model and digestion

1.3.1 Triple stage chemostat gut model

To evaluate microbial composition and diversity within the human gut, methodologies have focused on using faecal samples as a representation of the large intestine to closely resemble *in vivo* conditions (Jones et al., 2018). *In vitro* gut models are utilized to quantify bacterial communities, screen mechanistic and therapeutic outcomes, and have been validated for maintaining human gut microbiota (Macfarlane et al., 1998; Rajilić-Stojanović et al., 2010; Van den Abbeele et al., 2010). As such, a three stage chemostat gut model is ideal to assess and monitor bacterial communities. These multi-stage continuous culture reactors started with Gibson and Macfarlane in the 1980s who examined retention time of bacterial populations in the colon. The model consisted of three connected vessels that simulated the three stages of the colon (ascending, transverse and distal) that maintain parameters that reflect conditions within the gut, such as pH and temperature (Macfarlane et al., 1998). This allows for

reproducibility, ease of sampling and cost efficiency to study the effects of food either independently or as a combination. However, this system has not been utilized previously for monitoring bacterial communities in the same model during consecutive dietary interventions. Previous research using the triple stage gut model has been well documented in measuring the response to antimicrobial agents in *Clostridioides difficile* infection (CDI) models (Baines et al., 2005, 2013; Chilton et al., 2014; Etifa et al., 2023; Freeman et al., 2003). As such, the applications for extending the parameters of the three stage chemostat model past singular compounds and probiotics to include whole food diets have been under utilised and could easily incorporate additional interventions to further explore research questions such as, how diet affects the production of metabolites from commensal and pathogenic bacteria (Davis Birch et al., 2023; Gibson, 2022).

For instance, it may be possible to ascertain the effects of metabolites on microbial populations in the gut model in response to dietary interventions. Simulated digestion processes can be used to incorporate several modifications aimed at reducing cost and complexity. Resulting in a "modified original" method to better stimulate a fed state, by adding three times the amount of bile salts and digestive enzymes with comparable results (Garrett et al., 1999; Rodrigues et al., 2017; Rowland et al., 2018; Thakkar et al., 2007).

1.4 Metagenomics

Metagenomic sequencing of the human gut microbiome within the human gut gives new insight to microbial ecology and evolution (Hug et al., 2016; Nayfach et al., 2019). High throughput sequencing platforms like Illumina generate very accurate but restricted fragment lengths of around 100-300 base pairs (bp), reducing accuracy in reconstruction of strain-level genetically related organisms (Quince et al., 2017). Illumina sequencing has been the method of choice for years, high throughput short-read sequencing, but these failed to resolve repeated regions which is problematic in metagenome samples. In contrast, single-molecule sequencing platforms like Oxford Nanopore Technology (ONT) (MinION, GridION and PromethION) sequence very long fragments from high molecular weight DNA, with >10 kilobase pairs (Kb) - 2 mega base pairs (Mb) (Jain et al., 2018). Platform improvements have led to metagenomic final assemblies reaching ~99.5 to 99.8% of consensus accuracy (Latorre-Pérez et al., 2020). Specific long read alignment-based identification and classification was more accurate owing to their increased information content (Wommack et al., 2008). Long read sequences improves accuracy, by bridging shorter repetitive sequences that are often longer than the read length which are often shared by one or more taxa present within the microbial community, which can also contain related

species or subspecies in an unknown abundance. Producing long reads also allows the generation of genomes with a high degree of completeness in *de novo* assemblies (Bertrand et al., 2018; Latorre-Pérez et al., 2020). Long read sequencing in metagenomics allows retrieval of metagenome assembled genomes (MAGs) from high molecular weight (HMW) DNA whilst utilising short reads to polish and improve overall accuracy (Suzuki et al., 2019). Metagenomic pipelines use correction software to improve accuracy and reliability, such as frameshift-aware corrections, these can aid in producing high quality data for the taxonomic assignment of bacterial species found in the human microbiome through bioinformatic databases such as BLAST and Kraken2 (Cuscó et al., 2021). A comparative study investigated the alignment and assembly of mock microbial community standards using Illumina and nanopore sequencing. The 10 species within the mock community standards were recovered at anticipated abundances for both platforms, however, ultra long read nanopore data sets were recovered, improving genome assembly without additional processing steps. Results showed that ONT generated >30 Gigabytes - >300 Gb of data compared to 101-151 bp with Illumina. Long contiguous sequences generated by nanopore sequencing meant that whole microbial genome reconstruction from mixed samples without a binning step was possible (Nicholls et al., 2019).

Analysis of metagenomic data and bioinformatic pipelines are often complex, have long run times and are computationally resource intensive to process, especially when setting parameter boundaries. Therefore, automated metagenomic pipeline that do not require bioinformatics expertise nor high computing power was desirable. Chan-Zuckerberg-ID (CZ-ID) is a free cloud based genomic analysis platform that uses FASTAQ files of long reads to generate comprehensive and accurate *de novo* assembly of metagenomes. The CZ-ID metagenome next generation sequencing (mNGS) nanopore module within CZ-ID provides the characterization of complex microbial communities, with no need for prior knowledge of coding or computing resources. The pipeline executes quality control steps to remove reads of low quality, low complexity, and short length. Filters then exclude host DNA before subsampling non-host reads, into de novo assemblies, saving time and cost (Simmonds et al., 2024). The output of CZ-ID can then be analysed using common metrics for microbiome studies.

To measure and compare differences in microbiome data alpha and beta diversity metrics are used. It is possible to quantify between microbial communities at two different levels, the alpha diversity (within-sample) and the beta diversity (between sample). Alpha diversity metrics commonly used such as Shannon-Weiner diversity index (Shannon, 1948) which summarises the structure of the microbial communities by richness (number off taxonomic groups) and evenness (distribution of abundance of the

taxonomic groups). And Simpson's index (Simpson, 1949) which summarises diversity of taxa whilst taking into consideration richness and evenness. Beta diversity metrics commonly used such as Bray-Curtis dissimilarity index (Bray & Curtis, 1957) summarises how samples are different from one another by considering the dissimilarity of sequence abundance between two different samples (Kers & Saccenti, 2022). In microbiome studies, it is important to measure diversity, to understand bacterial composition, function, and dynamics of the microbiota between treatments and controls.

1.5 Hypothesis, Aims and Objectives

1.5.1 Hypothesis

The hypothesis of this study is that a change in diet from a commercial formula diet to a home blended tube-feeding diet will have an effect on the richness and diversity of bacterial communities within the gut microbiome derived from healthy children aged 2-15 years old.

1.5.2 Aim

The aim of this study is to investigate how a change in enteral feed from a sterile commercially prepared formula to a nutritionally comparable feed made from blended food impacts the gut microbiome using an *in vitro* gut model.

1.5.3 Objectives

I. Design a protocol for digestion that reflects enteral tube feeding for children.
II. Investigate the response of gut microbial communities on dietary interventions using a triple-stage chemostat model that simulates the human gut, inoculated with faecal slurry from health children aged 2-15 years old
III. Analyze the response of gut microbial communities by selective agar and using gDNA of microbial culture for long-read Nanopore sequencing and metagenomic analysis.

2 Materials and Methods

2.1 Commercial Formula and Blended Diet recipe

A survey conducted on a closed parent support group on Facebook showed that Paediasure (Abbott, Maidenhead), was the most frequently used commercial formula before switching to a home blended diet. Foods used for the blended diet recipe were those most frequently described as included in home blended feeding regimes by parents. The blended feed recipe was developed to nutritionally match the commercial formula (Table 1) for energy and macronutrients in a 500 ml volume using nutrient software (Nutritics, Dublin).

Table 1. Blended recipe and calculated nutritional composition to match the commercial formula to make up to 500ml.

Ingredients	**Weight (g)**
Oil olive	10
Honey	20
Bananas flesh only	100
Broccoli green steamed	50
Avocado average flesh only	30
Potatoes old boiled in unsalted water flesh only	80
Chicken pieces white meat deli style	15
Milk whole pasteurised average	80
Ground almonds	10

	Commercial Formula[1]		**Blended feed**[2,3]
Volume (ml)	100	500	500
Energy (kcal)	100	500	500
Protein (g)	2.8	14.0	14.5
Carbohydrate (g)	11.2	56.0	56.0
Sugar (g)	3.92	19.6	39.0
Fat (g)	4.98	24.9	24.9
Fibre	0	0	6.9

[1] Composition per 100 ml taken from BNF for Children 2020-2021 (2020) Borderline substances, appendix 2, page 1106. London: Pharmaceutical Press.
[2] Composition determined using Nutritics nutrient analysis software https://www.nutritics.com/en/
[3] Blended recipe was provided by registered dietitian Dr Angela Madden

2.2 Preparation of blended feed foods

All food ingredients were purchased from a local supermarket (Hatfield, UK). Procedures reflected those used in a normal kitchen environment with high level of hygiene practice. All kitchen utensils and removable blender parts (Vitamix Professional Series 750; Vitamix, Olmsted Falls, OH, USA) were washed in hot soapy water before use. Potatoes were peeled weighed and boiled in unsalted water. Broccoli was steamed for approximately 15 minutes until tender. All other ingredients were chopped roughly and placed directly in the blender. The blended feed was made by combining all the ingredients and blended until smooth, without sieving, then decanted into a sterile 1L Duran bottle. The blended recipe was prepared in conditions that reflected those normally found within a domestic environment and included ingredients that were frequently available and gave the right consistency to be easily administered by tube. The recipe consisted of raw, boiled and steamed foods which would reflect preparation within a domestic environment for children receiving blended enteral tube feeds. The ingredients were nutritionally matched to the commercial formula in energy, fats, sugars, fibre and carbohydrate content. Food choices were based on a poll conducted on a closed group of parents with tube-fed children on social media (Facebook), to identify the most frequently used foods given to tube fed children and these foods were included within the study recipe. Food was prepared and cooked to reproduce typical blended feed preparation at home and to achieve consistency that could easily be administered by tube. Cooking techniques implemented in the preparation of the BTF were boiling and steaming of ingredients such as potatoes and broccoli.

2.3 *In vitro* digestion of Commercial Formula and Blended feed

The *In vitro* digestion method used was based on the work by Failla et al in 2014. Rodrigues et al (2017) modified the “original method” by Failla et al. to better simulate fed conditions in the gut and compared this process to the Minekus et al, 2014 INFOGEST digestion model. The simulated digestion fluids (SDF) were made up as shown in Table 2. This protocol excludes the oral stage and simulated salivary fluid (SSF) to better reflect conditions of enterically fed individuals. The volumes of both SGF and SIF were calculated to a final volume of 500mL, the volume was made up to 400 mL with distilled water to a 1:25x concentration. The electrolyte stock (4 parts stock solution + 1 part water) had the correct ionic composition in the simulated digestion fluids (SDF). The addition of additional enzymes, bile salts Ca^{2+} solution and distilled water to the correct electrolyte concentrations in the final digestion mixture (Minekus et al., 2014; Rodrigues et al., 2017).

Table 2. Electrolyte stock solutions for Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluids (SIF) made up to a 1:25x concentration.

			SGF		SIF	
			pH 3		pH 7	
Constituent Stock volume and concentration in simulated digestion fluids (SDF)			**Vol of stock**	**Conc. in SGF**	**Vol of stock**	**Conc. In SIF**
	g L^{-1}	**Mol L^{-1}**	**mL**	**Mmol L^{-1}**	**mL**	**Mmol L^{-1}**
KCl	37.3	0.5	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	12.5	25	42.5	85
NaCl	117	2	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.5	0.5	-	-
pH adjustment						
	mol L^{-1}		**mL**	**mmol L^{-1}**	**mL**	**mmol L^{-1}**
NaOH	1		-	-	-	-
HCl	6		1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$						
	g L^{-1}	**Mol L^{-1}**		**Mmol L^{-1}**		**Mmol L^{-1}**
$CaCl_2(H_2O)_2$	44.1	0.3		0.15 (0.075*)		0.6 (0.03*)

*** Brackets show corresponding Ca^{2+} concentration in the final digestion mixture**

2.3.1 Gastric Phase

Five parts of liquid food was added to four parts of the SGF electrolyte stock solution (75 µm $CaCl_2$, 2000 U mL^{-1} pepsin, pH 3) and made up to 200mL with water. Time of digestion process was 2 hours in a shaking incubator at 37°C at 90 rpm immediately after the enzymes were added, the mixture was digested in a conical flask to allow for sufficient mixing during digestion. The reaction vessel was capped loosely with foil to avoid leaking of gases and an increase in pH. The pH was monitored at half hour intervals and adjusted throughout the digestion process with 5M HCL (Rodrigues et al., 2017).

2.3.2 Intestinal Phase

Five parts of the gastric chime is mixed with four parts of the SIF electrolyte stock solution (300 µm $CaCl_2$, 1.2mg/mL pancreatin, 8.5mg/mL bile salts, pH 6.5) made up to 400mL with water. Further monitoring at half hourly intervals, with pH adjustments was made during the SIF digestion phase. After the digestion process both digested solutions were aliquoted into 10mL volumes and frozen immediately at -20°C.

2.4 Inoculation and installation of the triple stage chemostat model

2.4.1 Gut Model

The triple stage chemostat model of the human gut is based on the work of Macfarlane et al. (1998), who validated a human gut model based on the quantification of the physicochemical and microbiological conditions within the intestinal contents of sudden death victims. The system was designed to reproduce *in vitro* the microbiome of bacteria in the human gut in a spatial and temporal manner. The model consists of three doubled walled vessels V1, V2 and V3; with V1 having an inner chamber volume of 280 mL and V2 and V3 operating on a slightly larger volume of 300 mL. The vessels are operated on a weir cascade system with growth medium being top-fed by a peristaltic pump into V1 at a controlled rate (D = 0.013 h^{-1}), with contents progressively moving through to V2 and V3 where the overflow runs into a waste vessel. Growth medium reservoirs and all three vessels were continually stirred, and each of the vessels was sparged with oxygen free nitrogen. The model was temperature controlled (37°C) and the pH of each vessel was automatically controlled to pH 5.50, 6.20 and 6.80 (±0.1) respectively, to reflect the increasing alkalinity within the human gut, from ascending to descending colon. The model was conducted in duplicate, model 1 as a control and model 2 as a test model.

2.4.2 Growth Medium

Growth medium for the gut model was prepared in 2L volumes (Table 3). After sterilization of the growth medium by autoclaving (15 mins, 121°C), resazurin (0.005g/L, R7017, Sigma-Aldrich) and glucose

(0.4g/L,41095-5000, Fisher) were added by sterile filtration through 0.22 µm syringe filters into the growth medium before use.

Table 3. Constituents of the gut model growth medium as a continuous culture system first described by Macfarlane et al. (1998).

Growth Medium	
Constituents	**g/L**
Peptone water	2.0
Yeast extract	2.0
Sodium chloride	0.1
di-Potassium hydrogen phosphate (K_2HPO_4)	0.04
Potassium di-hydrogen phosphate (KH_2PO_4)	0.04
Magnesium sulphate ($MgSO_4$. $7H_2O$)	0.01
Calcium chloride ($CaCl_2$. $2H_2O$)	0.01
Sodium hydrogen carbonate ($NaHCO_3$)	2.0
Tween 80	2.0 mL/L
Haemin	0.005
Cysteine HCl	0.5
Bile salts	0.5
Glucose	0.4
Arabinogalactan	1.0
Vitamin K_1	10 µL/L
Pectin	2.0
Starch (Potato)	3.0

2.4.3 Sample collection and inoculation of the model

Parents of seven healthy children were recruited to take part in this study, the children's ages ranged from 2 to 15 years old, with no history of antimicrobial treatment in the previous three months. Faeces were collected by parents and packaged to a UN3373 category B standard. Prior to sample collection informed written consent was obtained from each parent. Ethical approval for this study was granted by

the Health Science, Engineering & Technology EDCA at the University of Hertfordshire (UH protocol number LMS/PGR/UH/04981). Faecal samples were transported under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) to the laboratory within 12 hours of sample collection. A 10% (w/v) faecal slurry was obtained by pooling faecal samples and emulsifying in pre-reduced PBS pH 7.4 (70011044, Fisher), a smooth slurry was obtained by stomaching (Seward, Worthing, UK) followed by a coarse filtration through sterile muslin to a volume of 1L. The slurry was then used to inoculate both models to half volume in V1 and to a third volume in V2 and V3. The growth medium pump was started immediately after inoculation, with no further interventions. The model was left to equilibrate for ~15 days so that bacterial populations could attain a steady state, bacterial populations were continuously monitored during this time. Foaming within vessels was prevented by the addition of 1 mL of 10% polypropylene glycol (VWR International, Lutterworth, UK) *ad libitum*. Sampling was conducted at approximately the same time each day, and always in between interventions where applicable.

2.4.4 Dietary interventions in the gut model

Two models were run simultaneously, the test model and the control model. The control model had no interventions, while the test model after reaching a state where bacterial communities were stable had interventions introduced separately and sequentially. Interventions were introduced to the test model at day 16 of the experiment (Table 4), the volume administered at each intervention point was limited to 3mL. This volume was determined pragmatically on the limits of the gut model so as not to cause wash out of the bacteria within V1. As such a 3 mL volume of digested commercial formula (intervention one) was administered directly into V1, three times a day. These were administered at intervals meant to represent mealtimes (e.g. 09.00, 13.00 and 18.00). Intervention one (CF) lasted for 14 days, this time period was used to capture any changes in bacterial communities, and therefore sampling of the model would then reflect changes caused by diet. The second intervention followed immediately after the first, with no resting period, to represent the transition from commercial formula to a blended diet in a real-life scenario. The second intervention started on day 30 of the model, for another 14 days where 3 mL of the digested blended recipe was also administered into V1. This was also done three times a day at regular intervals as mentioned previously.

Table 4. Dietary interventions within the test model

Steady State (No intervention)	Intervention one (Commercial formula)	Intervention two (Blended tube feed)

Days 1-16	Days 16 to 30	Days 30 to 44
N/A	3mL into V1 3 times a day	3mL into V1 3 times a day

2.4.5 Enumeration of faecal bacteria

Sampling by selective agar viable counting was conducted every other day for the first two weeks, then bi-weekly for the following 4 weeks. At each sample point, 2 mL of sample was removed from vessel one, 0.5 mL was then serially diluted 10-fold to 10^{-7} in 4.5 mL of pre-reduced peptone water within an anaerobic cabinet (A35 HEPA, Don Whitley Scientific, Bingley, UK). A modified Miles and Misra viable counting technique was used as previously described (Baines et al., 2005; Miles et al., 1938), with 20 µL of each dilution inoculating the following selective agars in triplicate as shown in Table 5.

Table 5. Selective agars for isolation of bacterial species in both gut model**s**

Media and Incubation atmosphere	Supplements	Target species
MacConkey agar, no 3 (O_2)		Total facultatively anaerobic lactose fermenters
Aesculin Azide agar (O_2)	5 g/L agar technical and supplemented with 20 mg/L Kanamycin	*Enterococcus* spp.
Fastidious anaerobe agar (ANO_2)	5% horse blood	Total anaerobes
Beeren's agar (ANO_2)	42.5 g/L Columbia agar base; 5 g/L agar technical; 5 g/L glucose; 0.5 g/L cysteine hydrochloride; 5mL/L propionic acid	*Bifidobacterium* spp.
LAMVAB agar (ANO_2)	**Solution A**; 52.2 g/L MRS broth; 0.5 g/L cysteine hydrochloride, **Solution B**; 40 g/L agar technical; 0.05 g/L Bromocresol green, when mixed solution is supplemented with 20 mg/L vancomycin	*Lactobacillus* spp.

