Isolation and characterisation of *Faecalibacterium prausnitzii* in New Zealand calves

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Abstract

Faecalibacterium is an oxygen-sensitive bacterium found in the intestines of many animals and is of considerable interest due to the benefits it contributes to gastrointestinal tract (GIT) health and homeostasis. *Faecalibacterium* produces the short-chain fatty acid, butyrate, which is an important energy source for gut epithelial cells and acts as an anti-inflammatory agent in the GIT. In New Zealand, artificially reared calves that are raised in the absence of their dams are highly susceptible to gastrointestinal diseases which can result in morbidity and mortality. Understanding the roles of beneficial GIT microorganisms, and how these may enhance GIT health in calves, is highly desirable to improve calf health, growth and development. This project aimed to isolate and characterise *Faecalibacterium* strains from whole milk-fed New Zealand calves. Cultivation trials from calf faecal samples were conducted on *Faecalibacterium* enriching agar media in Petri plates or roll tubes and more than 300 colonies were screened to identify *Faecalibacterium*. A polymerase chain reaction (PCR) screen selective for *Faecalibacterium* was developed using a combination of universal and *Faecalibacterium*-specific 16S rRNA gene primers, and only one isolate was identified as *Faecalibacterium via* 16S rRNA gene sequencing. However, other closely related members of the *Ruminococcaceae* have also been isolated: two *Fournierella massiliensis* isolates and one member of *Gemmiger*. Genome sequencing and analyses showed that in both the *Faecalibacterium* and *Gemmiger* isolates, genes in the pathway from pyruvate to butyrate were present, consistent with their ability to produce butyrate. The *Fournierella* isolates were not observed to produce butyrate, and instead, ethanol was a major fermentation end-product. The genome of the *Faecalibacterium* isolate had an average nucleotide identity (ANI) of 96.05% compared to that of the human-derived *F. prausnitzii* strain A2-165, and an ANI of 85.01% compared to the *F. prausnitzii* type strain 27768^{T}, suggesting genomic differences between calf and human-derived isolates. This study will provide a better understanding of microbial diversity and function within New Zealand calves, and the bacteria isolated in this study may serve as beneficial microbes to help reduce scours or other gastrointestinal diseases.

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Abbreviations

ACK	acetate kinase
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ANI	average nucleotide identity
AOR	aldehyde:ferredoxin oxidoreductase
Bcd	butyryl-CoA dehydrogenase
BHI	brain heart infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
Buk	butyrate kinase
But	butyryl-CoA:acetate CoA transferase
CFU	colony forming unit
CoA	coenzyme A
COG	Clusters of Orthologous Groups of genes
CRISPR	clustered regularly interspaced short palindromic repeats
Cro	crotonase
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
g	gram
GC	guanine and cytosine
GIT	gastrointestinal tract

GTDB	Genome Taxonomy Database
Hbd	β-hydroxybutyryl-CoA dehydrogenase
HKY	Hasegawa, Kishino and Yano
IMG/M	Integrated Microbial Genomes & Microbiomes
kb	kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	litre
LAB	lactic acid-producing bacteria
(L)YBHI	brain heart infusion medium supplemented with 0.5% yeast extract
M	molar
M2GSC	modified Med2 of Hobson
Mb	megabase
µg	microgram
µL	microlitre
µm	micrometre
mm	millimetre
mM	millimolar
mL	millilitre
mRNA	messenger ribonucleic acid
N/A	not applicable
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor κB
ng	nanogram
nm	nanometre
nr/nt	non-redundant nucleotide
NZD	New Zealand dollar

OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDH	phenylacetaldehyde dehydrogenase
pmol	picomole
POR	pyruvate:ferredoxin oxidoreductase
PTA	phosphotransacetylase
Ptb	phosphate butyryltransferase
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SCFA	short-chain fatty acid
TAE	tris base, acetic acid and ethylenediaminetetraacetic acid
TE	tris-EDTA
Thl	thiolase
Tm	melting temperature
tRNA	transfer ribonucleic acid
V	volt
v/v	volume/volume
VTR2RF	VersaTREK™ REDOX™ 2 Media enriched with rumen fluid
w/v	weight/volume
× g	times gravity
YCFAG	yeast extract, casitone, fatty acid and glucose

Chapter One. Introduction

Faecalibacterium prausnitzii is a highly oxygen-sensitive, Gram-negative-staining, non-spore-forming rod-shaped bacterium found in the intestines of multiple animal species, including humans (Suau et al., 2001), mice (Nava & Stappenbeck, 2011) and bovines (Uyeno et al., 2010). In humans, *Faecalibacterium* has a lower prevalence in patients with inflammatory bowel disease (Sokol et al., 2008) and in obese as compared to lean individuals (Remely et al., 2014). *Faecalibacterium* has been identified as a next-generation probiotic in humans, as it produces butyrate, an important energy source for enterocytes (Bergman, 1990) and confers anti-inflammatory properties to the gastrointestinal tract (GIT) (Plöger et al., 2012), contributing to overall intestinal health and the maintenance of GIT homeostasis.

Artificially reared calves are at risk of gastrointestinal diseases, notably scours (diarrhoea)-related mortality, which commonly occurs in New Zealand and can be caused by bacteria, viruses and other pathogens (DairyNZ, 2022a). Currently, there is very little information on bovine strains of *F. prausnitzii*, particularly in New Zealand, and its effect in preventing or reducing the harmful effects of calves' scours, *via* butyrate production. However, phylogenetic analyses have suggested that the diversity of *Faecalibacterium* is very broad, and there are many *Faecalibacterium* species, and *F. prausnitzii* sub-groups, yet to be identified in culture (Fitzgerald et al., 2018). This diversity can be explored to identify new strains of *F. prausnitzii* or other closely related species that could be used as beneficial microorganisms (Foditsch et al., 2015) that support the health of artificially reared New Zealand calves.

1.1 The New Zealand agricultural industry

1.1.1 Calf rearing in New Zealand

In 2021, the New Zealand dairy industry earned approximately 19 billion NZD per annum and is currently forecasted to earn approximately 21 billion NZD in 2022 (Ministry for Primary Industries, 2021). About 5 million dairy cows are reared artificially each year (Livestock Improvement Corporation & DairyNZ, 2021), thus it is critical to maintain the health and productivity of calves under these New Zealand rearing conditions. KiwiCross (Friesian-Holsteins × Jersey) cattle made up ~50% of the dairy cattle in New Zealand from 2019 to 2020. Holstein-Friesian cattle made up approximately one-third of the New Zealand herd, while both Jersey cattle and Angus cattle (amongst others) were ~10%, and Ayrshires at 0.4% (Livestock Improvement Corporation & DairyNZ, 2021). Artificial rearing of calves is a common practice worldwide and involves separation of the calf from its mother soon after birth, often within 24 hours, and calves are reared together in facilities using whole milk or milk replacers with access to various solid feeds. This method of calf rearing is popular for many reasons, but primarily as artificial rearing promotes faster rumen development (Cantor et al., 2019).

The rumen is the fermentative forestomach of ruminant animals where rumen microorganisms ferment ingested solid feed and the short-chain fatty acids (SCFA) generated from fermentation can provide up to 70% of the mature ruminant’s daily energy requirements (Van Soest, 1994). However, at birth, the rumen is underdeveloped and non-functional, thus it needs to develop before the calf can be fully weaned (Warner et al., 1956). Feeding strategies that encourage the calf to eat more solid feed sooner promote faster rumen development and earlier weaning. Weaning calves earlier on to pasture diets is more economic for farmers as it reduces the cost associated with milk or milk replacer feeding (Khan et al., 2016). Artificial rearing also enables farmers to better manipulate the quality and quantity of colostrum that the calf receives after birth and, therefore, it is possible to increase the amount of immunoglobulins that the calf receives through the colostrum, which promotes stronger immune systems and better health (Weaver et al., 2000).

1.1.2 Calf scours and treatment

Scours is deadly and common amongst preweaned calves in New Zealand. It can be caused by various factors including viruses and bacteria (Smith, 2019) and lack of colostrum intake within the first 1-2 days following birth (Beef + Lamb New Zealand, 2017). Symptoms include diarrhoea and fatigue and can result in death from dehydration within a day (Al-Alo et al., 2018). A study conducted in 2014 in the United States of America found that 56.4% of pre-weaned heifer deaths were caused by diarrhoea (United States Department of Agriculture, 2014).

In New Zealand, dams may be vaccinated before birth to enable antibodies present in colostrum to be passed on to the calf. However, upon presentation of calf scours, it is treated by isolation of the animal, followed by feeding the affected animal oral electrolytes with milk (DairyNZ, 2022a), and may require treatment by antibiotics (South Waikato Veterinary Services, 2020). In 2019, in New Zealand, dairy and beef cattle accounted for 35.2% of the use of veterinary antibiotics (Agricultural Compounds and Veterinary Medicines Team, 2021). However, the New Zealand Ministry of Health and Ministry of Primary Industries recently published a five-year action aiming to reduce the use of antibiotics in New Zealand livestock by preventing the spread of harmful microorganisms (Ministry of Health & Ministry for Primary Industries, 2017). These measures have been taken to reduce the proliferation of antibiotic-resistant bacteria, which are already prevalent in very high numbers around the world, such as *Shigella* and *Neisseria gonorrhoeae* (World Health Organization, 2014). Thus, the use of beneficial microbes to promote GIT health, including probiotics, is of interest for the treatment of calf scours and overall calf health.

1.1.3 Gastrointestinal tract development and microbiota establishment in calves

Unborn calves are thought to have sterile GITs that are quickly colonised by microorganisms from their mothers and the environment during and following birth (Alexander & Lysons, 1971; Klein-Jöbstl et al., 2019; Soares et al., 1970; Tavernor et al., 1971). The GIT supports the growth of a variety of microorganisms, with the rumen and colon hosting the highest densities of bacteria, at approximately 10^{12} cells per gram of content (Yeoman & White, 2014). The bacterial community profiles of calf GIT microbiota change as they age and develop into adulthood (Jami et al., 2013). Diversity measurements from faecal samples show that the phyla Proteobacteria and Firmicutes were the most dominant in Holstein-Friesian calves, fed colostrum 2-hours after birth and thereafter kept on a milk diet, up to a day from birth, while in adult cattle, Bacteroidetes and Firmicutes dominated (Klein-Jöbstl et al., 2019). The rumen of calves is underdeveloped when they are born, and milk bypasses the rumen *via* the oesophageal groove, therefore the abomasum and intestine serve as the main sites of milk digestion until solid foods are introduced, then the rumen begins to develop (Drackley, 2008). For this reason, bacteria in the hindgut play an important role in the nutrition and health of the calf during the early stages of its life.

