Bananas grown in Aotearoa for feeding the infant gut microbiota

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Abstract

The human gut microbiota is established immediately at birth and thereafter is modulated by a range of environmental influences including the nutrients available within breast milk (and microbiota) or formula which support selective colonisation. Upon weaning to solid foods, the infant gut microbiota begins to become more akin to that of an adult in both microbial composition and metabolite production. Bananas are recommended by New Zealand health officials as an appropriate first food, however at present the only commercial variety available is Cavendish, imported largely from Ecuador and the Philippines. Although, in Northland and Gisborne regions other banana varieties are successfully cultivated, they have not been recognised by the commercial sector as valuable produce. Here we identify the nutritional composition of an array of banana cultivars grown in New Zealand, which are hybrids of acuminata (A) and balbisiana (B) species of banana and how these varieties impact SCFA production by the infant gut microbial population. We further identified the distinct shifts in microbial populations between three banana cultivar genome groups represented as AAA, AAB and ABB. Cavendish is AAA. Genomic mapping of unknown banana cultivars provided insight into the diversity of banana cultivars growing in New Zealand, in which the ecophysiology of bananas growing in this climate has presented different phenotypic characteristics to those of countries where banana horticulture is prevalent. These findings will contribute toward particularly, Māori communities building an emerging commercial banana industry and creating a nutritional database for New Zealand grown banana.

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My nieces and nephews, aunty is out here trying to be an ancestor you will be proud of.

"Aunty…you have a moustache"

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Ngā Kupu Māori

Māori language is used throughout this thesis. The reader is advised that Te reo Māori is a metaphorical language where a single word can have multiple meanings. This glossary provided below in Table 1 lists the Māori terms used throughout and their general meanings within the specific context of this thesis.

Table 1. Ngā Kupu Māori

Māori word/ term	Translation
Aotearoa	The land of the long white cloud, New Zealand
Kupu	Word, vocabulary
Māori	The first settlers, and the indigenous people of New Zealand
Mātauranga	Education, understanding, skill, wisdom
Rangatahi	Youth, younger generation
Tairāwhiti	East coast – Gisborne, North-eastern district in New Zealand
Te Tai Tokerau	The northern tide – Northland district in New Zealand
Whānau	Family, extended family, a term that addresses several people
Whenua	Land, territory

Abbreviations

Table 2. Abbreviations

Abbreviation	Definition
AMG	Amyloglucosidase
ANCOMBC	Analysis of composition of microbiomes with bias correction
ANOVA	Analysis of variance
DF	Dietary fibre
DNA	Deoxyribonucleic acid
FOC	*Fusarium oxysporum* f. sp. *Cubense*
FOS	Fructooligosaccharides
FP	Fruit pulp
FSANZ	Food Standards Australia & New Zealand
GC	Gas Chromatography
GC-FID	Gas Chromatography Flame Ionisation Detection
GOS	Galactooligosaccharides
HCl	Hydrochloride
HMOs	Human milk oligosaccharides
KEGG	Kyoto Encyclopedia of Genes and Genomes
NaOH	Sodium hydroxide
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PPR	Fruit pulp to skin ratio
PW	Peel weight
RDI	Recommended daily intake
RS	Resistant starch
SCFA	Short chain fatty acids
SD	Standard deviation
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SNPs	Single Nucleotide Polymorphisms
SSF	Simulated salivary fluid
TS	Total starch
USDA	United States Department of Agriculture

1. Literature review

1.1. Introduction

The most diverse and dense microbial community within the human anatomy resides in the gastrointestinal tract and is known as the gut microbiota. The gut microbiota consists of a vast array of microorganisms including bacteria, fungi, viruses, protozoa, and archaea. The majority of the microbes inhabiting the gastrointestinal tract are bacterial species [1]. The microorganisms within the intestine, known as the gut microbiota coexist alongside the host's intestinal cells, forming a symbiotic relationship with the human host [2]. The microbiota promotes epithelial growth and development, and thus homeostasis; and in turn receives nutrients when food is consumed. The gut microbiota plays critical roles in human health including aiding in nutrient and drug metabolism, maintaining structural integrity of the gut mucosal barrier, immunomodulation, and inhibition of pathogenic bacteria [3-5].

1.2. The immature gut microbiota

The initiation of the gut microbiota is believed to occur immediately after birth when bacteria from the environment are introduced using nutrients supplied in the internal and external environment of the maternal microbial reservoir [6, 7]. This early colonisation is influenced by mode of birth. During Caesarean section deliveries, the infants are not directly exposed to maternal faecal and vaginal microbes, and are instead colonised by the maternal skin and other bacteria present in the environment, resulting in a gut microbiota distinct from that of infants delivered vaginally[8-10]. Another important factor in the establishment of the infant gut microbiota is the gestational age at birth. Some preterm infants need to overcome serious and sometimes multiple health challenges. Often, they have a low birth weight and an immature gut, along with respiratory and neurological issues while also being exposed to antibiotics and other necessary drug treatments. Neonates require in-patient around the clock care and observation, so they usually endure longer periods in hospitals and may require artificial feeding and respiration. Research shows that these factors influence the infant gut microbiota composition, as preterm infants also exhibit less diversity in their microbiota composition in comparison to infants carried to full term [11-13]. In addition, the premature infant gut microbiota may have increased abundances of opportunistic pathogenic bacteria [11].

1.2.1. Implications of breast milk / formula

Milk provides all the nutrients required to nurture a growing infant from birth and throughout the first months of life and is usually the only source of nutrients until they are introduced to weaning foods [14]. The development of the gut microbiota is modulated by the nutrients available within breast milk or formula which support selective colonisation [15-17]. Human breast milk consists of a diverse matrix of nutritional compounds including fat, carbohydrates, proteins, vitamins, minerals and water alongside bioactive compounds, providing an optimal balance of components that are specifically tailored to the infant's needs [14, 18, 19]. Milk is a natural prebiotic source necessary for the development of the infant gut microbiota and the nutrition of the infant. Prebiotics are discussed further in section 1.3. After lactose and fat, human milk oligosaccharides (HMOs) are the third most abundant solid within breast milk. HMOs are emulsified solids comprised of short polymers of simple sugars that are able to withstand the enzymatic and acidic conditions of digestion[20]. The majority reach the colon intact, therefore acting as nutrients for the gut microbiota. Infant gut bacteria which have the capacity to utilise HMOs include *Bifidobacterium longum* subspecies *infantis, Bacteroides fragilis* and *Bacteroides vulgatus* [21, 22].

Infant formula may be ruminant, or plant based and has been developed as an alternative to breast milk when this is not available. Formula is usually made from hydrolysed proteins broken down so that it is easier to digest. Formula fed infants harbour a more diverse faecal microbiota but with less than half of the abundance of *Bifidobacterium* in comparison to breast fed infants [10, 23]. Only some HMOs are added to infant formula, and it is likely that this is a major contributor to the microbial community differences between breast fed and formula fed infants [24]. Although gestational age, mode of birth and breast/formula feeding are not always within parental or medical professional control, one controllable factor is when infants are introduced to weaning foods to complement the milk diet. Regardless of the initial infant gut microbiota composition, it is imperative that upon weaning from milk to solids that appropriate food is supplied that is safe for infant consumption, containing necessary nutrients for development and which promotes and sustains the gut microbiota function.

1.3. Prebiotics

Prebiotics are food ingredients that contain nutrients resistant to digestion, reach the large bowel intact and subsequently feed the colonic microbiota [25]. Examples of prebiotic compounds include plant fibre such as inulin, pectin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), resistant starch (RS) and also HMO [26-28]. Addition of prebiotics to the diet increases calcium and magnesium absorption and has an impact on lipid regulation [29, 30].A possible mechanism is that due to the administration of prebiotics, bacterial fermentation occurs, producing organic acids which lower the colonic pH therefore increasing mineral absorption. Although some mechanisms of the effects of prebiotic consumption are not yet determined, there are multiple which have been confirmed, as discussed further below.

1.3.1. Dietary fibre, resistant starch, and organic acid production by the microbiota

Resistant starch and dietary fibre can escape digestion and undergo anaerobic fermentation by the microbiota in the terminal small and large intestine [31]. Upon the introduction to solid food, bacteria which have a greater capacity to degrade these nutrients proliferate [8]. As a result of microbial metabolism, saccharolytic bacteria within the intestine secrete organic acids including short chain fatty acids (SCFAs) as by-products. Apical membranes within the luminal epithelium of the colon contain receptors which, when activated by SCFAs via anion transport, induce an anti-inflammatory response by the colonic phagocytes [32-34].

In addition, the presence of SCFAs reduces the luminal pH which inhibits colonisation by pathogenic bacteria [35]. The roles of specific SCFAs are discussed further in 1.3.2. Although SCFAs represent the end-product of metabolism for some bacteria, they also can be a substrate for further metabolism by another. In the first 2 years of human life, microbial SCFAs production changes over time. In the first few months there are elevated concentrations of succinate, lactate, and formate but these reduce until 1 year of age [36]. The gut microbiota is less diverse at this time, so these SCFAs are accumulated, whereas in the adult gut microbiota they are used as substrates for further metabolism. This is shown when infants are introduced to solid foods: the gut microbiota diversity is increased and the bacterial population that adapt to the new food, produce acetate, butyrate and propionate having the capability to recycle the SCFAs lactate, formate and succinate [6, 37]. Some of the bacteria responsible for the increase of these SCFAs that are introduced during weaning are *Bilophila wadsworthia*, *Lachnospiraceae*, and *Bacteroidete*s [38, 39].

1.3.2. Microbial organic acids (SCFAs) are beneficial to human health.

In adulthood the three most abundant SCFAs produced by the gut microbiota are acetate, butyrate, and propionate. Butyrate is the intestinal cell’s preferred source of energy as 95% of bacterial produced butyrate is transported across the epithelium for absorption via passive diffusion [40]. Through nourishing the intestinal epithelial cells, mucin production is stimulated, forming a protective barrier between the gut bacteria and the human host [41]. Butyrate plays crucial roles in immunity, as it induces an anti-inflammatory response via signalling regulatory T cells [42, 43]. Furthermore, butyrate supports ion absorption and intestinal motility, thus supporting regular bowel movement [44, 45].

Acetate is used by the liver as an energy source and as a substrate for the synthesis of other compounds including cholesterol [46, 47]. Acetate can improve glucose tolerance, which in turn could prevent diabetes for an infant later in life [48-50]. Acetate crosses the blood brain barrier where it can directly stimulate neurons within the hypothalamus, the structure within the brain that is responsible for maintaining homeostasis, by stimulating or inhibiting many of the body's key processes [3]. For appetite, acetate causes the enteroendocrine L- cells to secrete incretin, a hormone that is normally activated when the body has an energy surfeit [50, 51] . The incretin hormone signals neurons within the hypothalamus that induce satiety in the host. Although incretin hormone is secreted within the intestinal epithelium, there is evidence that in mice, acetate accumulates in the hypothalamus, causing pro-opiomelanocortin cells to stimulate neurons within the hypothalamus responsible for decreasing the appetite response without hormonal signalling [52]. Furthermore, in mice, colonic acetate has a hastened and increased effect on host satiety in comparison to acetate administered by injection directly to a cerebral ventricle [52]. This suggests that colonic acetate-induced incretin secretion is more effective than blood-acetate pro-opiomelanocortin cell stimulation of the hypothalamus for appetite reduction.

Propionate, another abundant SCFA in the adult gut after acetate, may also play a role in appetite. A pig model based study has indicated that in response to propionic stimuli, incretin is secreted within the gut, however evidence of this pathway is yet to be evidenced in human clinical trials [53]. Other roles that propionate play in the gut-liver-axis include promotion of lipid metabolism, and enhanced insulin sensitivity [54, 55].

In understanding the extensive impact SCFAs play in human health, it is important that when infants are introduced to solid foods, they contain sufficient amounts of dietary fibre and starches for the gut microbiota to metabolise and produce these important by-products.

1.4. Bananas as a complementary food

The New Zealand Ministry of Health recommends infants to be introduced to solid foods around the age of 6 months [56]. By this age, an infant has developed tongue and mouth movements sufficient for greater swallowing. At around 6-9 months old, infants also require more energy and nutrients that a diet consisting only of milk can provide [57]. The complementary feeding period is when foods (other than milk) are introduced to the infant diet to supplement milk. Humans have an innate preference for a sweet taste which they experience first from their mother's milk [58]. Thus, during the complementary feeding period, infants prefer foods sweet in taste also, so a commonly suggested first solid food for infants is bananas [59]. Bananas are fruit from the *Musa* genus of perennials, are palatable for the infant taste and can be easily mashed for safe solid food introduction, and as infants grow older, the flesh can be consumed whole without any preparation. Bananas are considered a prebiotic as they contain RS and dietary fibre which as discussed previously can be fermented by the gut microbiota. The Cavendish variety of banana, which is the cultivar variety available in New Zealand, contains various nutrients in adequate quantities which are vital to infant health. This includes potassium, magnesium, and vitamins C and B6 [60, 61]. A single 100g serving of ripe Cavendish significantly contributes toward various nutrient recommended daily intake (RDI) constituents as shown in Table 3.

*Table **3**. Nutrients within Cavendish banana which are vital to infant development [62, 63].*

Nutrient	Amount in 100g serving of ripe Cavendish	RDI for infants aged 0-6 months	% RDI in 100g serving
Potassium	326 mg	400 mg	81.5%
Magnesium	28 mg	30 mg	93.33%
Vitamin C	12.3 mg	40 mg	30.75%
Vitamin B6	0.209 mg	0.1 mg	209%

Green or unripe bananas contain more resistant starch in comparison to ripe bananas however as bananas ripen endogenous amylase breaks down these starches into simple sugars (shown in Figure 1) which are easily digested in the gastrointestinal tract [64]. Considering the benefits of resistant starch and its impact on the intestinal microbiota, green bananas would add benefits to the infant diet. However, due to the decreased sugar content, these may not appeal to the sweet palate of infants. Instead, a semi-ripe banana could serve as a more appropriate option during the complementary feeding period for optimum RS content while catering to a degree of the sweetness that infants desire. In understanding that bananas appeal to the sweet taste which infants find palatable, are safe for infant consumption, are easy to prepare and cater toward age specific nutritional needs; this is therefore an appropriate first food for infant nourishment during complementary feeding.

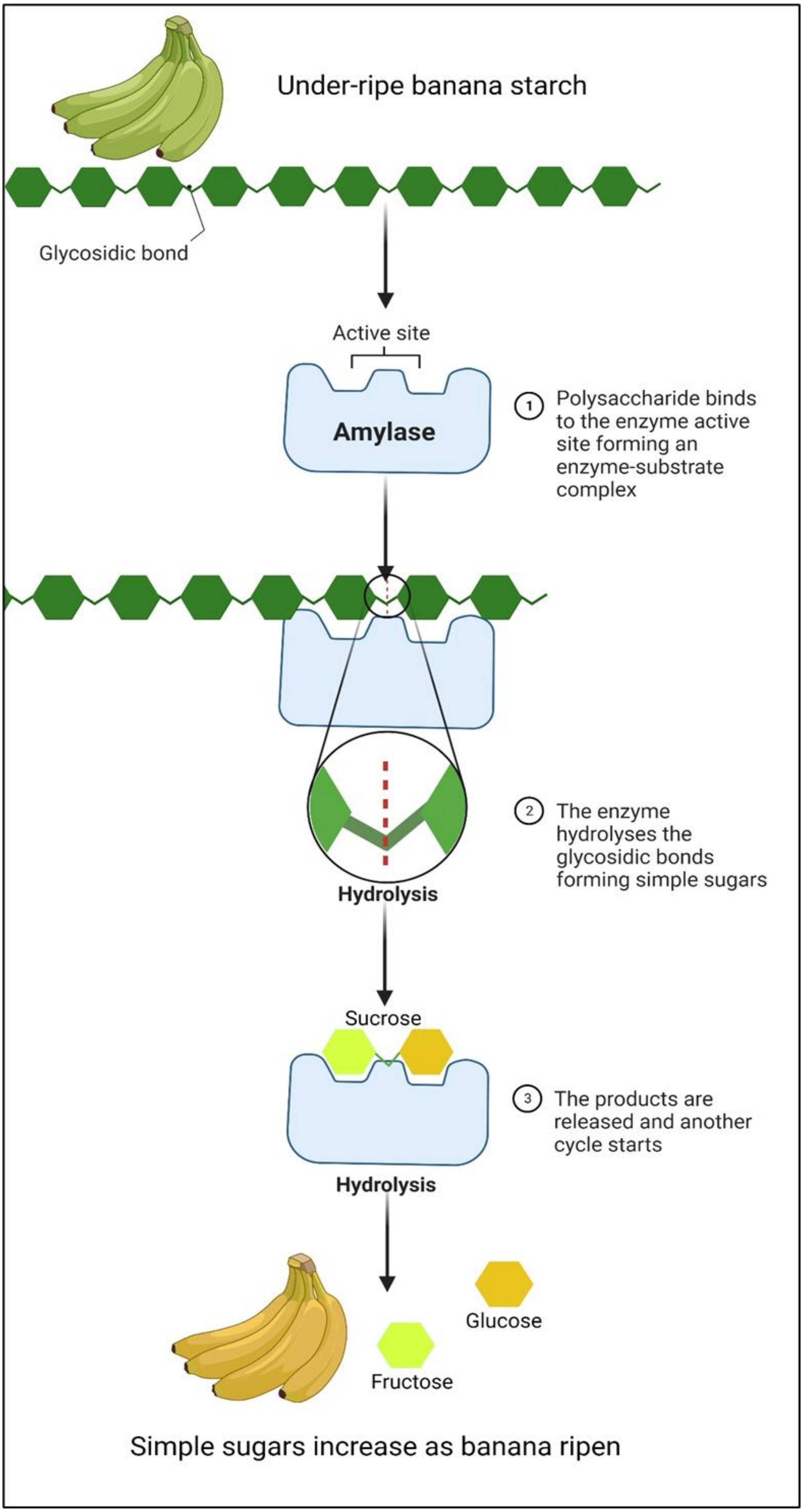

Figure 1. Banana starch hydrolysis by endogenous amylase during ripening. Created with Bio Render .com

1.5. Understanding banana genetics which determine flavour and starch characteristics.

Banana belongs to the genus *Musa* L within the family Musaceae. *Musa* have over 60 wild species which are diploid, the majority having 11 or 10 pairs of chromosomes [65]. Not all bananas are edible with some considered ornamental because they are full of large seeds. Most edible bananas originate from one diploid species or diploid or triploid hybrid with another diploid species (Figure 2). A consensus document on the biology of crops states "The *Musa acuminata* haploid genome is designated with the letter A, the *M. balbisiana* genome with B. The dessert or sweet bananas often (but not always) are triploids and diploids of *M. acuminata*, and the plantains and other starchy cooking bananas generally (but not always) are triploids derived from hybridisations between *M. acuminata* and *M. balbisiana*" [65]. This provides genomic classification of the wealth of cultivars within the *Musa* genus.