Agar plates used in the culture of anaerobic bacteria were pre-reduced for 24 h under anaerobic (ANO_2) conditions at 37^{o}C prior to inoculation. Additionally, agar plates used in the culture of aerobic bacteria were placed in an incubator overnight to reach 37^{o}C before inoculation. After inoculation all plates were then further incubated for 48 h, enumeration of colony forming units (CFU); colonies of 20-300 were counted to calculate the number of CFU/mL of the original sample. As each dilution was done in triplicate, the mean was used to calculate the CFU/mL of the sample. Colonies were identified to a genus level based on colony morphology and species level by MALDI-TOF (Matrix assisted laser desorption ionization time of flight) mass spectrometry (AXIMA-Confidence, Shimadzu Corporation, Kyoto, Japan) and the spectral archive and microbial identification system (SARAMIS system AnagnosTec GmbH, Potsdam-Golm, Germany). Sample analysis by MALDI TOF, a small amount of bacterial colony was smeared onto one well within a FlexiMass-DS 48 well polymeric MALDI plate, 1µL of 75% formic acid was pipette mixed into the sample, when nearly dry, 1µL of MALDI matrix solution was added and mixed by pipetting. *E. coli* DH5α was used as a control (99.9%) on spot wells between samples and plate was loaded onto plate carrier in the mass spectrometer for automated measurement. Bacterial identification is based on mass spectral signature of ribosomal proteins, nucleic acid proteins and cold shock proteins,

these spectra fingerprints are compared to and assigned to genus, species and subspecies using the SARAMIS commercial database.

2.4.6 Sampling for metagenomics

From both models, 5 mL samples were taken from each of the vessels every other day, for a total of 44 days. The samples were centrifuged at 7,000 rcf for 10 minutes at 20°C, to pellet the bacterial cells. The supernatant was removed to a sterile tube, both pellet and supernatant were frozen as soon as possible at -20°C for downstream analysis. A total of 36 samples were selected for gDNA extraction described below, one from each vessel, from both the test (Table 6) and the control (Table 7) models as shown below (Barcode NB02 was unavailable). Samples were pooled with the aim to provide data that is representative of the whole of the gut.

Table 6. Sampling of test model for metagenomics

Parameter	**Treatments**					
	None		Commercial Formula		Blended diet	
Day	**6**	**14**	**20**	**28**	**34**	**42**
Test V1 (pH5.5)	1	1	1	1	1	1
Test V2 (pH 6.2)	1	1	1	1	1	1
Test V3 (pH6.8)	1	1	1	1	1	1
Nanopore DNA library	Pooled and barcoded as NB01		Pooled and barcoded as NB04		Pooled and barcoded as NB06	

Table 7. Sampling of control model for metagenomics

Parameter	**No treatment**					
Day	**6**	**14**	**20**	**28**	**34**	**42**
Control V1 (pH5.5)	1	1	1	1	1	1
Control V2 (pH 6.2)	1	1	1	1	1	1
Control V3 (pH6.8)	1	1	1	1	1	1
Nanopore DNA library	Pooled and barcoded as NB03		Pooled and barcoded as NB05		Pooled and barcoded as NB07	

2.5 Bacterial gDNA extraction and quantification

Using the MagAttract® HMW (High Molecular Weight) DNA kit (67563, Qiagen), gDNA was extracted following manufacturer's instructions with the following amendments: RNase incubation for 30 minutes at 37^{o}C, lysates of buffer AL were centrifuge at 13,800 rcf for 30 seconds and supernatant used for the rest of the protocol, beads were washed three times, DNA was eluted after 10 minutes of incubation without mixing. Eluted DNA were quality assessed using Nanodrop spectrophotometer (ND-100, Thermo Fisher Scientific) for 260/280 and 260/230 ratios, Qubit 3 Fluorometer and Qubit Broad Range double-stranded DNA (dsDNA) quantification kit (both Thermo Fisher Scientific) for DNA concentration, Agilent TapeStation 4150 and Agilent Genomic DNA ScreenTape (Agilent Technologies) for DNA size distribution. Thirty-six DNA samples were pooled into six samples as seen in Table 6 and 7 for preparing DNA libraries for Nanopore sequencing as described below.

2.6 Library preparation and sequencing

The DNA library was prepared for Nanopore sequencing on the R9 SpotON flow cell and MinION Mk1C sequencer. The protocol (NBE_9065_v109_revAC_14Aug2019) for native barcoding genomic DNA using the Native Barcoding Expansion 1-12 (EXP-NBD104) in conjunction with the Ligation Sequencing Kit (SQK-LSK109) was followed. Input DNA was adjusted on basis of DIN value from TapeStation to calculate total mass of 1 µg or 100-200 fmol. The protocol incorporates steps for DNA repair and end preparation, native barcode ligation, and adapter ligation and clean up each step includes a AMPure beads step to improve sample purity before sequencing. After priming the R9 SpotON flow cell the library was loaded, sequencing was started using default parameters for data acquisition and fast basecalling in real time.

Sequencing status and real time data was observed using the MinKNOW UI, sequencing was stopped at ~24 hours and when active sequencing pores fell below 10 in the flow cell.

2.7 Bioinformatics

FastQ files were generated using Guppy (version 6.5.7) fast basecalling setting. Files were uploaded to Chan-Zuckerberg ID platform (CZ-ID: previously known as IDseq), an open-sourced cloud-based metagenomics pipeline for the initial assessment of the microbial content and species identification using NCBI databases. The pipeline (Nanopore mNGS Pipeline v0.7) filters out reads mapped to a human host, removes low quality and low complexity reads, duplicate reads and adapter sequences. Unique bases per million (bPM) mapped to specific microbial genus and species (Kalantar et al., 2020).

Richness, diversity and dissimilarity were carried out in R (version 4.3.3) using the Vegan package (2.6-5). For both model's alpha-diversity was carried out using the Shannon Wiener index and Simpson index, beta-diversity distance matrices were obtained using the Bray-Curtis method (Bray & Curtis, 1957; Shannon, 1948; Simpson, 1949).

3 Results

3.1 Commercial formula and blended tube food digestion

The digestion of CF and BTF including gastric and small intestinal phase with conditions simulating the fed state, the oral phase was omitted from the protocol to reflect tube fed conditions (Table 2). Bioavailability of micronutrients and phytochemicals from both feeding conditions was not assessed. In this study digestion parameters applied from the modified original method and were estimated to provide similar results for the bacterial metabolism of digested foods (Rowland et al., 2018). The final digestion state is shown in figure 3 for both conditions, showing change of consistency and lipid coagulation from the original consistency.

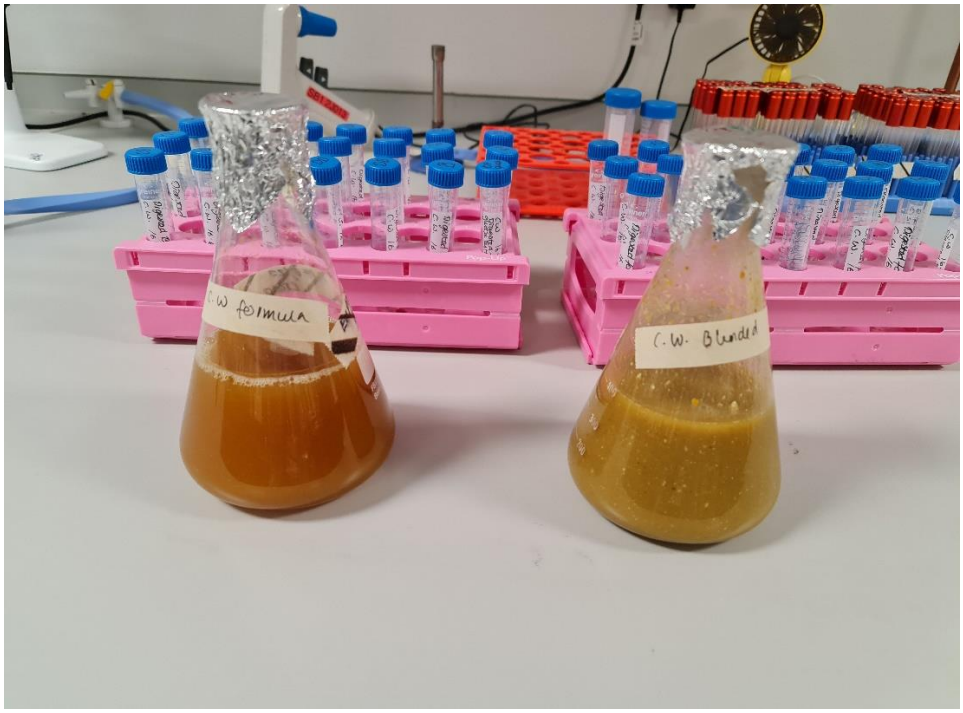


Figure 3. ***In vitro*** **pre-digested feed.** Commercial formula (left) and blended diet (right) after final digestion was complete. Flasks show a change of consistency after the digestion protocol, resulting in a watery consistency and lipids forming small lumps. Not all ingredients were digested from the blended condition where small bundles of vegetable fibers were seen.

3.2 In vitro gut model experiments

3.2.1 Enumeration of gut microflora by selective agar

Shown below is data from both the control model and the test model, results observed were from vessel one. Observations of bacteria cultured on FAA were observed from day two onwards. In the control model (Figure 4) during the first 14-16 days of the equilibrium period *Lactobacillus* spp., B*ifidobacterium* spp. and *Enterococcus* spp. declined compared to those observed on the first day of the experiment, with lactose fermenting Enterobacteriaceae and total anaerobes increasing compared to counts on the first day of the experiment. A reduction of lactose fermenting *Enterobacteriaceae* was observed during day 34 as was an increase of *bifidobacterium* spp. Viable counts by the end of the experiment were largely stable.

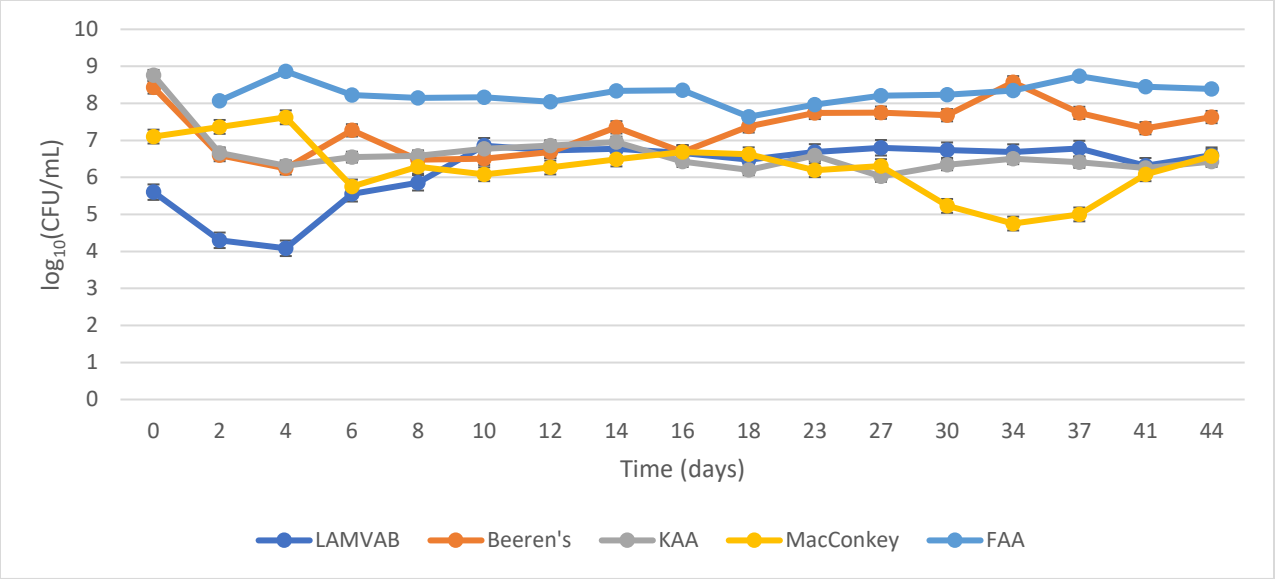


Figure 4. Bacterial trend in the control model. Mean viable counts of cultured *lactobacillus* spp (LAMVAB), *bifidobacterium* spp (Beeren's), *enterococcus* spp (KAA), lactose fermenting *Enterobacteriaceae* (MacConkey) and total anaerobes (facultative and obligate, FAA) within the control model were determined.

The plot shows that the test model during the first 14-16 days or the equilibrium period all bacterial groups decreased compared to counts on the first day of the experiment (Figure 5). A large reduction was seen in *Lactobacillus* spp at day 0 to days 2-4, from 8.6 $\log_{10}$(CFU/mL) to 4.4 CFU (Log), with a recovery at day 6 to 6.6 CFU(Log). A reduction was also seen in *Enterococcus* spp from day 0 to day 6 from 8.7 CFU(Log) to 4.3 CFU(Log) with a recovery at day 8 to 6.8 CFU(Log). By the end of the steady state period (Days 0-16) all bacterial groups were largely stable. During intervention one (CF) period (Days 16-30), all bacterial groups remained steady, with a small decrease in total anaerobes from 8.3 to 7.6 CFU(Log). During the intervention two (BTF) period total anaerobes increased from 7.8 CFU(Log) to 9.4 CFU(Log), whilst all other bacterial species remained largely stable.

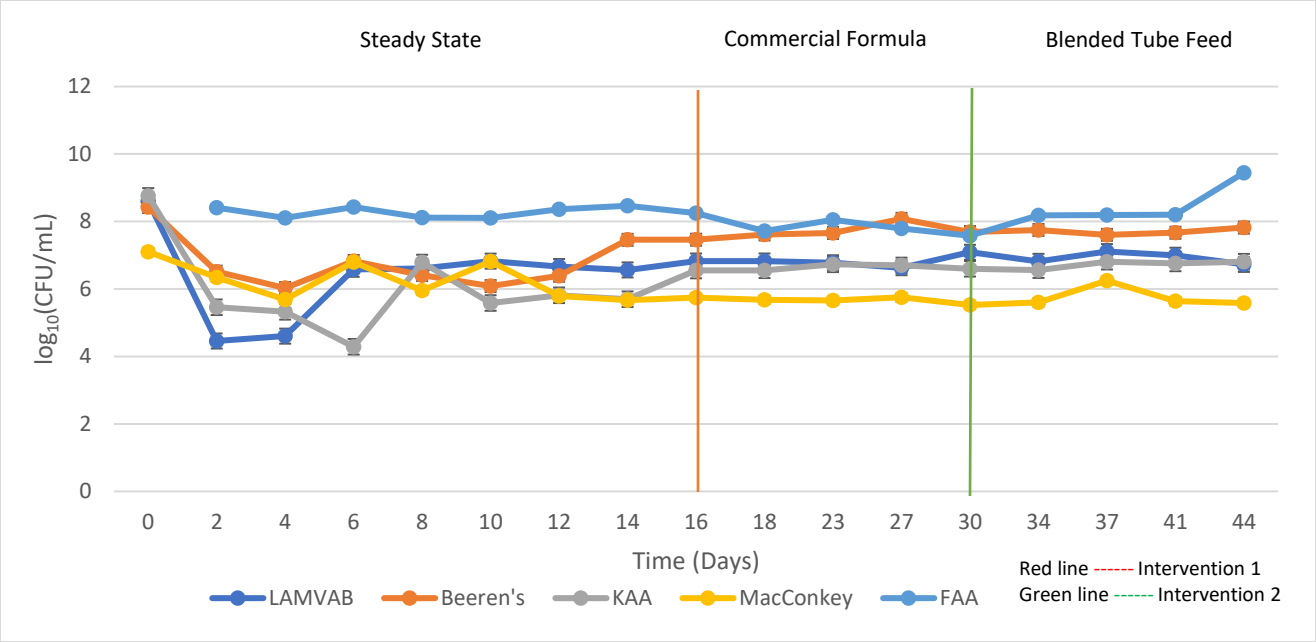


Figure 5. **Bacterial trend in test model**. Mean viable counts (log_{10} CFU/ml) of cultured *Lactobacillus* spp (LAMVAB), *Bifidobacterium* spp (Beeren's), *Enterococcus* spp (KAA), lactose fermenting *Enterobacteriaceae* (MacConkey) and total anaerobes (facultative and obligate, FAA) within the test model were determined. Red line denotes start of intervention one at day 16 (CF) and green line denotes start of intervention two (BTF) at day 30 (BTF).

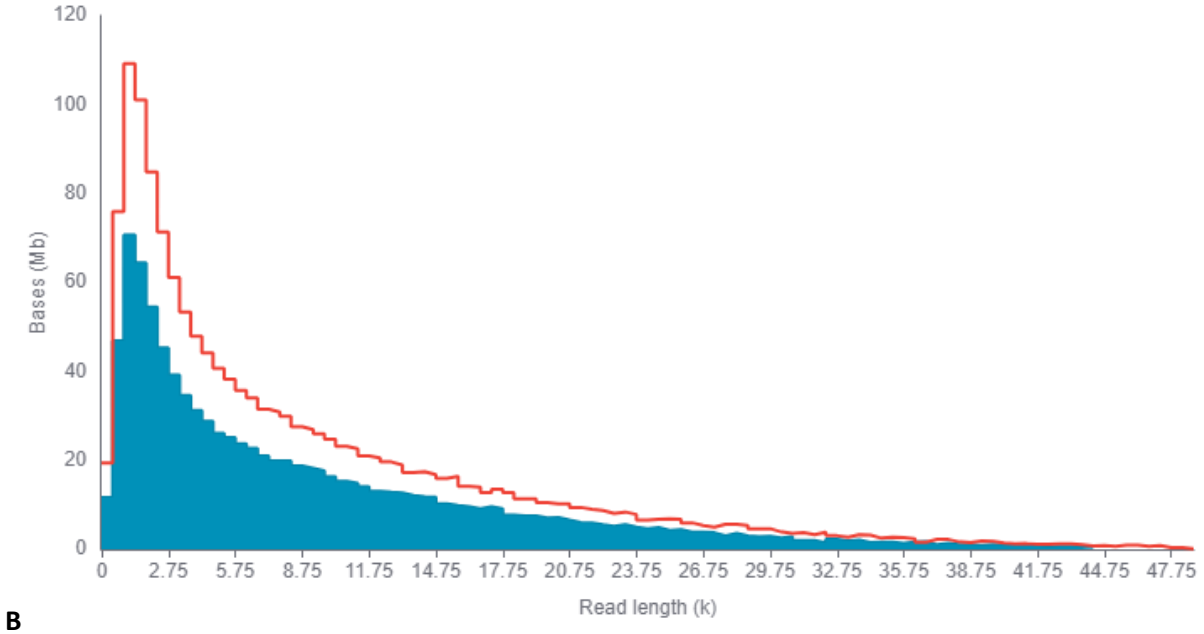
3.2.2 Nanopore sequencing

Genomic DNA (gDNA) was extracted from 36 gut model culture samples taken throughout the duration of the model using the MagAttract High Molecular Weight DNA kit (Qiagen GmbH, Hilden, Germany). The purity of the extracted gDNA ranged from 1.69-194 (A260/280 ratio), and quantities ranged from 15.8-155.2 ng/µL. The average DNA integrity number was 6.4 Kilobases (Kb). For barcoding, the samples were pooled (Table 6 and 7) into six barcodes, 117ng of gDNA was taken from each barcoded sample to produce a pooled sample to a total of 700ng, which was used to prepare the library for sequencing.