When a calf is raised with its mother, it receives important gastrointestinal commensal and protective microorganisms from her that help promote both rumen and hindgut colonisation and development. However, artificially reared calves are separated from their mother soon after birth, and so are unable to obtain these important anaerobes in the same way (Khan et al., 2016). Microbiota establishment is therefore highly influenced by colonisers from the environment, and there is an increased risk of pathogen colonisation. This highlights that the addition (direct-fed microbials) or enrichment of beneficial microorganisms through prebiotics, during microbiota establishment in artificially reared calves, could be a new preventative and therapeutic tool to protect them from pathogens including those that cause scours (Khan et al., 2019).

1.2 Beneficial microorganisms

The GIT of both monogastric and ruminant animals contains one of the most complex microbiotas, harbouring bacteria, archaea, fungi and protozoa in high abundance (Bull & Plummer, 2014; McCann et al., 2014). These GIT microorganisms have important roles in different areas of the GIT, such as fermenting host non-digestible substrates like dietary fibres in both the rumen and hindgut (Firkins & Yu, 2015), as the host does not produce the digestive enzymes required for this, and additionally educating the immune system in the hindgut (Belkaid & Hand, 2013). Fermentation results in energy substrates that are used by the host for energy and nutrition. The interactions between the microorganisms and the host range from mutualistic to predatory or pathogenic (Pickard et al., 2017). For healthy individuals, the GIT microbiota are mutualistic symbionts that also influence the immune system, host metabolism and GIT health and function.

1.2.1 Gut homeostasis and probiotics

Dysbiosis is referred to as an imbalance in the diversity and abundance of the microorganisms in the microbiome and can be caused by external factors (e.g. diet, antibiotics, disease) (Pickard et al., 2017). It has been linked to many intestinal disorders, such as the inflammatory bowel diseases Crohn’s disease (Seyedian et al., 2019) in humans and Johne’s disease (DairyNZ, 2022b) in cattle, and scours in calves. Dysbiosis can be associated with a range of other diseases in cattle, including bovine respiratory disease (Zeineldin et al., 2019), which can be caused by viruses and bacteria (Hoque et al., 2019). During disbalance, or as a preventive measure, the addition and/or enrichment of beneficial microorganisms should be considered.

Dysbiosis is associated with decreased abundances of beneficial microorganisms and overgrowth of microorganisms that can produce waste products that can negatively impact the host or beneficial microbiota. In comparisons of the GIT microbiota of healthy calves and diarrheic calves, bacteria such as *Faecalibacterium*, which produces butyrate, and *Bifidobacterium*, which may promote butyrogenesis when cross feeding with butyrate-producers such as *Faecalibacterium*, have been associated with decreased diarrhoea (Gomez et al., 2017; Oikonomou et al., 2013; Rivière et al., 2016). Restoration of GIT homeostasis can be achieved by promoting the growth of, or administering, beneficial microbes. Beneficial microorganisms can be exogenous (not originating from or persisting in the host GIT) or endogenous (originating from or persisting in the host GIT) and include probiotics, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agricultural Organization of the United Nations and World Health Organization, 2001). Traditionally probiotics include lactic acid-producing bacteria (LAB) such as *Lactobacillus* and *Bifidobacterium*, which are exogenous and are also found in many fermented foods such as yoghurt (Aryana & Olson, 2017) and miso paste (Murooka & Yamshita, 2008). In addition to lactic acid production, which causes a decrease in pH to levels that are unfavourable to the majority of microorganisms, many LAB synthesise and excrete antimicrobial compounds (Alakomi et al., 2000). Next-generation probiotics, however, is a term given to endogenous GIT-derived microorganisms that have positive effects on host health and may have potential as live biotherapeutics (O'Toole et al., 2017).

1.2.2 Mechanisms beneficial for host health

Microorganisms can be beneficial to host health through a variety of mechanisms, including immune-modulation (e.g. blocking the activation of nuclear factor κB (NF-κB) that regulates pro-inflammatory responses), maintenance of the epithelial mucous layer in the GIT, competitive adhesion to exclude pathogens, and the production of bacteriocins. The NF-κB family of transcription factors regulate cellular responses including innate and adaptive immune responses (Rahman & McFadden, 2011). NF-κB signalling can be activated by a range of stimuli, such as cytokines, and bacterial and viral antigens, and may proceed through “classical” or “alternative” pathways. Mutations in the receptors at the beginning of the NF-κB pathways can lead to transcription of pro-inflammatory genes, or mutations in these genes themselves cause persisting gene activation, resulting in inflammatory bowel diseases (Liu et al., 2017). The inflammation caused by these mutations may be reduced by beneficial bacteria, however. Recently, a microbial anti-inflammatory molecule (MAM) was discovered and appeared to inhibit activation of NF-κB when a MAM-encoded plasmid was inserted into *Lactobacillus lactis* and then fed to colitic mice (Quévrain et al., 2016).

Goblet cells are the epithelial cells of the intestine that produce the protective mucous layer that acts as the host’s first line of defence against microorganisms present in the GIT (Pelaseyed et al., 2014). Maintaining the integrity of this mucous layer is essential for maintaining host health and various beneficial bacteria, including *Lactobacillus reuteri*, *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii*, have been shown to improve host immunity *via* effects on goblet cells and/or mucin formation in rats (Wrzosek et al., 2013) and chickens (Xie et al., 2019).

Beneficial microorganisms may efficiently bind to the host mucosa resulting in the inability of pathogen binding, preventing disease to ensure. This is referred to as competitive adhesion. *Lactobacillus rhamnosus* strain GG expresses SpaCBA pili on its cell walls that enable it to adhere to the host’s intestinal mucosa and outcompete the multi-drug resistant pathogen *Enterococcus faecium* (Miller et al., 2014) that has highly similar pili (Tytgat et al., 2016). Competitive adhesion is also seen between *Bifidobacterium pseudolongum* PV8-2 and *Bifidobacterium kashiwanohense* PV20-2 against *Salmonella* Typhimurium N15, which is also a main salmonellosis-causing pathogen in cattle (DairyNZ, 2022c), and *Escherichia coli* O157:H45 (Vazquez-Gutierrez et al., 2016).

The production of bacteriocins, ribosomally-synthesised proteins that are antibiotic, are another mechanism used by potentially beneficial microorganisms. For example, *Escherichia coli* strain H22 produces the antimicrobials microcin C7, and colicins Ib and E1, which inhibited the growth of several pathogenic bacteria, including *Shigella*, *Yersinia* and *Salmonella*, and in a gnotobiotic mouse model was able to also inhibit the growth of *Shigella flexneri* 4 (Cursino et al., 2006).

1.2.3 Prebiotics

Prebiotics are substrates that are selectively utilised by host microorganisms conferring a health benefit (Gibson et al., 2017), and are typically carbohydrates indigestible by the host but fermentable by the GIT microorganisms. Consequentially, prebiotics can act by increasing the effect of probiotic organisms and aid in preventing colonisation of the GIT by pathogens (Gibson et al., 2005). Overall, a variety of mechanisms can be used by potentially beneficial bacteria to give them potential as probiotic organisms. *Faecalibacterium* is a beneficial microbe that has been of interest as a next-generation probiotic, mainly due to its mechanisms of conferring health benefits, such as the production of the anti-inflammatory SCFA butyrate.

1.3 *Faecalibacterium*

Faecalibacterium is an obligate anaerobe that was first isolated by Carl Prausnitz in 1922 from human pleural cavity pus (Prausnitz, 1922). This bacterium has since been found in many animals, including humans (Suau et al., 2001), bovines (Uyeno et al., 2010) and mice (Nava & Stappenbeck, 2011). Species *Faecalibacterium prausnitzii* underwent several name changes: originally named *Bacillus mucosus anaerobius* (Prausnitz, 1922), it was renamed *Fusobacterium* *prausnitzii* (Moore & Holdeman, 1973) and then *Faecalibacterium prausnitzii* after rDNA analysis (Duncan, 2002).

The original strain isolated by Prausnitz no longer exists, however, *F. prausnitzii* was isolated from human faeces in 1974 (Cato et al., 1974). This *F. prausnitzii* strain was designated as a type strain (ATCC 27768 = VPI C13-51 = NCIMB 13872) and described as a gram-negative, slender, non-motile, non-spore-forming, round-ended rod-shaped bacterium that was 0.5–0.9 µm × 2.4–14 µm and could appear as chains. A thick capsule was visible with fuchsin staining and grew optimally at 37°C. In *F. prausnitzii* strain HTF-F, a biofilm forms in liquid YCFAG (yeast extract, casitone, fatty acid and glucose) medium (Rossi, Khan, et al., 2015). On rumen fluid-supplemented carbohydrate media, cells reached 35 µm in length, colonies were transparent to semi-transparent, circular and convex; on blood agar colonies were white to colourless. Under electron microscopy, cell wall protrusions described as "swellings" were visible in some, but not all, strains when grown on (L)YBHI (brain heart infusion medium supplemented with 0.5% yeast extract) (Martín et al., 2017; Miquel et al., 2013), or M2GSC (modified Med2 of Hobson) (Hu et al., 2022). The genome size of *Faecalibacterium* species was later determined to be approximately 2.68–3.32 Mb (Fitzgerald et al., 2018; Zou et al., 2021) with a GC% of 52–57% (Cato et al., 1974), but more recently Zou et al. (2021) described a wider range of 47–63%.

Recently, *F. prausnitzii* A2-165 (DSM 17677 = JCM 31915) has been identified as a potential next-generation probiotic in humans due to its ability to confer anti-inflammatory effects in the GIT (**Figure 1-1**) (Miquel et al., 2015), and the ability of *F. prausnitzii* HTF-F to modulate the immune system. Immune modulation occurs *via* TLR2 (toll-like receptor 2), which induces the production of the anti-inflammatory cytokine IL-10 (interleukin 10) and white blood cell-regulating IL-2 (Rossi, van Berkel, et al., 2015). A beneficial effect on the GIT health and overall immunity of the host by *Faecalibacterium* spp.-produced SCFA butyrate was also reported in several studies (Bergman, 1990; Geirnaert et al., 2017; Wang et al., 2020). Miquel et al. (2015) showed that sodium butyrate reduced IL-8 production in TNF-α-stimulated HT-29 cells (human colorectal adenocarcinoma cells) and the levels of 4-hydroxybutyric acid were increased in mice harbouring both *F. prausnitzii* and *Escherichia coli* (in comparison to mice harbouring only *E. coli*). Butyrate is also thought to inhibit NF-κB, thus reducing inflammation by reducing the mRNA expression of tumour necrosis factors and preventing the NF-κB complex from entering the nucleus to begin transcription (Segain et al., 2000).