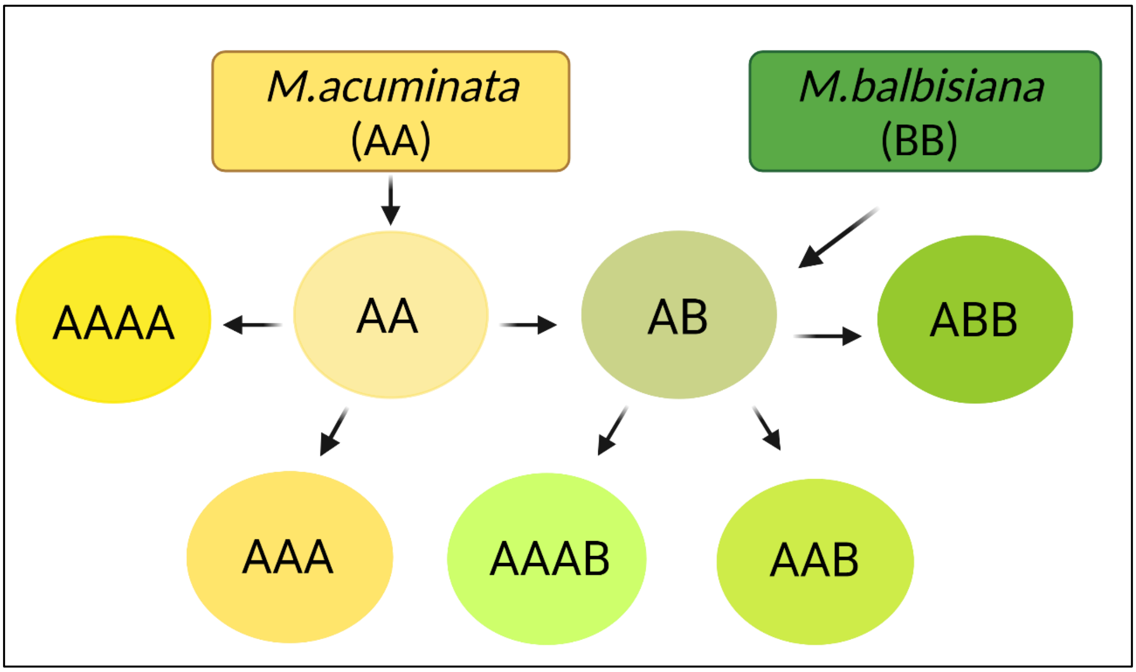

Figure 2. Banana genome configuration. Figure adapted from OECD,2010 [65] and created with Biorender.com.

Although there are other edible species including but not limited to *M. aurantiaca, M. basjoo* and *M. flaviflora*, the main edible varieties are derived by either *M. acuminata* or a combination of *M. balbisiana* and *M. acuminata* [66]. For example, in Cavendish, comprised from a *M. acuminata* triploid, the genome is classed as AAA, whereas the FHIA-01 Goldfinger banana is a synthetic hybrid of *M. acuminata* and *M. balbisiana* and tetraploid carrying three acuminata genomes and one Balbisiana so is classed as AAAB. Goldfinger is one of several FHIA synthetic hybrids which were developed by the Honduran Agricultural Research Foundation in the 1980s and have some resistance to diseases and pests. The genome arrangement and growing conditions impact the taste, texture, nutrition, yield, and disease resistance of bananas. These are factors that need to be considered when farming banana in New Zealand.

1.6. A Māori-led banana industry

Mātauranga in literal terms translates to knowledge, but there is so much more to this construct. Mātauranga is holistic, everything is interconnected and this is a body of knowledge that is upheld by the Māori world view and practices retaining and conveying information from ancestors [67]. For Māori, the whenua is a key part of identity and one's connection with the land has great significance in an individual's wellbeing. Across multiple generations Māori have protected and treasured multiple banana varieties which have been introduced to New Zealand. These cultivars have been passed down from generation to generation, however have not been recognised by the horticulture or business sector as a commercial product. Banana cultivation in New Zealand and its cooler climate has several advantages, including the absence of specific banana pests and disease as discussed in 1.7.1. Māori have developed and continue to develop the mātauranga based on first-hand experiences of growing in these conditions. By further cultivating and passing down this knowledge Māori can maintain the important connection with the land while providing a nutritious food source for our families. While mātauranga does not require validation by science, further research on these banana cultivars can continue to evolve this mātauranga, and whānau can utilise this knowledge to achieve their goals and aspirations in a pathway they see fit. Māori have the knowledge and experience to lead the country toward a successful commercial banana industry.

1.7. Banana grown in New Zealand climate

The only commercially available banana in New Zealand is imported Cavendish as, due to the temperate climate in New Zealand, locally grown bananas have to date never been economically or commercially viable. In Te Tai Tokerau and Tairāwhiti regions, however, there are areas where the climate has shown promise in cultivating bananas and other sub-tropical fruit. Whānau in the Northland region grow several banana varieties which differ in genomic composition to that of the popularly consumed Cavendish.

The Köppen-Geiger system is used to classify climate zones across the world. Based on the seasonal fluctuations of temperature and precipitation, it divides climate conditions into five main zones. These are tropical, arid , temperate , continental and polar [68]. New Zealand as a country is classified by this system as temperate zone. However, the Northland region alone is classed as subtropical as this area does not suffer as harsh a winter in comparison to the rest of the country, with an annual average daily high temperature of 20°C and humid summers with maximum air temperatures ranging from 22°C to 26°[69]. The banana is a plant of the tropics and subtropics; they can and do thrive in the warm conditions within areas of Northland New Zealand that the majority of the rest of the country does not experience. However currently there is no published literature on the nutritional composition of banana cultivars grown in New Zealand, or which varieties are well suited to our cooler climate and soil conditions. It is possible that climate change may even further enhance the ability to grow bananas in New Zealand, with rising temperatures providing suitable conditions for banana plants to thrive in regions outside of Northland and Gisborne .

1.7.1. Pests and diseases that jeopardise the banana industry

Banana horticulturalists outside of New Zealand face several pests and diseases that jeopardise plant health and fruit yield. This includes the banana weevil, the banana scab moth, and Panama disease [70-72]. Their absence within New Zealand is beneficial to the commercial banana industry.

The banana weevil (C*osmopolites sordidu*s) tunnels into the plants corm site, causing damage to the vascular and root system. This injury results in a restriction of nutrient and water uptake, promotes fungal infection and decreases the plants structural stability [73] . Infection of banana weevil in young plants can cause plant death and in mature plant's this insect causes stunted growth. Heavy infestations reduce fruit yield and impact the structural integrity so much that fruit bunches or the entire plant are susceptible to toppling in windy weather. A Ugandan study estimated an annual average total banana yield loss of 42% after high infestations of banana weevil, showing the significant impact this insect poses to the commercial banana industry [71]. Native to Southeast Asia, this pest is spread worldwide to most banana growing countries, however there are no reported cases of the banana weevil in New Zealand.

The banana scab moth (*Nacoleia octasema*) is an insect in which females lay eggs on the exterior of the distal male flower bud and nearby leaves. Once hatched, the larvae migrate under the flowering bracts to feed on the young banana bunches [70]. This causes deformation to the developing fruit and black scarring that can extend to the entire fruit surface. This distortion of appearance makes the fruit unmarketable, resulting in economic loss for commercial plantations. Sometimes the moth will eat through the banana skin to feed on the pulp, contaminating the fruit and causing premature rotting, again making the fruit unfit for the commercial market. This pest is present in our Tasman fellow, Australia, but like the banana weevil, there are no reported cases within New Zealand.

The most destructive disease to banana horticulture is fusarium wilt, otherwise known as Panama disease. This disease is caused by the fungus *Fusarium oxysporum* f. sp. *Cubense* (FOC) and can persist in soil for years [74]. There are 4 races used to classify different strains of FOC, 3 of which attack banana varieties. The fungus infects the plant by entering the root system and blocking the vessels responsible for recovering nutrients and water from the soil. As the disease advances the leaves collapse, the pseudostem becomes discoloured and splits before the plant eventually dies [75]. Unfortunately, this plant disease is untreatable and easily spread. In the early 1900's Panama disease devastated the banana industry when the dominating cultivar for export, Gros Michel, was infected in several commercial plantations, resulting in a shift to the race 1 resistant Cavendish cultivar that is known today [76]. However Cavendish is not resistant to tropical race 4 which is now present in Asia and northern Australia [72]. To date FOC has not been reported in New Zealand soil.

The absence of the banana weevil, the banana scab moth and FOC within New Zealand presents an advantage to the prospective commercial banana industry, in that additional pesticide application is not required to target these specific insect pests and there is a reduced risk of diminished plant health and fruit yield. Furthermore, because horticulturalists in Northland are growing a vast array of banana cultivars that are not Cavendish variety, if any diseases including Panama disease were to breach the New Zealand boarder, these varieties may be impacted requiring a shift to more resistant cultivars such as the FHIA ones.

1.7.2. The nutritional composition of New Zealand grown banana

There is some variation in nutritional composition between different banana cultivars [77-79]. The variation includes but is not limited to starches, provitamin A carotenoids, water, potassium, and antioxidants. At present there is no published literature available in the public domain regarding the nutritional composition of New Zealand grown bananas. It is undetermined if this variation is similar or significantly different to that of those grown overseas. It can be presumed that because the climate and soil conditions in New Zealand differ from those in places where bananas are a staple food (e.g. Pacific Islands, Ecuador, India, Philippines, Brazil), that the nutritional composition of bananas will differ also. This shows the need for research to determine the nutritional properties of bananas grown in New Zealand, so that these potential differences or similarities in banana nutrition can be identified and compared. When choosing a New Zealand grown banana cultivar that contributes toward infant health, it would be strategically beneficial to identify banana varieties that contain the optimal nutrients and that also grow well in our conditions to identify the most appropriate for a future commercial banana industry.

1.8. Future prospects for the Māori community

During complementary feeding the infant gut microbiota is subject to a dramatic transition [6, 8, 11]. As the diet which has solely consisted of milk is slowly introduced to solid foods, the gut microbiota has newfound nutrients available for metabolism which are not in breast milk or formula [37, 38]. The gut microbiota has several implications toward infant development regarding immunity, intestinal mobility, nutrient absorption, appetite and glucose tolerance [32, 34, 41, 51, 55]. Bananas are recommended as a first food for weaning infants due to the appeal they have to the infant taste, significant nutrients for nourishment, safe feeding texture and prebiotic content [56, 62]. The only commercial variety available in New Zealand is imported Cavendish. However, Māori in Northland are growing various varieties of banana for which, to date where neither the nutritional composition nor the cultivar is known. By gaining an understanding of what varieties thrive in New Zealand conditions and how these different varieties impact infants at this crucial stage of development, Māori in regions that can support banana growing can develop high value foods that support the health of their whānau through nutrition, not just in a physical sense but through further building the important connection with the land and passing down their important mātauranga to the next generation of rangatahi.

2. In-vitro digestion and fermentation of New Zealand grown bananas to understand their impact on the infant gut microbiota and functional potential.

2.1. Abstract

The human gut microbiota is established immediately at birth and thereafter is modulated by the nutrients available within breast milk or formula which support feeding the baby and selective colonisation. Upon weaning to solid foods, the infant gut microbiota begins to become more akin to that of an adult in both microbial composition and metabolite production. Bananas are recommended by New Zealand health officials as an appropriate first food, however at present the only commercial variety available is imported Cavendish. Although in Northland and Gisborne regions other banana varieties are successfully cultivated, they have not been recognised by the commercial sector as valuable produce. Here we identify the nutritional compositions of an array of banana cultivars grown in New Zealand and how these varieties impact SCFA production by the infant gut microbial community. We further identified the distinct shifts in microbial populations between three banana cultivar genome groups represented as AAA, AAB and ABB. Genomic mapping of unknown banana cultivars provided insight into the diversity of banana cultivars growing in New Zealand, in which the ecophysiology of bananas growing in this climate has presented different phenotypic characteristics to those of countries where banana horticulture is prevalent. These findings will contribute toward Māori communities building a commercial banana industry and creating a nutritional database for New Zealand grown banana.

2.2. Introduction

Within the first year of human life, the gut microbiota undergoes dramatic changes in microbial composition and function and thereafter stabilizes, becoming more akin to that of the adult microbiota [8]. Upon introduction to solid foods, the infant gut is subject to newfound nutrients that were not available in the diet consisting only of milk. This increases gut microbiota diversity and SCFA production. As SCFAs play multiple roles in human health, including but not limited to immunity, intestinal mobility and nutrient absorption, it is important that upon weaning to solid foods infants are supplied with foods that encourage the right SCFAs production [33, 35, 45]. Bananas are recommended by the New Zealand Ministry of Health as they are safe for infant feeding and provide nutrients essential for infant development [56]. Bananas also contain the prebiotics, dietary fiber and resistant starch that the gut microbiota metabolise, resulting in SCFA production [62, 64]. At present the only bananas available commercially are of the Cavendish variety, however whānau within the Te Tai Tokerau region have been growing several varieties of banana that differ in genomic composition to that of Cavendish. Bananas are a tropical plant, so not all varieties will be able to withstand the more temperate climate of the winters experienced in Te Tai Tokerau. Having a commercial banana industry in New Zealand has several advantages, including the absence of pests and diseases that jeopardize the banana industry in countries outside of New Zealand. This includes the banana weevil, scab moth and Panama disease, all of which are yet to breach the New Zealand border [70, 73, 74].

Toni Austen's (Te Rawawa/Ngāti Kahu) whānau have, over four generations, developed the mātauranga of which varieties grow successfully on her whenua-whānau in Te Tai Tokerau. The Austen family takes pride in their land and they educate the community about conservation and horticulture. Toni Austen aspires to lead whānau to develop a commercial banana food industry. Her whānau already have firsthand experience in growing several cultivars successfully in New Zealand soil and climate conditions. However, the genomic composition of some of these cultivars remains unknown. Utilising New Zealand grown banana for an ingredient in a product for an infant first food has the potential to improve the health of infants at this crucial stage of gut microbiota development, provide job opportunities for the Te Tai Tokerau community, achieve Māori aspirations, and expand New Zealand horticulture to a yet undeveloped commercial banana industry. There is as yet no published literature available regarding the nutritional compositions of New Zealand grown bananas and no existing knowledge of how our home-grown banana varieties impact the developing infant gut microbiota.

This research therefore aims to identify the nutritional compositions of different banana cultivars grown in New Zealand, and how these cultivars impact the microbial community and SCFA production within the gut of infants during the transition from milk to solid food diet. This study also aims to identify the genomic composition of the unknown banana cultivars being grown by the Austen whānau so that they can make informed choices about which varieties to focus on for commercial banana growing and perform further research on these varieties that thrive in Northland conditions.

Based on literature confirming differences in nutritional components between banana cultivars, and how climate also affects the nutritional profile, we hypothesized that New Zealand grown banana cultivars would differ in nutritional composition from imported Cavendish [77-79]. These differences will be reflected in distinct profiles in an *in-vitro* digestion and fermentation system with an infant faecal microbiota.

The commercial banana variety available for purchase in New Zealand is the imported Cavendish. Other imported banana varieties are either not available or not easily accessible nationwide. Furthermore, due to the New Zealand climate, Cavendish does not thrive in outdoor weather and is not widely cultivated. The lack of imported banana varieties available, and the insufficient amount of New Zealand grown Cavendish, meant it was not possible to effectively compare imported banana varieties to those grown in New Zealand. We instead chose to compare banana cultivars containing a combination of *M. acuminata* and *M. balbisiana* genomes with those cultivars containing only triploid or tetraploid *M. acuminata* genomes. As the *M. balbisiana* hybrids have a greater tolerance to wind and the colder climate, most New Zealand grown bananas carry one or two copies of *M. balbisiana* within their genome. Thus, we instead hypothesised that significant differences would be observed within the nutritional composition between a banana cultivar comprised of only the *M. acuminata* genome and the banana cultivars that also contain *M. balbisiana.* Further differences will also be reflected in distinct profiles in an *in-vitro* digestion and fermentation system with pooled infant faecal microbiota.

2.3. Materials and methods

This study was designed by the thesis author, Simone Frame, senior scientist and research associate Dr Jane Mullaney, the leader of vision mātauranga Māori, in the High Value Nutrition National Science Challenge, and Professor Warren McNabb, the leader of the Sustainable Nutrition Initiative at the Riddet Institute.

The study aimed to identify differences within the nutritional composition between banana cultivars comprised of only the *M. acuminata* genome and the banana cultivars that also contain *M. balbisiana*. We also aimed to find any cultivar differences reflected in distinct profiles in an *in-vitro* digestion and fermentation system with pooled infant faecal microbiota.

The study originally aimed to investigate the nutrition and fermentation profile of imported Cavendish alongside an array of 12 different banana cultivars grown within New Zealand, representing an array of banana genomic classifications, including a New Zealand grown Cavendish. Of each cultivar, the banana would be harvested from three separate plants. However, due to the time schedule and seasonal availability at the time of this study, it was not possible to obtain enough fruit to represent the 12 New Zealand grown banana cultivars.

Instead, seven New Zealand grown banana cultivars were harvested and investigated. Although a New Zealand grown Cavendish variety was supplied, it was not of an amount which would meet requirements for full analysis. Bananas were harvested from three separate plants of the same cultivar for all New Zealand varieties except for that of the Monalisa, Blue java and Tonga cultivars as stated in 2.3.1.

All laboratory processes were undertaken by the author unless otherwise indicated. This research was funded by the High-Value Nutrition National Science Challenge.

The reagents used were purchased from ThermoFisher, and Sigma-Aldrich unless otherwise indicated.