A flow cell check before sequencing detected 1,441 pore were available, and pore scans were performed every 1.5 hrs. to determine status within flow cell channels. Translocation speed of DNA travelling through the pores at 400 bases per second remained constant throughout the duration on the sequencing run. Sequencing was stopped when <10 pores were available to sequence, run time was 27 hours and 16 minutes, with a minimum read length of 200 base pairs (bp). The Q score of the generated reads was ~10.45 and remained stable throughout the duration of the sequencing, the minimum quality score to be accepted as a pass read was 8. The average read length was represented by N50 and estimated to be 6.53 kb (Figure 6A) with 485.4 k reads generated. An estimated 1.55 Giga bases (Gb)

were sequenced, 1 Gb of bases passed, and 534.63 Mega bases (Mb) failed (Figure 6B), The longest 1% of reads were classified as outliers, and were excluded from the analysis.

A



B

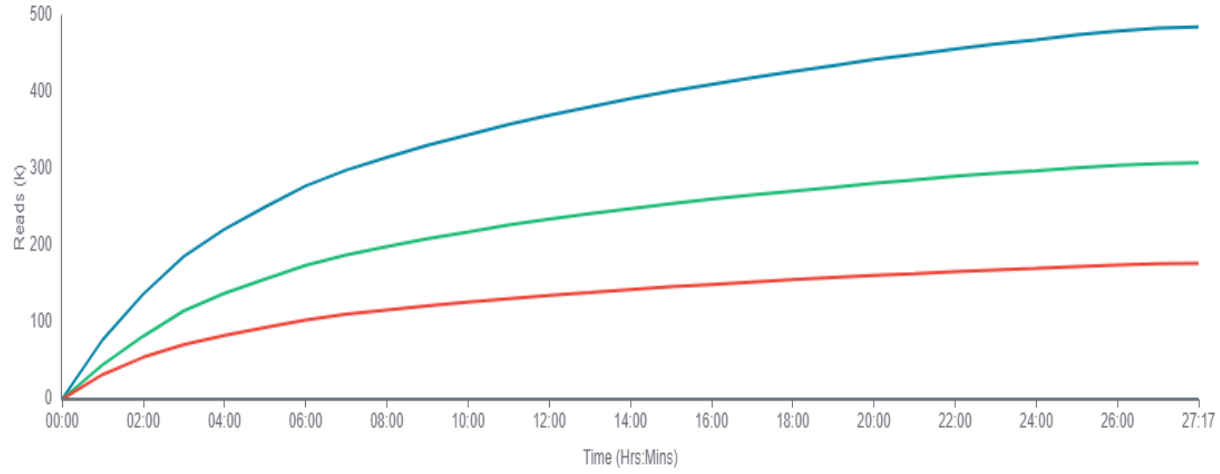


Figure 6. Summary of Nanopore reads. A, Read length graph shows total number of bases versus read length, the number of passed strands basecalled is represented by solid blue, the number estimated is represented by the red line. **B,** Cumulative total amount of reads sequenced, the red line shows the number of reads that failed the quality score threshold, the green line shows reads that passed or equal to the quality score threshold and the blue line shows total number of reads including passed and failed.

The total number of bases for each barcode is shown in Figure 7, the test model (Figure 7A) represented 65% of the total bases over six barcodes, while the control model (Figure 7B) represented 35% of the total bases over all six barcodes.

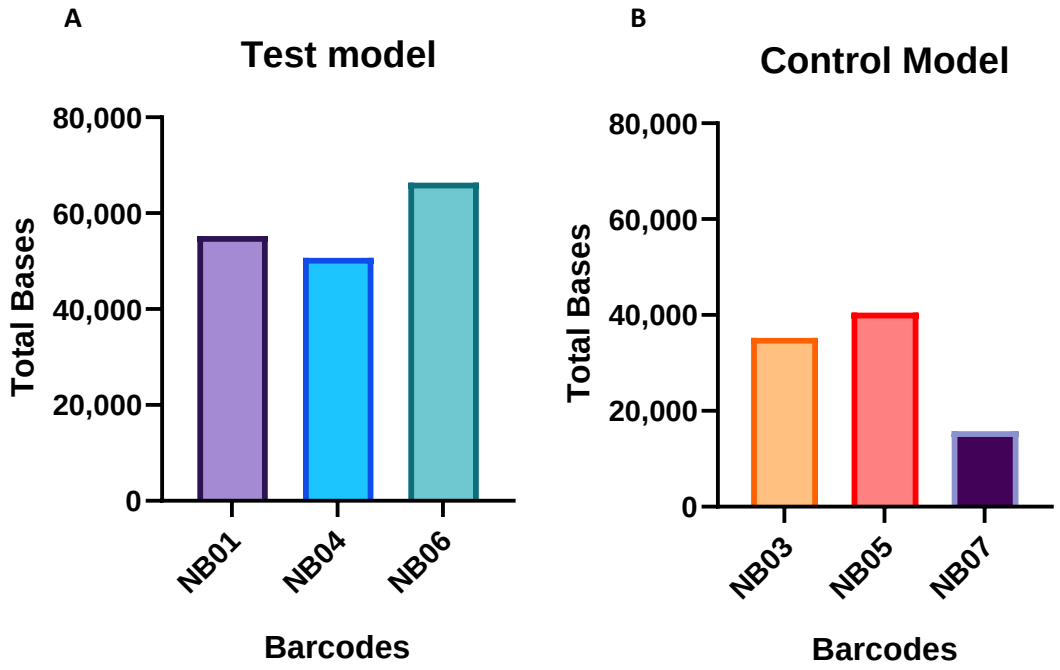


Figure 7. Sequence summary for barcoded bases. A, Total bases for test model barcodes NB01 (55,266), NB04 (50,700), and NB06 (66,370)**. B,** Total bases for control model barcodes NB03 (35,263), NB05 (40,497) and NB07 (15,741).

Nanopore sequencing barcode distribution within each model is shown in Table 8. Barcodes within the control model represented 35% of the whole sequencing sample, with barcode NB07 accounted for 17% within the model itself. All other barcodes were generally evenly distributed within each model.

Table 8. Distribution percentages of sequenced barcodes in the test and control model.

Test Model		Control Model	
Barcode	Distribution (%)	Barcode	Distribution (%)
NB01	32	NB03	39
NB04	29	NB05	44
NB06	39	NB07	17

3.2.3 Nanopore and CZ-ID

To include a diverse range of bacteria, the relative abundance cutoff was selected as 0.001%, which accounted for low bases per million rates. Within those parameters 38 bacterial genera were included in the analysis from the test model and 41 bacterial genera from the control model. The microbial profile used as a control was selected from the first two-week period, where microbial communities had received no interventions.

3.2.4 Validation of viable bacterial species and metagenomics of samples

Mean viable counts from selective agar were matched and compared to barcoded metagenomic data. Bacterial taxa from barcodes NB01, NB04, and NB06 were matched to cultured species (figure 8), bacterial species included within the counts for both lactose fermenting Enterobacteriaceae and total anaerobes can be found in Table 9. Compared to mean viable counts, the barcoded matched sequenced data was very low in comparison. Selective media results from the test model showed a significant difference (p-value < 0.05) between Beeren's viable counts in weeks 1 & 2 and weeks 5 & 6, but no other significant differences were found in selective media within the test model. Bacterial genera *(Lactobacillus, bifidobacterium, enterococcus*, lactose fermenting *enterobacteria* and total anaerobes) from barcoded metagenomic data were matched to the same time periods as the selective media, bacterial counts (bPM) were very low. Extremely low values for *enterococcus* and lactose fermenting *enterobacteria* were not visible on the graph (Figure 8), however, differences within genus prevalence between all conditions (week 1 & 2, week 3 & 4, week 5 & 6) of the weeks were significant (Tukey's multiple comparison test; p= 0.0001).

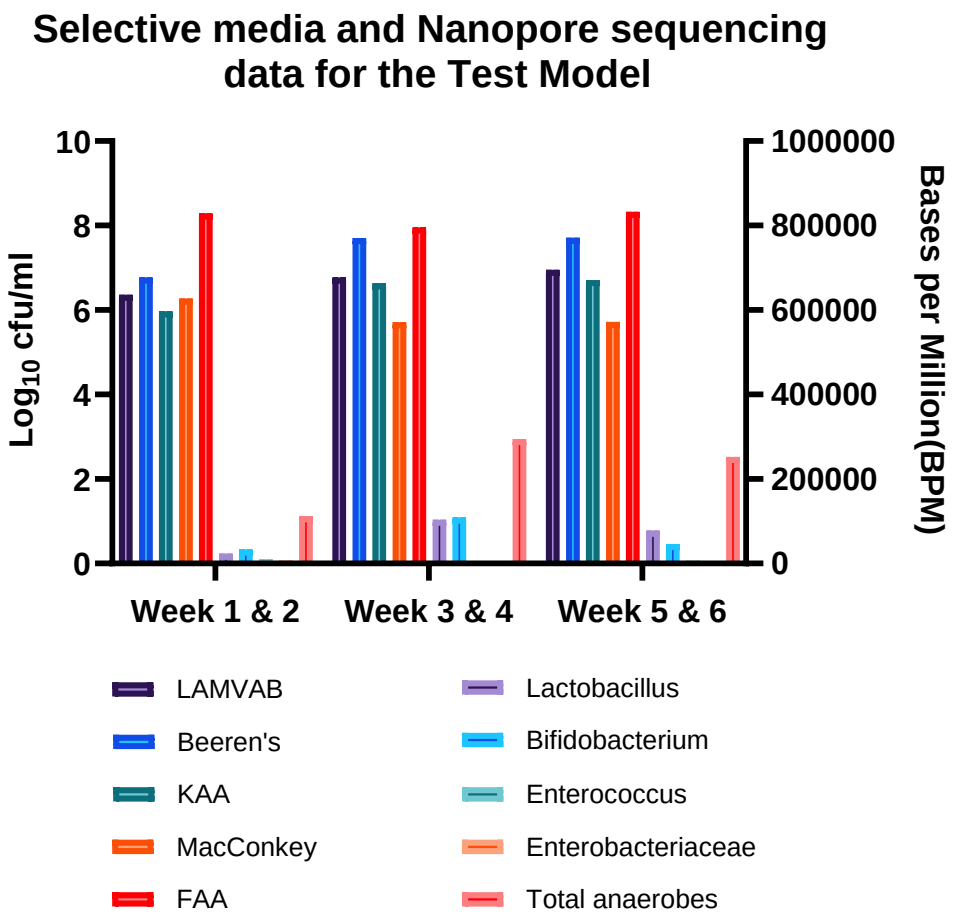


Figure 8. **Bacterial viable counts and sequence reads in the test model.** Mean viable counts from cultured bacteria (log_{10} CFU/ml) LAMVAB, Beeren's, KAA, MacConkey and FAA), significant differences in Log_{10}CFU/mL were found in Beeren's media between weeks 1 & 2 and weeks 5 & 6 (Tukey's multiple comparison test; *p*-value <0.05). Matched bacterial genera (*Lactobacillus, bifidobacterium, enterococcus,* lactose fermenting *enterobacteria* and total anaerobes) from barcoded metagenomic data, a significant difference was found within all genera over the three sets of weeks (Tukey's multiple comparison test; *p*-value <0.0001).

Bacterial taxa from barcodes NB03, NB05, and NB07 were matched to cultured species (figure 9), bacterial species included within the counts for both lactose fermenting Enterobacteriaceae and total anaerobes can be found in Table 9. Compared to mean viable counts, the barcoded matched sequenced data was very low in comparison. Selective media results from the control model showed a significant difference (*p*-value < 0.05) between LAMVAB and Beeren's viable counts in weeks 1 & 2 and weeks 5 & 6, with significant differences in MacConkey agar viable counts between weeks 3 & 4 and weeks 5 & 6 (*p*-value < 0.05) and weeks 1 & 2 and weeks 5 & 6 (*p*-value < 0.01). Bacterial genera *(Lactobacillus, bifidobacterium, enterococcus*, lactose fermenting *enterobacteria* and total anaerobes) from barcoded

metagenomic data were matched to the same time periods as the selective media, bacterial counts (bPM) were also low. Extremely low values for *Lactobacillus* (week 3 & 4, week 5 & 6) and lactose fermenting *enterobacteria* (week 5 & 6) were barely visible on the graph (Figure 9), however, differences within genus prevalence between all conditions (week 1 & 2, week 3 & 4, week 5 & 6) of the weeks were significant (Tukey's multiple comparison test; p= 0.0001).

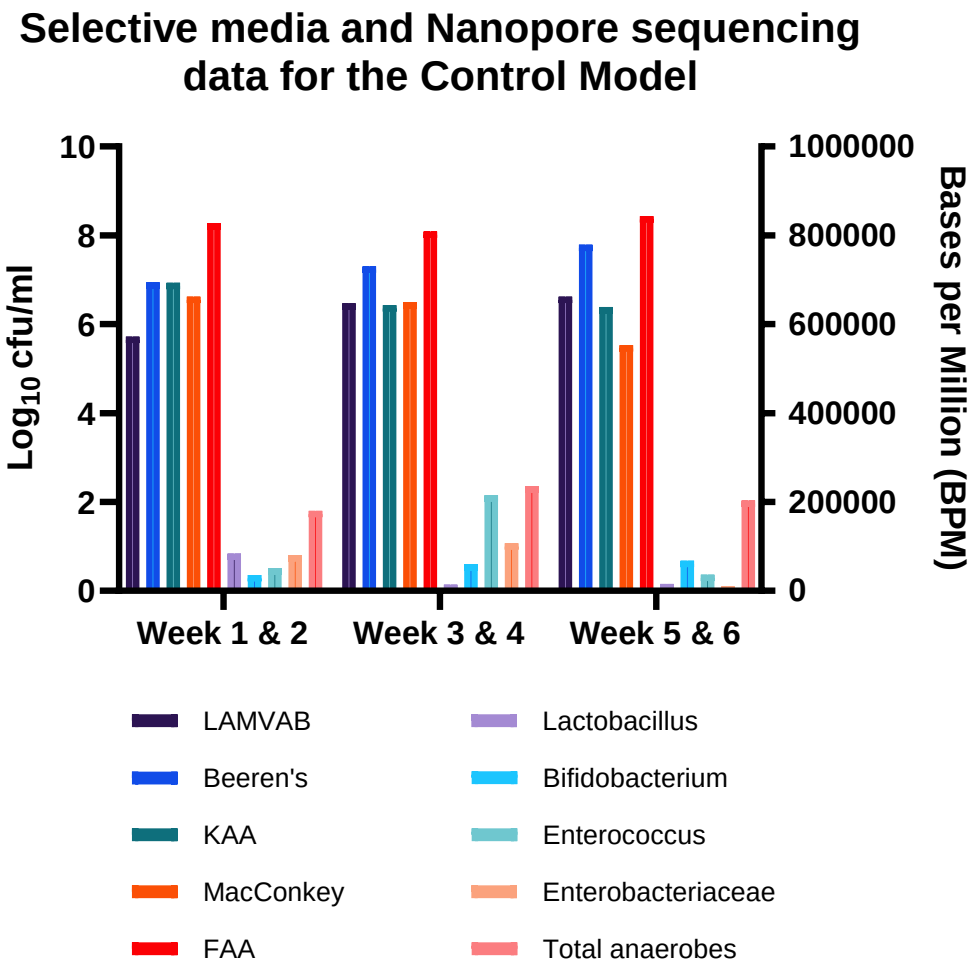


Figure 9. Bacterial viable counts and sequence reads in the control model. Mean viable counts from cultured bacteria (log_{10} CFU/ml) LAMVAB, Beeren's, KAA, MacConkey and FAA), significant differences in Log_{10}CFU/mL LAMVAB and Beeren's media between weeks 1 & 2 and weeks 5 & 6 (Tukey's multiple comparison test; p-value <0.05), significant differences in Log_{10}CFU/mL MacConkey media between weeks 3 & 4 and weeks 5 & 6 (Tukey's multiple comparison test; p-value <0.05), and between weeks 1 & 2 and weeks 5 & 6 (Tukey's multiple comparison test; p-value <0.01). Matched bacterial genera (*Lactobacillus, bifidobacterium, enterococcus*, lactose fermenting *enterobacteria* and total anaerobes) from barcoded metagenomic data, a significant difference was found within all genera over the three sets of weeks (Tukey's multiple comparison test; p-value <0.0001).

Table 9. Genera of bacteria generated from nanopore sequencing used to match to selective media for lactose fermenting Enterobacteriaceae (MacConkey) and total anaerobes (FAA).

Lactose fermenting Enterobacteriaceae	Total anaerobes
Escherichia	Fusobacterium
Enterobacter	Prevotella
Klebsiella	Bacteroides
Citrobacter	Clostridium
	Bifidobacterium
	Actinomyces

3.2.5 Control and Test model diversity

Despite the different barcode proportions and the low bases per million ratios for bacterial genera from the nanopore sequencing there is similarity between the species richness and evenness of distribution of bacterial genus in both conditions and between both models, as indicated by the Shannon-Weiner index. The index indicates the abundance or richness of bacterial species and evenness which is the distribution of the bacterial species present within the community, but not the identity of bacterial communities present. The Shannon-Weiner diversity index for the test model (Figure 10A) ranged from 2.17 to 2.42. The control model (Figure 10B) showed a range of 2.33 to 2.71 which is higher than the test model, suggesting a possibility the interventions had in impact on bacterial species richness and evenness.

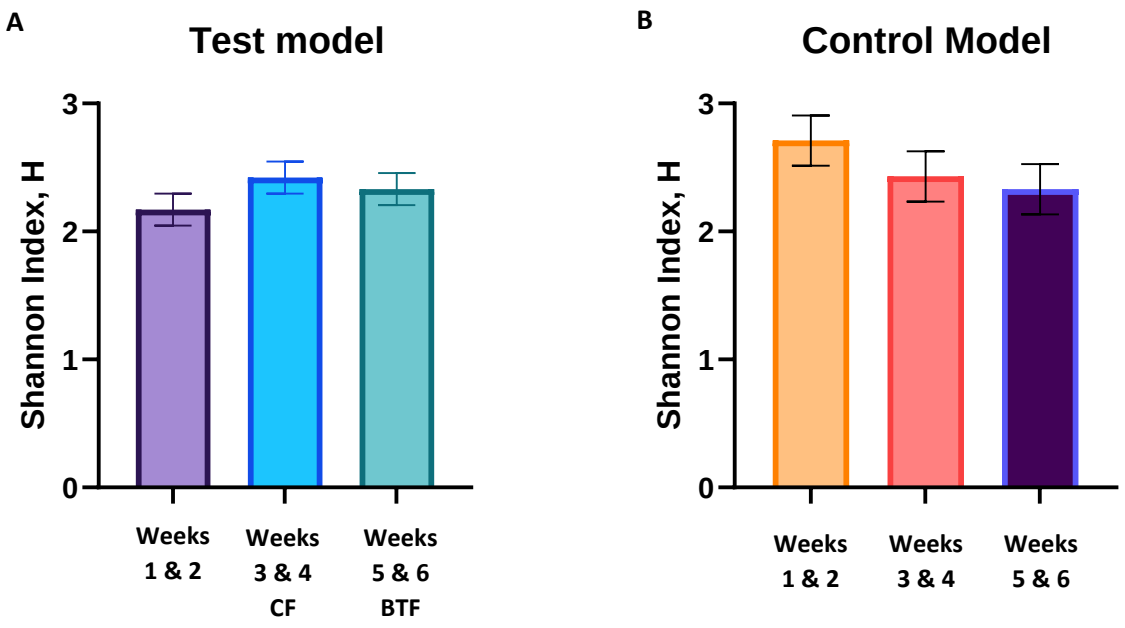


Figure 10. Bacterial diversity in test and control models. A Shannon diversity index for weeks 1 & 2 (Steady state, 2.17), weeks 3 & 4 (intervention one, CF, 2.42) and weeks 5 & 6 (intervention two, BTF, 2.33) within the test model. **B** Shannon diversity index for weeks 1 & 2 (2.71), weeks 3 & 4 (2.43) and weeks 5 & 6 (2.33) within the control model.