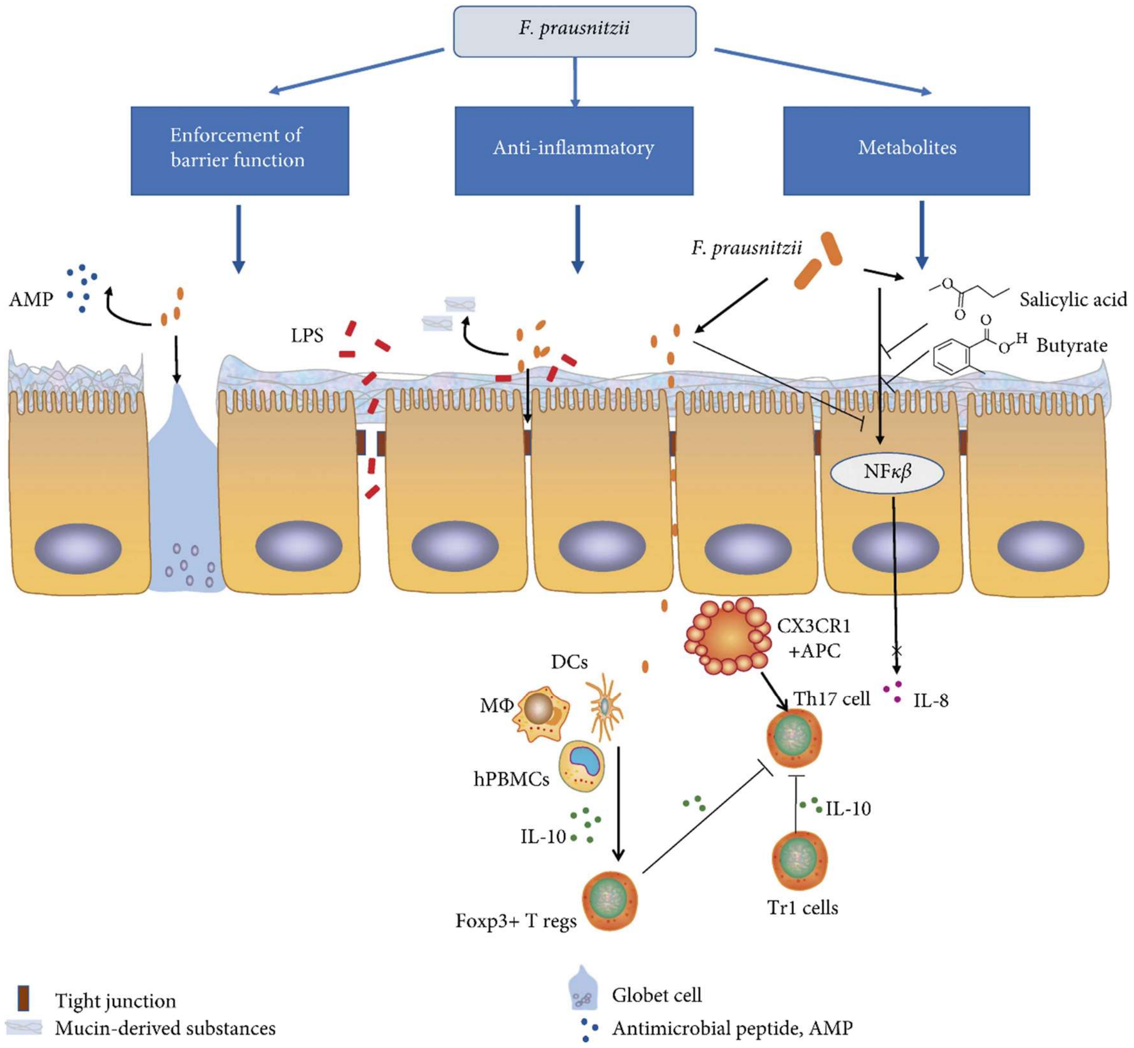


Figure 1-1. GIT barrier enhancement and anti-inflammatory mechanisms mediated by *F. prausnitzii*. Image obtained with permission from He et al. (2021).

1.3.1 *Faecalibacterium* butyrate production

Faecalibacterium spp. produce butyrate, an SCFA that is an important energy source for enterocytes (Bergman, 1990) which may enhance the GIT barrier integrity and contribute to its anti-inflammatory properties. Bacterial fermentation of dietary fibres produces butyrate through one of the two metabolic pathways (**Figure 3-15**). Both metabolic pathways start with acetyl-CoA but the end of butyrate production differs. In the first pathway, an intermediate butyryl-phosphate is formed by phosphorylation (phosphate butyryltransferase (Ptb)) of butyryl-CoA which is then transformed to butyrate *via* butyrate kinase (Buk). In the second pathway, the CoA moiety of butyryl-CoA is transferred to acetate *via* butyryl-CoA:acetate CoA-transferase (But), leading to the formation of butyrate and acetyl-CoA.

A recent study found that butyrate may have a role in promoting wound healing in T84 cells (transplantable human carcinoma cells) and induces the expression of the synaptopodin, an epithelial tight junction protein, which has a role in maintaining the epithelial barrier while allowing permeation of water, ions and nutrients (Wang et al., 2020). In a study by Geirnaert et al. (2017), faecal microbiota from human Crohn's disease patients inoculated with a mix of butyrate-producing bacteria (*F. prausnitzii, Butyricicoccus pullicaecorum* 25-3^T*, B. pullicaecorum* 1.20*, Eubacterium hallii, Anaerostipes caccae, Roseburia hominis* and *R. inulinivorans*) in a fed-batch culture improved the integrity of the epithelial barrier.

There has also been interest in the use of *F. prausnitzii* as a probiotic for calves, though compared to humans, studies in the calves are more limited. Oikonomou et al. (2013) found that increased microbial diversity (which was dominated by *Faecalibacterium* spp. in one-week-old calves) was associated with a lower incidence of diarrhoea and increased weight gain in calves fed 6 L of milk daily with free access to calf starter. Foditsch et al. (2015) created a probiotic treatment using four genetically distinct *Faecalibacterium prausnitzii* isolates that they had previously isolated and fed this to 3-7-day-old calves, while on a diet of whole milk, once a week for two weeks to allow *F. prausnitzii* to colonise the lower GIT. It was found that the oral administration of *F. prausnitzii* to pre-weaned dairy heifers reduced the likelihood of severe diarrhoea and associated death and promoted weight gain when compared to the control (no bacterial supplementation) calves. However, whether reduced diarrhoea and increased weight gain was the result of one, a few or all four of the isolated *Faecalibacterium* strains is unknown. The mechanisms and potential for *Faecalibacterium* as a beneficial probiotic in the calf industry is still to be determined.

1.3.2 *Faecalibacterium* ethanol production

Bacterial fermentation of sugars may also lead to the production of alcohols such as ethanol, and this can occur through four different fermentation pathways (**Figure 3-16**). In the first pathway, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase (Pdc) and then converted into ethanol by alcohol dehydrogenase (Adh). In the other three pathways, pyruvate is converted to acetyl-CoA by either phenylacetaldehyde dehydrogenase (Pdh) or pyruvate:ferredoxin oxireductase (Por). If Pdh is used, acetyl-CoA is then dehydrogenated by aldehyde dehydrogenase (Aldh) into acetaldehyde and then to ethanol by alcohol dehydrogenase (Adh). Two pathways use Por. In the first Por-mediated pathway, acetyl-CoA is converted into ethanol using the same enzymes and intermediates as the Pdh pathway. In the second Por-mediated pathway acetyl-CoA is converted into acetyl-phosphate, acetate, and lastly acetaldehyde, before being converted into ethanol by Adh (Nissen & Basen, 2019).

1.3.3 *Faecalibacterium* diversity

In the past, there has been debate on whether there were two (Lopez-Siles et al., 2012) or three (Benevides et al., 2017) phylogroups of *F. prausnitzii*. In the recent study by Fitzgerald et al. (2018), it was proposed that there were two phylogroups of *F. prausnitzii,* based on 16S rRNA (ribosomal ribonucleic acid)-encoding gene sequencing. These phylotypes differed in their methods of defence, such as multidrug efflux pumps, and nutrient catabolism (e.g. of carbohydrates and amino acids). However, both phylogroups of *Faecalibacterium*, one containing the *F. prausnitzii* type strain ATCC 27768^T and three other *F. prausnitzii* strains and the other containing *F. prausnitzii* A2-165 and 12 other *F. prausnitzii* strains, have not differed in the amount of butyrate produced (~29 mM) when grown on YCFA media supplemented with 0.5% glucose (Lopez-Siles et al., 2012).

In recent years, the amount of genomic information for *Faecalibacterium* has increased considerably. The National Center for Biotechnology Information (NCBI) Genome database (National Center for Biotechnology Information, 2022) currently lists 166 genome assemblies for *Faecalibacterium* sp., and 197 strains of *Faecalibacterium prausnitzii.* One strain of the newly proposed species *Faecalibacterium longum* (Zou et al., 2021), and seven Candidatus *Faecalibacterium* (Candidatus *F. faecigallinarum,* Candidatus *F. faecipullorum,* Candidatus *F. gallistercoris,* Candidatus *F. intestinigallinarum,* Candidatus *F. avium,* Candidatus *F. intestinipullorum* and Candidatus *F. intestinavium*) are available. The genome assembly of the newly proposed species *F. butyricigenerans* (Zou et al., 2021) is not yet available on NCBI.

Multiple *Faecalibacterium* phylotypes that have been detected in a single cow, and different cows within the same herd can host unique combinations of different phylotypes (Savin et al., 2019). A study by Savin et al. (2019) additionally reported that there were possibly two unidentified phylotypes of *Faecalibacterium*, which had less than 96% sequence identity, by 16S rRNA-encoding gene sequence variable region V4, to *F. prausnitzii* ATCC 27768^T. This diversity suggests the presence of potentially unidentified *Faecalibacterium* species or phylotypes that are of uniquely bovine origin.

Faecalibacterium is closely related to several other butyrate-producing species such as *Gemmiger formicilis* (Salanitro et al., 1976), *Fournierella massiliensis* (Togo et al., 2017) and *Subdoligranulum variabile* (Holmstrøm et al., 2004) (**Figure 1-2**), making them potentially beneficial microorganisms. However, there is little information available on both *G. formicilis* and *F. massiliensis*, particularly from the calf environment. Like *Faecalibacterium* both *F. massiliensis* and *G. formicilis* are extremely oxygen-sensitive, non-motile and Gram stain negative bacteria (Gossling & Moore, 1975; Salanitro et al., 1976; Togo et al., 2017). *F. massiliensis* AT2^T cells are rod-shaped, while *G. formicilis* has been observed to occur in pairs (or chains) of spherical or tear-drop-shaped cells, or as bowling pin cells (which were constricted in the middle). From 16S rRNA gene analysis, the sequences from *F. massiliensis* and *G. formicilis* share 95.2% identity (Togo et al., 2017), and the %identity to *Faecalibacterium* has not yet been determined.