2.3.1. Banana cultivar sample collection

Fore each cultivar, a minimum 2 two kg of banana were harvested from three separate growing banana stems (banana are referred to as ‘stems’, not trees). However, due to availability at the time of the study, not all banana cultivar fruit could be harvested from three separate plants. Cultivar fruit which were harvested from less than three separate plants were Blue java (1), Tonga (1), Mona lisa (2). The fruit were harvested in bunches and maintained together where possible during the ripening process explained further in 2.3.2. In total eight cultivars were used for this study. Other than the Cavendish variety, which is available as a commercial import, all cultivars were harvested in Northland New Zealand between February and August 2022. The Cavendish was purchased from two local supermarkets in Palmerston North. Table 4 indicates the banana cultivars used within this study, harvest location, genomic assembly, and other known names. Some of the banana cultivars used in this study are pictured in Figure 3.

Table 4. Banana cultivars, location of harvest, respective genome and other name(s) [65, 80, 81].

Banana cultivar	Harvest location	Genome	Other name(s)
Cavendish	Ecuador, Philippines, and Mexico	AAA	
Mona Lisa	Kotare Farm	AAAA	FHIA-02
Goldfinger	Kotare Farm, Austen Nursery.	AAAB	FHIA-01
High noon	Kotare Farm	AAAB	SH-3640
Tonga	Whangarei	AAB	Hopa
Misiluki	Kotare Farm	AAB	Lady finger, Pisang Keling
Rajapuri	Kotare Farm	AAB	Raja puri
Blue java	Whangarei	ABB	Ney Mannan, Ice cream

Figure 3. Banana Cultivars. (Left to right: Goldfinger, Monalisa, Misiluki, Highnoon and Rajapuri).

2.3.1.1. Banana sample ripening, pulp:skin ratio and storage

Bananas were harvested green and hung at room temperature until they were before-ripe as shown in Figure 4.

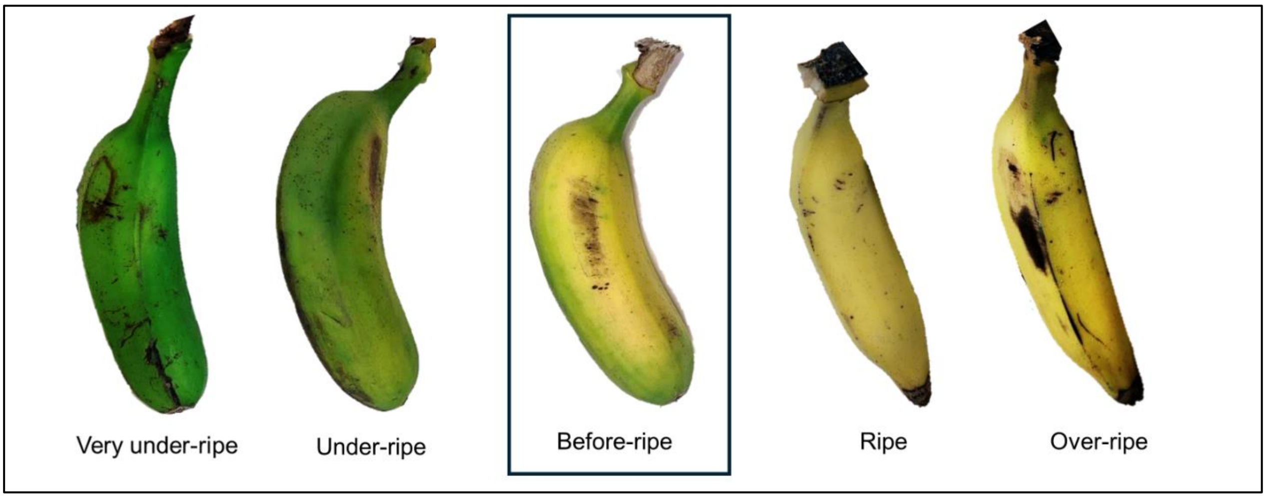

Figure 4. Banana ripeness scale.

Once bananas were before-ripe as described, they were separated from the bunch and weighed using a two decimal point scale. The weight recorded were of the whole banana and the individual fruit pulp (FP) and peel (PW). These were used to calculate the pulp to peel ratio (PPR) as follows:

$$\text{PPR} = \frac{FP}{PW}$$

The banana pulp was sliced into approximately 5 g pieces and frozen at -20°C.

2.3.2. Dry matter analysis

Of each cultivar, three bananas from three separate plants were used for dry matter analysis where possible explained in 2.3.1. Once bananas were ‘before-ripe’ (as explained previously in 2.3.1.1) the pulp and fruit were separated and weighed on a two decimal point scale. The banana peel and pulp were sliced into smaller pieces and oven dried on tinfoil trays at 120°C for a minimum of 72 hours. The weights of the tinfoil trays were recorded after oven drying and the samples were re-weighed. Dry matter was determined as follows:

$$Dry\ matter\ (\%) = 100\ x\ \frac{Dried\ weight\ - weighboat}{Fresh\ weight}$$

2.3.3. Nutritional analysis

Of each cultivar, 200 (+/-5) g of banana fruit from three separate plants were sliced into smaller pieces and frozen at -18°C for a minimum of 24 hours. The frozen samples were then freeze dried. The freeze-dried material was ground into a fine powder using an electric blender. The powdered samples of each cultivar were sent to the Massey Nutrition Laboratory for the analyses shown in Table 5.

Table 5. Banana nutritional analyses components. All nutritional analyses are measured in % unless otherwise specified.

Massey Nutritional analysis		
Ash	Moisture	Organic matter
Nitrogen	Crude protein	Total energy (kj/100g)
Lipids	Carbohydrates	Starch
Total dietary fiber	Resistant starch	Total sugar % m/m[1]
Sugars % m/m[1]	Potassium (mg/kg)	Magnesium (mg/kg)

[1] *m/m is mass percent per 100g*

2.3.3.1. Nutritional data conversion

To compare the nutritional analysis findings with those in literature and food databases, the dry matter results of each cultivar were used to determine the nutritional components in a 100g serving. The calculation determines how much of the nutrients are in 100g of a fresh serving containing the water that was removed during freeze-drying prior to analysis shown as follows:

$$\text{grams of nutional component in 100g serving} = \% - \left[\frac{H_2O}{100}\, x\ m/m^1\right]$$

Magnesium and potassium were calculated as above then divided by 10 so that they were measured as mg/100g.

2.3.4. Banana DNA extraction and sequencing

In total, 22 banana leaf samples with an array of known banana genomic compositions and four unknowns were harvested for DNA analysis. Samples were harvested from young leaves where possible and were stored at -20°C before extraction. The banana leaf samples were extracted for DNA using Bioline Isolate II Plant DNA kit with a minor modification. To remove polyphenols which interfere with DNA extraction quality 1 mL 70% ethanol was added to each sample after liquid nitrogen homogenization, this was mixed well and centrifuged at 11,000g for two minutes at room temperature. The supernatant containing excess polyphenols was disposed of and the pellet proceeded to further Bioline Isolate II Plant DNA kit extraction steps as per manufacturer instructions. The extracted DNA quantity was checked using Nanodrop. The extracted DNA was run on a 1% agarose electrophoresis gel alongside a 10,000bp mass ruler ladder to check DNA quality and fragmentation. Samples were sent to Custom Bioscience Ltd (Auckland NZ) who carried out quality checks, library preparation, library QC and sequencing for 3G data per sample using 150PE reads. Data were supplied as both raw and filtered data and sequenced for 3G data using Illumina HiSeq platform for 150 paired end reads by Next Generation Sequencing. Samples supplied as metagenomic DNA. Service includes sample QC, library prep,

2.3.5. In-vitro digestion

Each banana cultivar was subject to *in-vitro* digestion in triplicate, based on the methods of Minekus et al.[82]. Modifications to this method are that the digested fluids were prepared in concentrates as explained further in 2.3.5.1. To ensure the *in-vitro* conditions are more akin to that of an infant *in-vivo* digestive system, further modifications were applied by decreasing the gastric pH to 3 and adding porcine amyloglucosidase (AMG) in the intestinal phase with a final concentration of 100 U/mL [83].

2.3.5.1. In-vitro digestion - Simulated digestive fluids and digestive enzymes

The digestive fluids were simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). These were prepared so that reagent molarities were equal to the Minekus *in-vitro* protocol within each digestive phase. Banana samples used were fresh, therefore had a high-water content. To ensure adequate dry matter content for fermentation, the digestive fluids were concentrated so that the organic material was not diluted. For the benefit of solution storage, the Minekus method utilizes 1.25x concentrated digestive fluids which are reduced to 1x by adding water before each digestive phase. In this method the water was added directly to the digestive fluids for storage so additional water was not required within each digestive phase as shown in 2.3.5.2. The simulated fluids were prepared as shown in Table 6. The simulated fluids were used to dissolve the enzyme stock solutions in their respective phases e.g. pepsin dissolved in simulated gastric fluid. The α-amylase was obtained from *Bacillus licheniformis* (EC 3.2.1.1) supplied at a concentration of 500 U/mg and was dissolved in 1-part SSF and 1-part sterile water. Pepsin was obtained from porcine gastric mucosa (EC 3.4.23.1) supplied at a concentration of 250 U/mg. AMG was obtained from *Aspergillus niger* (EC 3.2.1.3 Megazyme) and was supplied at a concentration of 3260 Starch U/mL. Pancreatin was obtained from porcine pancreas (EC 232.468.9) supplied at 8x USP.

2.3.5.2. Infant in-vitro digestion method of fresh banana cultivars

Each banana cultivar was digested in triplicate alongside potato starch as a control. Sample order was randomized using Microsoft Excel. Of each sample 94.5 g (+/- 0.05 g) of frozen banana was defrosted overnight at 4°C. The defrosted sample was blended well with 40 mL of sterile water using an electric blender. To this mixture, an equal amount of SSF was added and blended thoroughly.

Oral phase: 47.375 mL of this mixture was added to 0.125 mL of 0.3 M $CaCl_2(H_2O)_2$, and pH adjusted to 7*, 2.5 mL α-amylase was added to achieve 75 U/mL The sample was left to digest for 2 minutes in a shaking incubator at 37°C. To halt enzyme activity and reduce pH, 0.285 mL of 6 M HCl was added and mixed well. **Gastric phase:** the oral bolus was mixed well with 0.015 mL 0.3 M $CaCl_2(H_2O)_2$, 4.9 mL of SGF and 4.8 mL porcine pepsin to achieve 2000 U^{-mL}. The sample was pH adjusted to 3* and left to digest for 2 hours in a 37°C-shaking incubator. **Intestinal phase:** 0.475 mL of 1 M NaOH was added to increase the pH. To the gastric bolus, 3 mL of bile (resulting in 10 mM), 0.075 mL of 0.3 M $CaCl_2(H_2O)_2$, 3 mL amyloglucosidase to achieve 100 U/mL and 3.5 mL of pancreatin to achieve 100 U/mL of amylase activity. The sample was pH adjusted to 7* before digesting for 2 hours in a 37°C-shaking incubator. The sample vessels were suspended in an 80°C water bath for 3 minutes or until internal sample temperature reached a minimum of 65°C to halt enzyme activity. Each sample was poured into individual dialysis tubing which was clipped at both ends and submerged in 10 L buckets of reverse osmosis water. Over a 24-hour period the water was refreshed three times. Samples were emptied from dialysis tubing into plastic storage containers and stored at -18°C.

**pH adjustments were made using 2 M NaOH or 3 M HCL.*

Table 6. Simulated fluid constituents

Showing the reagent volumes within each in-vitro digestive phase. The volumes made were 2200 mL of 2x concentrated simulated salivary fluid (SSF), 500 mL of 3.5x concentrated simulated gastric fluid (SGF) and 500mL of 4.6x concentrated simulated intestinal fluid (SIF). Calcium chloride was excluded from digestive fluid preparation but was added individually to each digestive phase to avoid precipitation.

			2x SSF			3.5x SGF			4.6x SIF		
Reagent	gL^{-1}	Stock mol L^{-1}	mL in SSF	conc in SSF mMol L^{-1}	conc in **50mL** digest mMol L^{-1}	mL in SGF	conc in SGF mMol L^{-1}	conc in **60mL** digest mMol L^{-1}	mL in SIF	conc in SIF mMol L^{-1}	conc in **70mL** digest mMol L^{-1}
KCl	37.3	0.5	132.88	30.2	0.228	12.08	12.51	0.048	15.64	22.168	0.046
KH_2PO_4	68	0.5	32.56	7.4	0.014	1.58	1.63	0.001	1.84	2.608	0.001
$NaHCO_3$	84	1	59.84	27.20	0.092	21.88	45.31	0.31	97.75	277.1	3.6
NaCl	117	2	-	-	-	20.65	85.55	0.56	22.08	125.184	0.37
$MgCl_2(H_2O_6)$	30.5	0.15	4.40	0.3	0.000075	0.70	0.22	0.000048	2.53	1.0758	0.00036
$NH_4(CO_3)$	48	0.5	0.53	0.12	0.000004	0.88	0.91	0.00025	-	-	-
Water	/	/	1969.8	-	-	442.25	-	-	360.16	-	-
Total volume			2200			500			500		

2.3.6. Infant faecal sample collection, preparation, and storage

Ethical approval for involving human participants for stool donation was obtained from the Human Ethics Southern A Committee (Application: SOA 21/69). A total of 17 infants in the Tararua and Manawatu regions were recruited via parental consent, and then screened by filling the questionnaire attached in Appendix 1. Of those that completed the questionnaire, five were not eligible. For this study, two separate faecal samples (and one from one participant) were collected from six healthy infants that met the following criteria:

- Aged five to seven months old
- Free of known metabolic and gastrointestinal diseases
- Had not been administered prebiotics
- Had begun eating solid foods

A bamboo liner was placed inside the infant's diaper, this liner was removed containing the faecal sample, placed inside a snap lock bag, and stored in a cooler bin containing a frozen ice pack. The samples were stored at 4°C until being processed within the same day. Faecal samples were placed inside a filter bag with a known volume of potassium phosphate buffer 50 mM (pH 7.2). The bag was manually palpitated to homogenise and filter the sample. The sample was aliquoted into 1.5 mL Eppendorf tubes and stored at -80°C until used for fermentation.

2.3.6.1. In-vitro fermentation

The method for fermentation of the digested banana was based on the human faecal *in-vitro* fermentation method used by Edwards, et al [84].The infant faecal inoculum was defrosted in 4°C refrigerator overnight prior to fermentation. In a sterile 10mL Hungate tube, 3 mL of defrosted digest retentate was added to 1 mL of 150 mmol/L phosphate buffer and mixed well. A nitrogen gas probe was used to eliminate oxygen. 50 µL of filtered 3% cysteine was added, mixed well, and left for 10 minutes at room temperature. The Hungate tube headspace was filled with CO_2 and the Hungate was capped tightly. 1 mL of pooled faecal inoculum was added. Time zero samples were immediately put on ice and the 24-hour time point tubes were incubated for 24 hours at 37°C then put on ice before sample recovery explained in 2.3.6.2.

2.3.6.2. In-vitro fermentation sample recovery

For SCFA analysis and DNA extractions, 1 mL from each Hungate was centrifuged in a microcentrifuge tube at 13,000 for 2 minutes, 900 mL of supernatant was used for SCFAs analysis and the pellet was used for DNA extractions. Both supernatant and pellet were stored separately at -80°C until further use.

2.3.6.3. Infant faecal DNA extraction, sequencing and metagenomic analysis

Faecal DNA was extracted from the pellet (recovery explained in 2.3.6.2) after defrosting on ice. Extraction was performed using Macherey-Nagel NucleoSpin® Soil kit as per manufacturer's instructions with minor modifications, whereby the sample is left to lyse in the lysis buffer for 20 minutes before homogenization on Mini-Beadbeater 96 for four minutes. Another modification was that the eluted flow-through was transferred back into the same column to repeat elution. The extracted DNA quantity was checked using Nanodrop. The extracted DNA was run on a 1% agarose electrophoresis gel alongside a 10,000 bp mass ruler ladder (Thermo Fisher cat#SM0403) to check DNA quality and fragmentation. Approximately 500 ng of metagenomic DNA was sent to Custom Biosciences (Auckland New Zealand) who carried out quality control, constructed and prepared libraries for the Illumina HiSeq platform Next Generation Sequencing with 3G data per sequence reads, and 150-pairended sequences. FastQC version 0.11.9 was used to QC the raw data [85].The software Trimmomatic v.0.36 was used for removal of adapters , low quality (Phred scores < 30), and short (< 36 bp) sequencing reads [86]. The detection of host reads was done using the “mem” algorithm of bwa version 0.7.17-r1188 [87] and fastq files were generated from the unmapped sequencing reads using the “fastq” function of samtools version 1.8 [88]. Read pairs were joined using PEAR version 0.9.6 [89] with default settings. Read pairs that did not join were pasted together with a string of N’s using the "fuse" function from the BBMAP package version 38.22-0 [90]. Joined and fused reads from different lanes from the same sample were then compiled into a final “clean” read sample file. The "blastx" function of diamond version 0.9.22 [91] was used to map the reads against the "nr" NCBI database [92]

2.3.7. Banana genome analysis

The genome mapping of the banana sample DNA extractions was performed by Dr Jane Mullaney, Dr Ambarish Biswas and Paul Maclean (AgResearch) based on methods used by Christelová et al [93]. Paired-end Illumina FASTQ files from 30 samples were merged using PEAR (version: v0.9.6, parameters: default) with a quality threshold value of 30 (i.e., -q 30). The resulting reads from the three FASTQ files per sample (i.e., merged, forward-unmerged, and reverse-unmerged) were concatenated into a single FASTQ file. Subsequently, this FASTQ file underwent further processing with trimmomatic (version: 0.39, parameters: LEADING:30 TRAILING:30 SLIDINGWINDOW:4:30 MINLEN:36) to trim low-quality bases from both ends of the reads. The resulting 30 merged-trimmed FASTQ files were mapped against two reference genomes *Musa acuminata* (NCBI assembly ID: GCF_000313855.2) and *Musa balbisiana* (NCBI assembly ID: GCA_004837865.1) using bwa (Version: 0.7.17-r1188) that generated the ‘sam’ files. The sam files were converted to sorted ‘bam’ files using samtools sort function (Version: 1.16.1) and reference genome specific SNP profiles in VCF format were constructed using mpileup function (parameters: --max-depth 8000 -I -Ou -a AD, DP) of bcftools (Version: 1.9).