Simpson's diversity index has a range of 0-1; the higher the score the more diverse and stable the environment. The Simpson's diversity index for the test model (Figure 11A) showed a range 0.19 to 0.35, indicating a low diversity and unstable environment, week 1 & 2 showed the highest diversity, week 3 & 4 shows a sharp decrease in diversity, week 5&6 shows a slight increase in diversity. The Simpson's diversity index for the control model (Figure 11B) showed a range of 0.11 to 0.22, over time the diversity and stableness of the control model does increase.

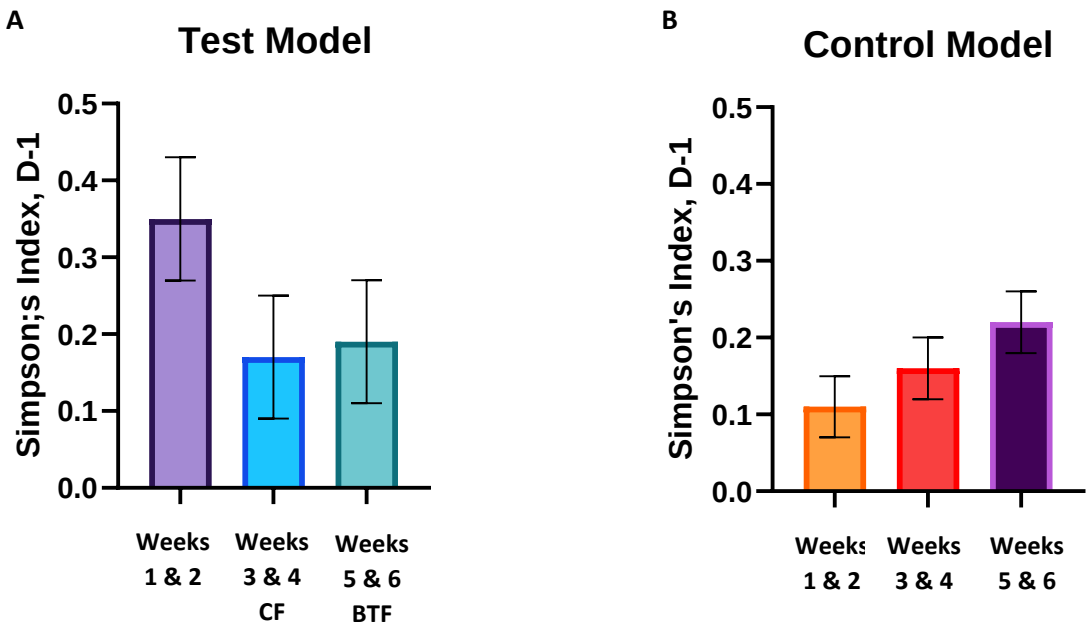


Figure 11. Bacterial diversity in test and control models. A Simpson's diversity index for weeks 1 & 2 (0.35), weeks 3 & 4 (CF, 0.17) and weeks 5 & 6 (BTF, 0.19) within the test model. **B** Simpson's diversity index for weeks 1 & 2 (0.11), weeks 3 & 4 (0.16) and weeks 5 & 6 (0.22) within the control model.

The Bray-Curtis dissimilarity index ranges between 0-1, where 0 indicates that the two samples share the exact number and type of bacterial species, and 1 indicates that the two samples share none of the same bacterial species. The index was calculated pairwise between samples represented by barcodes within each model. The dissimilarity of species composition between microbial communities within the test model (Figure 12A) showed that week's 1 & 2 (0.474) and weeks 3 & 4 (CF, 0.473) were moderately dissimilar from each other, while weeks 5 & 6 (BTF, 0.349) was less dissimilar. Both week's 1 & 2 and weeks 3 & 4 shared around the same dissimilarity between each other, suggesting higher variation of bacterial species between these two samples. In contrast weeks 5 & 6 shared less dissimilarity to both weeks 1 & 2 and weeks 3 & 4, suggesting that week's 5 & 6 shared a moderate number of bacterial species between both barcodes. The differences in bacterial composition may be a consequence of the interventions as this was not seen in the control model. In the control model (Figure 12B) week's 1 & 2 (0.35) a moderate dissimilarity is seen between weeks 3 & 4 (0.334) and week's 5 & 6 (0.37), whereas weeks 3 & 4 shared less dissimilarity between the other samples represented by barcodes. week's 5 & 6 showed the highest dissimilarity of shared microbial species and presence between barcodes, however the difference in values between barcodes was 0.036 indicating small shifts in dissimilarity and stability during the duration of the control model that was not seen in the test model.

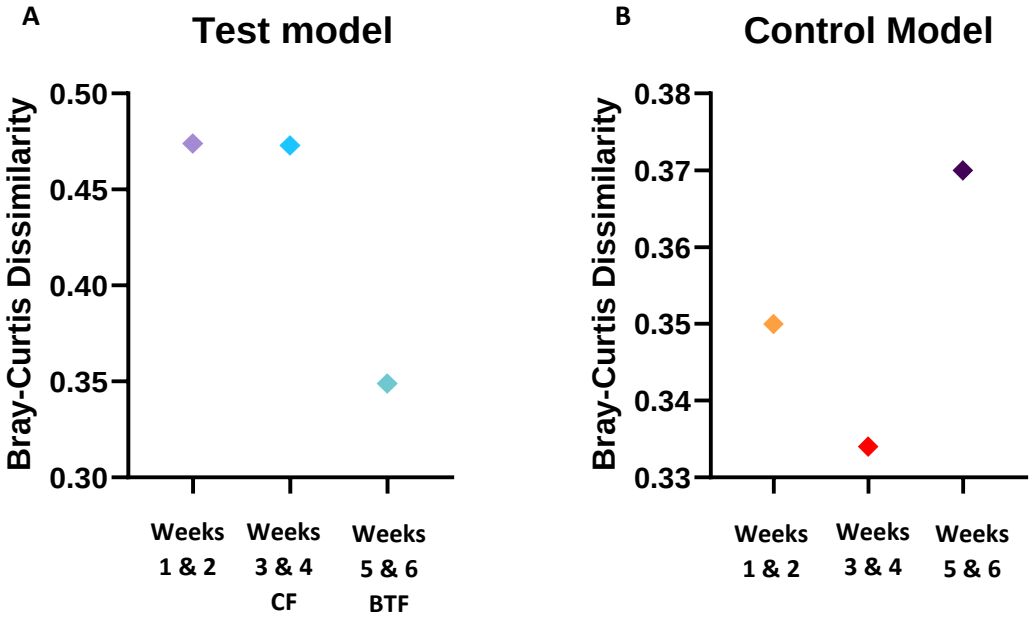


Figure 12. Bacterial similarity in test and control models. A, Bray-Curtis dissimilarity showing the difference in bacterial species between barcodes weeks 1 & 2 (NB01, 0.474), NB04 (NB04, 0.473) and NB06 (NB06, 0.349) within the test model. **B,** Bray-Curtis dissimilarity for barcodes NB03 (NB03, 0.35), NB05 (NB05, 0.334) and NB07 (NB07, 0.37) within the control model.

3.2.6 Prevalence of genera between models

The abundance of bacterial genera and species in both the control model and the test model showed a good degree of variation over the experiment duration (Table 10 and 11).

The highest number of reads assigned to a given genus within the test model during week 1 & 2 (preintervention) was aligned to the genus *Collinsella* at 48.2% (Table. 10). With the most predominant species within those reads being *Collinsella aerofaciens*. However, a reduction in reads was reported with the commencement of CF to 12.11% (weeks 3 & 4) then a further reduction was reported to 11.48% during BTF (weeks 5 & 6). A contrast was seen in the prevalence of *Collinsella* in the control model where prevalence in week 1 & 2 was reported as 15.70%. A reduction was reported in week 3 & 4 to 12.53% with a sharp rise in weeks 5 & 6 to 35.10% (Table. 11). In the test model the genus *Bacteroides* contained the highest number of species with 28-30 bacterial species identified throughout the duration of the model including *B. ovatus, B. uniformis and B. fragilis*. The prevalence of *Bacteroides* in the test model increased from 11.45% in weeks 1 & 2, to 26.86% during CF and decreased during BTF

to 15.97%, however, prevalence exceeded preintervention levels (Table. 10). In contrast prevalence of *Bacteroides* within the control model remained stable ranging from 17.29-19.28%, suggesting interventions influenced variability in prevalence. Interestingly, a similar trend in prevalence was reported in *Bifidobacterium* (*B. longum* and *B. breve*), and *Lactobacillus* (L. *gasseri* and *L jensenil*). Where an increase in prevalence was reported during CF (weeks 3 & 4) followed by a decrease in BTF (weeks 5 & 6) but exceeding preintervention levels (Table.10). The most noticeable increase in prevalence was seen in the *Lachnospiraceae* family, (*Eubacterium rectale*) where prevalence at weeks 1 & 2 (preintervention) was reported at 6.04% decreasing during CF (weeks 3 & 4) to 1.04% and increased during BTF (weeks 5 & 6) to 31.37%. This dramatic increase was not seen within the control model where levels of *Lachnospiraceae* dropped and remained low during the duration of the model. Prevalence of *Enterocloster* (*C. bolteae* and *C. clostridioforme*) in the test model in weeks 1 & 2 was 3.33% increasing to 3.96% during CF and decreasing by around half to 1.97% during BTF (weeks 5 & 6). As seen in Table. 10 similar prevalence trends to *Enterocloster* were reported in *Lachnoclostridium* (*C. scindens*), *Lacrimispora* (*C. sphenoides*) and *Hungatella hathewayi*. A reduction in *Alistipes* (*A. shahii* and *A. communis*) from 2.64% in weeks 1 & 2 to 1.10% during CF and increasing slightly to 1.16% during BTF in the test model, with a similar prevalence trend reported in control model. A reduction in prevalence was seen within the test model for *Lacticaseibacillus* (*L. paracasei*), *enterococcus* (*E. faecalis*) and *blautia* (*R. obeum*) where prevalence was reduced in CF and BTF compared to preintervention. Within the control model only the genus *blautia* showed the same prevalence trend, suggesting that interventions had no effect on prevalence levels (Table.11). *Lacticaseibacillus* (*L.* paracasei) reported a decreased prevalence in the control model from 0.56% in weeks 1 & 2, decreasing to 0.25% in weeks 3 & 4 increasing to 0.56% in weeks 5 & 6 (Table. 11). The reverse was seen in genera *Enterococcus* (*E. faecalis*) in the control model where prevalence was 6.91% in weeks 1 & 2 rising sharply to 26.87% in weeks 3 & 4 and then decreasing to 5.32% in weeks 5 & 6. This prevalence data from the control model suggests that changes of prevalence within the test model could be a result of the interventions administered. In the test model prevalence of *Faecalibacterium* (*F. prausnitzii*) ranged from 1.22% during preintervention to 1.11% in CF, increasing to 5.95% during BTF, in contrast prevalence within the control model was 2.47% in weeks 1 & 2 decreasing to 0.41% in weeks 3 & 4 and increasing to 0.88% in weeks 5 & 6 (Table.11). This contrasting data between models suggests that BTF had an effect on prevalence. In the test model prevalence of *Dialister* (*D. hominis*) stayed evenly distributed, however, in the control model prevalence decreased from 1.18% in weeks 1 & 2 to 0.32% in weeks 3 & 4, increasing to 0.96% in weeks 5 & 6 (Table.11), this also suggests interventions had an effect on prevalence. In the test model Megasphaera

(*M. hexanoica*) increased from 0.46% at preintervention to 4.25% in CF decreasing to 3.60% during BTF, in contrast prevalence in the control model increased from 0.19% in weeks 1 & 2 to 0.58% in in weeks 3 & 4 decreasing to 0.35% in weeks 5 & 6. This supports the suggestion that prevalence within the test model could also be a result of the interventions administered. Within both models prevalence of a genus was not dependant on the number of species within that genus. For example, the genus *Bacteroides* showed 28 species at both 11.45% and at 26.86%, and 30 species within a prevalence of 15.97%.

Table 10. Bacterial genera present in the triple-stage chemostat model of the human gut. Inclusion criteria is based on prevalence to ~1% and is based on data during the first two weeks of the model where bacterial populations reached a steady state. Additional genera were included where prevalence was seen above 1% during intervention one, CF (weeks 3 & 4, NB04) and intervention two, BTF (weeks 5 & 6, NB06).

Taxon	Weeks 1 & 2 (No intervention)		Weeks 3 & 4 (CF)		Weeks 5 & 6 (BTF)	
	N° species	Prevalence %	N° species	Prevalence %	N° species	Prevalence %
Collinsella	17	48.18	7	12.11	14	11.48
Bacteroides	28	11.45	28	26.86	30	15.97
Lachnospiraceae	14	6.04	12	1.39	13	31.37
Bifidobacterium	8	5.07	10	15.96	10	6.16
Lactobacillus	12	3.47	11	15.18	14	10.40
Enterocloster	3	3.33	2	3.96	3	1.97
Lachnoclostridium	5	3.10	5	3.82	6	2.56
Alistipes	7	2.64	7	1.10	6	1.16
Lacticaseibacillus	4	2.23	4	1.06	3	0.37
Enterococcus	10	1.49	7	0.77	10	0.83
Faecalibacterium	1	1.22	1	1.11	4	5.95
Blautia	8	1.17	9	0.56	6	0.55
Dialister	3	0.99	3	0.85	3	0.98
Lacrimispora	2	0.62	1	1.83	2	0.19
Megasphaera	5	0.46	5	4.25	7	3.60
Hungatella	1	0.42	2	2.20	1	0.12

[1] Percentage prevalence data from CZ-ID with barcodes that represented samples from the test model.

Table 11. Bacterial genera present in the triple-stage chemostat model of the human gut. Inclusion criteria is based on prevalence to ~1% and is based on data during the first two weeks (NB03) of the model where bacterial populations reached a steady state. Additional genera were included where prevalence was seen above 1% during weeks 3 & 4 (NB05) and weeks 5 & 6 (NB07).

Taxon	Weeks 1 & 2		Weeks 3 & 4		Weeks 5 & 6	
	N° species	Prevalence %	N° Species	Prevalence %	N° Species	Prevalence %
Bacteroides	24	17.29	27	19.28	21	18.82
Collinsella	24	15.70	15	12.53	18	35.10
Lactobacillus	10	11.25	8	1.76	8	2.32
Escherichia	5	9.99	2	7.03	1	0.19
Lachnospiraceae	11	9.91	13	0.75	9	2.60
Enterococcus	10	6.91	14	26.87	10	5.32
Bifidobacterium	8	4.83	10	7.40	7	9.85
Lachnoclostridium	7	3.16	5	2.68	4	4.20
Enterocloster	2	2.54	5	1.91	2	3.09
Faecalbacterium	2	2.47	1	0.41	1	0.88
Clostridium	16	2.05	20	2.60	6	0.83
Alistipes	7	1.94	8	0.94	3	0.94
Blautia	9	1.32	7	0.86	7	0.88
Dialister	3	1.18	3	0.32	3	0.96
Eubacterium	6	0.52	4	1.20	4	0.58
Enterobacter	6	0.45	12	3.71	6	0.52
Schleiferilactobacillus	1	0.44	1	3.42	1	6.01
Klebsiella	1	0.21	9	1.70	1	0.21

[1] Percentage prevalence data from CZ-ID with barcodes that represented samples from the control model.

4 Discussion

Many tube fed children suffer from gastro intestinal dysbiosis with symptoms such as diarrhea and reflux, little is known on how artificial nutrition affects the gut microbiota in children and whether gut dysbiosis is associated with diet.

The aim of this study was to- examined whether commercial formula and blended tube feeds altered gut microbial communities in tube fed children, using an *in vitro* gut model of microbial communities derived from healthy children. The objectives were: i) to design a protocol for digestion that reflects enteral tube feeding for children; ii) to investigate the response of gut microbial communities on two dietary interventions using a triple-stage chemostat model that simulates the human gut inoculated with faecal slurry from healthy children iii) to examine and analyze the response of gut microbial communities following a change in feed, by selective culture and using gDNA from microbial culture for long read Nanopore sequencing and metagenomic analysis.

The initial composition of the microbiota defined the microbial signature and was used to compare any changes in bacterial composition during dietary interventions. Also, a modified protocol for digestion for use within a three stage chemostat *in vitro* gut model, omitting the oral digestion phase to mimic the digestion process of tube fed children. Metagenomic analysis of microbial data was over three time points spanning the duration of the experiment, and pooled samples were used so that changes in relative abundance of bacterial composition could be attributed to either a formula or blended feed within the seeded microbiota.

4.1 Blended recipe and digestion process

The ingredients and preparation of the blended feed (see sections 2.1 & 2.2) were designed to replicate typical feeds that might be prepared for children in their own homes so that any study findings might have potential relevance to real life situations. In the absence of precise guidelines for the content or preparation of blended feeds, details from parents who made blended feeds, and a published in vivo study (Madden et al 2019) were used to inform the content and composition of the blended feed used in this study. It is noted that blended feeds given to children in real life are usually not based on a single recipe (reference) and this difference may limit the extrapolation of the microbial findings. This is a study limitation that could be investigated in future studies by expanding the range of blended feeds tested. In addition, the impact of the cooking process in this study was not assessed, however, the study by Lerma-Aguilera et al (2024) assessed whether any measurable changes within the microbiota could be attributed to cooking method. The study investigated both the cooking method and the effect of raw and cooked

food on microbial abundances using batch models. The study excluded steaming food, but the method of boiling potatoes was examined, which was shown to lower abundance of the Firmicutes phylum, this may be a result of hydro-soluble nutrient loss, limiting the growth of some bacteria and impacting the stability and diversity of the microbiome (Lerma-Aguilera et al., 2024).Taking into consideration these findings and how transferable they would be to this study, the impact alone of boiling potatoes that were included within the blended recipe on the overall abundance of Firmicutes within the test model would be insignificant. The proportions used within the Lerma-Aguilera study were greater as potatoes were tested as a singular food source, in comparison to the smaller proportions used within the whole food recipe in this study (Lerma-Aguilera et al., 2024).

The digestion process of both the commercial formula and the blended feed was modified from a method by Rodrigues at al. (2017) to include the gastric and small intestinal phases, simulating a fed state condition. Efficiency of digestion was not quantified by measuring the products of digestion due to time constraints (Rodrigues et al., 2017). The BTF in this study was a single recipe matched nutritionally to the CF, and although tailored to represent a real life BTF, it cannot reflect all BTFs. This is a study limitation that could be addressed in future experiments.