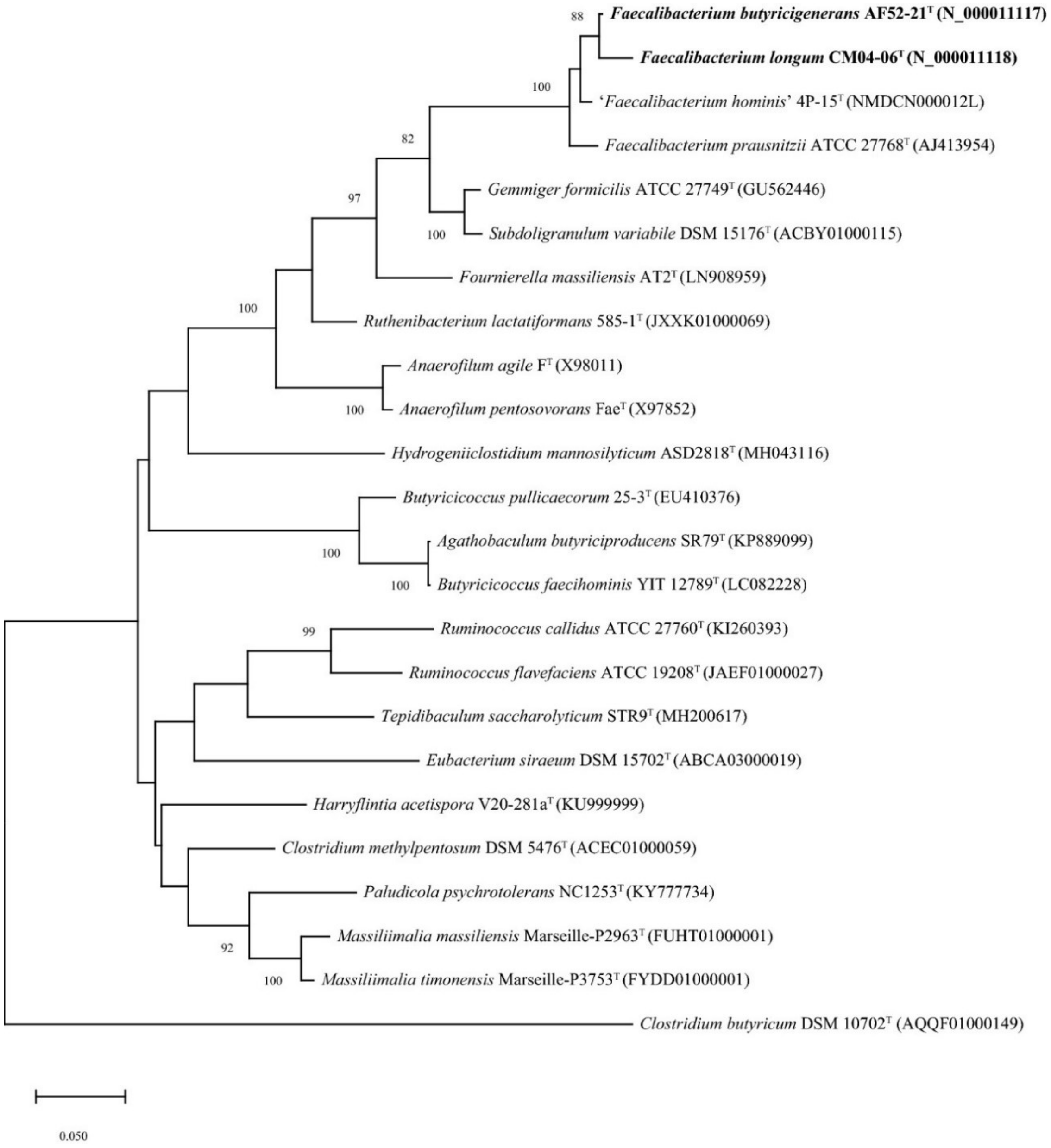


Figure 1-2. The phylogenetic relationship between 16S rRNA genes of *Ruminococcaceae*, in comparison to *F. prausnitzii* ATCC 27768^{T} and two newly proposed species *F. longum* and *F. butyricigenerans*. Image obtained with permission from Zou et al. (2021).

1.3.4 Impact of diet on *Faecalibacterium* prevalence

Numerous studies on different animals have shown that diet has a great effect on GIT microbiota abundance (Loor et al., 2016; Zmora et al., 2019). There are few studies that examine the effects of different diets on the prevalence of *F. prausnitzii* in calves. For example, analysis of the effect of inulin in humans (Ramirez-Farias et al., 2009) and the effect of arabinoxylan in pigs (Nielsen et al., 2014) where both substances lead to an increase *of F. prausnitzii* in the faeces.

Our research group has previously observed that calves fed higher allowances of whole milk had a higher abundance of caecal *F. prausnitzii* (~8%) than calves fed on lower milk allowances (4%) (Maclean et al., 2018). Whole milk was shown to contain approximately 3.1−3.6% protein in the first two months of lactation (Edwards et al., 2014; Morton et al., 2016). In other studies, *Faecalibacterium* abundance was highest at ~20% in one-week-old calves fed milk replacer (Uyeno et al., 2010) and ~15% in three-week-old calves on whole milk (Malmuthuge et al., 2014) or one-month-old calves fed acidified milk (Hennessy et al., 2020). This is in contrast to a recent study by Kumar et al. (2021), where *Faecalibacterium* was present at an abundance of ~2.6% in 2-month-old pre-weaned calves fed milk replacer. Diet has been shown to affect *Faecalibacterium* abundances, e.g. for calves fed milk replacer with 55% (w/v) crude protein or 74% crude protein, there was a higher relative abundance of *Faecalibacterium* in calves fed the higher protein milk replacer at one-week-old (~12% in higher crude protein-fed calves *vs.* ~2% in low crude protein-fed calves) (Badman et al., 2019). In a different trial comparing the effect of feeding newborn Holstein calves four different types of waste milk (untreated waste milk, pasteurised waste milk, waste milk acidified with formic acid and untreated bulk milk) for 42 days, the highest abundance of *Faecalibacterium* (~20% in colon digesta and ~15% in colon mucosa) was found in calves fed acidified waste milk, although *Faecalibacterium* was also detected in the other three milk diets at lower levels (~5-10%) (Deng et al., 2017). Thus, *Faecalibacterium* appears to have a prominent role in the GIT microbial community, particularly on a whole milk diet, in the early weeks of life.

1.4 Approaches for the isolation and characterisation of *Faecalibacterium*

To better understand the function of calf *Faecalibacterium* isolates and their potential for use as probiotic candidates, cultivation is necessary to enable physiological characterisation and genome sequencing. To culture a wide variety of microorganisms, general media are often used, such as Brain Heart Infusion (BHI) medium (Haden, 1923; Rosenow, 1919). Other general media also include YCFAG medium (Duncan, 2002; Lopez-Siles et al., 2012) and modified VersaTREK™ REDOX™ 2 Media enriched with rumen fluid (VTR2RF) medium (Balch et al., 1979; Foditsch et al., 2014; Scott & Dehority, 1965), which have both been successfully used in the past to isolate *Faecalibacterium* from the faeces of calves, piglets and humans (Duncan, 2002; Foditsch et al., 2014; Khan et al., 2014; Lopez-Siles et al., 2012). These media can be used in liquid form, to culture high cell densities of microorganisms whose cellular features (such as Gram stain type, motility and cell shape) can later be determined under a microscope, or in solid form (by adding agar to the medium) to culture and be able to select individual colonies of bacteria and assess their colony morphologies. Two different collection and microbiological culturing methods have been used: (i) samples collected using polybags, with the air manually excluded, then plated on agar plates within an anaerobic chamber followed by incubation inside modified AnaeroPack™ rectangular jars containing Mitsubishi™ AnaeroPack™-Anaerobic Gas Generator pouches (Delaney & Onderdonk, 1997; Takeuchi et al., 1992); (ii) samples collected in air-tight PBS-buffered bottles which are then inoculated into agar roll tubes (Hungate, 1969) at a gassing bench and incubated accordingly. For the former method, air-tight bottles do not need to be prepared and making the agar plates and isolating single colonies from them is less laborious in comparison to roll tubes. However, a possible disadvantage of this method is the risk of the agar plates drying out sooner than the roll tubes, especially as they go through the anaerobic chamber airlock multiple times. For the latter method, while both the collection and culture methods are more strictly anaerobic, it is more difficult to isolate colonies that are deep inside the tubes, and it is still unknown if the higher levels of humidity in the roll tubes affect the growth of *Faecalibacterium.*

To identify specific bacteria from amongst the cultured bacteria or in environmental samples, a common practice is to include a type or model reference strain in subsequent analyses. Such characterised strains can be used for phenotypic comparisons of colonies for selection and also in polymerase chain reaction (PCR) (Mullis & Faloona, 1987) screening of colonies using a universal marker gene present in all microorganisms, e.g. 16S rRNA-encoding gene. A human-derived *F. prausnitzii* strain, A2-165, has been used in previous studies as a reference strain (Khan et al., 2012; Rossi, van Berkel, et al., 2015; Sokol et al., 2008), particularly in phylogenetic studies where a variable region, V4, of the 16S rRNA-encoding gene was used for comparison. Currently, there are no cattle or calf-derived *Faecalibacterium* type species. Therefore, in this study, strain A2-165 was used to compare to calf-derived *F. prausnitzii* isolates. To identify *Faecalibacterium, Faecalibacterium*-specific 16S rRNA gene primer pairs such as FPR-2F (Wang et al., 1996)/Fprau645R (Ramirez-Farias et al., 2009) are available.

Genome sequencing provides the ability to understand the metabolic and functional potential of an organism *via* its gene complement and metabolic pathway reconstruction. Initiatives such as the Hungate1000 project (Seshadri et al., 2018), which have obtained over 500 reference genome sequences from bacteria and archaea cultivated from the rumen environment, have enabled a much deeper understanding of the biology of these microorganisms. Next-generation sequencing technologies (e.g. Illumina) have accelerated our ability to sequence whole genomes and interpret the metabolic and functional potential of microbes. The Hungate1000 study (Seshadri et al., 2018), for example, used either Illumina (Mavromatis et al., 2012) or Pacific Biosciences RS (Eid et al., 2009) technology to sequence the genomes of 501 bacteria and archaea cultivated from ruminants. Once a whole genome sequence is obtained, a multitude of analyses can be performed, such as gene prediction and function *via* comparison to databases such as Clusters of Orthologous Genes (COG) (Galperin et al., 2015) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa, 2000), and comparative genome analyses between organisms to understand their relatedness and functional differences. The availability of reference genome sequences also facilitates the interpretation of microbiome function *via* metagenome sequencing, by enabling taxonomic identities to be attributed to genes and functions that are detected in the metagenome.

1.5 Aims of this thesis and hypothesis

The main aim of the study is to isolate, and phenotypically and genetically characterise *Faecalibacterium* strains from calves reared on New Zealand farms. We hypothesise that calf-derived *Faecalibacterium* differs to human-derived *Faecalibacterium*.

To test the hypothesis and achieve the proposed aim we will:

- Isolate bacteria anaerobically from calves on diets that promote *Faecalibacterium*. A selective PCR screen will be developed to identify *Faecalibacterium* using specific 16S rRNA gene primers. The morphology and fermentation profiles of isolates will be determined, to assess the production of butyrate.
- The genetic diversity of New Zealand calf *Faecalibacterium* isolates will be assessed by 16S rRNA gene phylogenetic analyses. The genomes of calf *Faecalibacterium* isolates will be sequenced and analysed, with emphasis on butyrate production. This will enable us to compare and contrast the genetic diversity of NZ calf *Faecalibacterium* isolates with other known *Faecalibacterium* strains*.*

Chapter Two. Materials and Methods

2.1 Materials

2.1.1 Oligonucleotides

Oligonucleotides were purchased from Invitrogen (Massachusetts, USA) and Integrated DNA Technologies (Iowa, USA). All oligonucleotides used in the study are listed in **Table 2-1**.