2.3.8. SCFAs extraction and derivatisation

The 900 μL fermentation supernatant (recovery explained in 2.3.6.2) was defrosted on ice and added to 100 μL of 50 mmol 2-ethyl butyric acid. Of this mixture,500 μL was added to 250 μL 12 M HCl and 1000 μL of diethyl ether and mixed well. Once solvent phase and liquid phase had separated, 200 μL of ether were added to a 2 mL glass gas chromatography (GC) vial with 40 μL N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide and capped firmly with a 11 mm crimp top. The vial was incubated for 20 minutes at 80°C then 24 hours at room temperature. Sample was transferred to a new GC vial with a glass insert and capped and left for 72 hours to completely derivatise.

2.3.8.1. SCFAs analysis

SCFAs extracts and standards were run on a Nexus GC-2010 Gas Chromatography Flame Ionisation Detection (GC-FID) system with a HP-1 column (30m x 0.25mm ID,0.25 μm film thickness) obtained from Agilent Technologies, USA. The carrier gas was helium at a pressure of 131.2 kPA at a flow rate of 52.3 mL/min at a split ratio of 30:1. The detector temperature was 310°C. The initial temperature was 70°C with an increase of 6°C/min to 115°C, then an increase of 60°C/min to 280°C and sustaining for three minutes before decreasing at 120°C/min to 70°C. Shimadzu LabSolutions software (v5.98) was used for data acquisition and analysis. After peak integration, the data were exported to Microsoft Excel spreadsheets. SCFAs content within each sample was normalised by the internal standard peak area and a standard curve generated. Line of best fit was used to determine the concentration of each SCFAs within the samples against external standards.

2.3.9. Statistical analysis

All statistical analyses were performed in GraphPad Prism software (version 10.0.2) and/or Microsoft Excel (version 2304) unless otherwise specified. Where statistical tests measure significant differences, they are reported within figures as follows: not significant (ns), p-value <0.05 (*), p-value <0.01 (**). p-value <0.001 (***).

2.3.9.1. Banana cultivar composition statistical analyses

Replicate values (n=3 for all cultivars except Blue java and Tonga were n=2) were used to determine the mean dry matter and standard deviation (SD) of each cultivar. To observe differences in banana fruit pulp weight between genome groups the banana cultivar were sorted into genome groups (AAA, AAAA, AAAB, AAB and ABB) and checked for normality using a Shapiro-Wilk test. This indicated the data were not normally distributed so were tested for differences using a Kruskal Wallis test. Differences were identified by an Uncorrected Dunn's post-hoc test. The same tests were used to observe differences between dry matter content of cultivars. The same test was used to determine differences between genome groups of dry matter content, macronutrients, potassium, and magnesium. To identify differences of total sugar content between genome groups a Kruskal-Wallis test was used.

2.3.9.2. Banana cultivar post-fermentation SCFA statistical analysis

To identify significant differences of post-fermentation SCFA between cultivars containing the *M. balbisiana* genome and those without, first the SCFA data were checked for normality using a Shapiro-Wilk test. This test showed that the SCFAs were of normal distribution, therefore a Brown-Forsythe ANOVA test determined if there were differences among cultivar means. An unpaired t-test with Welch's correction was used to determine which cultivars containing the *M. balbisiana* genome differed from those without. The mean and SD of each SCFA across all cultivars was calculated to determine the most dominant SCFA produced post fermentation.

2.3.9.3. Taxonomic analysis of in-vitro fermentations

For analysing high-dimensional microbiome data the ANCOM-BC (Analysis of Composition of Microbiomes with Bias Correction) package in R was used (Version 3.18) [94]. Using the

ANCOM-BC results, bacterial taxa were filtered by global q value <0.01, global p value <0.05 and global w value >50. Any identified differentially abundant taxa underwent post-hoc testing using an uncorrected Dunn's test. To observe correlations between post-fermentation bacterial taxa and the produced SCFA a Spearman's Correlation test was used.

2.4. Results

2.4.1. Banana cultivar composition

2.4.1.1. Pulp:peel ratio and average fruit weight

Shown in Table 7 below is the measured pulp:peel ratio and average weight of the banana cultivars used in this study. Significant differences of fruit pulp weight were observed between Cavendish and Blue java (p=0.0495).

Table 7. Banana cultivar, genome, pulp:peel ratio, mean fruit weight and number of bananas cultivar.

Cultivar	Genome	Pulp:Peel ratio	Mean fruit weight (g)	(n)
Monalisa	AAAA	1.6	90.4	15
Imported Cavendish	AAA	1.7	160.0	23
Goldfinger	AAAB	1.8	65.0	54
Highnoon		1.7	102.9	30
Tonga	AAB	1.4	84.5	8
Misiluki		1.4	81.3	54
Rajapuri		1.7	92.4	30
Blue java	ABB	1.5	37.5	27

2.4.1.2. Banana cultivar dry matter

Dry matter shown in Table 8 below indicates that banana pulp dry matter of cultivars varied between 20-40% with a mean of 27.8%. Banana peel had a lower dry matter content regardless of cultivar.

Table 8. Banana Dry matter. Showing % dry matter of banana cultivar pulp.

Cultivar	Genome	Pulp
Monalisa	AAAA	20%
Imported Cavendish	AAA	23%
Goldfinger	AAAB	26%
Highnoon		24%
Tonga	AAB	32%
Misiluki		28%
Rajapuri		29%
Blue java	ABB	40%

When comparing cultivar pulp dry matter to Cavendish, significant differences were observed for Rajapuri (p=0.0483), Tonga (p=0.0171) and Blue java (p=0.0171), as shown in Figure 5 (A) below. When comparing dry matter of fruit pulp between genome groups, the genome groups AAB and ABB had significantly more dry matter than the Cavendish genome group AAA (p=0.0135 and p=0.0171 respectively), as shown in Figure 5 (B). Greater statistically significant differences were observed when comparing the genome group AAAA with genome groups AAB (p=0.0014) and ABB (p=0.0036) as shown in Table 9.

Table 9. Uncorrected Dunn's test comparing dry matter pulp content of banana genome groups.

Uncorrected Dunn's test	P Value	Summary
AAA vs. AAAA	0.5535	ns
AAA vs. AAAB	0.2695	ns
AAA vs. AAB	0.0135	*
AAA vs. ABB	0.0171	*
AAAA vs. AAAB	0.0773	ns
AAAA vs. AAB	0.0014	**
AAAA vs. ABB	0.0036	**
AAAB vs. AAB	0.1284	ns
AAAB vs. ABB	0.1013	ns
AAB vs. ABB	0.5238	ns

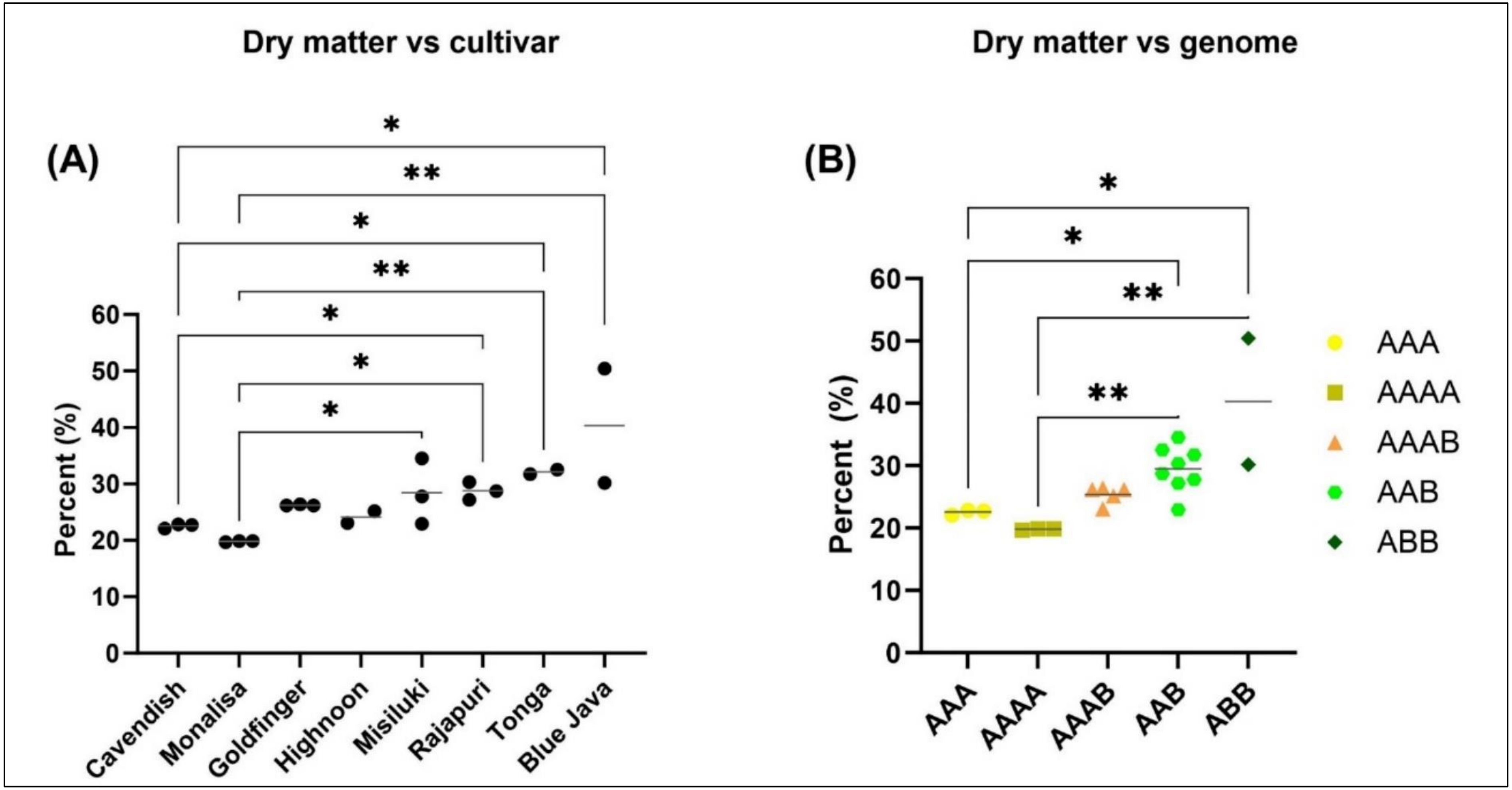

Figure 5.(A) Dry matter content vs cultivar. Monalisa vs Misiluki (p=0.0212), Monalisa vs. Rajapuri (p=0.0102), Monalisa vs. Tonga (p=0.0036),Monalisa vs. Blue Java (p=0.0036). Cavendish vs Rajapuri (p=0.0483), Cavendish vs. Tonga (p=0.017, Cavendish vs. Blue Java (p=0.0171). (B) Dry matter content vs genome. AAAA vs. AAB (p=0.0014), AAAA vs. ABB (p=0.0036) AAA vs. AAB (p=0.0135), AAA vs. ABB (p=0.0171), Differences are represented as: not significant (ns), p-value <0.05 (*), p-value <0.01 (**) and p-value <0.001, (***)

2.4.1.3. Banana cultivar nutritional composition – essential minerals

The potassium and magnesium contents of those banana cultivars assessed in this study are shown in Table 10.

Table 10. Banana cultivar potassium and magnesium content

Cultivar	Genome	Potassium (mg/100g)	Magnesium (mg/100g)
Monalisa	AAAA	317	27
Cavendish	AAA	389	34
USDA Cavendish		326	28
Goldfinger	AAAB	396	38
Highnoon		375	31
Tonga	AAB	347	33
Misiluki		276	29
Rajapuri		361	33
FSANZ Lady finger		320	38
Blue java	ABB	447	54

The USA Department of Agriculture (USDA) food database indicates that the mean magnesium (Mg) and potassium (K) content for ripe/slightly ripe Cavendish banana is 28 mg/100g and 326 mg/100g, respectively [62]. The USDA does not state the country where the bananas they tested were grown, however because the Cavendish analysed in this study was imported it was expected to be comparable to that analysed in the US. Furthermore, Food Standards Australia & New Zealand (FSANZ) reports a 100g serving of ladyfinger banana to contain 38mg/100g of magnesium and 320mg/100g potassium [95]. When banana cultivars were sorted into genome groups significant differences were not present when comparing potassium content. However, when comparing magnesium content significant differences were observed between AAAA and AAB genome groups ($p<0.04$) (Dunns test).

2.4.1.4. Banana cultivar nutritional composition – Total starch, resistant starch, and dietary fibre

Shown in Figure 6 are the total starch, resistant starch, and dietary fibre of fresh banana cultivars. No significant differences were found in resistant starch between genome groups. AAAA had significantly less dietary fibre and total starch than ABB (p=0.043).

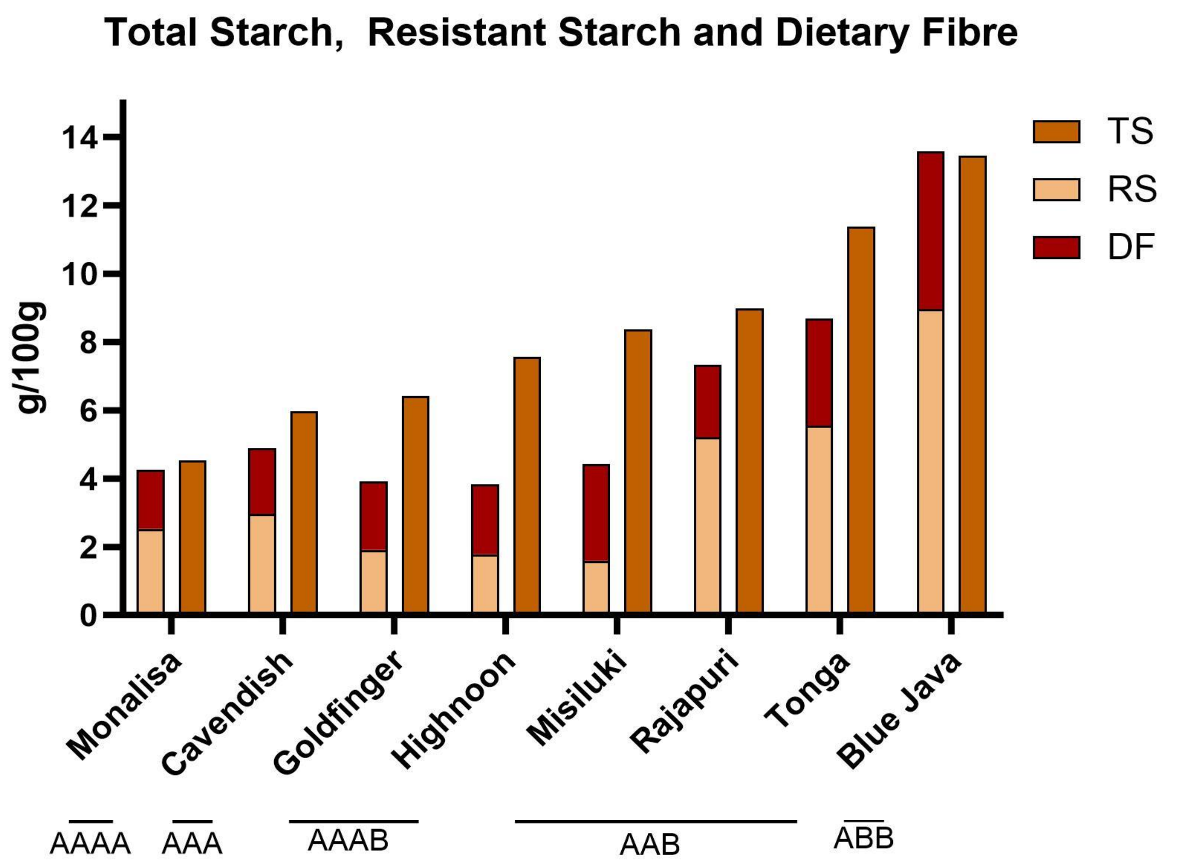

Figure 6. Total starch (TS), resistant starch (RS) and dietary fibre (DF) content g/100g serving of fresh banana cultivars

2.4.1.5. Banana cultivar nutritional composition – macronutrients

The protein, fat, carbohydrates, and energy content for each banana cultivar (including FSANZ and USDA databases) are shown in Table 11 below. No significant differences were detected between genome groups for fat content. Dunns tests showed that significant differences were only observed between the genome groups AAAA and AAB in protein (p=0.049), carbohydrates (p=0.049) and energy content (p=0.049).

Table 11. Fresh banana cultivar macronutrients in a 100g serving.

Cultivar	Genome group	Protein (g)	Fat (g)	Carbohydrates (g)	Energy (kJ/100g)
Monalisa	AAAA	0.79	0.28	18.93	359
Imported Cavendish	AAA	1.15	0.11	22.42	421
USDA Cavendish		0.74	0.29	23.0	410
Goldfinger	AAAB	1.15	0.60	24.49	474
Highnoon		1.02	0.17	23.06	432
Misiluki	AAB	0.85	0.36	26.54	502
Rajapuri		0.97	0.22	26.46	492
Tonga		1.07	0.04	29.49	546
FSANZ Lady finger		1.0	0.3	21.7	397
Blue java	ABB	1.53	0.55	33.87	660

2.4.1.6. Banana cultivar nutritional composition – sugars

The main sugars found across all cultivars are fructose and glucose. Only Mona Lisa and imported Cavendish contained >0.1g/100g of sucrose in fresh banana. Table 12 shows all sugars analysed in the fresh banana cultivars and the USDA and FSANZ databases. Galactose information was not available for the FSANZ lady finger banana. No significant differences of total sugars were observed between banana genome groups.