4.2 Gut model and dietary interventions.

There has been limited or no published research utilizing a three stage chemostat fermentation model to measure whole food dietary interventions identified during this research study. The use of chemostat models to measure community dynamics, cross-feeding mechanisms, treatment responses on gut microbiota seeded from targeted donor populations, makes it possible to monitor temporal colon dynamics in an environment that simulates basic parameters of the gut. Most nutritional studies use batch fermentation to ascertain the impact of food containing beneficial components or dietary fibre on gut microbiota. These cultures are typically maintained for 24-48 hrs. Although batch fermenters are simple to set up and operate, they are limited by depletion of nutrients, pH changes and accumulation of fermentation and growth inhibitors over time (Kang et al., 2022), this makes batch fermentation models not ideal for monitoring the effects of nutrients on gut microbiome, particularly beyond 24 h (Pérez-Burillo et al., 2021; Venema & van den Abbeele, 2013). Unsuitability to study whole foods in batch fermenters arises partly due to the ability of gut microbes to ferment carbohydrates, which lowers the pH of the simulated gut environment. Since SCFA production correlates with a highly acidic production in the gut, inhibition of SCFA synthesis would occur, resulting in an unrepresentative metabolite production compared to three-stage chemostat *in vitro* gut environments. An important

consideration is that changes in diet could take more than 24 h to have a measurable effect on the gut microbiome *in vivo*, it would be ideal to have a model that allows the assessment of gut microbiome changes over longer periods. In the three stage chemostat model used here, the test and control model pH was regulated automatically to replicate in vivo conditions as closely and as consistently as possible. The gut models (control and test) were run in parallel, seeded with the same faecal inoculate to keep results as standardized and robust as possible, both models were maintained for 44 days, capturing gut microbiome dynamics in a time frame that was physiologically relevant to measure changes in the gut microbiota due to dietary interventions.

4.3 CZ-ID mNGS Nanopore pipeline

Metagenomic next generation sequencing (mNGS) is a powerful tool that uses sequencing technologies to analyze the genomic content of samples. As such, metagenomics can potentially detect all microbes present in a sample, whether or not they have been previously characterized or cultivated (Vijay & Valdes, 2022). In this study it was essential to quantify the microbial communities within and between samples, and to be able to gain an insight into changes of diversity, so that inferences could be made on whether diet had an influence on microbial content. When sequencing generating long reads provides an advantage in mNGS, enabling a detailed view of microbial communities within a given sample by providing non-biased, quantitative, and comprehensive *de novo* assemblies (Trigodet et al., 2022). In this study the reads on average were 6,000 base pairs which is above the threshold classified as a long read (>4,000 base pairs) (Wang et al., 2021). This allowed for reliable and accurate recovery of partial or complete metagenome assembled genomes (MAGs), that were mapped to NCBI nucleotide (NT) and non-redundant protein (NR) databases within the CZ-ID mNGS nanopore pipeline, giving accurate characterization of complex microbial communities within gut model samples (Ciuffreda et al., 2021).

CZ-ID is a fully automated, code-free open-source platform for characterizing microbes within a sample, it is designed as a tool to support researchers with limited computational capability. The pipeline removes the host sequencing leaving only the bacterial reads (Gu et al., 2019), and has been benchmarked against the well-used metagenomic system Kraken 2 (Simmonds et al., 2024). CZ-ID (IDseq) has been a key component in recent studies achieving taxonomic identification and relative abundance estimations comparable to other pipelines in the field. The default database of CZ-ID is NCBI (nucleotide (NT) and non-redundant protein (NR) databases). Kalatar et al (2020) benchmarked CZ-ID to reference datasets containing 525 bacterial species to measure performance metrics using area under the precision recall curve (AUPR). Where 1.0 indicates true positive species are identified with no false

positives species, datasets from CZ-ID (IDseq) observed AUPR generated means across all datasets of 0.9627-0.9633 (Kalantar et al., 2020). This is the first *in vitro* gut model study that applied CZ-ID for metagenomics.

4.4 Richness, Diversity and Enterotypes

Quantifying the Alpha and Beta diversity of microbial communities between the two models showed that microbial communities responded to interventions. Diet has been shown to have a modifying effect on the gut microbiota affecting the richness and diversity of microbial communities. In this study microbiota diversity was quantified using the Shannon-Weiner index, Simpson's index and Bray-Curtis dissimilarity index (Bray & Curtis, 1957; Shannon, 1948; Simpson, 1949). Data generated from the control model showed trends in bacterial community diversity without interventions. This data was used as a benchmark to assess whether changes in microbial communities within the test model could be a consequence of dietary interventions.

In the control model alpha diversity measured by Shannon-Weiner diversity index ranged from 2.71 to 2.33 and showed the overall diversity within each sample represented by each barcode. It is not surprising that these values for samples have a lower diversity, most studies use either fresh faecal samples or samples directly from the environment of interest where bacterial loss is at a minimum. Gut model studies culture microorganism's representative of the gut microbiota, but some species are unable to thrive and are therefore not represented, reducing the overall diversity within samples (Kers & Saccenti, 2022). The values for the control model indicated that over time the richness of species present and the relative abundance or the evenness of the bacterial species within each of the samples declined slightly. This was supported by Simpson's inverse diversity index which ranged from 0.11 to 0.22 showing a low diversity and that there were a few dominant species within the communities which causes low stability, however, diversity did increase slightly over time and reflected similar results to the Shannon index. This suggested that although the richness and diversity of the taxa declined as reported by the Shannon index, the proportion of dominant species within the sample increased as shown by the Simpsons index. The composition similarity of the samples within the control model was calculated using the Bray-Curtis dissimilarity index which ranges from 0-1 where 0 indicates the composition is identical and 1 indicates the composition is completely different. The dissimilarity index quantified the differences in the overall taxonomic composition between the three samples represented by the barcodes within the control model. Results showed that there was a moderate taxonomic dissimilarity

between the three samples, that all sites shared common taxa within the composition of the bacterial communities during the duration of the gut model.

In the test model alpha diversity measured by Shannon-Weiner diversity index ranged from 2.17-2.42 and showed the overall diversity in each sample represented by each barcode. The overall diversity for the model was quite low, however, there was an increase in diversity during intervention one (CF) compared to the steady state. This suggested that intervention one (CF) had a positive effect, increasing the number of species and evenness with which they are distributed throughout the bacterial community. Similar results were seen during intervention two (BTF) where diversity was slightly reduced compared to intervention one (CF) but was increased compared to the steady state. This suggests that intervention two (BTF) also had a positive effect by increasing the number of bacterial species and distribution throughout the bacterial community, especially as diversity indices value exceeded the steady state value. Interestingly, diversity in the test model calculated by Simpson's inverse diversity index ranged from 0.19 to 0.35 and values exceeded those of the control model suggesting that both interventions caused a positive effect on diversity and stability. Simpson's index values for the steady state showed the highest diversity overall and the highest stability, during intervention one (CF) diversity decreased from 0.35 to 0.17 showing diversity was reduced by half compared to the steady state. This suggests an increase in prominent taxa which in results in a decrease in diversity. During intervention two (BTF) the bacterial community diversity increased compared to intervention one (CF), however, diversity was still lower than reported in the steady state. This suggests that intervention two (BTF) had a positive effect by increasing diversity within the bacterial communities and reflected similar results to the Shannon index. The Bray-Curtis dissimilarity index for the test model calculated there was a moderate taxonomic difference in bacterial composition between intervention two (BTF) and both intervention one (CF) and the steady state. The dissimilarity for both intervention one (CF) and the steady state were very similar at 0.474 and 0.473, this result showed that between these time periods there was larger difference between taxonomic composition than during intervention two (BTF). Interestingly, intervention two (BTF) showed similar levels of taxonomic dissimilarity to the control model, suggesting that this could be a natural variation of composition within gut models.

The Shannon's index is more sensitive to the number of species within a sample, whereas Simpson's index is less sensitive to the number of rare species within samples and is more focused on abundance of dominant species. However, both indices provide insights into overall community diversity which was the focus of this study.

Donated faecal samples into the gut model were from healthy children aged 2-15years old, the mixing of microbiomes created an artificial community which was used to seed the gut model. The steady state allows for selective pressure from competing bacterial populations to create homeostasis within the microbial communities with a distinct microbial profile or signature. An analysis of gut microbial communities proposed three enterotypes which are not nation or continent specific, composed of species that together, contribute to a preferred community composition (Arumugam et al., 2011). Wu et al. (2011) linked long-term dietary patterns with gut microbial enterotypes, using dietary inventories and 16S rDNA sequencing to characterize faecal samples from 98 individuals. Based on the results, communities were clustered into enterotypes based on long-term diets of protein and animal fats (*Bacteroides*) versus carbohydrate (*Prevotella*). Predominant phyla that were positively associated with diets high in fat and negatively associated with fibre were Bacteroidetes and Actinobacteria, whereas, associations of high fibre and low fat were Firmicutes and Proteobacteria (Wu et al., 2011). Based on these enterotypes, the profile of the pooled faeces donated to this study was strongly associated with the *Bacteroides* enterotype, which is highly associated with the consumption of animal protein, saturated fats and a variety of amino acids, a profile accepted as from an industrialized nation or those consuming a western diet (Wu et al., 2011).

This was supported further by examining the prevalence of *Prevotella* spp. found within each of the models, the results showed that low levels were present in both models, in line with the profile for a *Bacteroides* enterotype and levels associated with healthy children (Zou et al., 2020). In this study, *Prevotella* spp prevalence in the control model ranged from 0.02-0.05% which was very low, in the test model during the steady state and intervention one (CF), prevalence of *Prevotella copri* remained at 0.15%, however, during intervention two (BTF) prevalence increased to 0.62%, suggesting intervention two (BTF) had a positive impact on prevalence.

Although pooling faeces would remove intersubject variation, little is understood on how the bacterial species would interact within the microbiota of several individuals and whether that would impact the time it takes to reach a state of homeostasis. Additionally, how the enterotype profile of the artificial microbiota would have had an impact on how interventions influenced microbial communities remains to be seen. Short term dietary interventions have been shown to change the microbiota of individuals significantly and rapidly, however, the magnitude of changes was modest and failed to switch enterotypes (Wu et al., 2011). In this study dietary interventions had an impact on diversity, however, extending the intervention period from 14 days to longer could result in greater diversity changes.

4.5 Differences in microbial abundances between models

Microbial differences between models showed that microbial communities responded to interventions. The increase of beneficial bacteria associated with intervention two (BTF) suggests that the use of the BTF diet could potentially be beneficial in improving tube fed children's health and wellbeing. Future clinical trials are needed to confirm this.

Microbes in the human gut undergo selective pressure from the host and microbial competitors typically leading to a stable community and ecosystem within the gut. This selective pressure for nutrition and space enables healthy commensal bacteria protect the gut from the overgrowth of pathogens. This regulation is affected by many factors, one of which is the nutritional composition of the diet (Mohr et al., 2024).

Gut microbiome composition mediated by diet has been implicated both directly and indirectly on human health (Valdes et al., 2018). Compositional and functional changes in gut microbiota have been well reported in other studies as factors associated with disease status and correlated with a low fibre diet (Cronin et al., 2021; Gomez-Arango et al., 2018). Microbial metabolites are derived from the fermentation of non-digestible dietary protein, carbohydrates and fats which have a major influence on host physiology. Fermentation of carbohydrates by bacteria produces SCFA which provide energy for host tissues, have anti-inflammatory and anti-apoptotic effects, determining the gut environment by influencing microbial balance, pH, nutrient uptake and gut transit (Sleeth et al., 2010). The two major phyla within the gut, Bacteroidetes and Firmicutes, are major utilizers of polysaccharides. *Bacteroides* carry out most of the hydrolysis of polysaccharides, while Firmicutes, such as *Faecalibacterium prausnitzii* and *Eubacterium rectale,* are prominent producers of butyrate. The two major mechanisms that impact species composition within the gut are: 1) certain bacterial species will be favored over others when substrates are available, with the capability to utilise non-digestible fibre determined on a genetic level, and 2) the gut environment plays a major role, with different species having different tolerances to high or low pH, bile salt concentrations or low micronutrient concentrations, which have an impact on microbial growth and community shifts (Flint et al., 2015).

Children on tube fed diets often have complex medical needs and up to 70% have severe chronic illnesses (Burdall et al., 2017). The associations between disease status and the gut microbiome have been well reported (Valdes et al., 2018). The role of the gut microbiome and gut derived metabolites in chronic diseases highlights the need for a diet which modulates or prevents chronic conditions. In the test model levels of *Collinsella* decreased during both interventions, however, intervention two (BTF)

had the greatest impact on prevalence. Bacterial communities in the test model during the steady state (0-16 days) showed similar prevalence to the control model during the same time period, with the exception of the genus *Collinsella.* Which accounted for almost half of the bacteria present at 48.18%, compared to the control model where prevalence was 15.7%. The large differences between the test model and the control model within the first 16 days cannot be explained, however, one possible cause is within the test model a contamination event at day eight. Where a technical issue resulting in an absence of growth medium pumping for ~4-6 hours and may have reduced the number of competing bacteria within the model, allowing the overgrowth of *Collinsella*. Interestingly, a dominant profile of *Collinsella* have been associated with a "Western diet" and lower dietary fibre consumption, a consequence of a higher prevalence of *Collinsella* is that abundance may alter the overall fermentation pattern in the microbiota, contributing alongside a lower prevalence of fibre utilising bacteria (Cronin et al., 2021).

In response to intervention one (CF), the prevalence of *Collinsella* decreased to 12.11%, however, with the introduction of intervention two (BTF) the prevalence decreased further to 11.48%. In response to the decrease in *Collinsella*, an increase was seen in *Bacteroides*, *bifidobacterium* and *lactobacillus* this suggested that both interventions had a positive influence on the grow of these beneficial bacteria, and a consequence being that they then regulated the prevalence of *Collinsella*. To support this, prevalence of *Collinsella* within the control model decreased from 15.70% in weeks 1 & 2 to 12.53% in weeks 3 & 4, however, prevalence increased to 35.10% at the end of the experiment in weeks 5 & 6. As the control model was without intervention, the cause of the increase is unknown and could be a consequence of *Collinsella* species needing a longer period to stabilise. However, a decrease was seen in *lactobacillus* suggesting a regulatory role in microbial community dynamics between these two genera. The prevalence of *Collinsella* in diseases associated with long term tube fed children where an increased prevalence is seen rheumatoid arthritis (RA) patients, contributing to gut permeability and inducement of IL-17A expression a key cytokine in the pathogenesis of RA (Chen et al., 2016). Furthermore, *Collinsella* has been shown to contribute to the development of non-alcoholic fatty liver disease (NAFLD), by metabolizing bile-acids to oxo-bile acid intermediates which may contribute to an increased intestinal permeability and the development of NAFLD (Maslowski et al., 2009). In conclusion, results suggested that both interventions, especially intervention two (BTF), increased prevalence of beneficial bacteria within the test model, and therefore, regulated *Collinsella* by decreasing its prevalence.

In contrast, in the test model increased prevalence was seen in genera *Lachnospiraceae*, *Bacteroides*, *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium* showing that this increase was a result of an impact of each of the interventions on the microbiota structure. Interestingly, higher prevalence of bile-tolerant *Bacteroides* which plays an important role in gut homeostasis was seen during intervention one (CF) than in intervention two (BTF), however, higher levels of *Bacteroides* are seen in individuals who tend to consume more protein and fat and are lower in those who consume more fibre. Higher prevalence of *Bacteroides* have been linked to a high fat or western diet, which is associated with changes in metabolic activity in the microbiota which is hypothesized to contribute to chronic illness such as obesity and diabetes, especially in children (Gurung et al., 2020). Indeed, intervention one (CF) contained no fibre, whereas intervention two (BTF) contained 6.9g fibre per 500ml, this suggested that a reduction in prevalence of *Bacteroides* may have been a consequence of fibre intake (Flint et al., 2015). Similar results have been seen in transitions from plant diets to animal based diets high in fats by characterizing the temporal patterns of microbial communities before and after interventions (David et al., 2014). This is further supported by the increased prevalence of *Lachnospiraceae,* a well-known fibre degrader, during intervention two (BTF) where prevalence increased substantially to 31.37% from 1.39%. However, during the steady state period the prevalence was 6.04% suggesting the high fat/low fibre content of intervention one (CF) was a factor influencing the decrease of *Lachnospiraceae* spp. A similar trend was seen in *Prevotella* which is hypothesized to be sensitive to long term fibre intake, where levels increased from 0.15% to 0.62% during intervention two, suggesting the BTF contained higher amounts of foods with un-digestible fibre, however, the overall prevalence was low which is a profile commonly found in a *Bacteroides* enterotype.

An increase in *Bifidobacterium* and *Lactobacillus*, especially during intervention one (CF) where levels increased from steady state (Table 10). This suggests constituents within the CF had a positive effect on bacterial growth, however, the prevalence decreased during intervention two (BTF) but did not fall below levels of the steady state stage. The prevalence of Palmitic acid, an important saturated fatty acid widely found in plant and animals and also added to commercial formulas, Paediasure which was used in this study. During digestion palmitic acid is esterified to β-palmitate which has been demonstrated to have a prebiotic effect on *Bifidobacterium* and *Lactobacillus* spp. and increases prevalence within the microbiota (Yaron et al., 2013). This suggests that the addition of Palmitic acid in CF could increase prevalence of *Bifidobacterium* and *Lactobacillus* in intervention one (CF), however, an increase was seen during both interventions. Similar results were found by Yaron et al. (2013) who investigated the prebiotic effect of β-palmitate on the microbiota of infants, high levels of β-palmitate included in infant

formula increased prevalence of bifidobacteria and *Lactobacillus* species whilst low levels of β-palmitate increased levels of *Lactobacillus* species and a significant decrease in bifidobacteria. These findings suggests that the β-palmitate contained within the CF had a positive effect on the growth of both *Bifidobacterium* and *Lactobacillus* (Yaron et al., 2013). Interestingly, the microbiota in healthy children after weaning and around the age of three years old has the same composition and diversity as those of an adult. Within an adult microbiota Firmicutes and Bacteroidetes are the dominant phyla, and that over time as the child grows the microbiota becomes more mature with levels of *Bifidobacterium* and *Lactobacillus* decreasing (Yatsunenko et al., 2012). Interestingly, infant formula/breast milk is a main source of nutrition for infants less than a year old, and would therefore consume greater concentrations of Palmitic acid, this would be similar for tube fed children consuming CF as their only source of nutrition. However, a consequence of these increased levels of Palmitic acid could be a reduction in diversity. In infants the microbiota is immature with limited diversity, it is possible that constituents within CF like palmitic acid aid in reducing diversity within tube fed children. By increasing prevalence of *Bifidobacterium* and *Lactobacillus* species whilst limiting the growth of other genera within the microbiota. Results showed that, during intervention two (BTF) prevalence was reduced compared to intervention one (CF) but increased compared to steady state. Suggesting that with a BTF the beneficial bacteria are present but in a prevalence that reflects a more stable microbiota, this is reflected in Simpson's diversity index, where intervention two (BTF) showed a higher diversity of dominant species than intervention one (CF).