Table 2-1. The universal and internal 16S rRNA gene-encoding primers used in this project.

Primer	Sequence (5'-3')	Tm[a]	Reference
27F	GAGTTTGATCMTGGCTCAG	50°C	Lane (1991)
1492R	GGYTACCTTGTTACGACTT	50°C	Lane (1991)
FPR-2F	GGAGGAAGAAGGTCTTCGG	43°C	Wang et al. (1996)
Fprau645R	AATTCCGCCTACCTCTGCACT	44°C	Ramirez-Farias et al. (2009)
Fprau 07	CCATGAATTGCCTTCAAAACTGTT	47°C	Sokol et al. (2009)
Fprau 02	GAGCCTCAGCGTCAGTTGGT	51°C	Sokol et al. (2009)
514R	CCGCGGCKGCTGGCAC	65.6°C	Lane (1991)
1100R	GGGTTGCGCTCGTTG	54.6°C	Lane (1991)
1382R	CGGTGTGTRCAAGGCCC	58.2°C	Dorsch and Stackebrandt (1992)

[a] Melting temperature (Tm) values were calculated for 50 mM monovalent cations using the following formula: 59.9 + 41[%GC] – [675/Primer Length], where "%" is referred to as a decimal value.

2.1.2 Anaerobic 1× PBS buffer

Anaerobic 1× phosphate-buffered saline (PBS) buffer (pH 7.3–7.5) was prepared using either PBS tablets (Life Technologies, California, USA), according to the manufacturer's recommendations, or made up with 137 mM NaCl, 2.7 mM KCl, 1.42 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. Five drops of 0.1% (w/v) resazurin solution were also added prior to boiling the 1× PBS. Following cooling to room temperature, 59.52 mM $NaHCO_3$ and 2.85 mM L-cysteine hydrochloride monohydrate were mixed in and allowed to reduce until the media turned pale purple or colourless. The buffer was used in both Duran® pressure plus (DWK Life Sciences GmbH, Germany) sample collection bottles and Hungate tubes (Chemglass Life Sciences, New Jersey, USA) for serial dilutions of samples. For use in collection bottles, 500 mL of PBS buffer was dispensed into 1 L bottles, while for serial dilutions, 9 mL of buffer was dispensed into 16.5 mL volume Hungate tubes. CO_2 was continuously flushed into the vessels *via* a cannula during dispensing and removed just prior to sealing the vessels with a rubber bung and screw-top cap. The PBS buffer was then sterilised by autoclaving.

2.1.3 Internal standard for SCFA analyses

The internal standard for SCFA analyses by gas chromatography contained 20 mM 2-ethylbutyrate in 20% (w/v) H_3PO_4 and stored at 4°C.

2.2 Bacterial strains and growth media

2.2.1 Bacterial strains

Faecalibacterium prausnitzii A2-165 was originally sourced from a human faecal sample (Barcenilla et al., 2000) and was used as a reference *Faecalibacterium* strain in this study.

Prevotella albensis strain M384 (Avguštin et al., 1997) or putative *Bacteroides* DNA (F2.002) was used as a control for PCR reactions.

2.2.2 General anaerobic media preparation

All media were prepared in 500 mL batches in 1,000 mL Duran® Pressure Plus bottles (DWK Life Sciences GmbH) unless otherwise stated. Anaerobic microbiological techniques were performed using a gassing station on the lab bench or in a Coy anaerobic chamber (Coy Laboratory Products, Michigan, USA) containing a gas mix of ~95% CO_2, <4% H_2 and <50 ppm O_2.

Generally, anaerobic media was boiled in a microwave oven prior to adding 2.85 mM L-cysteine hydrochloride monohydrate while flushing with CO_2 for at least 20 minutes and rapidly cooling in an ice water bath. Media prepared in Hungate tubes or Duran® pressure plus bottles were kept anaerobic under a stream of CO_2 and then sealed with rubber bungs and screw caps before sterilising.

Liquid media in Hungate tubes or serum bottles were prepared by flushing the empty vessels with CO_2, prior to anaerobically transferring the media using a sterile serological pipette that had been flushed with CO_2. The headspaces of the vessels were flushed with CO_2 until the resazurin oxygen indicator in the media indicated that they were anaerobic, and then the vessels were sealed.

All solid media contained 1.5% (w/v) bacteriological agar powder (Oxoid, UK) added to the liquid media and were flushed in the headspace with CO_2 for an additional 5–10 minutes before sterilising. Media were poured into Petri plates inside an anaerobic chamber and allowed to cool overnight at room temperature until dry. All media were steam sterilised at 121°C at 15 psi for 20 minutes.

2.2.3 Anaerobic BHI medium

Anaerobic Brain Heart Infusion (BHI) is a rich medium (Haden, 1923; Rosenow, 1919) used for the general cultivation of obligate and facultative anaerobic bacteria. The medium contained 18.5 g of dehydrated BHI broth (Oxoid), 2.5 g of granulated yeast extract (Merck Group, Germany), 5 mL of 0.05% (w/v) hemin solution (**Section 2.2.7.1**) (Sigma-Aldrich, Missouri, USA) and 0.5 mL of 0.1% (w/v) resazurin solution in a 500 mL volume. The mixture was boiled in a microwave oven and one gram of L-cysteine hydrochloride monohydrate was added once the medium was cold. The pH was adjusted to 7.0 ± 0.2 prior to the addition of 0.5 mL of vitamin K solution (**Section 2.2.7.3**). The medium was anaerobically transferred into Hungate tubes and sterilised.

2.2.4 Anaerobic YCFAG medium

Anaerobic YCFAG (Yeast extract, Casitone, Fatty Acid and Glucose) medium is commonly used for *Faecalibacterium* cultivation and was made as previously described by Duncan (2002) and Lopez-Siles et al. (2012) but modified with the addition of 33 mM of sodium acetate, rather than a mixture of SCFAs (Maier, 2017). To 500 mL of media (containing casitone, yeast extract, anhydrous sodium acetate, D-(+)-glucose, sodium bicarbonate, K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2$, hemin stock and resazurin dissolved in distilled water (dH_2O), as previously described), 500 µL of YCFAG vitamin stock 1 (**Section 2.2.7.4**) and 0.5 g of L-cysteine hydrochloride monohydrate were added, and the pH of the solution was adjusted to 6.5 ± 0.2. Hungate tubes were anaerobically filled with 9 mL of the media and sterilised by autoclaving (**Section 2.2.2**). A few hours prior to inoculation, 0.1 mL of vitamin stock 2 (**Section 2.2.7.5**) was anaerobically added to each 9 mL of media to give a 1× final concentration.

For YCFAG solid media, 1.5% (w/v) bacteriological agar was added to anaerobically prepared YCFAG broth and autoclaved. Vitamin stock 2 was added to the molten agar to obtain a 1× final concentration. The agar was slowly inverted a few times to mix and prevent the formation of air bubbles, then poured into sterile Petri plates inside an anaerobic chamber.

For YCFAG agar roll tubes, following the addition of bacteriological agar, the media was kept at 60°C on a heating magnetic stirrer (VELP Scientifica, Italy) until the agar had dissolved, then cooled to ~50°C. Vitamin stock 1 and L-cysteine hydrochloride monohydrate were added while stirring under CO_2 for 30 minutes. Approximately 4.5 mL of molten agar media was anaerobically dispensed into Hungate tubes that were continuously flushed with CO_2, sealed with a rubber bung and screw cap, and then sterilised. Prior to use, the agar in the roll tubes was melted by heating in an Optima™ T100 heated circulating water bath (Grant Instruments, England) set to 60°C. Immediately prior to inoculation, 0.1 mL vitamin stock 2 was anaerobically added into each tube.

2.2.5 Anaerobic modified VTR2RF medium

Anaerobic modified VTR2RF (VersaTREK™ REDOX™ 2 Media enriched with rumen fluid) agar, used for cultivation of *Faecalibacterium*, was modified from Foditsch et al. (2014), Balch et al. (1979) and Scott and Dehority (1965). To make 500 mL of modified VTR2RF agar roll tubes, 50 mL of clarified rumen fluid (**Section 2.2.7.2**), 37.5 mL mineral solution I (containing 34.44 mM K_2HPO_4), 37.5 mL mineral solution II (containing 44.08 mM KH_2PO_4, 90.81 mM $(NH_4)_2SO_4$, 205.34 mM NaCl, 9.97 mM $MgSO_4$ and 9.01 mM $CaCl_2$), 5 mL of vitamin solution (containing a final concentration of 1.95 mM pyridoxine hydrochloride, 1.06 mM riboflavin, 1.19 mM thiamine hydrochloride, 3.28 mM nicotinamide, 1.54 mM DL-calcium pantothenate, 0.15 mM *p*-aminobenzoic acid, 0.02 mM folic acid, 0.04 mM biotin and 7.38×10^{-4} mM vitamin B_{12}), 5 mL fatty acid solution (containing a final concentration of 2.21 M acetic acid, 74.9 mM isobutyric acid, 78.33 mM isovaleric acid and 86.16 mM valeric acid), 0.25 g yeast extract, 0.5 g tryptone, 1.0 g cellobiose, 1.0 g maltose, 0.5 mL of 0.1% (w/v) resazurin solution, 1 mL of 0.05% (w/v) hemin solution (**Section 2.2.7.1**), 7.5 g bacteriological agar and dH_2O were used. The media was brought to a boil, then cooled to 60°C with continuous stirring on a heating magnetic stirrer while the CO_2 was streamed into the headspace. Once the media had cooled to ~40°C, L-cysteine hydrochloride monohydrate and 1.25 g of $NaHCO_3$ were added and stirred for 20 minutes. Aliquots of 4.5 mL were anaerobically dispensed into Hungate tubes flushed with CO_2, then sterilised.

2.2.6 Anaerobic 40% (v/v) glycerol solution

Anaerobic 40% (v/v) glycerol solution contained approximately 1/6 volume of Salt Solution A (102.64 mM NaCl, 22.04 mM KH_2PO_4, 11.35 mM $(NH_4)_2SO_4$, 5.39 mM $CaCl_2.2H_2O$ and 4.87 mM $MgSO_4.7H_2O$) (Noel, 2013) and 1/6 volume of Salt Solution 2B (43.05 mM $K_2HPO_4.3H_2O$ or 43.06 mM K_2HPO_4) (AgResearch, New Zealand), in addition to 200 mL of glycerol, 2.5 g of $NaHCO_3$, 2 drops of 0.1% resazurin solution and 130 mL of dH_2O. The solution was anaerobically prepared for sterilisation by adding 0.25 g of L-cysteine hydrochloride monohydrate, aliquoting anaerobically into serum bottles and sterilised.