*Table 12. Sugars in banana cultivars measured in g/100g of fresh pulp (*Anhydrous and Monohydrate).*

	g/100g fresh banana							
Cultivar	Genome	Fructose	Glucose	Lactose*	Maltose	Sucrose	Galactose	Total sugar
Monalisa	AAAA	6.4	6.3	<0.1	<0.1	0.7	<0.1	13.5
Imported Cavendish	AAA	4.6	4.7	<0.1	<0.1	4.5	<0.1	13.9
USDA Cavendish		6.09	5.5	<0.25	<0.25	4.2	<0.1	15.8
Goldfinger	AAAB	8.7	8.6	<0.1	<0.1	<0.1	0.1	17.5
Highnoon		6.8	6.7	<0.1	<0.1	<0.1	0.1	13.5
Tonga	AAB	7.0	6.9	<0.1	<0.1	<0.1	<0.1	13.9
Rajapuri		5.3	5.2	<0.1	<0.1	<0.1	<0.1	10.5
Misiluki		8.2	8.3	<0.1	<0.1	<0.1	0.1	16.6
FSANZ Lady finger		5.1	5.1	<0.1	<0.1	5.0		15.2
Blue Java	ABB	8.4	8.4	<0.1	<0.1	<0.1	0.1	16.9

2.4.2. Post-fermentation SCFAs production of digested banana cultivars

The total concentrations of SCFAs produced in the glucose control sample were as expected when compared to infants within the same age demographic in a recent study, reflecting a mixed acid fermentation where acetate was the dominant SCFA [37]. The dominating SCFAs produced post-fermentation across all cultivars was acetate (mean =281.03 mmol/100g, SD=181.34) as shown in Figure 7. All mean SCFA's produced for each cultivar with standard deviations are shown in Figure 8 and are attached in Appendix 2.

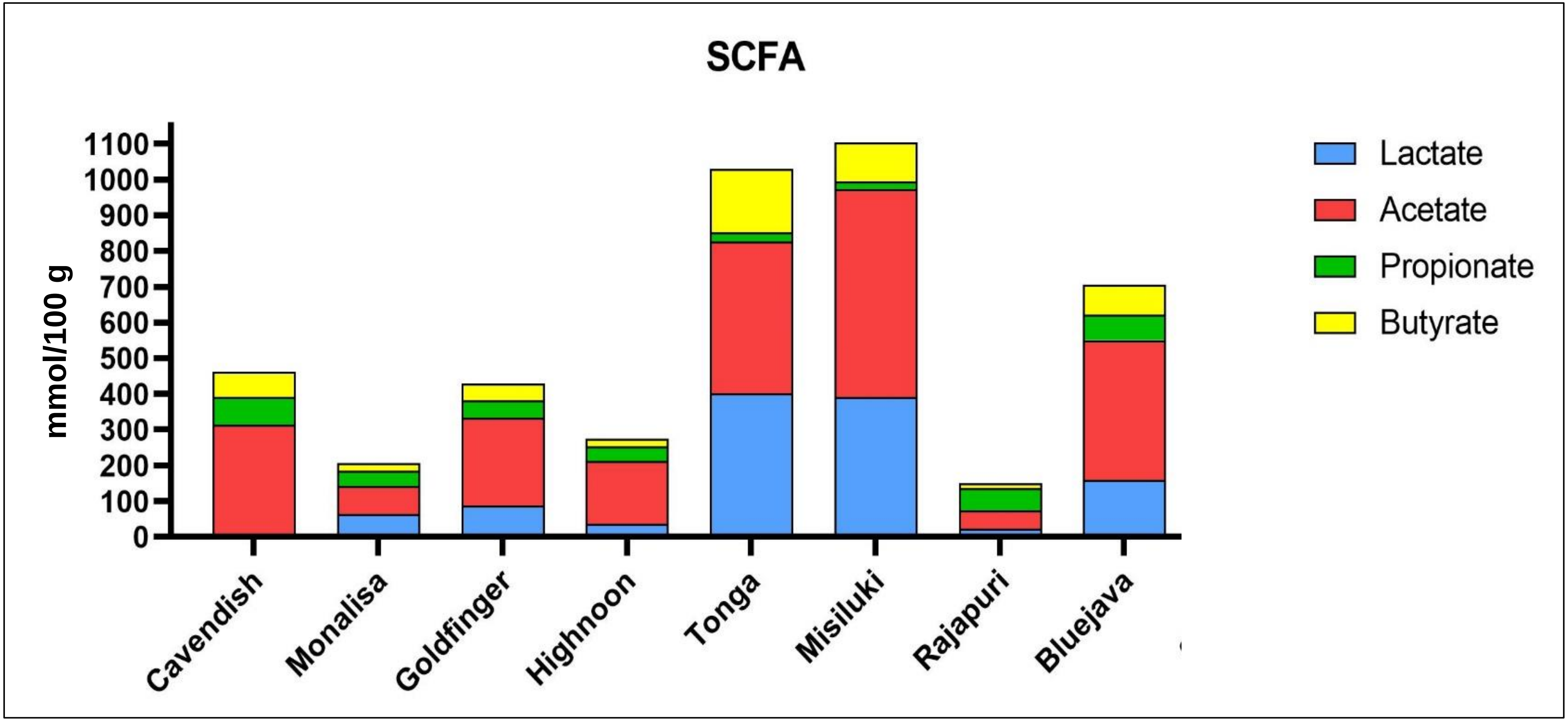

Figure 7. Mean SCFA's produced post 24-hour fermentation of in-vitro digested banana cultivars.

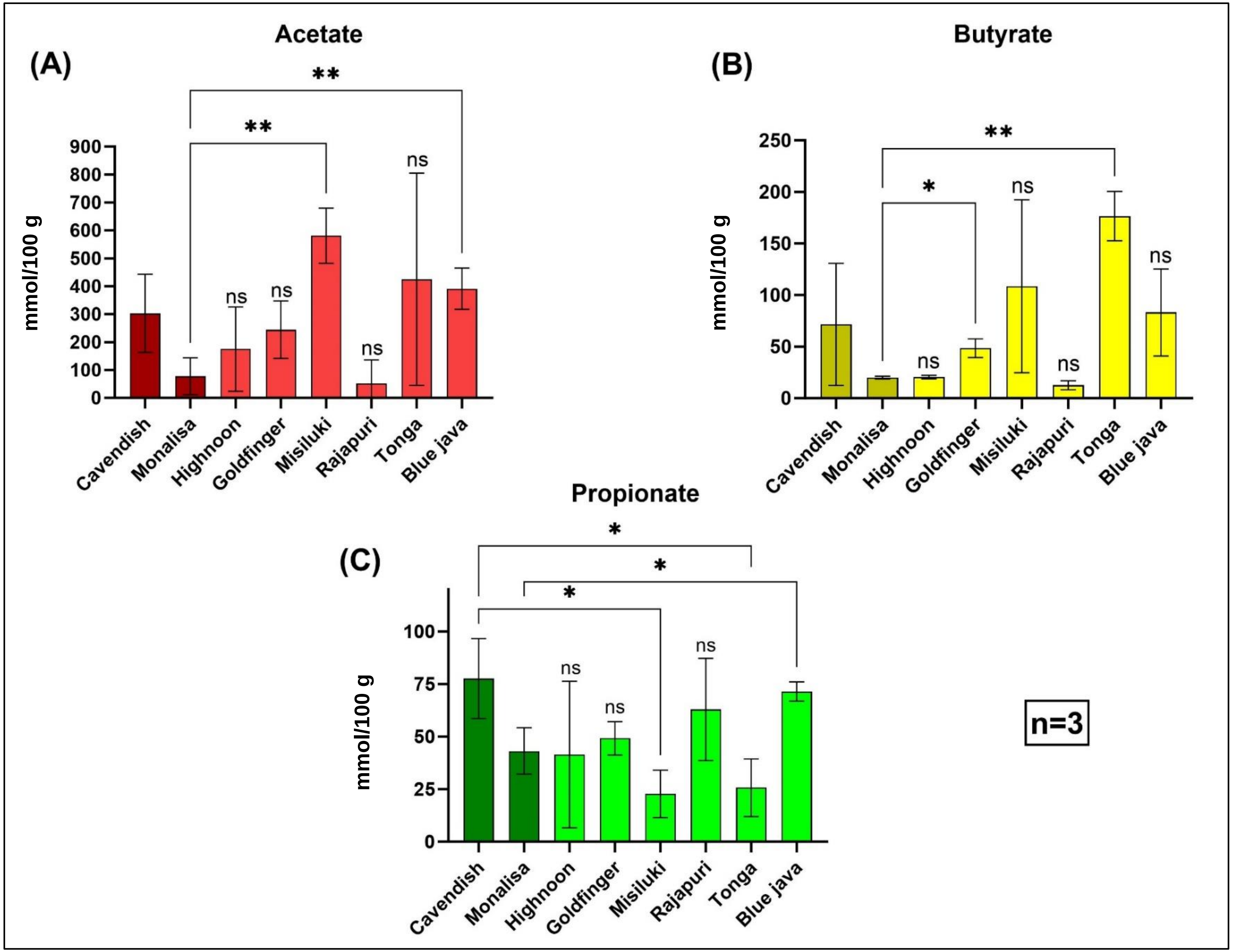

Figure 8. Individual major SCFAs produced and standard deviation (n=3). Post 24-hour fermentation of in-vitro digested banana cultivars. (A) Acetate, (B) Butyrate and (C) propionate. Differences of SCFA content between cultivars containing and without the Balbisiana genome are represented as: not significant (ns), p-value <0.05 (*), p-value <0.01 (**) and p-value <0.001, (***). (A) Acetate. Monalisa vs Bluejava (p=0.0056), Monalisa vs Misiluki (p=0.0031). (B) Butyrate. Monalisa vs Tonga (p=0.0076), Monalisa vs Goldfinger (p=0.0302). (C) Propionate. Cavendish vs Tonga (p=0.0221) Cavendish vs Misiluki (p=0.0196), Monalisa vs Bluejava (p=0.0327)

2.4.3. Taxonomic analysis of fermented banana

Due to issues with faecal DNA extractions, not all fermentations of digested banana cultivar were sequenced. The cultivars that were sequenced were Blue java (ABB), Rajapuri (AAB), Misiluki (AAB) and Cavendish (AAA). This means that we could still compare microbial populations between genome 3 groups, between cultivars of the same genome group (Misiluki and Rajapuri) and between cultivars with and without the *M. balbisiana* genome. The minimum number of reads per sample was 99,184,502 and the maximum number of reads per sample was 144,926,053. Across all samples the total number of reads was 9,212,363,434 with an average number of reads at 13,160,519. Sequencing of samples were checked for quality as shown in Appendix 3. Alpha diversity is shown in Figure 9 where Chao1 (A) indicates the richness of bacterial species, and the Shannon Index (B) indicates the communal diversity of species. Biological replicates and triplicates were used and indicated by each dot. At 24h, Blue java consistently had the highest Shannon score as shown in Figure 9 (B) but was similar to others in the CHAO1 diversity score. This indicates that although Blue java was similar in bacterial richness to the other banana cultivar fermentations, it presented a higher communal diversity.

A PCoA plot was drawn to examine the bacterial beta diversity differences between banana cultivar fermentations as shown in Figure which shows the abundance of the bacterial phyla at time zero and after 24 hours of fermentation which accounted for approximately 60% of the total abundances (unclassified are not shown). The 4 banana cultivars grouped in 3 distinct clusters representing the 3 genome groups (AAA, AAB and ABB). Tonga and Misiluki fermentations clustered close together; these two cultivars are from the same genome group (AAB). On the axis 1, 80.8% of the total variance could be explained where AAA, AAB and ABB stratified, clearly indicating that banana genome group strongly drives the bacterial community composition. Figure 11 and Figure 12 shows the relative abundances of bacterial taxa level from time 0 and after 24 hours at phylum and family level respectively. Figure 12 shows the top 31 taxa detected. In these figures, approximately 60% of relative abundances are shown, the remaining sequencing data could not be taxonomically assigned to bacterial phyla. Table 13 gives the ANCOM-BC analyses showing the differentially abundant bacterial taxa which have been filtered by global p-value (<0.05), global q-value (<0.001) and global w-value (>50). The global p-value measures differences in the data set where p=<0.05 represents significant. Differences that were observed between banana cultivar post fermentation are shown in Appendix 4. The q-value measures the rate of error or false discovery rate where the cut off was 1% and the global w-value measures how many times in the test the taxon (of interest) is differentially abundant when compared against the variable being tested. The higher the w-value, the more likely the difference is significant and the setpoint used in this experiment was >50. By taking the significant adjusted p value (<0.05) and a low false discovery or error rate (q<0.001) and a w value >50, we try to avoid results that are more likely to have occurred by chance. Detected Actinobacteria included taxa from the families *Eggerthellaceae*, *Bifidobacteriaceae* and *Coriobacteriaceae*. The most abundant phylum at genus level of fermented Cavendish (AAA) was *Bacteroidetes* where the *Bacteroides* genus represented >50% relative abundance which was significantly more than Misiluki (p <0.003). Actinobacteria which included was the most abundant phylum for both Misiluki and Tonga (AAB) at 61.3% and 52.8% respectively as shown in Table 13 and in appendix 4. Bonferroni testing was used due to the small sample size and number of replicates and indicated differences between banana digests regarding the two genera *Clostridium* and *Hungatella* (shown in Table 13), Dunn's post-hoc tests however, showed that these differences were not statistically significant.

Most of the differences in bacterial abundance post-fermentation were observed between Cavendish and the other banana cultivars as shown in Appendix 4. Notably, however, the Blue java fermentations contained significantly more *Veillonella* than Misiluki (p=0.023) and Tonga (p=0.007). There is evidence that the *Veillonella* genus played a role in propionate production, as the spearman's correlation indicated that there was a strong positive correlation between *Veillonella* and propionate content post-fermentation (r=0.6) as shown in Figure 12. The Cavendish cultivar produced significantly more propionate than Tonga (p=0.022) and Misiluki (p=0.020) as shown previously in Figure 8 (C) whereas Blue java fermentations produced a similar amount. Furthermore, Cavendish and Blue java were similar in *Veillonella* abundance where only differences were observed between Blue java vs Tonga and Misiluki. Also observed was a very strong negative correlation between *Veillonella* and lactate (r=-0.8). The genera *Bifidobacterium, Lactobacillus, Mediterraneibacter,* were more abundant in Tonga and Misiluki fermentations in comparison to Cavendish as shown in Table 13 and Appendix 4. These three genera had very strong positive correlations with butyrate production (r=0.8, r=1.0 and r=0.738 respectively) as shown in Figure 13. Notably, strong negative correlations were however shown between known butyrate producers including *Butyricicoccus, Anaerostipes, Blautia* and *Faecalibacterium* (r=-0.95, r=-0.77,r=-0.95 and r=-0.80 respectively). These genera in turn have strong positive correlations with propionate production (r=0.95, r=0.77, r=0.95 and r=1.00 respectively). Because propionate production is shown in Figure 13 to have a negative correlation with lactate, this could suggest that the lactate is being reduced to propionate via the acrylate pathway by these genera.

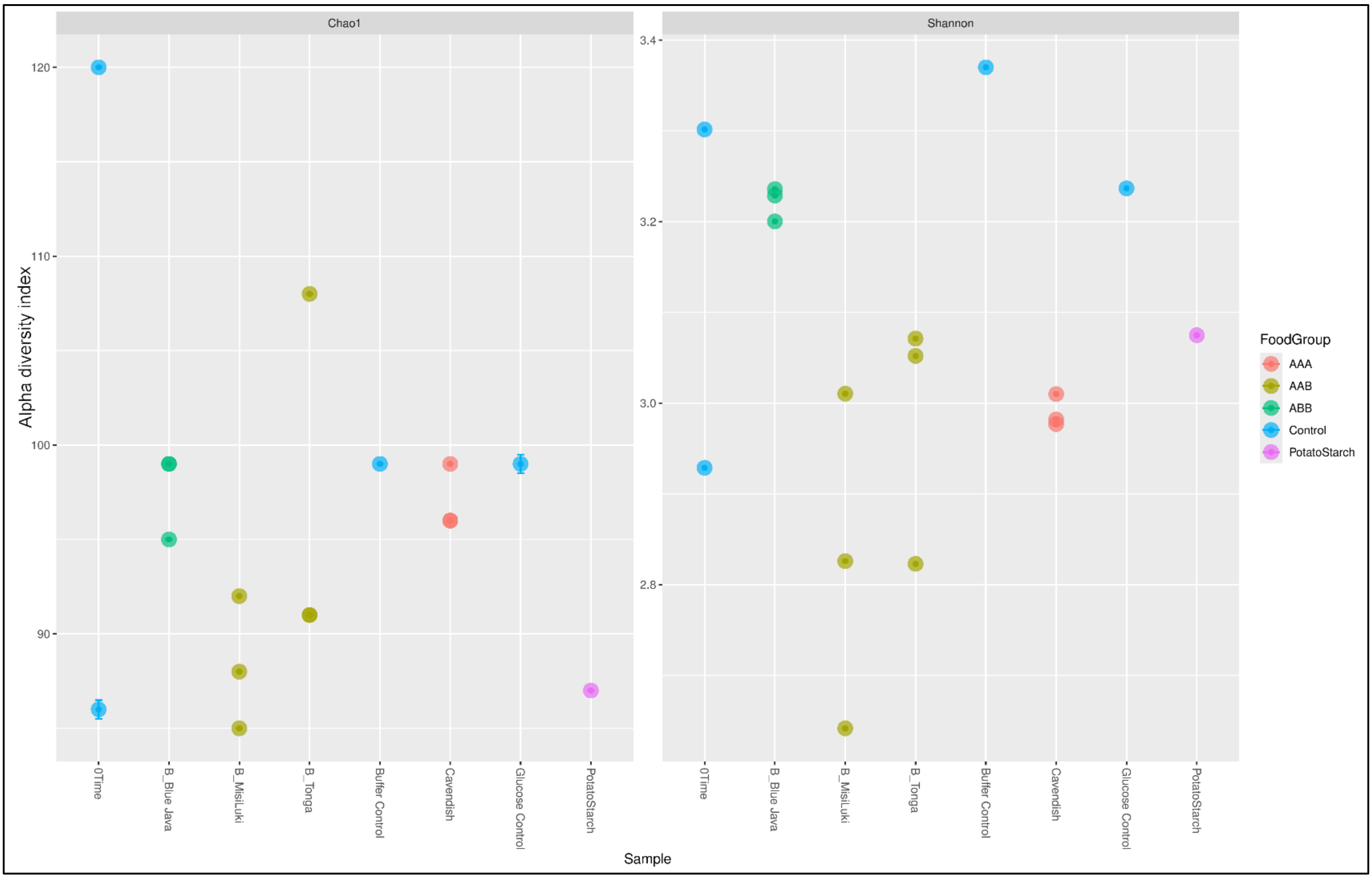

Figure 9. (Above) Alpha diversity of 24 hour fermented digests. (A) Chao1 species richness and (B) Shannon index diversity of species within the microbial community