In the control model, prevalence of *Bifidobacterium* spp. increased from 4.83 to 9.85% (Table 11) throughout the duration of the model, suggesting two things. Firstly, the data supports the suggestion that intervention one (CF) in the test model had an effect by rapidly increasing *Bifidobacterium* spp. prevalence. Secondly that intervention two (BTF) also had effect by reducing prevalence, that if either intervention had no effect, we would expect to see steady populations. Furthermore, *Lactobacillus* spp. prevalence in the control model dropped from 11.25% to 1.76% in weeks 4 & 5, with a small increased to 2.32% in weeks 5 & 6. A reduction in *Lactobacillus* during this period may have been a consequence of increased number of competing bacteria and nutrient depletion. Additionally, as levels of *Lactobacillus* spp. are lower in a *Bacteroides* enterotype, without nutrients to specifically increase prevalence such as metabolites from the digestion of fermented foods, it would be reasonable to assume levels would remain reasonably low or decline (Singh et al., 2017).

Diet is an important factor in the pathophysiology of chronic diseases in children, the imbalance of gut microbiota has not only been linked to a western diet but may also contribute to inflammation found in many of these diseases (Clemente-Suárez et al., 2023). Interestingly, a decline in prevalence of *Faecalibacterium prausnitzii,* one of the main producers of butyrate, that has been attributed with significant anti-inflammatory properties, was seen in the control model throughout the duration of the study ranging from 2.47-0.88%. Conversely, within the test model during the steady state and during intervention one (CF) prevalence remained stable 1.22-1.11% respectively. However, during intervention two (BTF) prevalence increased fivefold to 5.95%, indicating that intervention two (BTF) had a positive effect on *F. prausnitzii* growth and prevalence. As prebiotics are an important food source for commensal bacteria within the gut and Ingredients contained within the BTF that included honey, avocado, broccoli and almonds contain galactose. When fermented galactose produces diverse short chain galactooligosaccharides (GOS), which are prebiotics which influence the prevalence of *F. praunsnitzii*. Similar findings were observed by Davis et al. who investigated the effect of GOS on the entire bacterial community. In this *in vivo* study of healthy volunteers given increasing doses of GOS over four months, assessed by pyrosequencing of 16S rDNA from faecal samples, Davis et al. reported increased abundance of *F. praunsnitzii* however, the differences were not significantly different from base levels, but changes were significant between a 5g and 10g doses (Davis et al., 2011). Results suggest that ingredients making up the BTF during intervention two had a positive effect on *F. praunsnitzii* prevalence, which could benefit children with long term chronic diseases.

Other taxa influenced by GOS supplementation are bifidobacteria, however, high prevalence of *Bifidobacterium* species is associated with long term asthma, allergic diseases, irritable bowel syndrome (IBS) and mental health disorders (Arboleya et al., 2016; Vijay & Valdes, 2022). In contrast, lower levels of bifidobacteria have been associated with a variety of common diseases suggesting that bifidobacteria has an important role in health, with a normal prevalence (>2years of age) ranging from 2-14% abundance (Odamaki et al., 2016). Interestingly, a large increase was seen in prevalence within the test model during intervention one (CF) compared to the steady state (5.07% to 15.96%), this indicates that intervention one (CF) had an effect on prevalence. However, prevalence of *Bifidobacterium* species during intervention two (BTF) declined suggesting that GOS from food sources may have a small influence on *Bifidobacterium* abundance. In comparison an increase was seen in *Bifidobacterium* species over the duration of the study within the control model, however, those increases are not comparable with the test model as prevalence increased from 4.83-9.85%. It is interesting to note that intervention

one (CF) increased levels of bifidobacterium species to above normal levels, compared to both the steady state and intervention two (BTF) suggesting that prolonged use of a CF may influence disease states.

Pathogenic bacteria proliferate when levels of commensal bacteria are reduced, this leads to dysbiosis and contributes to disease states. In the test model, shifts in prevalence of were also reported in *Lacrimispora (Clostridium)*, *Hungatella* and *Megaspaera* species. An increased prevalence was seen in *Lacrimispora (Clostridium*) *sphenoides* and *L. saccharolyticum* also *Hungatella hathewayi* during intervention one (CF) compared to steady state. Interestingly, both genera were reclassified from the genus *Clostridium* to *Lachnospiraceae* and have been associated with high fat diets (HFD) and metabolic syndrome increasing the risk of diabetes and cardiovascular disease (CVD) (Haas & Blanchard, 2019; Vacca et al., 2020). Some *Clostridium* species are well known for their pathogenicity and toxin production, often associated with disease states. Both *L. sphenoides* and *L. saccharolyticum* showed a threefold increase during intervention one (CF) from steady state in the present study, *L. sphenoides* is one of the few clostridia to produce butyrate and metabolise bile acids, is part of normal microflora and is considered a commensal bacterium. However, *L. saccharolyticum* lacks the pathways to produce butyrate and has been associated with toxins linked to cardiovascular disease and kidney damage (Guzior et al., 2024). The prevalence of *H. hathewayi* also increased fivefold during intervention one (CF) from the steady state (Table 8). Although it has been defined as a core organism in human metagenomes and part of the normal gut microbiota (Bandoy et al., 2019), its importance has increased due to the association with severe diseases such as fatal septicemia, bacteremia and severity of COVID-19. However, little is known about this organism despite it being classified as non-pathogenic (Dababneh et al., 2014; Linscott et al., 2005; Zuo et al., 2020). Interestingly, the association with severe human diseases and misclassification suggests further investigation (Hernández-Juárez et al., 2021). Prevalence for both *Lacrimispora* and *Hungatella* saw a reduction below steady state during intervention two, this suggests that the BTF diet contained fats considered beneficial for health like olive oil, and as such was reflected in the prevalence rates. The increase of pathogenic bacteria during intervention one (CF) is concerning, especially as prevalence increased up to 5-fold, this suggests that intervention one (CF) impacted microbial community dynamics, leaving opportunistic pathogenic bacteria opportunity to proliferate. In contrast, reductions are observed during intervention two (BTF), suggesting that these community dynamics are changeable over a short period of time to regulate pathogenic bacteria.

Increased prevalence of *Megaspaera* spp. have been associated with high fat diet (HFD) profile. Further, emerging evidence is in support of the involvement of gut microbiota in regulation of blood lipids and hyperlipidemia (HLP) one of the main contributing factors in the development of hypertension, diabetes and fatty liver and metabolic syndromes increasing the risk of stroke, coronary heart disease (Gao et al., 2022). An increased prevalence of *Megasphaera* spp. was seen from the steady state during intervention one (CF) from 0.46% to 4.25%, this indicates that increased prevalence was a response to high fat contained within intervention one (CF). During intervention two (BTF), prevalence decreased to 3.60% also suggesting a response to dietary fats. Similar results were seen by Gao et al (2022) that used a non-human primate model fed HFD, to examine the gut microbiota associated with changes in lipid profiles by metagenomic sequencing of faecal samples. Results revealed long term dietary changes to a HFD in monkeys changed the composition of the gut microbiota. Interestingly, 40% of the monkeys developed a tolerance to the HFD with blood lipid levels returning to normal levels. Within the HFD tolerant group the profile of the microbiota differed to the remaining 60%. In the remaining monkeys the microbiota profile showed that the most abundant phyla was Bacteroidetes, with a decrease in Firmicutes. Whereas the most abundant genera in the gut microbiota of the HFD tolerant monkeys was *Megaspaera* including species *M. elsdenii*, *M. hexanoica* and *M. stantonii*, with an increase in *Prevotella*, *Bacteroides* and *Lactobacillus*. To validate those findings, a faecal transplant of the HFD tolerant gut microbiota was administered into rats fed a HFD, the rats receiving the facial transplant showed markedly inhibited lipid accumulation (Gao et al., 2022). The results showed the increase of *Megasphaera* spp. in intervention one seems to be related to the increase of specific fats contained within the CF, in contrast the reduction of prevalence during intervention two (BTF) suggests that the type of fat maybe main cause of response. As the CF and the BTF were matched for fat content and not fat type, then it is reasonable to suggest this as a reason for prevalence differences across interventions.

There is a strong influence of long-term dietary intake upon relative abundance of bacterial communities within a microbiome, however, a change in diet can switch enterotypes in as little as five days, a more permanent change in enterotype results from a long-term change in diet (David et al., 2014; Wu et al., 2011).

The enumeration of bacteria by selective agar in triple stage chemostat models is crucial for tracking microbial population shifts after faecal inoculation. Aiding in identifying core microbial species, evaluating when bacterial populations are in a steady state and tracking microbial shifts in response to dietary interventions which can alter the microbiotas structure and function (Li et al., 2019; Rowland et

al., 2018). During the equilibrium period (days 0-16) both models saw a reduction in most of the bacterial groups, this state of flux is expected and only when the bacterial populations settle and become steady can you commence with interventions. Interestingly in the test model a contamination event occurred on day eight, consequences of such an event cannot be clarified, or the impact quantified due to pooling of samples for metagenomics. How the reduction of nutrients within the model affected the microbial community dynamics is uncertain, however, an overgrowth of opportunistic bacteria could lead to skewed results. However, after day 16 viable counts for both models were largely stable, although the counts for the test model were more consistent compared to the control model. Whilst enumeration of bacterial populations via selective media provides valuable data, it is limited and cannot fully capture complex microbial communities, however, paired with metagenomics a more complex and more robust characterization of the microbiota during interventions (Child et al., 2006; Li et al., 2019).

The human gut microbiome is dynamic and modifiable the symbiotic nature of host interaction and diet on bacterial species make the microbiota unique to each person. Interactions between bacteria species create homeostasis within the gut environment, disruptions can result in dysbiosis, each unique microbiota will react differently based on diet, genetics, and environment.

4.6 Considerations and future work

The inoculum seeded within the models was pooled from several children age ranging from 2 to 15 years old, a significant amount of faecal inoculum is needed for gut models, this results in the necessity for multiple donors. Pooling faecal microbiota from multiple donors removes interindividual differences and gives a more diverse and representative sample. However artificial communities created can be unpredictable, creating imbalance and competition among taxa which can bias results (Pérez-Burillo et al., 2021). Using fresh faeces to seed *in vitro* chemostat models is gold standard, stored in an oxygen deprived environment to protect bacterial viability and recovery. Whilst other researchers who do not have access to fresh faecal material use frozen faecal samples, the freeze-thaw cycle is known to induce stresses (osmotic and mechanical), resulting in cell death, and can add significate bias to the model (Isenring et al., 2023). With susceptibility of bacteria to stress-inducing conditions favored upon reactivation than sensitive taxa. However, the proportion of faecal material need from the same person to inoculate the model may be challenging, the possibility of introducing inoculum over the course of a few days should be considered, especially when samples are from children. The possibility or

miniaturising gut models is being considered by other researchers, this will reduce the amount of faecal inoculum need, enable the use of multiple systems and focus of interests to examined at the same time.

Whilst gut models are able to reproduce all or part of the physiochemical and microbial parameters of the human gut, they are unable to replicate the complex interaction between the gut and the host. This is a limitation of the model which exclude interactions between bacteria, the metabolites they produce in the gut which interacts with the nervous, immune, or endocrine systems. The model is unable to absorb water or have an epithelial surface for bacteria to adhere onto, meaning that whilst chemostat models are good for reproducing gut like conditions they cannot replicate the fine balance you would see within an *in vivo* model. Additionally, lower values can be expected when measuring alpha and beta diversity as the model is only able to support a limited community of microbes, due to physiological and chemical limitations (Van den Abbeele et al., 2010).

Biofilm formation in the gut model was present but limited. The double walled vessels in a gut model have smooth surfaces and offers no adherence for extensive biofilm formation, due to this bacterial abundance may have been impacted. As, many bacterial species produce biofilms, it would be beneficial to add a matrix within the vessels to facilitate any biofilm formation and increase abundance values.

During the experiment both models ran consecutively for 44 days, which in hindsight may be too short for the bacterial communities to reach a steady state before introducing interventions. MacDonald et al. (2013) examined composition and stability of microbial communities within a human distal gut chemostat model and found that all five models did not reach a steady state until days 30-36 post inoculation. This suggests that during this study when interventions started at day 16 both models may have been unstable, that microbial composition may have been stable but metabolic activity was still unstable. This perhaps could account for the low diversity within models, however, diversity and stableness increased over time in the control model. This demonstrated that the models needed at least 30 days to reach a steady state, before introducing each of the interventions (McDonald et al., 2013).

On reflection and for future studies, individual models for each of the interventions would show the impact of each of the interventions on community dynamics. Not only would you be able to ascertain when an intervention has an effect the microbiota after initiation, but also how long the intervention has an active effect on the microbiota after cessation. Additionally, whilst barcoding pooled samples is an economic solution to maximizing data collection, combining time points over each intervention reducing the possibility of seeing the impact of each intervention. Whilst pooling samples from all three

vessels allows an overview of the whole simulated gut, there is a risk of diluting any significant impact dietary interventions would have on community dynamics within the simulated environment. Ideally if the experiment were repeated, sampling for metagenomic sequencing would be at the end of the steady state and each intervention, with each vessel being assigned its own barcode. Amending to these time points for each section of the simulated gut, would give an accurate overview of microbial community dynamics for each section.

The novel use of the gut model to successfully assess changes in the microbiota due to various sources of artificial nutrition open an avenue for broader scope research. This study provides the basis for larger trails to address the impact a variety of commercial formulas, and different blended feed recipes on the microbiota of children, this in turn addresses the impact and benefits of using BTF for medically complex children. Additionally, research into sterile foods and the impact on the human gut microbiota and metabolite production.

The artificial microbiota in this study had an *Bacteroides* enterotype, it would be beneficial in future studies to examine how different enterotypes are impacted by changes in diet.

A limitation of the study was that metabolites were not quantified, for future work SCFA should be quantified against bacterial populations to make findings more robust.

5 Conclusion

The results highlight several trends of bacterial prevalence within the microbiota when comparing commercial formula to blended tube feeds. This study demonstrated that blended tube feeds promoted prevalence of bacterial species beneficial within the gut microbiota compared with commercial formula and alters the gut microbial composition to a healthy state.

Despite limitations, this study achieved its aims and objectives to investigate how the gut microbiome in an *in vitro* gut model with enteral feed change from a sterile commercial formula to blended food. Given that BTF is gaining interest from parents of children with complex medical needs, evidence of benefits of using BTF is valuable to this community. This novel research is the first step towards evidence-based knowledge, demonstrating that BTF have the potential to promote the prevalence of beneficial bacteria within the gut microbiome.

6 References

Arboleya, S., Watkins, C., Stanton, C., & Ross, R. P. (2016). Gut Bifidobacteria Populations in Human Health and Aging. *Frontiers in Microbiology*, *7*, 1204. https://doi.org/10.3389/fmicb.2016.01204

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J.-M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., … Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature*, *473*(7346), 174–180. https://doi.org/10.1038/nature09944

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M. T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y. S., Kotowska, D., Colding, C., … Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host & Microbe*, *17*(6), 852. https://doi.org/10.1016/j.chom.2015.05.012

Baines, S. D., Crowther, G. S., Todhunter, S. L., Freeman, J., Chilton, C. H., Fawley, W. N., & Wilcox, M. H. (2013). Mixed infection by Clostridium difficile in an in vitro model of the human gut. *Journal of Antimicrobial Chemotherapy*, *68*(5), 1139–1143. https://doi.org/10.1093/jac/dks529

Baines, S. D., Freeman, J., & Wilcox, M. H. (2005). Effects of piperacillin/tazobactam on Clostridium difficile growth and toxin production in a human gut model. *Journal of Antimicrobial Chemotherapy*, *55*(6), 974–982. https://doi.org/10.1093/jac/dki120

Bajinka, O., Tan, Y., Abdelhalim, K. A., Özdemir, G., & Qiu, X. (2020). Extrinsic factors influencing gut microbes, the immediate consequences and restoring eubiosis. *AMB Express*, *10*, 130. https://doi.org/10.1186/s13568-020-01066-8

Bakewell, C., Corlett, C., Sharrard, A., Howarth, L. J., & Lee, A. C. (2021). Current trends in paediatric enteral tube feeding. *Paediatrics and Child Health*, *31*(8), 308–315. https://doi.org/10.1016/j.paed.2021.05.002

Bandoy, D. D. R., Huang, B. C., & Weimer, B. C. (2019). *Misclassification of a whole genome sequence reference defined by the Human Microbiome Project: A detrimental carryover effect to microbiome studies* (p. 19000489). medRxiv. https://doi.org/10.1101/19000489

Batsis, I. D., Davis, L., Prichett, L., Wu, L., Shores, D., Au Yeung, K., & Oliva- Hemker, M. (2020). Efficacy and Tolerance of Blended Diets in Children Receiving Gastrostomy Feeds. *Nutrition in Clinical Practice*, *35*(2), 282–288. https://doi.org/10.1002/ncp.10406

Bertrand, D., Shaw, J., Kalathiappan, M., Ng, A. H. Q., Muthiah, S., Li, C., Dvornicic, M., Soldo, J. P., Koh, J. Y., Tek, N. O., Barkham, T., Young, B., Marimuthu, K., Rei, C. K., Sikic, M., & Nagarajan, N. (2018). *Nanopore sequencing enables high-resolution analysis of resistance determinants and mobile elements in the human gut microbiome* (p. 456905). bioRxiv. https://doi.org/10.1101/456905

Blumenstein, I., Shastri, Y. M., & Stein, J. (2014). Gastroenteric tube feeding: Techniques, problems and solutions. *World Journal of Gastroenterology : WJG*, *20*(26), 8505–8524. https://doi.org/10.3748/wjg.v20.i26.8505

Bobo, E. (2016). Reemergence of Blenderized Tube Feedings. *Nutrition in Clinical Practice*, *31*(6), 730–735. https://doi.org/10.1177/0884533616669703

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., Korecka, A., Bakocevic, N., Ng, L. G., Kundu, P., Gulyás, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B. T., Diamond, B., & Pettersson, S. (2014). The gut microbiota influences blood-brain barrier

permeability in mice. *Science Translational Medicine*, *6*(263), 263ra158. https://doi.org/10.1126/scitranslmed.3009759

Bray, J. R., & Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349. https://doi.org/10.2307/1942268

Burdall, O. C., Howarth, L. J., Sharrard, A., & Lee, A. C. H. (2017). Paediatric enteral tube feeding. *Paediatrics and Child Health*, *27*(8), 371–377. https://doi.org/10.1016/j.paed.2017.05.001

Chen, J., Wright, K., Davis, J. M., Jeraldo, P., Marietta, E. V., Murray, J., Nelson, H., Matteson, E. L., & Taneja, V. (2016). An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. *Genome Medicine*, *8*(1), 43. https://doi.org/10.1186/s13073-016-0299-7

Child, M. W., Kennedy, A., Walker, A. W., Bahrami, B., Macfarlane, S., & Macfarlane, G. T. (2006). Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques. *FEMS Microbiology Ecology*, *55*(2), 299–310. https://doi.org/10.1111/j.1574-6941.2005.00016.x

Chilton, C. H., Crowther, G. S., Baines, S. D., Todhunter, S. L., Freeman, J., Locher, H. H., Athanasiou, A., & Wilcox, M. H. (2014). In vitro activity of cadazolid against clinically relevant Clostridium difficile isolates and in an in vitro gut model of C. difficile infection. *Journal of Antimicrobial Chemotherapy*, *69*(3), 697–705. https://doi.org/10.1093/jac/dkt411

Cho, I., Yamanishi, S., Cox, L., Methé, B. A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I., Li, H., Alekseyenko, A. V., & Blaser, M. J. (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature*, *488*(7413), Article 7413. https://doi.org/10.1038/nature11400