2.2.7 Media components

2.2.7.1 Hemin solution

Hemin solution, 0.05% (w/v), was made by dissolving 50 mg of hemin (Sigma-Aldrich) in 100 mL of 4% (w/v) NaOH in an ice water bath. The solution was stored in the dark at 4°C.

2.2.7.2 Clarified rumen fluid

Clarified rumen fluid was collected from fistulated hay-fed cattle and strained through a cheesecloth. The fluid was centrifuged at 10,000 × *g* at 4°C for 20 minutes, and the supernatant was frozen at −20°C. For use in media, the frozen rumen fluid was thawed in hot water then transferred into a Duran® pressure plus bottle and heated in a microwave until it reached boiling point. The rumen fluid was then gassed through with 100% N_2 for at least 20 minutes, loosely capped and sterilised. The sterilised, cooled, rumen fluid was transferred back into a container and frozen at −20°C overnight. The following day, the rumen fluid was thawed and centrifuged at 15,000 × *g* at 4°C for 15 minutes, and the supernatant was used for making media. The remaining supernatant was stored at −20°C.

2.2.7.3 Vitamin K solution for BHI media

Vitamin K solution, 0.05% (v/v), was made by mixing 5 µL of vitamin K_1 (Sigma-Aldrich) in 10 mL of 95% ethanol. The solution was stored in the dark at 4°C.

2.2.7.4 1000× vitamin stock 1

1000× vitamin stock 1 for YCFAG media was made by dissolving 10 mg of biotin, 10 mg of cobalamin, 30 mg of *p*-aminobenzoic acid, and 150 mg of pyridoxamine dihydrochloride separately in 1 mL of Milli-Q® water (Merck Group) and dissolving 50 mg of folic acid in 20 mL of 1 M NaOH. The vitamin solutions were combined and made up to 1 L with Milli-Q® water. The solution was stored at −20°C.

2.2.7.5 100× vitamin stock 2

100× vitamin stock 2 was made by dissolving 5 mg of thiamine hydrochloride (Sigma-Aldrich) and 5 mg of riboflavin (VWR Chemicals BDH, UK) in 100 mL of Milli-Q® water. The vitamin solution was filter sterilised by filtering through a sterile 22 µm syringe filter into sterile N_2-filled serum bottles that were continuously flushed with CO_2. The serum bottles were then sealed with a rubber bung and aluminium crimp seal and stored in the dark at 4°C.

2.2.7.6 Vitamin solution for modified VTR2RF agar

To make vitamin solution for modified VTR2RF, 100 mg of pyridoxine hydrochloride, 100 mg of riboflavin, 100 mg of thiamine hydrochloride, 100 mg of nicotinamide, 100 mg of calcium-d-pantothenate, 5 mg of p-aminobenzoic acid, 2.5 mg of folic acid, 2.5 mg of biotin and 0.25 mg of vitamin B_{12} were dissolved in with Milli-Q® water to a volume of 250 mL and stored in the dark at 4°C.

2.2.7.7 Fatty acid solution for modified VTR2RF agar

To make the fatty acid stock solution, 26.6 g of acetic acid, 1.32 g of isobutyric acid, 1.6 g of isovaleric acid and 1.76 g of valeric acid were dissolved in Milli-Q® water to 200 mL. The solution was stored in the dark at 4°C.

2.3 Methods

An overview of the approach used to isolate and characterise New Zealand calf *Faecalibacterium* isolates in this research project has been summarised in **Figure 2-1**.

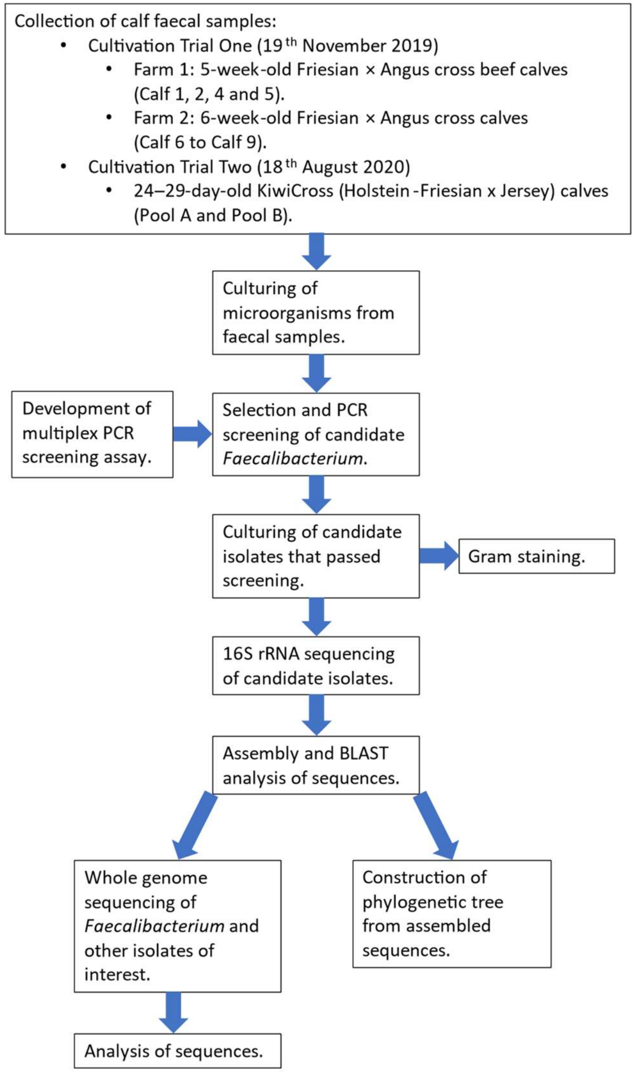


Figure 2-1. Schematic diagram of isolation and identification of calf faecal bacteria.

2.3.1 Calf sampling from farms

Freshly voided faecal samples were collected from healthy whole milk-fed calves on farms in Manawatu, New Zealand. Collections took place during the morning milk feeding, and freshly voided faecal samples were immediately collected from the ground and stored anaerobically for transportation back to the laboratory at AgResearch Grasslands, Palmerston North, for cultivation. Where available, the identification numbers of the calves were recorded.

Cultivation Trial One was conducted on 19th November 2019 at two farms in Palmerston North, New Zealand. Farm 1, located at 1640 Tennent Drive, had nine 5-week-old Friesian × Angus cross beef calves group-fed 40 L whole milk per day. The calves also had free access to pasture, and pellets had recently been introduced into their diet. Faecal samples from four different calves were collected (samples Calf 1, 2, 4 and 5). Farm 2, located at 617 Fitzherbert East Road, Aokautere, had approximately thirty 6-week-old Friesian × Angus cross calves, group-fed a diet of 100 L of whole milk per day with access to pasture and pellets. Four faecal samples were collected (samples Calf 6 to Calf 9). For both collections, polybags were used to pick up freshly voided faeces from the ground, avoiding any soil or plant material. Samples were individually collected and air in the bag was manually excluded before tying, closing and storing n a polystyrene box to minimise heat loss.

Cultivation Trial Two was conducted on 18th August 2020 at Dairy Farm 1, Massey University, Tennent Drive, Palmerston North. Freshly voided faecal samples were collected from 24–29-day-old KiwiCross (Holstein-Friesian x Jersey) calves, fed approximately 7 L whole milk per calf per day, with access to a plantain, chicory and clover pasture. For this collection, faecal samples were collected and immediately placed into pre-weighed bottles containing 500 mL of anaerobically prepared 1× PBS (**Section 2.1.2**) in 1 L gas-tight bottles. Two samples of approximately 15–20 g material per sample, were pooled per bottle. Two pooled groups (Pool

A and Pool B) were collected. The bottles were again weighed to accurately determine the weight of faecal samples added to each Pool.

2.3.2 Isolation of bacteria on agar plates

Cultivation of isolates in Cultivation Trial One (**Section 3.2**) was performed in an anaerobic chamber. Each calf faecal sample was added to a Hungate tube containing 9 mL of YCFAG broth to a final volume of 10 mL, which was resuspended using a vortex mixer (Labnet International, New Jersey, USA), resulting in a 10^{-1} dilution. The 10^{-1} dilutions from each calf were pooled with samples from the same Farm (samples Farm 1 and Farm 2) in YCFAG broth to make a pooled 10^{-2} dilution. Each pooled sample was 10-fold serially diluted to 10^{-10} in YCFAG broth.

In Cultivation Trial One, aliquots, 100 µL, of the pooled faecal 10^{-6}, 10^{-8}, 10^{-9} and 10^{-10} dilutions were plated onto YCFAG and BHI agar plates in triplicate. In addition, 100 µL aliquots of the 10^{-5}, 10^{-6} and 10^{-7} dilutions of faecal material from Calf 1 (Farm 1) and Calf 6 (Farm 2) were spread onto YCFAG agar plates (**Section 2.2.4**). In parallel, *Faecalibacterium prausnitzii* A2-165 was streaked onto YCFAG and BHI agar plates to enable the comparison of colony morphologies and growth rates. Spread plates were incubated at 37°C in a Precision Incubator (Contherm Scientific, New Zealand). For isolations from individual calves (Calf 1 from Farm 1 and Calf 6 from Farm 2), the 10^{-1} diluted frozen faecal samples were serially diluted in YCFAG broth, and 100 µL of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread plated onto YCFAG agar plates and incubated at 37°C. These isolations enabled the screening of more colonies and evaluation of the effect of freezing faecal samples in glycerol on the viability of the bacteria in the samples.

Agar plates were stored upside down in modified AnaeroPack™ 2.5L rectangular jars (Thermo Fisher Scientific, Massachusetts, USA) with a hole and removable rubber bung to release pressure accumulated from passing through the anaerobic chamber airlock. A Mitsubishi™ AnaeroPack™-Anaerobic Gas Generator pouch was placed into each jar used (Thermo Fisher Scientific). Colonies that were morphologically similar to *F. prausnitzii* A2-165 colonies on YCFAG agar, i.e. creamy white in colour, smooth and shiny, growing on agar plates were picked inside an anaerobic chamber using a sterile plastic inoculating loop and patched onto a YCFAG agar plate, as a backup, and inoculated into tubes of anaerobic YCFAG broth. In Cultivation Trial One, broth cultures were incubated at 37°C and frozen at –80°C with a final concentration of 10% anaerobic glycerol (**Section 2.2.6**). The original calf faecal material from Cultivation Trial One was diluted to 10^{-1} anaerobically in cryotubes also with a final concentration of 10% anaerobic glycerol and frozen at –80°C.