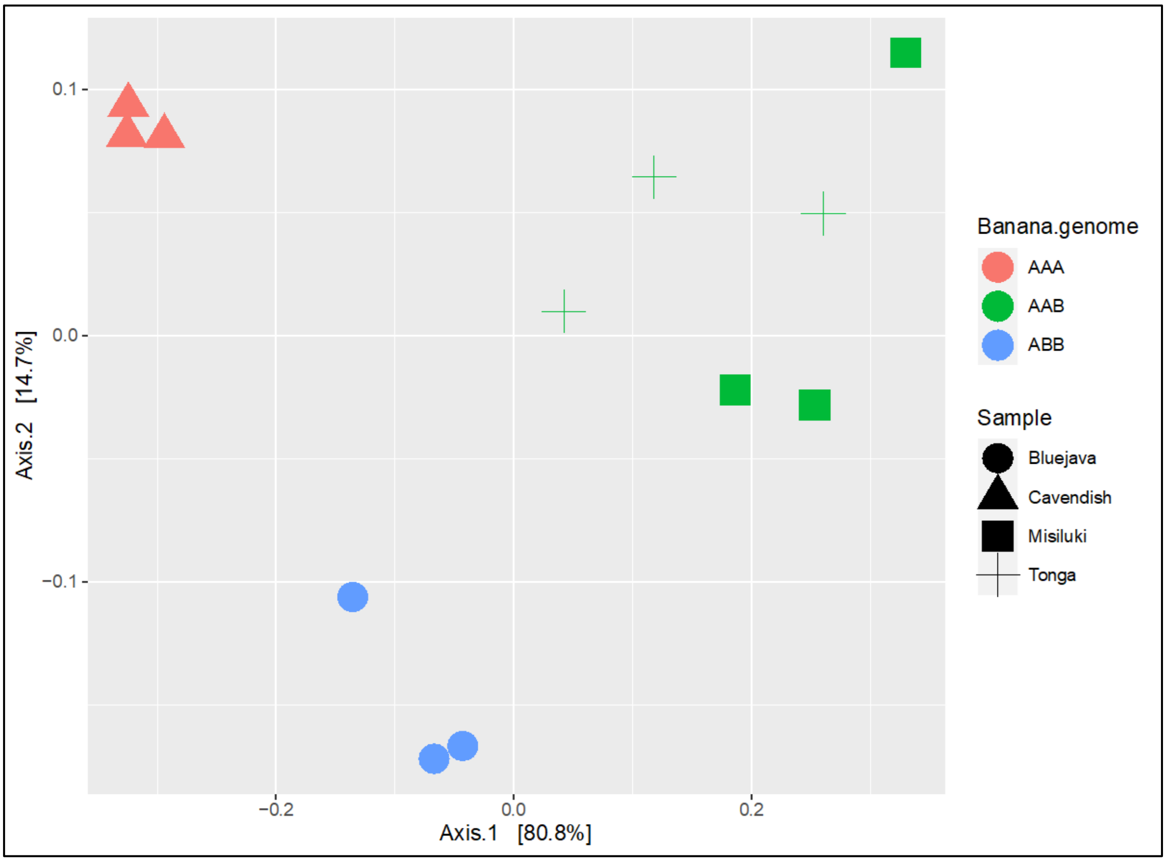

Figure 10. Principal Coordinates Analysis (PCoA) of beta diversity post fermentation, showing differences between banana genome groups.

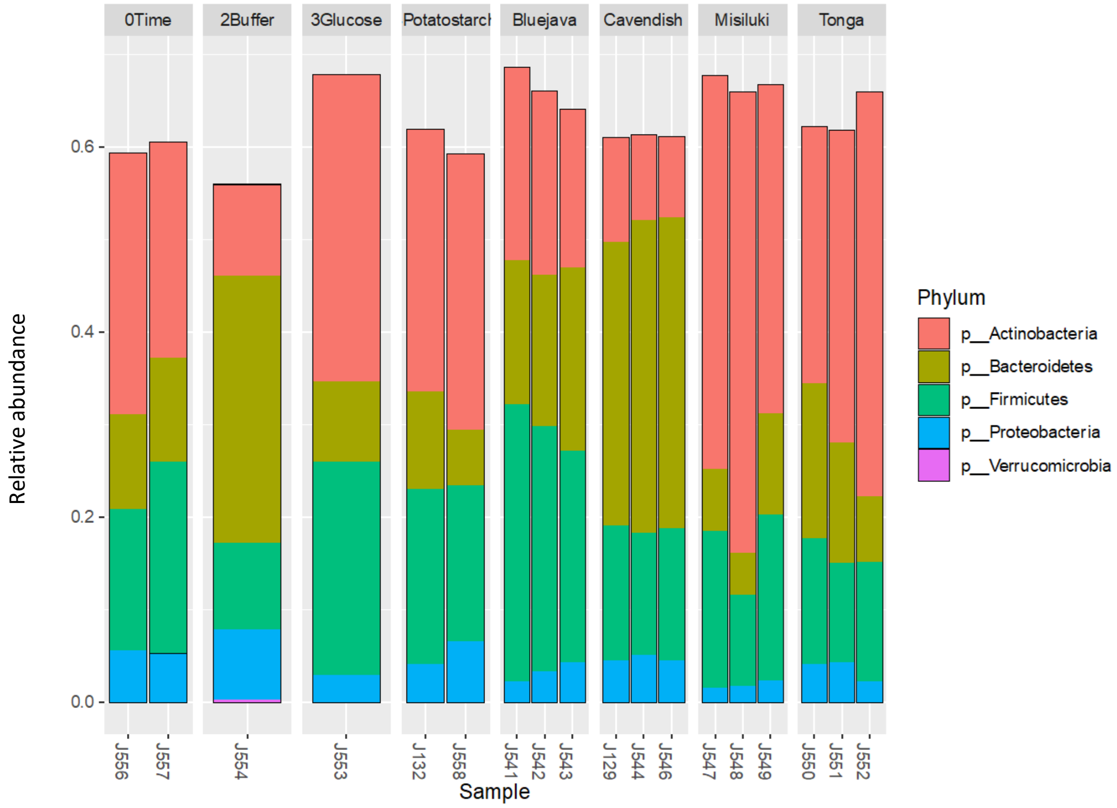

Figure 11. Stacked bar plot of identified phyla gives compositional abundances of bacterial phyla post fermentation by banana cultivar and includes controls (time 0, buffer, glucose and potato starch).

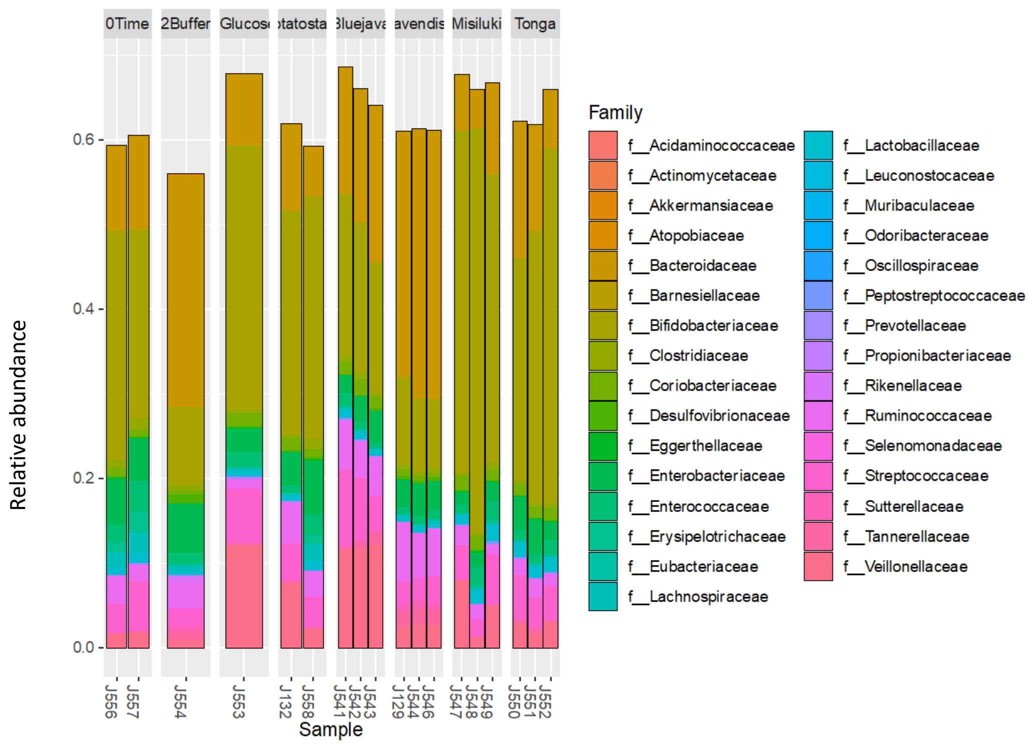

Figure 12.. Stacked bar plot of the top 31 taxa gives compositional abundances of bacterial families post-fermentation by banana cultivar and includes controls (time 0, buffer, glucose and potato starch).

Table 13. ANCOM-BC of differentially abundant bacterial taxa post-fermentation sorted by lowest p value

Phylum	Family	Genus	Cavendish	SEM	Misiluki	SEM	Tonga	SEM	Bluejava	SEM	global p	global q	global w
p__Actinobacteria	f__Bifidobacteriaceae	*g__Bifidobacterium*	14.83	1.11	61.26	6.24	52.83	6.18	26.34	1.11	<0.0001	<0.0001	61
p__Bacteroidetes	f__Bacteroidaceae	*g__Bacteroides*	50.53	1.51	10.90	2.76	18.89	4.50	24.88	2.07	<0.0001	<0.0001	525
	f__Tannerellaceae	*g__Parabacteroides*	3.03	0.07	0.21	0.05	0.66	0.24	1.21	0.41	<0.0001	<0.0001	530
p__Firmicutes	f__Clostridiaceae	*g__Butyricicoccus*	0.49	0.08	0.00	0.00	0.00	0.00	0.11	0.11	0	0	3619
		g__Clostridium	0.36	0.02	0.65	0.24	0.58	0.08	0.80	0.28	<0.0001	<0.0001	62
		g__Hungatella	0.10	0.00	0.08	0.04	0.04	0.04	0.00	0.00	0	0	11056
	f__Eubacteriaceae	*g__Eubacterium*	0.14	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0	0	12331
	f__Lactobacillaceae	*g__Lactobacillus*	0.66	0.02	2.34	0.36	2.49	0.15	1.16	0.13	<0.0001	<0.0001	187
	f__Lachnospiraceae	*g__Anaerostipes*	0.18	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0	0	17334
		g__Blautia	0.20	0.01	0.00	0.00	0.00	0.00	0.05	0.05	0	0	6270
		g__Dorea	0.31	0.02	0.00	0.00	0.10	0.05	0.41	0.07	0	0	1616
		g__Mediterraneibacter	0.00	0.00	0.14	0.03	0.12	0.00	0.00	0.00	0	0	10274
	f__Ruminococcaceae	*g__Faecalibacterium*	9.78	0.90	2.52	0.50	2.98	0.42	7.30	0.54	<0.0001	<0.0001	75
	f__Veillonellaceae	*g__Megasphaera*	0.24	0.01	4.48	2.51	1.45	0.38	7.88	2.03	<0.0001	<0.0001	961
		g__Veillonella	3.31	0.17	2.37	0.56	2.08	0.25	8.84	1.24	<0.0001	<0.0001	52
p__Proteobacteria	f__Desulfovibrionaceae	*g__Bilophila*	0.64	0.05	0.18	0.02	0.19	0.01	0.22	0.09	<0.0001	<0.0001	101
	f__Enterobacteriaceae	*g__Escherichia*	5.09	0.29	2.12	0.32	4.59	1.00	3.83	0.71	<0.0001	<0.0001	104
	f__Sutterellaceae	*g__Sutterella*	1.41	0.14	0.00	0.00	0.16	0.08	0.42	0.16	0	0	1947

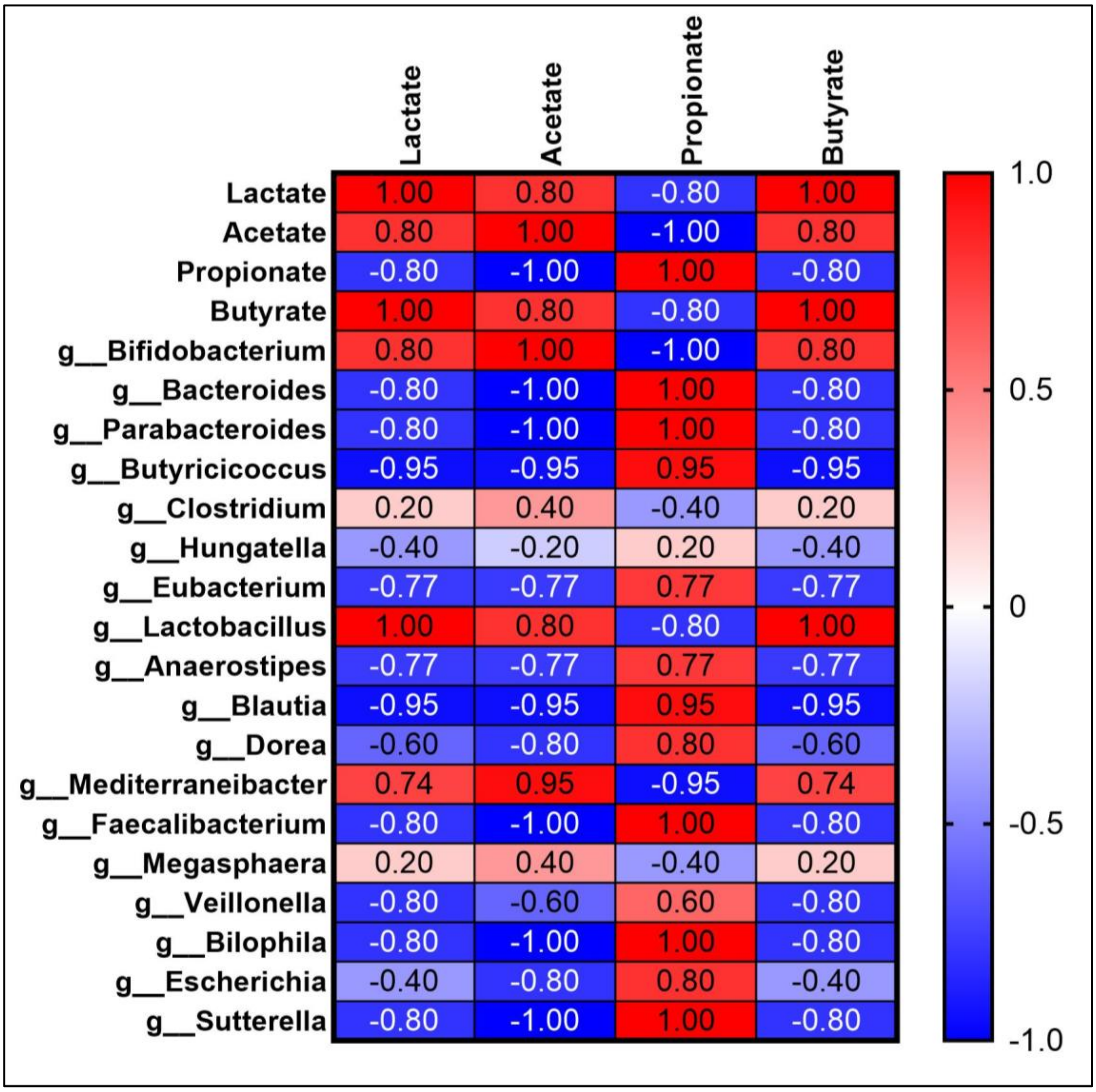

Figure 13. Spearman's correlation between bacterial genus and SCFA production

2.4.3.1. Microbial functional analysis of fermented banana

Several KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were significantly differentially expressed post fermentation between banana containing the *M. balbisiana* genome (ABB and AAB) and those that only contained copies of *M. acuminata* (AAA), as shown in Figure 14 and Appendix 5. KEGG 5240 is an ABC pathway L-cystine transport system permease protein; KEGG 5238 is an L-cystine transport system substrate-binding protein; KEGG 9836 is a major facilitator superfamily (MFS) transporter, DHA1 family, a putative efflux transporter; KEGG 11254 is a glycoside disaccharide hydrolase; KEGG 10081 is a metal transporter CBS-pair domain divalent metal transport mediator (CNNM), and KEGG 9790 is an MFS transporter, of the SP family of proteins and an inositol transporter.

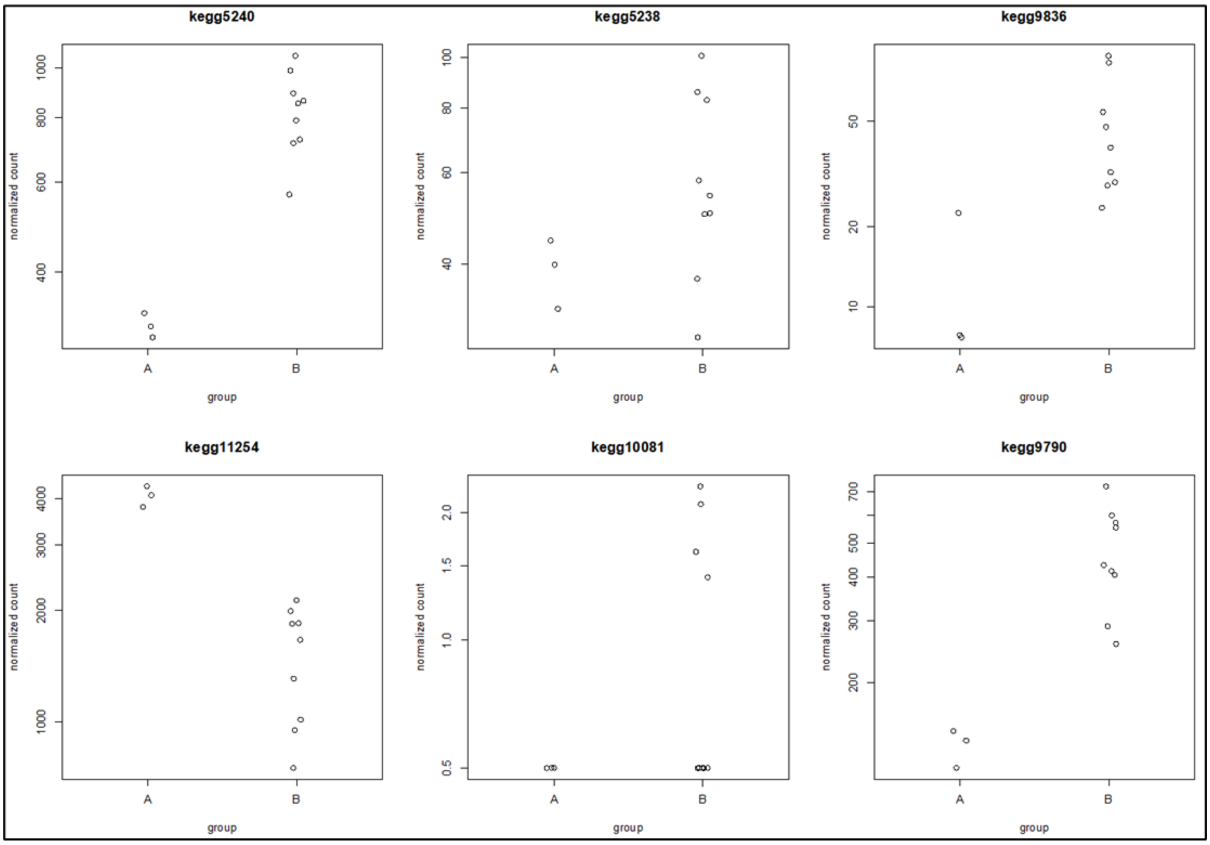

Figure 14. KEGG pathway genes normalised count differences between banana genome groups containing only M. acuminata copies (A) and those containing copies of M. balbisiana (B).