Ciuffreda, L., Rodríguez-Pérez, H., & Flores, C. (2021). Nanopore sequencing and its application to the study of microbial communities. *Computational and Structural Biotechnology Journal*, *19*, 1497–1511. https://doi.org/10.1016/j.csbj.2021.02.020

Clemente-Suárez, V. J., Beltrán-Velasco, A. I., Redondo-Flórez, L., Martín-Rodríguez, A., & Tornero-Aguilera, J. F. (2023). Global Impacts of Western Diet and Its Effects on Metabolism and Health: A Narrative Review. *Nutrients*, *15*(12), 2749. https://doi.org/10.3390/nu15122749

Cronin, P., Joyce, S. A., O'Toole, P. W., & O'Connor, E. M. (2021). Dietary Fibre Modulates the Gut Microbiota. *Nutrients*, *13*(5). https://doi.org/10.3390/nu13051655

Cuscó, A., Pérez, D., Viñes, J., Fàbregas, N., & Francino, O. (2021). Long-read metagenomics retrieves complete single-contig bacterial genomes from canine feces. *BMC Genomics*, *22*(1), 330. https://doi.org/10.1186/s12864-021-07607-0

Dababneh, A. S., Nagpal, A., Palraj, B. R. V., & Sohail, M. R. (2014). Clostridium hathewayi bacteraemia and surgical site infection after uterine myomectomy. *Case Reports*, *2014*, bcr2013009322. https://doi.org/10.1136/bcr-2013-009322

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

Davis, L. M. G., Martínez, I., Walter, J., Goin, C., & Hutkins, R. W. (2011). Barcoded Pyrosequencing Reveals That Consumption of Galactooligosaccharides Results in a Highly Specific Bifidogenic Response in Humans. *PLoS ONE*, *6*(9), e25200. https://doi.org/10.1371/journal.pone.0025200

Davis Birch, W. A., Moura, I. B., Ewin, D. J., Wilcox, M. H., Buckley, A. M., Culmer, P. R., & Kapur, N. (2023). MiGut: A scalable in vitro platform for simulating the human gut microbiome—Development, validation and simulation of antibiotic-induced dysbiosis. *Microbial Biotechnology*, *16*(6), 1312–1324. https://doi.org/10.1111/1751-7915.14259

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(33), 14691–14696. https://doi.org/10.1073/pnas.1005963107

de Meij, T. G. J., Budding, A. E., de Groot, E. F. J., Jansen, F. M., Kneepkens, C. M. F., Benninga, M. A., Penders, J., van Bodegraven, A. A., & Savelkoul, P. H. M. (2016). Composition and stability of intestinal microbiota of healthy children within a Dutch population. *The FASEB Journal*, *30*(4), 1512–1522. https://doi.org/10.1096/fj.15-278622

Derrien, M., Alvarez, A.-S., & de Vos, W. M. (2019). The Gut Microbiota in the First Decade of Life. *Trends in Microbiology*, *27*(12), 997–1010. https://doi.org/10.1016/j.tim.2019.08.001

Devkota, S., Wang, Y., Musch, M., Leone, V., Fehlner-Peach, H., Nadimpalli, A., Antonopoulos, D. A., Jabri, B., & Chang, E. B. (2012). Dietary fat-induced taurocholic acid production promotes pathobiont and colitis in IL-10−/− mice. *Nature*, *487*(7405), 104–108. https://doi.org/10.1038/nature11225

Diamanti, A., Di Ciommo, V. M., Tentolini, A., Lezo, A., Spagnuolo, M. I., Campanozzi, A., Panetta, F., Basso, M. S., Elia, D., & Gambarara, M. (2013). Home enteral nutrition in children: A 14-year multicenter survey. *European Journal of Clinical Nutrition*, *67*(1), Article 1. https://doi.org/10.1038/ejcn.2012.184

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., & Relman, D. A. (2005). Diversity of the Human Intestinal Microbial Flora. *Science (New York, N.Y.)*, *308*(5728), 1635–1638. https://doi.org/10.1126/science.1110591

Etifa, P., Rodríguez, C., Harmanus, C., Sanders, I. M. J. G., Sidorov, I. A., Mohammed, O. A., Savage, E., Timms, A. R., Freeman, J., Smits, W. K., Wilcox, M. H., & Baines, S. D. (2023). Non-Toxigenic Clostridioides difficile Strain E4 (NTCD-E4) Prevents Establishment of Primary C. difficile Infection by Epidemic PCR Ribotype 027 in an In Vitro Human Gut Model. *Antibiotics*, *12*(3), Article 3. https://doi.org/10.3390/antibiotics12030435

Fabiani, A., Sanson, G., Bottigliengo, D., Dreas, L., Zanetti, M., Lorenzoni, G., Gatti, G., Sacilotto, M., Pappalardo, A., & Gregori, D. (2020). Impact of a natural versus commercial enteral-feeding on the occurrence of diarrhea in critically ill cardiac surgery patients. A retrospective cohort study. *International Journal of Nursing Studies*, *108*, 103605. https://doi.org/10.1016/j.ijnurstu.2020.103605

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society*, *74*(1), 13–22. https://doi.org/10.1017/S0029665114001463

Freeman, J., O'Neill, F. J., & Wilcox, M. H. (2003). Effects of cefotaxime and desacetylcefotaxime upon Clostridium difficile proliferation and toxin production in a triple-stage chemostat model of the

human gut. *Journal of Antimicrobial Chemotherapy*, *52*(1), 96–102. https://doi.org/10.1093/jac/dkg267

Fujimura, K. E., Demoor, T., Rauch, M., Faruqi, A. A., Jang, S., Johnson, C. C., Boushey, H. A., Zoratti, E., Ownby, D., Lukacs, N. W., & Lynch, S. V. (2014). House dust exposure mediates gut microbiome Lactobacillus enrichment and airway immune defense against allergens and virus infection. *Proceedings of the National Academy of Sciences*, *111*(2), 805–810. https://doi.org/10.1073/pnas.1310750111

Gallagher, K., Flint, A., Mouzaki, M., Carpenter, A., Haliburton, B., Bannister, L., Norgrove, H., Hoffman, L., Mack, D., Stintzi, A., & Marcon, M. (2018). Blenderized Enteral Nutrition Diet Study: Feasibility, Clinical, and Microbiome Outcomes of Providing Blenderized Feeds Through a Gastric Tube in a Medically Complex Pediatric Population. *Journal of Parenteral and Enteral Nutrition*, *42*(6), 1046–1060. https://doi.org/10.1002/jpen.1049

Gao, J.-M., Rao, J.-H., Wei, Z.-Y., Xia, S.-Y., Huang, L., Tang, M.-T., Hide, G., Zheng, T.-T., Li, J.-H., Zhao, G.-A., Sun, Y.-X., & Chen, J.-H. (2022). Transplantation of Gut Microbiota From High-Fat-Diet-Tolerant Cynomolgus Monkeys Alleviates Hyperlipidemia and Hepatic Steatosis in Rats. *Frontiers in Microbiology*, *13*, 876043. https://doi.org/10.3389/fmicb.2022.876043

Garrett, D. A., Failla, M. L., & Sarama, R. J. (1999). Development of an in Vitro Digestion Method To Assess Carotenoid Bioavailability from Meals. *Journal of Agricultural and Food Chemistry*, *47*(10), 4301–4309. https://doi.org/10.1021/jf9903298

Gibson, G. R. (2022). Commentary on: Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition*, *127*(4), 554–555. https://doi.org/10.1017/S0007114521004608

Glick-Bauer, M., & Yeh, M.-C. (2014). The Health Advantage of a Vegan Diet: Exploring the Gut Microbiota Connection. *Nutrients*, *6*(11), 4822–4838. https://doi.org/10.3390/nu6114822

Gomez-Arango, L. F., Barrett, H. L., Wilkinson, S. A., Callaway, L. K., McIntyre, H. D., Morrison, M., & Dekker Nitert, M. (2018). Low dietary fiber intake increases Collinsella abundance in the gut microbiota of overweight and obese pregnant women. *Gut Microbes*, *9*(3), 189–201. https://doi.org/10.1080/19490976.2017.1406584

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J. T., Spector, T. D., Clark, A. G., & Ley, R. E. (2014). Human genetics shape the gut microbiome. *Cell*, *159*(4), 789–799. https://doi.org/10.1016/j.cell.2014.09.053

Gormican, A., & Catli, E. (1972). Nutritional and clinical responses of immobilized patients to a sterile milk-base feeding. *Journal of Chronic Diseases*, *25*(5), 291–303. https://doi.org/10.1016/0021-9681(72)90165-8

Graf, D., Di Cagno, R., Fåk, F., Flint, H. J., Nyman, M., Saarela, M., & Watzl, B. (2015). Contribution of diet to the composition of the human gut microbiota. *Microbial Ecology in Health and Disease*, *26*, 10.3402/mehd.v26.26164. https://doi.org/10.3402/mehd.v26.26164

Gramlich, L., Hurt, R. T., Jin, J., & Mundi, M. S. (2018). Home Enteral Nutrition: Towards a Standard of Care. *Nutrients*, *10*(8), 1020. https://doi.org/10.3390/nu10081020

Gu, W., Miller, S., & Chiu, C. Y. (2019). Clinical Metagenomic Next-Generation Sequencing for Pathogen Detection. *Annual Review of Pathology*, *14*, 319–338. https://doi.org/10.1146/annurev-pathmechdis-012418-012751

Gurung, M., Li, Z., You, H., Rodrigues, R., Jump, D. B., Morgun, A., & Shulzhenko, N. (2020). Role of gut microbiota in type 2 diabetes pathophysiology. *EBioMedicine*, *51*, 102590. https://doi.org/10.1016/j.ebiom.2019.11.051

Guzior, D. V., Okros, M., Shivel, M., Armwald, B., Bridges, C., Fu, Y., Martin, C., Schilmiller, A. L., Miller, W. M., Ziegler, K. M., Sims, M. D., Maddens, M. E., Graham, S. F., Hausinger, R. P., & Quinn, R. A. (2024). Bile salt hydrolase acyltransferase activity expands bile acid diversity. *Nature*, *626*(8000), 852–858. https://doi.org/10.1038/s41586-024-07017-8

Haas, K., & Blanchard, J. (2019). Reclassification of the Clostridium clostridioforme and Clostridium sphenoides clades as Enterocloster gen. Nov. And Lacrimispora gen. Nov., including reclassification of 15 taxa. *International Journal of Systematic and Evolutionary Microbiology*, *70*. https://doi.org/10.1099/ijsem.0.003698

Harkness, L. (2002). The History of Enteral Nutrition Therapy: From Raw Eggs and Nasal Tubes to Purified Amino Acids and Early Postoperative Jejunal Delivery. *Journal of the American Dietetic Association*, *102*(3), 399–404. https://doi.org/10.1016/S0002-8223(02)90092-1

Hernández-Juárez, L. E., Camorlinga, M., Méndez-Tenorio, A., Calderón, J. F., Huang, B. C., Bandoy, D. D. R., Weimer, B. C., & Torres, J. (2021). Analyses of publicly available Hungatella hathewayi genomes revealed genetic distances indicating they belong to more than one species. *Virulence*, *12*(1), 1950–1964. https://doi.org/10.1080/21505594.2021.1950955

Hopwood, N., Elliot, C., Moraby, K., & Dadich, A. (2020). Parenting children who are enterally fed: How families go from surviving to thriving. *Child: Care, Health and Development*, *46*(6), 741–748. https://doi.org/10.1111/cch.12808

Hron, B., Fishman, E., Lurie, M., Clarke, T., Chin, Z., Hester, L., Burch, E., & Rosen, R. (2019). Health Outcomes and Quality of Life Indices of Children Receiving Blenderized Feeds via Enteral Tube. *The Journal of Pediatrics*, *211*, 139-145.e1. https://doi.org/10.1016/j.jpeds.2019.04.023

Hug, L. A., Baker, B. J., Anantharaman, K., Brown, C. T., Probst, A. J., Castelle, C. J., Butterfield, C. N., Hernsdorf, A. W., Amano, Y., Ise, K., Suzuki, Y., Dudek, N., Relman, D. A., Finstad, K. M., Amundson, R., Thomas, B. C., & Banfield, J. F. (2016). A new view of the tree of life. *Nature Microbiology*, *1*(5), Article 5. https://doi.org/10.1038/nmicrobiol.2016.48

Hugon, P., Dufour, J.-C., Colson, P., Fournier, P.-E., Sallah, K., & Raoult, D. (2015). A comprehensive repertoire of prokaryotic species identified in human beings. *The Lancet Infectious Diseases*, *15*(10), 1211–1219. https://doi.org/10.1016/S1473-3099(15)00293-5

Hurt, R. T., Edakkanambeth Varayil, J., Epp, L. M., Pattinson, A. K., Lammert, L. M., Lintz, J. E., & Mundi, M. S. (2015). Blenderized Tube Feeding Use in Adult Home Enteral Nutrition Patients. *Nutrition in Clinical Practice*, *30*(6), 824–829. https://doi.org/10.1177/0884533615591602

Imhann, F., Bonder, M. J., Vich Vila, A., Fu, J., Mujagic, Z., Vork, L., Tigchelaar, E. F., Jankipersadsing, S. A., Cenit, M. C., Harmsen, H. J. M., Dijkstra, G., Franke, L., Xavier, R. J., Jonkers, D., Wijmenga, C., Weersma, R. K., & Zhernakova, A. (2016). Proton pump inhibitors affect the gut microbiome. *Gut*, *65*(5), 740–748. https://doi.org/10.1136/gutjnl-2015-310376

Isenring, J., Bircher, L., Geirnaert, A., & Lacroix, C. (2023). In vitro human gut microbiota fermentation models: Opportunities, challenges, and pitfalls. *Microbiome Research Reports*, *2*(1), 2. https://doi.org/10.20517/mrr.2022.15

Jain, M., Koren, S., Miga, K. H., Quick, J., Rand, A. C., Sasani, T. A., Tyson, J. R., Beggs, A. D., Dilthey, A. T., Fiddes, I. T., Malla, S., Marriott, H., Nieto, T., O'Grady, J., Olsen, H. E., Pedersen, B. S., Rhie, A., Richardson, H., Quinlan, A. R., … Loose, M. (2018). Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nature Biotechnology*, *36*(4), 338–345. https://doi.org/10.1038/nbt.4060

Jernberg, C., Löfmark, S., Edlund, C., & Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME Journal*, *1*(1), Article 1. https://doi.org/10.1038/ismej.2007.3

Johnson, T. W., Milton, D. L., Johnson, K., Carter, H., Hurt, R. T., Mundi, M. S., Epp, L., & Spurlock, A. L. (2019). Comparison of Microbial Growth Between Commercial Formula and Blenderized Food for Tube Feeding. *Nutrition in Clinical Practice*, *34*(2), 257–263. https://doi.org/10.1002/ncp.10226

Jones, R. B., Zhu, X., Moan, E., Murff, H. J., Ness, R. M., Seidner, D. L., Sun, S., Yu, C., Dai, Q., Fodor, A. A., Azcarate-Peril, M. A., & Shrubsole, M. J. (2018). Inter-niche and inter-individual variation in gut microbial community assessment using stool, rectal swab, and mucosal samples. *Scientific Reports*, *8*(1), Article 1. https://doi.org/10.1038/s41598-018-22408-4

Kalantar, K. L., Carvalho, T., de Bourcy, C. F. A., Dimitrov, B., Dingle, G., Egger, R., Han, J., Holmes, O. B., Juan, Y.-F., King, R., Kislyuk, A., Lin, M. F., Mariano, M., Morse, T., Reynoso, L. V., Cruz, D. R., Sheu, J., Tang, J., Wang, J., … DeRisi, J. L. (2020). IDseq—An open source cloud-based pipeline and analysis service for metagenomic pathogen detection and monitoring. *GigaScience*, *9*(10), giaa111. https://doi.org/10.1093/gigascience/giaa111

Kang, J., Yin, S., Liu, J., Li, C., Wang, N., Sun, J., Li, W., He, J., Guo, Q., & Cui, S. W. (2022). Fermentation models of dietary fibre *in vitro* and *in vivo*—A review. *Food Hydrocolloids*, *131*, 107685. https://doi.org/10.1016/j.foodhyd.2022.107685

Katagiri, S., Ohsugi, Y., Shiba, T., Yoshimi, K., Nakagawa, K., Nagasawa, Y., Uchida, A., Liu, A., Lin, P., Tsukahara, Y., Iwata, T., & Tohara, H. (2023). Homemade blenderized tube feeding improves gut microbiome communities in children with enteral nutrition. *Frontiers in Microbiology*, *14*, 1215236. https://doi.org/10.3389/fmicb.2023.1215236

Kers, J. G., & Saccenti, E. (2022). The Power of Microbiome Studies: Some Considerations on Which Alpha and Beta Metrics to Use and How to Report Results. *Frontiers in Microbiology*, *12*, 796025. https://doi.org/10.3389/fmicb.2021.796025

Klimenko, N. S., Tyakht, A. V., Popenko, A. S., Vasiliev, A. S., Altukhov, I. A., Ischenko, D. S., Shashkova, T. I., Efimova, D. A., Nikogosov, D. A., Osipenko, D. A., Musienko, S. V., Selezneva, K. S., Baranova, A., Kurilshikov, A. M., Toshchakov, S. M., Korzhenkov, A. A., Samarov, N. I., Shevchenko, M. A., Tepliuk, A. V., & Alexeev, D. G. (2018). Microbiome Responses to an Uncontrolled Short-Term Diet Intervention in the Frame of the Citizen Science Project. *Nutrients*, *10*(5), 576. https://doi.org/10.3390/nu10050576

Latorre-Pérez, A., Villalba-Bermell, P., Pascual, J., & Vilanova, C. (2020). Assembly methods for nanopore-based metagenomic sequencing: A comparative study. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-70491-3

LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., & Ventura, M. (2013). Bacteria as vitamin suppliers to their host: A gut microbiota perspective. *Current Opinion in Biotechnology*, *24*(2), 160–168. https://doi.org/10.1016/j.copbio.2012.08.005

Leeming, E. R., Johnson, A. J., Spector, T. D., & Le Roy, C. I. (2019). Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. *Nutrients*, *11*(12), 2862. https://doi.org/10.3390/nu11122862

Lerma-Aguilera, A. M., Pérez-Burillo, S., Navajas-Porras, B., León, E. D., Ruíz-Pérez, S., Pastoriza, S., Jiménez-Hernández, N., Cämmerer, B.-M., Rufián-Henares, J. Á., Gosalbes, M. J., & Francino, M. P. (2024). Effects of different foods and cooking methods on the gut microbiota: An in vitro approach. *Frontiers in Microbiology*, *14*. https://doi.org/10.3389/fmicb.2023.1334623

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature*, *444*(7122), Article 7122. https://doi.org/10.1038/4441022a

Li, L., Abou-Samra, E., Ning, Z., Zhang, X., Mayne, J., Wang, J., Cheng, K., Walker, K., Stintzi, A., & Figeys, D. (2019). An in vitro model maintaining taxon-specific functional activities of the gut microbiome. *Nature Communications*, *10*(1), 4146. https://doi.org/10.1038/s41467-019-12087-8

Linscott, A. J., Flamholtz, R. B., Shukla, D., Song, Y., Liu, C., & Finegold, S. M. (2005). Fatal septicemia due to *Clostridium hathewayi* and *Campylobacter hominis*. *Anaerobe*, *11*(1), 97–98. https://doi.org/10.1016/j.anaerobe.2004.10.002