2.3.3 Isolation of bacteria using agar roll tubes

In Cultivation Trial Two, the bottles of anaerobic PBS buffer with faecal samples were shaken vigorously to resuspend the faecal material. The suspensions were anaerobically serially diluted in Hungate tubes containing anaerobic 1× PBS buffer to 10^{-11}, using sterile needles and syringes pre-flushed with CO_2 to transfer samples. To inoculate agar roll tubes, three tubes at a time were removed from the water bath and kept at room temperature to cool slightly. A sterile 16G PrecisionGlide™ needle tip (BD, New Jersey, USA) was bent to 90°, attached to a 0.22 µm filter (Merck Group) and connected to a CO_2 gas line. A sterile needle and syringe, that had been flushed with sterile filtered CO_2, was used to draw up 0.9 mL of faecal material diluted in PBS buffer and 0.3 mL was gently inoculated into each agar roll tube, preventing the formation of bubbles within the molten agar. Each inoculated roll tube was gently inverted once to mix the diluted faecal material into the agar, then laid on its side in a tray of ice water and quickly rolled back and forth until the agar evenly coated the entire surface of the Hungate tube. Dilutions from 10^{-7} to 10^{-9} were plated on YCFAG agar plates (**Section 2.3.2**) and incubated at 39°C to compare the differences between CFUs and the time taken for colonies to appear between agar roll tubes versus agar plates.

YCFAG (**Section 2.2.4**) and modified VTR2RF (**Section 2.2.5**) media agar roll tubes were inoculated with the 10^{-7}, 10^{-8} and 10^{-9} dilutions and incubated upright at an ~45° angle, to allow condensation to pool at the bottom of the tubes. Tubes were monitored daily until no more new colonies appeared, then refrigerated at 4°C when the colonies were ~2 mm in diameter. To pick colonies, the agar roll tubes were opened on the bench at a gassing station, and CO_2 was immediately streamed into the tube *via* a sterile needle to maintain the anaerobic environment within the tube. Colony selection was largely random but still aimed for colonies that were mostly creamy white in colour, smooth and shiny. Tubes of YCFAG broth to be inoculated were similarly opened and CO_2 streamed into the headspace. Roll tube colonies were picked using a sterile, bent glass Pasteur pipette tip (Poulten & Graf, Germany) and transferred to YCFAG broth before resealing the tube. Broth cultures were incubated at 39°C and frozen at –80°C with a final concentration of 10% glycerol for long-term storage.

2.3.4 Bacterial culture growth curves

To generate growth curves, bacterial cultures were revived from frozen stocks by anaerobically inoculating into Hungate tubes of YCFAG medium (**Section 2.2.4**) pre-warmed to 37°C. Cultures were grown at 37°C for ~24 hours, then passaged at least twice. For the growth curve experiment, 0.1 mL of overnight culture was inoculated into 10 mL of YCFAG medium in Hungate tubes, pre-warmed to 37°C. The optical densities (ODs) of the cultures were measured by absorbance at 600 nm (OD_{600}) using a SPECTRONIC™ 200 Spectrophotometer (Thermo Fisher Scientific) at inoculation and every 3 hours for 24 hours. Once cultures reached stationary phase, indicated by two similar OD readings in a row, 1.5 mL samples were taken and processed for

SCFA analyses (**Section 2.3.5**). Samples from all cultures were also taken at 24 hours for SCFA analyses.

2.3.5 SCFA analysis

Aliquots of cultures (**Section 2.3.4**) were centrifuged at 14.1 × *g* for 4 minutes in a microcentrifuge (Eppendorf, Germany) to pellet the cells. An internal standard (**Section 2.1.3**), 100 µL, was added to 900 µL of the culture supernatant, mixed by inversion, and frozen at −20°C. Samples were analysed for aqueous and derivatised SCFA and alcohol concentrations by gas chromatography using established protocols, based on Kumar et al. (2021) and Richardson et al. (1989), at AgResearch, New Zealand.

2.3.6 DNA extraction

2.3.6.1 NucleoSpin® Soil kit

The NucleoSpin® Soil kit (Macherey-Nagel, Germany) was used according to the manufacturer's recommendations to extract DNA from bacterial cultures. DNA was stored at −20°C for long-term storage.

2.3.6.2 InstaGene™ Matrix

The InstaGene™ Matrix (Bio-Rad Laboratories, California, USA) was used according to the manufacturer's recommendations to prepare DNA templates from bacterial samples for PCR amplification. DNA was stored at −20°C for long-term storage.

2.3.6.3 High molecular weight DNA extraction

Extraction of high molecular weight DNA for whole genome sequencing was performed using a modified version of the standard phenol-chloroform DNA extraction method described by Saito and Miura (1963). Bacterial cells from 30–100 mL late log phase cultures were pelleted in 50 mL centrifuge tubes by centrifugation at 5,000 × *g* for 5 minutes at 4°C in a Sigma 3-18KS centrifuge (Sigma Laborzentrifugen GmbH, Germany). The cell pellet was washed once with saline-EDTA solution (150 mM NaCl, 10 mM EDTA) and centrifuged at 5,000 × *g* for 5 minutes at 4°C. Saline-EDTA solution, 4–8 mL, containing 1 mg/mL lysozyme from chicken egg white (Sigma-Aldrich) and 0.2 µL/mL RNase A (Qiagen, Germany), was added to the cell pellet and gently inverted. The solution was incubated stationary at 37°C or gently shaken on an orbital shaker (Ratek Instruments, Australia) at 39°C for 1.5 to 2 hours. To each millilitre of cell lysate-lysis buffer solution, 54 µL of 20% SDS (sodium dodecyl sulfate) solution (Invitrogen) and 22 µL of Ambion™ proteinase K (Life Technologies) were added and incubated for a further 1.5 hours at 60°C. To each millilitre of cell lysate-lysis buffer solution, 400 µL of TE buffer (10 mM Tris base, 1 mM EDTA, pH 7.4) was added and inverted gently. An equal volume of UltraPure™ phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) (Invitrogen) was added to the lysate in a fume hood with gentle inversions for ~1 minute, then centrifuged at 13,000 × *g* for 5 minutes at 4°C. The aqueous supernatant was transferred to a new centrifuge tube using a sterile wide-bore pipette tip and again extracted with an equal volume of phenol:chloroform:isoamyl alcohol followed by centrifugation. The supernatant was transferred to a new centrifuge tube and extracted with an equal volume of chloroform (VWR Chemicals BDH), then centrifuged. This step was repeated. To precipitate the DNA, the supernatant was transferred into a new sterile centrifuge tube and 0.1 volumes of 5 M ammonium acetate (pH 5.2, Invitrogen), and 3 volumes of absolute ethanol were added, and the tube inverted gently. The sample was chilled at −20°C for 18–24 hours. If a large amount of DNA was observed, a sterile bent glass Pasteur pipette was used to hook the DNA out, where it was washed twice in 70% ethanol by dipping up and down into the solution. The washed DNA was deposited into a new sterile microcentrifuge tube. However, if there was little or no DNA precipitate visible, the sample was centrifuged at 12,000 × *g* for 30 minutes at 4°C to pellet the DNA. The pellet was washed twice with 1 mL of 70% ethanol. Following the 70% ethanol washes, the DNA samples were dried at 37°C, gently resuspended in 100-400 µL of UltraPure™ DNase/RNase-free dH_2O (Invitrogen) and stored at 4°C.

2.3.6.4 DNA concentration and quality assessment

DNA quality was assessed on an N50 NanoPhotometer (Implen, Germany). For DNA extracts intended for whole genome sequencing, a Qubit® Fluorometer (Invitrogen), using the Qubit™ dsDNA BR (broad range) assay kit, was used to quantify the DNA. For low-quality DNA samples (low A_{260}/A_{280} ratio), dialysis was performed by placing an aliquot of the DNA sample on a 0.025 µm pore size MF-Millipore™ membrane filter (Merck Group), floated on MilliQ water for 1 hour, to allow any impurities (e.g., inhibitory substances, leftover buffer) from the DNA extraction process to be filtered out of the sample.

2.3.7 PCR screen for *Faecalibacterium*

A multiplex PCR screening method was developed to identify *Faecalibacterium* among isolates derived from the calf faecal samples. This PCR assay employed Taq PCR master mix (Qiagen), universal 16S rRNA gene primers, 27F and 1492R, to provide an amplification control for all samples, and *Faecalibacterium*-specific 16S rRNA gene primers, FPR-2F and Fprau645R (**Table 2-1**), to further identify *Faecalibacterium*-like isolates (**Figure 2-2**). DNA extracted from A2-165 cells using the NucleoSpin® Soil kit (Macherey-Nagel) was used as a *Faecalibacterium*-

positive control. DNA from *Prevotella albensis* M384 was used as a *Faecalibacterium*-negative bacterial control, and RNAse-free water was used as a PCR negative control. PCR (**Table 2-2**) was performed in an Eppendorf® Mastercycler® Pro S thermal cycler (Eppendorf).