2.4.4. Unknown banana cultivar genome mapping

The banana cultivar principal component analysis (PCA) mapping the known and unknown banana cultivar to *M. acuminata* by their Single Nucleotide Polymorphisms (SNPs) profiles is shown in Figure 15. Genomic sequencing and mapping to SNPs determined that some banana cultivars identified by phenotype characteristics may have been mislabelled as other cultivars. For example, Blue java GM appears to be identical to the Mysore JU variety while Blue java_JM mapped closely to Pacific plantain. The two Red Dacca at the top left in blue were identical samples from the same plant to allow us to visualise the degree of noise in the SNP mapping.

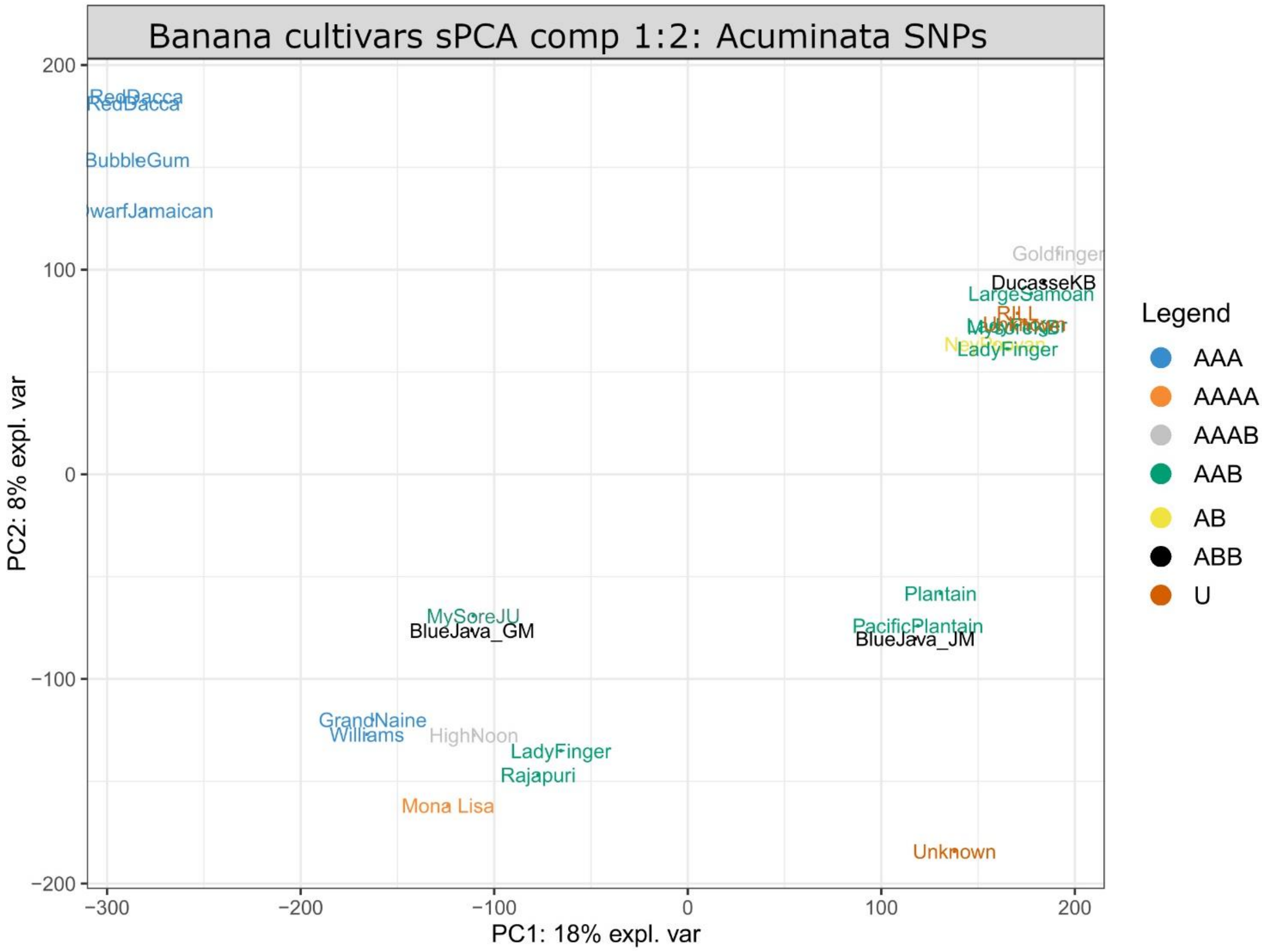

Figure 15. Banana cultivar PCA plot mapping banana cultivars by their SNP profiles aligned to the Acuminata (A) genome.

3. Discussion

3.1. Banana cultivar composition and nutrition

The results suggest that the addition of the *M. balbisiana* genome to banana varieties appears to increase the dry matter content of banana fruit pulp (Figure 5). The *M. acuminata* tetraploid (AAAA) Mona Lisa had the least dry matter content, with a mean of 19.8%, and the hybrid triploid genome group containing two *M. balbisiana genomes* (ABB) contained the most dry matter content, with a mean of 40.3% (Figure 5). Significant differences were observed between genome groups AAAA and both AAB ($p=0.001$) and ABB ($p=0.004$). Other significant differences were observed between genome groups AAA and both AAB ($p=0.013$) and ABB ($p=0.017$) as shown in Figure 5 (B). Within Figure 5 (B) genome groups containing more copies of the *M. acuminata* genome trend towards a reduced dry matter content, and those with increased *M. balbisiana* trend towards an increase in dry matter. Although the banana genome group ABB contained the greatest amount of dry matter it had the least amount of fresh pulp per fruit, with a mean of 37.5 g being significantly lower compared to the genome group AAA ($p=0.0495$) with a mean of 160 g. These factors need to be considered by the potential New Zealand banana industry, as although it is clear the Cavendish AAA variety has a greater weight per fresh fruit, these fruits contain more water, rather than nutrients (g/100g), in comparison to banana cultivars from genome groups AAB and ABB. When comparing dry matter results to overseas studies, this research had a similar mean pulp dry matter content of 27.8% to Aquino et al. [96], where they observed an average dry matter of 28.6% ,however, of their 15 cultivars, some were those from the genome group AA that was not included in this research. A 2009 study which observed four synthetic hybrids from the genome group AAAB were FHIA-18, FHIA-01, FHIA-21 and PV03-44 which had similar dry matter to the cultivar from the same genome group in this study (24%, 25%, 26% and 26% respectively) where Goldfinger had 26% and Monalisa had 24% [97].

The Massey Nutrition Laboratory data indicated that no banana genome groups used in this study were significantly different from US and New Zealand/Australia food databases when comparing magnesium and potassium content in fresh banana [62, 95]. However, the genome groups AAAA and ABB were significantly different (p=0.037) from each other, containing 27 g/100 g and 54 g/100 g of magnesium content respectively. The USDA and FSANZ database bananas belonged to genome groups AAA and AAB respectively and were similar to the banana cultivar within the same genome groups that were analysed in this study. There were no differences in total sugar content between genome groups. However, the only cultivar containing >1.0 g/100 g of sucrose was Cavendish with 4.5g/100 g, which was similar to the USDA database Cavendish and the FSANZ database ladyfinger with 4.2 g/100 g and 5 g/100 g respectively. We know that, as bananas ripen, the endogenous amylase hydrolyses starches into simple sugars as depicted in Figure 1 [64]. The endogenous enzymes convert to glucose and fructose. The bananas analysed in this study contained <1 g of sucrose but the total sugars were comparable with databases (USDA and FSANZ) suggesting they had already converted available sucrose to the monosaccharides. Regarding macronutrients, significant differences were observed between the genome groups AAAA and AAB in protein (p=0.0495), carbohydrates (p=0.0495) and energy content (p=0.0495). No significant differences were observed between cultivar fat content which aligns with previous research where similar genome groups have been grown outside of New Zealand [97].

The data supports our hypothesis that the significant differences in nutrition occur between banana cultivar genome groups, particularly between those cultivars with more copies of *M. acuminata* and those with less *M. acuminata* such as AAA and AAAA vs ABB. Further, the few observed similarities of New Zealand grown banana and the cultivar belonging to the same genome groups grown overseas suggest that perhaps the New Zealand growing conditions are not the driving factor of dry matter and nutritional composition, but instead the cultivar genome group. More research is needed to adequately compare New Zealand grown cultivar to those grown overseas. It should be noted that it is difficult to compare the nutritional composition of the banana cultivar within this study to others, as often in other studies banana are assessed at ripe or unripe stages of fruit maturity, whereas this study aimed to assess bananas at a more intermediate stage to obtain optimum RS content while catering to a degree of the sweetness that infants prefer.

3.2. Infant colonic microbiota

Genomic DNA sequencing indicated that after 24 hours of fermentation the bacterial community of the banana digests decreased in microbial diversity compared to time zero, as shown in Figure 9. This was to be expected in a static fermentation as the faecal microbiota present at the beginning of fermentation are not all viable and not all can grow in a static system even after incubation anaerobically with the digest substrate. Once initiated, bacterial populations also begin to shift by outcompeting in nutrient uptake and produce microbial byproducts including gases, SCFAs and subsequent reduction in pH that inhibit competing taxa [98]. Post fermentation, microbial alpha diversity of each banana cultivar faecal fermentation clustered together, indicating that the microbial diversity was comparable over time as shown in Figure 9. In this regard, the fermentations acted as expected, as between biological replicates the same nutrients of each cultivar were available for bacterial metabolism. Although four different banana cultivars were fermented, the post-fermentation microbial beta diversity between banana cultivars was grouped in three distinct clusters representing the three genome groups (AAA, AAB and ABB). The two banana cultivars that came together to form one of the three clusters were Tonga and Misluki, which belong to the same genome group (AAB). There were no statistically significant differences in bacterial taxa abundances observed between the cultivar fermentations Tonga and Misiluki. These two cultivars have similar nutritional compositions as indicated in 2.4.1 where the differences in nutrition are instead found between genome groups. Both Misiluki and Tonga fermentations were dominated by the Actinobacteria phylum (61.3% and 52.8% respectively).

The most abundant phylum of fermented Cavendish (AAA) was Bacteroidetes, where the *Bacteroides* genus represented >50% relative abundance. The majority of the differences in bacterial abundance post-fermentation were observed between Cavendish and the other banana cultivars as shown in Appendix 4. Notably, however, Blue java contained significantly more *Veillonella* genus than Misiluki (p=0.023) and Tonga (p=0.007). Spearman's correlation indicated that there is a strong positive correlation between *Veillonella* and propionate production (r=0.6). Furthermore, there was a very strong negative correlation with *Veillonella* and lactate (r=-0.8). Members of the *Veillonella* genus have a limited capability to convert glucose into pyruvate, instead the intermediate fermentation product, lactate, is utilised as a substrate resulting in propionate excretion [99]. Blue java and Cavendish have similar *Veillonella* abundances with a corresponding similarity in propionate production where lactate is more than 2-fold less than both Misiluki and Tonga. The Cavendish cultivar produced more propionate than Tonga (p=0.022) and Misiluki (p=0.020). Spearman's correlation showed that the genus most abundant in Cavendish fermentations (*Bacteroides)* was also strongly positively correlated (r=1.0) with propionate production. The genera *Bifidobacterium, Lactobacillus, Mediterraneibacter,* were more abundant in Tonga and Misiluki fermentations in comparison to Cavendish as shown in Table 13 and Appendix 4. These three genera had very strong positive correlations with butyrate production (r=0.8, r=1.0 and r=0.738 respectively). Known butyrate producers did not act as expected with regard to butyrate production, as strong negative correlations were shown in Figure 13 between butyrate production and *Butyricicoccus, Anaerostipes, Blautia* and *Faecalibacterium* (r=-0.95, r=-0.77, r=-0.95 and r=-0.80 respectively). It is possible that known butyrate producers are playing a role in reducing lactate to propionate via the acrylate pathway, and in turn have strong positive correlations with propionate production (r=0.95, r=0.77, r=0.95 and r=1.00 respectively). Further supporting this proposal is the negative correlation between propionate and lactate, suggesting that when lactate content has been decreased, it has been used as a substrate for propionate synthesis. The most abundant SCFA produced post-fermentation across all cultivars was acetate (M=281.03 mM/100g, SD=181.34) as shown in Figure 8. Previous research indicates that acetate is the dominating SCFA within the infant gut at this stage of life so our fermentations acted as expected and support this theory [36] The cultivar Monalisa (AAAA) fermentation produced significantly less acetate than Misiluki (p=0.003) and Blue java (p=0.005). Monalisa produced significantly less butyrate than Tonga (p=0.008) and Goldfinger (p=0.030). Blue java had an increased propionate production when compared to Monalisa (p=0.033). The differences in cultivar post-fermentation SCFA content and microbiota composition supports the hypothesis that the difference in cultivar nutrition between genome groups containing *M. balbisiana* would result in distinct profiles in an *in-vitro* digestion and fermentation system with a pooled infant faecal microbiota.

3.3. Banana cultivar genetics

The PCA shown in Figure 15, which was used to discriminate banana cultivars by their SNP profiles mapped to the Acuminata triploid genome, resulted in 5 distinct clusters. The two known Red Dacca samples (AAA) were harvested from the same plant and provided a control for sequencing noise but also helped to determine where AAA would map to. These two samples overlaid one another on the plot, indicating they are likely the same cultivar. Grand Naine and Williams are both Cavendish varieties or AAA. They did not map near the AAA but were close to others that were thought to be AAAB. We also saw that the two Blue java's (ABB), one which had been phenotypically identified (by fruit colour and texture and growth characteristics) (sample ID Blue java_JM), and the other which had not (sample ID Blue java_GM) did not cluster together, with one mapping more closely to AAAB. The two banana varieties suspected by growers to be plantain varieties (AAB) clustered near the phenotypically confirmed Blue java (ABB). The PCA plot indicates that some banana varieties labelled by growers (using phenotype characteristics) are not genetically closely related and may even be mislabelled. There is a banana variety, Saba (ABB), with similar characteristics to plantain due to its starchy texture, thick skin, and ability to maintain a firm structure after cooking. The Saba variety has a genome of ABB akin to that of Blue java. The phenotypic characteristics of the banana cultivar labelled Blue java, paired with the genomic mapping, suggest that these cultivars are not plantain (AAB) but from the Saba family (ABB) which are also known to grow in Northland areas. Two of the three unknown banana samples were able to be mapped to the AAB genome group while one unknown banana had a unique SNP profile (shown on bottom right of Figure 15). We could not determine the identity of this banana cultivar. To do so would involve collecting the phenotypic information about fruit including number of fingers per hand, shape and texture, stem growth, colour, flower structure and this might help guess the genome group involved. DNA of candidate varieties for which identities are known could also be used as reference varieties to compare against this unknown banana sample. All we can say at this point is that it is not a Cavendish nor an AAB variety that we have sequenced. Limiting factors in genomic identification include resolution of the data and lack of reference cultivars. It also should be noted that multi allelic genetics can make it more challenging to determine banana genome.

The KEGG database assists with the understanding of the functions within the cell. The sequencing approach we used was shotgun metagenomics, which allowed for KEGG analysis as well as identification of taxa due to its coverage of the 16s region. The KEGG analysis indicated that there were functional differences between banana cultivars containing *M. balbisiana* and those only comprised of *M. acuminata* as shown in Figure 14 and Appendix 5. Functional genes which differentiated between these two groups were genes which play roles in carbohydrate metabolism, outer membrane protein transport, disaccharide hydrolysis, membrane fusion protein and inositol transport. These differences in functional genes require further investigation as it is unknown what the implications of this may be, but perhaps the microbial communities are adapting to the nutrients, and this is seen through their metabolism. The differences observed may be related to the nutritional differences found as different pathways may have been activated depending on substrate availability.

3.4. Potential for the Māori community

This research was undertaken by Māori, to achieve aspirations within the Māori community. The findings of this research will be used by the Māori community as they see fit, their mātauranga can continue to evolve and remain protected.