Lu, K., Cable, P. H., Abo, R. P., Ru, H., Graffam, M. E., Schieper, K. A., Parry, N. M. A., Levine, S., Bodnar, W. M., Wishnok, J. S., Styblo, M., Swenberg, J. A., Fox, J. G., & Tannenbaum, S. R. (2013). Gut microbiome perturbations induced by bacterial infection affect arsenic biotransformation. *Chemical Research in Toxicology*, *26*(12), 1893–1903. https://doi.org/10.1021/tx4002868

Macfarlane, G. T., Macfarlane, S., & Gibson, G. R. (1998). Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon. *Microbial Ecology*, *35*(2), 180–187. https://doi.org/10.1007/s002489900072

Madden, A. M., Baines, S., Bothwell, S., Chen, E., Goh, S., Jerome, L., Sommariva-Nagle, C., & Szychta, M. (2019). A laboratory-based evaluation of tube blocking and microbial risks associated with one blended enteral feed recipe. *Journal of Human Nutrition and Dietetics*, *32*(5), 667–675. https://doi.org/10.1111/jhn.12685

Martin, K., & Gardner, G. (2017). Home Enteral Nutrition: Updates, Trends, and Challenges. *Nutrition in Clinical Practice*, *32*(6), 712–721. https://doi.org/10.1177/0884533617701401

Martin-Gallausiaux, C., Marinelli, L., Blottière, H. M., Larraufie, P., & Lapaque, N. (2021). SCFA: Mechanisms and functional importance in the gut. *Proceedings of the Nutrition Society*, *80*(1), 37–49. https://doi.org/10.1017/S0029665120006916

Maslowski, K. M., Vieira, A. T., Ng, A., Kranich, J., Sierro, F., Di Yu, Schilter, H. C., Rolph, M. S., Mackay, F., Artis, D., Xavier, R. J., Teixeira, M. M., & Mackay, C. R. (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature*, *461*(7268), 1282–1286. https://doi.org/10.1038/nature08530

McCormack, S., Patel, K., & Smith, C. (2023). Blended diet for enteral tube feeding in young people: A systematic review of the benefits and complications. *Journal of Human Nutrition and Dietetics:*

The Official Journal of the British Dietetic Association, *36*(4), 1390–1405. https://doi.org/10.1111/jhn.13143

McDonald, J. A. K., Schroeter, K., Fuentes, S., Heikamp-deJong, I., Khursigara, C. M., de Vos, W. M., & Allen-Vercoe, E. (2013). Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model. *Journal of Microbiological Methods*, *95*(2), 167–174. https://doi.org/10.1016/j.mimet.2013.08.008

Menni, C., Jackson, M. A., Pallister, T., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. *International Journal of Obesity (2005)*, *41*(7), 1099–1105. https://doi.org/10.1038/ijo.2017.66

Mills, S., Stanton, C., Lane, J. A., Smith, G. J., & Ross, R. P. (2019). Precision Nutrition and the Microbiome, Part I: Current State of the Science. *Nutrients*, *11*(4), Article 4. https://doi.org/10.3390/nu11040923

Milton, D. L., Johnson, T. W., Johnson, K., Murphy, B., Carter, H., Hurt, R. T., Mundi, M. S., Epp, L., Spurlock, A. Y., & Hussey, J. (2020). Accepted Safe Food-Handling Procedures Minimizes Microbial Contamination of Home-Prepared Blenderized Tube-Feeding. *Nutrition in Clinical Practice*, *35*(3), 479–486. https://doi.org/10.1002/ncp.10450

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., … Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food Funct.*, *5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Mohr, A. E., Sweazea, K. L., Bowes, D. A., Jasbi, P., Whisner, C. M., Sears, D. D., Krajmalnik-Brown, R., Jin, Y., Gu, H., Klein-Seetharaman, J., Arciero, K. M., Gumpricht, E., & Arciero, P. J. (2024). Gut microbiome remodeling and metabolomic profile improves in response to protein pacing with intermittent fasting versus continuous caloric restriction. *Nature Communications*, *15*(1), 4155. https://doi.org/10.1038/s41467-024-48355-5

Murri, M., Leiva, I., Gomez-Zumaquero, J. M., Tinahones, F. J., Cardona, F., Soriguer, F., & Queipo-Ortuño, M. I. (2013). Gut microbiota in children with type 1 diabetes differs from that in healthy children: A case-control study. *BMC Medicine*, *11*(1), 46. https://doi.org/10.1186/1741-7015-11-46

Nayfach, S., Shi, Z. J., Seshadri, R., Pollard, K. S., & Kyrpides, N. C. (2019). New insights from uncultivated genomes of the global human gut microbiome. *Nature*, *568*(7753), 505–510. https://doi.org/10.1038/s41586-019-1058-x

Nicholls, S. M., Quick, J. C., Tang, S., & Loman, N. J. (2019). Ultra-deep, long-read nanopore sequencing of mock microbial community standards. *GigaScience*, *8*(5), giz043. https://doi.org/10.1093/gigascience/giz043

Novak, P., Wilson, K. E., Ausderau, K., & Cullinane, D. (2009). The Use of Blenderized Tube Feedings. *ICAN: Infant, Child, & Adolescent Nutrition*, *1*(1), 21–23. https://doi.org/10.1177/1941406408329196

O'Connor, G., Watson, M., Van Der Linde, M., Bonner, R. S., Hopkins, J., & Saduera, S. (2022). Monitor gastrointestinal tolerance in children who have switched to an "enteral formula with food-

derived ingredients": A national, multicenter retrospective chart review (RICIMIX study). *Nutrition in Clinical Practice*, *37*(4), 929–934. https://doi.org/10.1002/ncp.10812

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J., Abe, F., & Osawa, R. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: A cross-sectional study. *BMC Microbiology*, *16*, 90. https://doi.org/10.1186/s12866-016-0708-5

Odermatt, A., Da Cunha, T., Penno, C. A., Chandsawangbhuwana, C., Reichert, C., Wolf, A., Dong, M., & Baker, M. E. (2011). Hepatic reduction of the secondary bile acid 7-oxolithocholic acid is mediated by 11β-hydroxysteroid dehydrogenase 1. *Biochemical Journal*, *436*(3), 621–629. https://doi.org/10.1042/BJ20110022

Oparaji, J.-A., Sferra, T., & Sankararaman, S. (2019). Basics of Blenderized Tube Feeds: A Primer for Pediatric Primary Care Clinicians. *Gastroenterology Research*, *12*(3), 111–114. https://doi.org/10.14740/gr1192

Park, A. J., Collins, J., Blennerhassett, P. A., Ghia, J. E., Verdu, E. F., Bercik, P., & Collins, S. M. (2013). Altered colonic function and microbiota profile in a mouse model of chronic depression. *Neurogastroenterology and Motility*, *25*(9), 733-e575. https://doi.org/10.1111/nmo.12153

Parrish, C. R. (2014). Blenderized Tube Feeding: Suggested Guidelines to Clinicians. *PRACTICAL GASTROENTEROLOGY*, 7.

Pérez-Burillo, S., Molino, S., Navajas-Porras, B., Valverde-Moya, Á. J., Hinojosa-Nogueira, D., López-Maldonado, A., Pastoriza, S., & Rufián-Henares, J. Á. (2021). An in vitro batch fermentation protocol for studying the contribution of food to gut microbiota composition and functionality. *Nature Protocols*, *16*(7), 3186–3209. https://doi.org/10.1038/s41596-021-00537-x

Phillips, G. (2022). Introduction of blended diet for enteral tube feeding in paediatrics: A case report. *Nutrition and Health*, *28*(4), 483–487. https://doi.org/10.1177/02601060211054662

Phillips, G., & Coad, J. (2023). Blended diets for tube-fed children and young people: A rapid review update. *Archives of Disease in Childhood*, *108*(12), 1014–1018. https://doi.org/10.1136/archdischild-2023-325929

Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J., & Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology*, *35*(9), 833–844. https://doi.org/10.1038/nbt.3935

Rajilić-Stojanović, M., Maathuis, A., Heilig, H. G. H. J., Venema, K., de Vos, W. M., & Smidt, H. (2010). Evaluating the microbial diversity of an in vitro model of the human large intestine by phylogenetic microarray analysis. *Microbiology*, *156*(11), 3270–3281. https://doi.org/10.1099/mic.0.042044-0

Ramage, I. J., Geary, D. F., Harvey, E., Secker, D. J., Balfe, J. A., & Balfe, J. W. (1999). Efficacy of gastrostomy feeding in infants and older children receiving chronic peritoneal dialysis. *Peritoneal Dialysis International: Journal of the International Society for Peritoneal Dialysis*, *19*(3), 231–236.

Reber, E., Messerli, M., Stanga, Z., & Mühlebach, S. (2019). Pharmaceutical Aspects of Artificial Nutrition. *Journal of Clinical Medicine*, *8*(11), 2017. https://doi.org/10.3390/jcm8112017

Reyes, A., Haynes, M., Hanson, N., Angly, F. E., Heath, A. C., Rohwer, F., & Gordon, J. I. (2010). Viruses in the fecal microbiota of monozygotic twins and their mothers. *Nature*, *466*(7304), 334–338. https://doi.org/10.1038/nature09199

Rodrigues, D. B., Chitchumroonchokchai, C., Mariutti, L. R. B., Mercadante, A. Z., & Failla, M. L. (2017). Comparison of Two Static in Vitro Digestion Methods for Screening the Bioaccessibility of Carotenoids in Fruits, Vegetables, and Animal Products. *Journal of Agricultural and Food Chemistry*, *65*(51), 11220–11228. https://doi.org/10.1021/acs.jafc.7b04854

Rosen, D., Schneider, R., Bao, R., Burke, P., Ceballos, C., Hoffstadter-Thal, K., & Benkov, K. (2016). Home Nasogastric Feeds: Feeding Status and Growth Outcomes in a Pedriatric Population. *Journal of Parenteral and Enteral Nutrition*, *40*(3), 350–354. https://doi.org/10.1177/0148607114551967

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., & Tuohy, K. (2018). Gut microbiota functions: Metabolism of nutrients and other food components. *European Journal of Nutrition*, *57*(1), 1–24. https://doi.org/10.1007/s00394-017-1445-8

Schmidt, S. B., Kulig, W., Winter, R., Vasold, A. S., Knoll, A. E., & Rollnik, J. D. (2019). The effect of a natural food based tube feeding in minimizing diarrhea in critically ill neurological patients. *Clinical Nutrition*, *38*(1), 332–340. https://doi.org/10.1016/j.clnu.2018.01.007

Shannon, C. E. (1948). A mathematical theory of communication. *The Bell System Technical Journal*, *27*(3), 379–423. The Bell System Technical Journal. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x

Sienna, J. L., Saqan, R., Teh, J. C., Frieling, M. L., Secker, D., Cornelius, V., & Geary, D. F. (2010). Body size in children with chronic kidney disease after gastrostomy tube feeding. *Pediatric Nephrology*, *25*(10), 2115–2121. https://doi.org/10.1007/s00467-010-1586-y

Simmonds, S. E., Ly, L., Beaulaurier, J., Lim, R., Morse, T., Thakku, S. G., Rosario, K., Perez, J. C., Puschnik, A., Mwakibete, L., Hickey, S., Tato, C. M., Team, C. I., & Kalantar, K. (2024). *CZ ID: A cloud-based, no-code platform enabling advanced long read metagenomic analysis* (p. 2024.02.29.579666). bioRxiv. https://doi.org/10.1101/2024.02.29.579666

Simpson, E. H. (1949). Measurement of Diversity. *Nature*, *163*(4148), 688–688. https://doi.org/10.1038/163688a0

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*, 73. https://doi.org/10.1186/s12967-017-1175-y

Sleeth, M. L., Thompson, E. L., Ford, H. E., Zac-Varghese, S. E. K., & Frost, G. (2010). Free fatty acid receptor 2 and nutrient sensing: A proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. *Nutrition Research Reviews*, *23*(1), 135–145. https://doi.org/10.1017/S0954422410000089

Sturkenboom, M. C. J. M., Verhamme, K. M. C., Nicolosi, A., Murray, M. L., Neubert, A., Caudri, D., Picelli, G., Sen, E. F., Giaquinto, C., Cantarutti, L., Baiardi, P., Felisi, M.-G., Ceci, A., & Wong, I. C. K. (2008). Drug use in children: Cohort study in three European countries. *The BMJ*, *337*, a2245. https://doi.org/10.1136/bmj.a2245

Suzuki, Y., Nishijima, S., Furuta, Y., Yoshimura, J., Suda, W., Oshima, K., Hattori, M., & Morishita, S. (2019). Long-read metagenomic exploration of extrachromosomal mobile genetic elements in the human gut. *Microbiome*, *7*, 119. https://doi.org/10.1186/s40168-019-0737-z

Tanchoco, C. C., Castro, C. A. M., Villadolid, M. F., Casiño, G., Rodriguez, M. P., Roa, C., Cruz, C. M. A. D. L., & Tangcongco Jr, F. (2001). Enteral feeding in stable chronic obstructive pulmonary disease patients. *Respirology*, *6*(1), 43–50. https://doi.org/10.1111/j.1440-1843.2001.00295.x

Taylor, J., O'Neill, M., Maddison, J., Richardson, G., Hewitt, C., Horridge, K., Cade, J., McCarter, A., Beresford, B., & Fraser, L. K. (2019). 'Your Tube': The role of different diets in children who are gastrostomy fed: protocol for a mixed methods exploratory sequential study. *BMJ Open*, *9*(10), e033831. https://doi.org/10.1136/bmjopen-2019-033831

Thakkar, S. K., Maziya-Dixon, B., Dixon, A. G. O., & Failla, M. L. (2007). β-Carotene Micellarization during in Vitro Digestion and Uptake by Caco-2 Cells Is Directly Proportional to β-Carotene Content in Different Genotypes of Cassava1,2. *The Journal of Nutrition*, *137*(10), 2229–2233. https://doi.org/10.1093/jn/137.10.2229

Trigodet, F., Lolans, K., Fogarty, E., Shaiber, A., Morrison, H. G., Barreiro, L., Jabri, B., & Eren, A. M. (2022). High molecular weight DNA extraction strategies for long-read sequencing of complex metagenomes. *Molecular Ecology Resources*, *22*(5), 1786–1802. https://doi.org/10.1111/1755-0998.13588

Trollip, A., Lindeback, R., & Banerjee, K. (2020). Parental Perspectives on Blenderized Tube Feeds for Children Requiring Supplemental Nutrition. *Nutrition in Clinical Practice*, *35*(3), 471–478. https://doi.org/10.1002/ncp.10368

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., & De Angelis, M. (2020). The Controversial Role of Human Gut Lachnospiraceae. *Microorganisms*, *8*(4), Article 4. https://doi.org/10.3390/microorganisms8040573

Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *The BMJ*, *361*, k2179. https://doi.org/10.1136/bmj.k2179

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P., Rabot, S., Bruneau, A., El Aidy, S., Derrien, M., Zoetendal, E., Kleerebezem, M., Smidt, H., & Van de Wiele, T. (2010). Microbial Community Development in a Dynamic Gut Model Is Reproducible, Colon Region Specific, and Selective for Bacteroidetes and Clostridium Cluster IX. *Applied and Environmental Microbiology*, *76*(15), 5237–5246. https://doi.org/10.1128/AEM.00759-10

Venema, K., & van den Abbeele, P. (2013). Experimental models of the gut microbiome. *Best Practice & Research Clinical Gastroenterology*, *27*(1), 115–126. https://doi.org/10.1016/j.bpg.2013.03.002

Vijay, A., & Valdes, A. M. (2022). Role of the gut microbiome in chronic diseases: A narrative review. *European Journal of Clinical Nutrition*, *76*(4), 489–501. https://doi.org/10.1038/s41430-021-00991-6

Walters, K. E., & Martiny, J. B. H. (2020). Alpha-, beta-, and gamma-diversity of bacteria varies across habitats. *PLoS ONE*, *15*(9), e0233872. https://doi.org/10.1371/journal.pone.0233872

Wang, Y., Zhao, Y., Bollas, A., Wang, Y., & Au, K. F. (2021). Nanopore sequencing technology, bioinformatics and applications. *Nature Biotechnology*, *39*(11), 1348–1365. https://doi.org/10.1038/s41587-021-01108-x

Wilson, A. S., Koller, K. R., Ramaboli, M. C., Nesengani, L. T., Ocvirk, S., Chen, C., Flanagan, C. A., Sapp, F. R., Merritt, Z. T., Bhatti, F., Thomas, T. K., & O'Keefe, S. J. D. (2020). Diet and the Human Gut Microbiome: An International Review. *Digestive Diseases and Sciences*, *65*(3), 723–740. https://doi.org/10.1007/s10620-020-06112-w

Wommack, K. E., Bhavsar, J., & Ravel, J. (2008). Metagenomics: Read Length Matters. *Applied and Environmental Microbiology*, *74*(5), 1453–1463. https://doi.org/10.1128/AEM.02181-07

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A., & Jenkins, D. J. A. (2006). Colonic Health: Fermentation and Short Chain Fatty Acids. *Journal of Clinical Gastroenterology*, *40*(3), 235–243.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D., & Lewis, J. D. (2011). Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science (New York, N.y.)*, *334*(6052), 105–108. https://doi.org/10.1126/science.1208344

Yang, L., Bajinka, O., Jarju, P. O., Tan, Y., Taal, A. M., & Ozdemir, G. (2021). The varying effects of antibiotics on gut microbiota. *AMB Express*, *11*, 116. https://doi.org/10.1186/s13568-021-01274-w

Yang, M., Gu, Y., Li, L., Liu, T., Song, X., Sun, Y., Cao, X., Wang, B., Jiang, K., & Cao, H. (2021). Bile Acid–Gut Microbiota Axis in Inflammatory Bowel Disease: From Bench to Bedside. *Nutrients*, *13*(9), 3143. https://doi.org/10.3390/nu13093143

Yaron, S., Shachar, D., Abramas, L., Riskin, A., Bader, D., Litmanovitz, I., Bar-Yoseph, F., Cohen, T., Levi, L., Lifshitz, Y., Shamir, R., & Shaoul, R. (2013). Effect of High β-Palmitate Content in Infant Formula on the Intestinal Microbiota of Term Infants. *Journal of Pediatric Gastroenterology and Nutrition*, *56*(4), 376–381. https://doi.org/10.1097/MPG.0b013e31827e1ee2

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053

Zou, R., Xu, F., Wang, Y., Duan, M., Guo, M., Zhang, Q., Zhao, H., & Zheng, H. (2020). Changes in the Gut Microbiota of Children with Autism Spectrum Disorder. *Autism Research*, *13*(9), 1614–1625. https://doi.org/10.1002/aur.2358

Zuo, T., Zhang, F., Lui, G. C. Y., Yeoh, Y. K., Li, A. Y. L., Zhan, H., Wan, Y., Chung, A. C. K., Cheung, C. P., Chen, N., Lai, C. K. C., Chen, Z., Tso, E. Y. K., Fung, K. S. C., Chan, V., Ling, L., Joynt, G., Hui, D. S. C., Chan, F. K. L., … Ng, S. C. (2020). Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization. *Gastroenterology*, *159*(3), 944-955.e8. https://doi.org/10.1053/j.gastro.2020.05.048