This research shows the nutritional variation between banana cultivars grown here in Aotearoa. This nutritional variation causes distinct shifts in colonic microbial composition and metabolite production. Māori have firsthand experience and have developed an understanding of how the banana investigated within this study grow within New Zealand conditions. Māori horticulturalists can utilise the findings of this study combined with their continuously developing mātauranga; to contribute toward deciding which banana cultivar or combination of cultivars they can use to create a yet emerging commercial New Zealand banana industry. The banana genomic DNA mapping provided an insight into how some banana cultivars which horticulturalists have identified by phenotypic characteristics are not matched to their corresponding genomic profile, suggesting that some cultivars have been misidentified. This is to be expected as experienced New Zealand banana growers have found that some cultivars show different characteristics growing in our country conditions when compared to overseas and/or country of origin. This poses a risk to this new commercial industry, not only based on banana cultivar nutrition, but also banana cultivar disease resistance and optimum growing conditions. To manage these risks, before moving forward toward mass cultivation, banana cultivars should be appropriately identified through phenotypic characterisation and, if possible, by genomic sequencing. Growers should buy only varieties that are confirmed to be what their label says. This is difficult in an unregulated environment where some growers in their desire to sell plants, label them whatever they are being asked for. This means Misiluki, the most commonly grown NZ banana may be sold as a ladyfinger, Blue java or even plantain in the hands of unscrupulous sellers to obtain higher prices per plant.

3.5. Study strengths and limitations

This study investigated the nutritional and structural composition of eight different banana cultivars and how these components could impact the developing infant gut microbiota. Although seven of the eight banana cultivars were grown locally, there are still multiple varieties that are being grown successfully in the Northland and Gisborne districts that are yet to be better understood via scientific research. Some of these varieties include Ducasse, Lemon Lady Finger, Sweetheart, Hamoa, Ducasse and Plantain.

The bananas were frozen once they were before-ripe by comparing to a coloured scale. This is a qualitative assessment by the author. A quantitative analysis of banana ripeness such as dielectric spectroscopy, DA-meter or colorimeter would be suitable to ensure all cultivars are of similar ripeness [100, 101].

The banana cultivars used within this research were harvested from Northland nurseries across multiple seasons during 2022. It is unknown if seasonal weather changes in New Zealand impact banana nutrition. Harvesting all cultivars in the same season may be a better reflection of the differences in nutritional composition with implications for the gut microbiota and metabolites produced during fermentation. It would also be pertinent to compare banana nutritional compositions regionally (between Northland and Gisborne) and internationally as the soil and climate conditions differ, potentially reflected in distinct nutritional compositions within the same cultivar.

As shown in the banana cultivar genotype mapping results, it was revealed that some of the banana leaf samples supplied did not match the genomic classification to their identified cultivar. This poses a possibility that banana cultivar used for the *in-vitro* experiments may not be appropriately identified. We did obtain fruit from experienced knowledgeable growers, one of whom has had their bananas verified through DNA typing in a previous study called the Tai Pūkenga Banana Project (2018-2020). That said, harvesting banana fruit for experiments only from plants which have been previously confirmed by DNA sequencing would prevent any mismatching.

Misiluki and Tonga were two banana cultivars that were used in the *in-vitro* experiments and analyses thereafter, but banana leaf samples of these cultivar were not sent for genomic sequencing. Although DNA extractions of these cultivars were performed, the quality of DNA was poor due to degradation. The Misiluki cultivar is widespread throughout the Whangarei and Gisborne areas and was of less importance to the Māori community at this time, but it would be better to have included all of the varieties.

This research is an *in-vitro* based study. *In-vitro* based methods applied are useful for predicting potential outcomes *in-vivo* and pose a lower risk to infant health as they are less invasive. However, there are several limitations to mimicking the complexity that is the human gastrointestinal tract, including digestive enzyme supply. The Minekus et al. *in-vitro* digestion method used in this study is static, thus a dynamic digestion in which digestive enzymes are supplied consistently would more effectively predict outcomes similar to that *in-vivo*.

To simulate the infant colon fermenting digested food, infant stool was used. By recruiting infants once they had begun eating solid foods this research gained a greater understanding of how different banana cultivars impact the infant gut microbiota at this transitional stage of feeding. As ethnicity is a driving factor in differences in microbiota composition, it would be pertinent to recruit infants of Māori descent for stool donation to better reflect how these banana cultivars can impact the wellbeing of the Māori community [102, 103]. This study is also limited to assess the aerobic/ facultative aerobic bacterial population changes, since as soon as donor faecal samples are produced, the anaerobic bacterial taxa are subject to rapid population decline in the presence of oxygen once leaving the human body. As shown in Figure 8, some SCFAs were produced with large standard deviations between triplicate assays. To define experiment repeatability and possibly produce more consistent SCFA results the fermentation procedure needs to be repeated. The banana cultivar post fermentations that were sent for metagenomic sequencing were Blue java, Cavendish, Misiluki and Tonga. In future it would be insightful to have other cultivar fermentations sequenced including those from genome groups not assessed in this study such as cultivars with the genome AA .

4. . Concluding remarks

Several banana varieties have been growing successfully in New Zealand; however, the commercial market is limited to the imported Cavendish variety which is comprised of a triploid *M. acuminata* genome. Although we are growing several varieties here in New Zealand, this research gained a valuable insight regarding there identification, where some cultivars have been misidentified as we have shown that they do not match the reference cultivar. New Zealand grown banana varieties include a *M. acuminata* tetraploid and several combinations of both *M. acuminata* and *M. balbisiana*. This study indicates that nutritional differences occur between banana cultivar genome groups, particularly between those cultivars with more copies of *M. acuminata* and those with less *M. acuminata* such as AAA and AAAA vs ABB. Furthermore, between three banana genome groups (AAA, AAB and ABB) there are distinct microbiota fermentation profiles.

Before this study, there was no published literature available in the public domain regarding the nutritional composition of banana cultivars grown in New Zealand. This research is a forefront for a nutritional database for New Zealand grown bananas, which will be crucial for Māori banana horticulturalists in Te Tai Tokerau and Tairāwhiti to continue to develop their mātauranga and create a local commercial banana industry that can support whānau through business development and nutrition.

Appendix 1. Participant screening questionnaire

Riddet Institute
ADVANCING FRONTIERS IN FOOD SCIENCE

Riddet Institute (PN 445)
Massey University
Private Bag 11 222
Palmerston North 4442
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Infant Study:

Evaluating the digestion and fermentation of banana and kumara as complementary weaning foods by infant gut microbes.

SCREENING QUESTIONNAIRE

We ask that you complete this screening questionnaire so we can assess whether you and your baby are eligible to participate in this study. Any information you provide is confidential to the researchers.

In keeping with ethical protocols, please note that you have the right to:

- not answer any particular question
- ask for more information about these questions at any time

Please answer the following questions.

Today's date:	Click or tap here to enter text.
Your Name:	Click or tap here to enter text.
Your phone number:	Click or tap here to enter text.
Your email address:	Click or tap here to enter text.
Your home address:	Click or tap here to enter text.
Please indicate the best time and day to contact you on the phone:	Click or tap here to enter text.
Your baby's name:	Click or tap here to enter text.
Your baby's date of birth:	Click or tap here to enter text.
Your baby's current age:	Click or tap here to enter text.

Your baby's sex: Click or tap here to enter text.

Is your baby healthy today and been free of illness for the past week?	Yes☐	No☐
Was your baby born >32 weeks gestation? (i.e. full term)	Yes☐	No☐
Was your baby born (< 2500g or < 2.5kg) at birth? (i.e. small for gestational age)	Yes☐	No☐
Have you introduced any complementary food (i.e. solid food) to your baby yet?	Yes☐	No☐
Does your baby have any health conditions which affect feeding?	Yes☐	No☐
Does your baby have any digestive illnesses?	Yes ☐	No☐
Is your baby currently receiving or has received antibiotic treatment in the last 3 weeks?	Yes☐	No☐
Is your baby receiving a supplement with a pre- and/or probiotic?	Yes☐	No☐

Thank you for completing the questionnaire

A member of the research team will be in contact with you about your eligibility.

Appendix 2. Mean and standard deviation of SCFA's produced post-fermentation.

	mM/100g							
Cultivar	Lactate	SD	Acetate	SD	Propionate	SD	Butyrate	SD
Blue java	159.58	35.24	391.21	73.84	71.47	4.53	83.18	42.20
Cavendish	10.37	6.37	302.67	139.91	77.67	19.02	71.58	59.08
Misiluki	390.93	100.46	581.05	99.17	22.73	11.27	108.55	83.79
Tonga	401.26	98.96	425.13	379.94	25.71	13.76	176.58	24.00
Highnoon	36.71	15.90	174.96	151.26	41.52	34.85	20.52	1.60
Monalisa	64.88	13.69	77.52	66.04	43.20	11.01	20.03	1.31
Rajapuri	22.98	13.11	51.23	84.67	62.93	24.29	12.61	4.41
Goldfinger	88.18	22.45	244.47	103.04	49.21	7.91	48.51	9.10

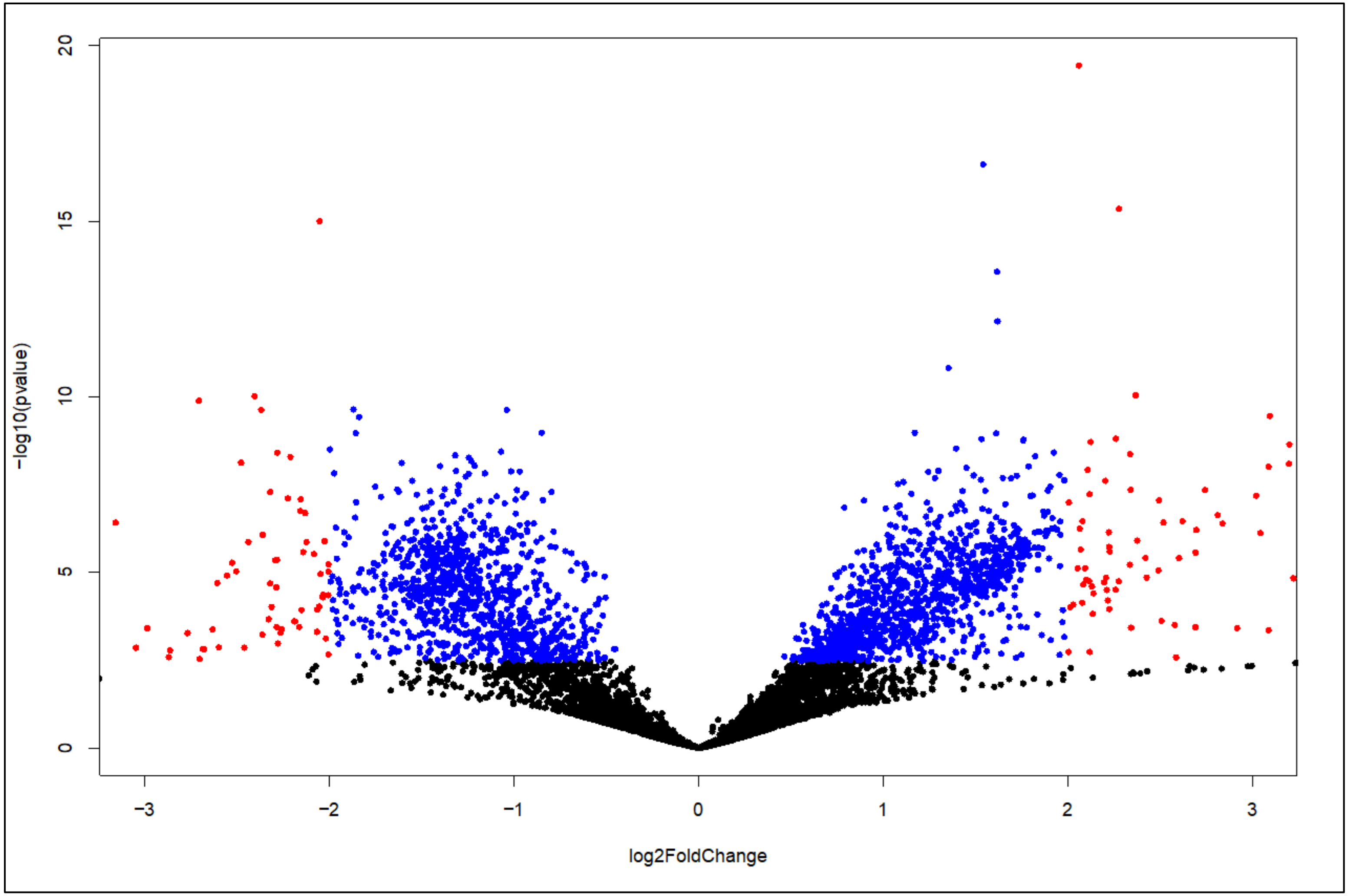
Appendix 3. Faecal fermentation DNA sequencing quality control

Sample	Raw Reads#	Raw Bases#	Clean Reads#	Clean Reads(%)	Clean Bases #	Low-quality Read#	Low-quality Rate(%)	Ns Reads#	Ns Read Rate(%)	Adapter Polluted Read#	Adapter Polluted Reads(%)	Raw Q30 Base(%)	Clean Q30 Base(%)	Raw GC(%)	Clean GC(%)
J129	29,941,628	4,491,244,200	29,643,004	99	4,446,450,600	47,754	0.16	4,292	0.01	246,578	0.82	94.16	94.22	49.26	49.24
J541	34,502,338	5,175,350,700	31,021,776	89.91	4,653,266,400	57,710	0.17	4,258	0.01	3,418,594	9.91	95.21	95.32	49.36	49.25
J542	30,640,174	4,596,026,100	29,647,026	96.76	4,447,053,900	49,882	0.16	3,186	0.01	940,080	3.07	94.86	94.94	49.72	49.69
J543	30,824,370	4,623,655,500	29,821,880	96.75	4,473,282,000	53,032	0.17	3,798	0.01	945,660	3.07	94.91	94.98	50.4	50.38
J544	32,529,640	4,879,446,000	30,605,864	94.09	4,590,879,600	48,536	0.15	5,324	0.02	1,869,916	5.75	94.59	94.68	47.99	47.88
J546	31,258,224	4,688,733,600	30,096,696	96.28	4,514,504,400	46,464	0.15	4,040	0.01	1,111,024	3.55	94.56	94.62	47.7	47.63
J547	32,620,456	4,893,068,400	31,241,528	95.77	4,686,229,200	90,516	0.28	4,708	0.01	1,283,704	3.94	94.54	94.62	55.43	55.43
J548	26,222,540	3,933,381,000	24,584,660	93.75	3,687,699,000	95,486	0.36	5,662	0.02	1,536,732	5.86	94.79	94.92	56.99	57.04
J549	33,100,434	4,965,065,100	30,641,070	92.57	4,596,160,500	72,618	0.22	5,776	0.02	2,380,970	7.19	94.13	94.24	52.77	52.72
J550	32,155,656	4,823,348,400	30,637,768	95.28	4,595,665,200	58,698	0.18	4,146	0.01	1,455,044	4.53	95.3	95.39	51.63	51.62
J551	31,444,738	4,716,710,700	28,964,446	92.11	4,344,666,900	64,532	0.21	4,106	0.01	2,411,654	7.67	95.35	95.47	53.53	53.53
J552	34,306,840	5,146,026,000	30,722,966	89.55	4,608,444,900	89,918	0.26	4,160	0.01	3,489,796	10.17	95.47	95.65	55.14	55.19
J553	31,006,386	4,650,957,900	29,360,652	94.69	4,404,097,800	55,632	0.18	3,614	0.01	1,586,488	5.12	95.27	95.35	52.68	52.68
J554	33,681,356	5,052,203,400	31,991,340	94.98	4,798,701,000	50,894	0.15	4,852	0.01	1,634,270	4.85	94.85	94.92	49.08	49.01
J556	32,320,742	4,848,111,300	31,167,000	96.43	4,675,050,000	87,686	0.27	5,108	0.02	1,060,948	3.28	93.76	93.82	52.88	52.89
J557	36,316,654	5,447,498,100	31,840,176	87.67	4,776,026,400	75,984	0.21	6,240	0.02	4,394,254	12.1	95.45	95.62	50.02	49.91
J558	31,906,728	4,786,009,200	29,633,566	92.88	4,445,034,900	87,590	0.27	4,720	0.01	2,180,852	6.84	95	95.16	53.5	53.53

Appendix 4. Post fermented banana cultivar differences in bacterial taxa

Phylum	Family	Genus	Uncorrected duns test	Significance	p value
p__Actinobacteria	f__Bifidobacteriaceae	*g__Bifidobacterium*	Cavendish vs. Misiluki Cavendish vs. Tonga	* **	0.017 0.007
p__Bacteroidetes	f__Bacteroidaceae	*g__Bacteroides*	Cavendish vs. Misiluki	**	0.003
	f__Tannerellaceae	*g__Parabacteroides*	Cavendish vs. Misiluki	**	0.005
p__Firmicutes	f__Clostridiaceae	*g__Butyricicoccus*	Cavendish vs. Blue Java Cavendish vs. Tonga Cavendish vs. Misiluki	* ** **	0.043 0.009 0.009
	f__Eubacteriaceae	*g__Eubacterium*	Cavendish vs. Blue Java Cavendish vs. Tonga Cavendish vs. Misiluki	** ** **	0.007 0.007 0.007
	f__Lachnospiraceae	*g__Anaerostipes*	Cavendish vs. Blue Java Cavendish vs. Tonga Cavendish vs. Misiluki	** ** **	0.007 0.007 0.007
		g__Blautia	Cavendish vs. Blue Java Cavendish vs. Tonga Cavendish vs. Misiluki	* ** **	0.043 0.009 0.009
		g__Dorea	Cavendish vs. Misiluki Blue Java vs. Misiluki	* **	0.025 0.009
		g__Mediterraneibacter	Cavendish vs. Tonga Cavendish vs. Misiluki Blue Java vs. Tonga Blue Java vs. Misiluki	* * * *	0.046 0.018 0.046 0.018
	f__Lactobacillaceae	*g__Lactobacillus*	Cavendish vs. Tonga Cavendish vs. Misiluki	** *	0.009 0.013
	f__Ruminococcaceae	*g__Faecalibacterium*	Cavendish vs. Tonga Cavendish vs. Misiluki	* **	0.017 0.007
	f__Veillonellaceae	*g__Megasphaera*	Cavendish vs. Blue Java	**	0.005
		g__Veillonella	Blue Java vs. Tonga Blue Java vs. Misiluki	** *	0.007 0.023
p__Proteobacteria	f__Desulfovibrionaceae	*g__Bilophila*	Cavendish vs. Blue Java Cavendish vs. Misiluki	* *	0.017 0.031
	f__Enterobacteriaceae	*g__Escherichia*	Cavendish vs. Misiluki	*	0.031
	f__Sutterellaceae	*g__Sutterella*	Cavendish vs. Tonga Cavendish vs. Misiluki	* **	0.044 0.003

Appendix 5. KEGG volcano plot



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