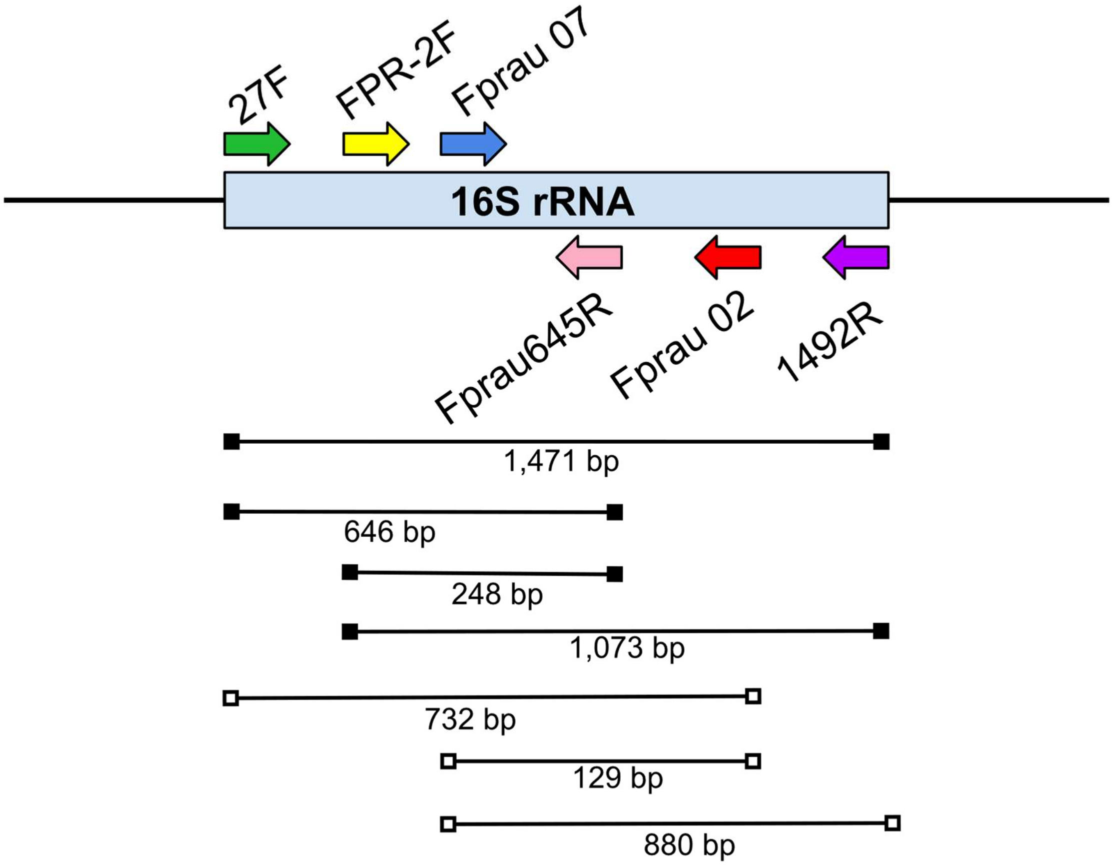


Figure 2-2. Schematic diagram of primer locations on the 16S rRNA-encoding gene used in the selective PCR screen. Two universal 16S rRNA gene primers, 27F (green arrow) and 1492R (purple arrow), and two pairs of *Faecalibacterium*-specific 16S rRNA gene primers, FPR-2F (yellow arrow) and Fprau645R (pink arrow), and Fprau 07 (dark blue arrow) and Fprau 02 (red arrow), were used to amplify the 16S rRNA gene region (pale blue box) of each faecal isolate. PCR samples that included fragments of 248, 646 and 1,471 bp indicated the amplification of the 16S rRNA gene of a putative *Faecalibacterium* isolate by the FPR-2F and Fprau645R primer pair, while samples that contained 129 bp, 732 bp and 880 bp fragments indicated amplification by the Fprau 07 and Fprau 02 pair; samples with only the 1,471 bp band indicated the amplification of the 16S rRNA region of either a *Faecalibacterium* or non-*Faecalibacterium* isolate.

Table 2-2. Optimised PCR composition for screening candidate *Faecalibacterium* isolates.

Components	**1× master mix template**	**Final concentration**
Taq PCR Master Mix	12.5 µL	1×
5 µM 27F	0.625 µL	0.125 µM
5 µM 1492R	0.625 µL	0.125 µM
5 µM FPR-2F[a]	0.625 µL	0.125 µM
5 µM Fprau645R[b]	0.625 µL	0.125 µM
Template (~5-10 ng/µL DNA or cells[c]) / Control	1 µL	N/A
RNAse-free water	9 µL	N/A
Total volume	25 µL	N/A

[a] Fprau 07 was used instead of FPR-2F when testing the Fprau 07 and Fprau 02 pair.

[b] Fprau 02 was used instead of Fprau645R when testing the Fprau 07 and Fprau 02 pair.

[c] Cells were either directly suspended in the PCR master mix or suspended in 50 µL Milli-Q® water and 1 µL of the suspension was added to the master mix.

The thermocycler conditions used were an initial denaturation once at 94°C for 4 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 2 minutes. A final extension at 72°C for 10 minutes was followed by cooling to 12°C. These conditions yielded the expected banding patterns for A2-165 and M384 (control DNA).

In the development of the screening assay, additional primer combinations, including *Faecalibacterium*-selective Fprau 07 and Fprau 02 (**Table 2-1**), were tested using thermocycling annealing temperatures appropriate for the primer sequences (**Table 2-1**). Annealing temperatures were also assessed using a gradient function on the PCR thermocycler to determine optimal temperatures for selective amplification. Annealing temperatures ranging from 43.3°C, which was ~5°C lower than the lowest melting temperature of the six primers (**Table 2-1**), to 49.7°C (above the highest melting temperature of the six primers) were tested.

A range of DNA template preparations from A2-165 colonies was assessed to facilitate high throughput PCR screening. These included: (Method 1) suspension of a fresh colony in 50 µL of Milli-Q® water; (Method 2) as per (Method 1) with heating at 100°C for 5 minutes to lyse cells and inactivate DNases; (Method 3) InstaGene™ Matrix preparation from colony material (**Section 2.3.6.2**); (Method 4) addition of colony material directly into the PCR mix; and (Method 5) heating a 0.2 mL aliquot of broth culture at 75°C for 10 minutes to lyse bacterial cells. Method 1 of template preparation resulted in the expected amplification banding patterns more frequently than the other methods tested, in addition to amplification of the expected band sizes in the A2-165 and M384 PCR controls, therefore was used for general screening of colony isolates.

2.3.8 Gel electrophoresis

Agarose gel electrophoresis was performed using either a Horizon® 58 agarose gel electrophoresis apparatus (LabRepCo, Pennsylvania, USA) or a wide mini-sub cell GT cell (Bio-Rad Laboratories). Agarose gels to visualise PCR products used 1.5% (w/v) agarose (Thermo Fisher Scientific) gels made up with 1× TAE (Tris-acetate-EDTA, containing 40 mM Tris, 20 mM acetic acid and 1 mM EDTA) buffer and containing 1× SYBR® Safe DNA gel stain (Invitrogen). Gels were submerged in 1× TAE running buffer in the electrophoresis apparatus. DNA samples were loaded with BlueJuice™ gel loading buffer or TrackIt™ cyan/orange loading buffer. A 1 Kb Plus DNA ladder (Invitrogen) was loaded in the end lanes of the gel to determine band sizes. Gels were electrophoresed for ~1 hour at 80 V, then visualised under UV (ultraviolet) light on a gel documentation system (Biolab Scientific, Canada) *via* a camera (Kodak, New York, USA). Images were digitally captured using Adobe Photoshop Lightroom (Adobe, California, USA).

To visualise high molecular weight genomic DNA samples, DNA was run on 0.4% (w/v) agarose gels in 1× TAE buffer for ~120 minutes at 50 V. A GeneRuler HR (High Range) DNA Ladder

(Thermo Fisher Scientific) was used according to the manufacturer's instructions. Alternatively, 0.7% (w/v) agarose gels in 1× TAE buffer with Lambda DNA/HindIII Marker 2 (Thermo Fisher Scientific) molecular weight marker, used according to the manufacturer's recommendations, was performed. These gels were run at 60 V for ~90 minutes in 1× TAE buffer. DNA staining and visualisation were as described above.

2.3.9 DNA sequencing

2.3.9.1 Sanger 16S rRNA-encoding gene sequencing

PCR products amplified using primers 27F and 1492R (**Section 2.3.7**) were purified using the QIAquick PCR Purification Kit (Qiagen) and quantified (**Section 2.3.6.4**). A 25–50 ng quantity of the purified PCR product and 4 pmol of one of either of the universal 16S rRNA gene primers (27F or 1492R) or internal primers (514R, 1100R, 1382R and FPR-2F) were brought to a total volume of 20 µL with PCR-grade water in 0.2 mL PCR tubes and sequenced at the Massey Genome Service (Massey University, Palmerston North, New Zealand) using the BigDye™ Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Waltham, Massachusetts, USA).

2.3.9.2 Whole genome sequencing

At least 10 µg of high molecular weight DNA (**Section 2.3.6.3**) and 50 µL of UltraPure™ DNase/RNase-free dH_2O were transferred using a sterile wide-bore pipette into either a 1.5 mL DNAstable® flip cap tube (Biomatrica, California, USA) or GenTegra-DNA 0.5mL screw-cap microtubes (GenTegra, California, USA). The sides of the tubes were gently flicked to mix the liquid with the DNA stabilising matrices, and dried at room temperature in a biological safety cabinet (Thermo Fisher Scientific), according to the manufacturers' recommendations, for ~118 hours. The dried samples were placed in a moisture-barrier foil pouch (Biomatrica) and sent for whole genome sequencing by GrandOmics (China). The DNA samples were quality checked and sequencing was performed using a PromethION instrument (Oxford Nanopore Technologies, United Kingdom) to a depth to achieve closed bacterial genomes.

2.3.10 Bioinformatic analysis

2.3.10.1 16S rRNA gene sequence analyses

16S rRNA gene sequence .ab1 files (**Section 2.3.9.1**) were manually trimmed of poor quality sequence, then aligned to form a consensus sequence by *de novo* assembly in Geneious version 2020.2.2 (Biomatters, 2020), which was then used as an input into the BLAST algorithm (Altschul et al., 1990) to search the GenBank non-redundant nucleotide (nr/nt) database for "somewhat similar sequences" using BLASTN.

2.3.10.2 Phylogenetic analyses

Faecalibacterium 16S rRNA-encoding gene sequences were aligned in Geneious using ClustalW (Thompson et al., 1994) and assembled into a neighbour-joining phylogenetic tree, using the HKY (Hasegawa, Kishino and Yano) (Hasegawa et al., 1985) genetic distance model and isolate C6.010 was used as an outgroup.

2.3.10.3 Genome sequence analyses

The closed genome sequence data and associated analyses, generated by GrandOmics (**Section 2.3.9.2**), were analysed. Bactopia version 1.7.X (Petit III & Read, 2020) was used to compare the genome sequences against the Genome Taxonomy Database (GTDB) (Chaumeil et al., 2020), using FastANI analysis in GTDB-tk version 1.5.0, with a reference radius of ≥95% average nucleotide identity (ANI) between genomes for classification as the same species (Jain et al., 2018). Genome-encoded proteins were annotated (BLASTP E value < 1e-05) using multiple databases, including the COG (Clusters of Orthologous Genes) (Tatusov et al., 1997) and KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa, 2000).

The presence or absence of genes in the production of butyrate from acetyl-CoA or pyruvate to ethanol was identified in the KEGG report provided by GrandOmics. Where candidate genes were not present in the KEGG report, they were manually searched for by using the reference protein sequences from *Faecalibacterium prausnitzii, F. prausnitzii* A2-165, *Faecalibacterium* sp. An58, *Faecalibacterium* sp. An121, *Fournierella massiliensis* and/or *Gemmiger formicilis* from GenBank or the Integrated Microbial Genomes & Microbiomes (IMG/M) system (Chen et al., 2021) to query the predicted protein sequences from each genome using Protein BLAST (BLASTP) (Altschul et al., 1990).

Chapter Three. Results

3.1 Development of a multiplex PCR screen to identify *Faecalibacterium*

To isolate *Faecalibacterium* from calf faecal samples, a multiplex PCR screening method to identify *Faecalibacterium* isolates was developed using two universal 16S rRNA gene primers and *Faecalibacterium*-specific 16S rRNA gene primers. Amplification of the positive control *Faecalibacterium* A2-165 DNA using 27F and 1492R with either FPR-2F and Fprau645R or Fprau 07 and Fprau 02, resulted in prominent bands consistent with the expected sizes (**Figure 2-2**) at an annealing temperature of 46.7°C (**Figure 3-1**). In contrast, amplification of the negative control *Prevotella* M384 DNA using the same primer combinations resulted in amplification of ca. 1,471 bp band only using annealing temperatures of 46.7 and 49.7°C, respectably (**Figure 3-1**). Higher yields of amplicons were generated with the FPR-2F and Fprau645R primer pair than with the Fprau 07 and Fprau 02 primer pair at the highest annealing temperature tested of 49.7°C. Therefore, the FPR-2F and Fprau645R pair was selected for screening for *Faecalibacterium* isolates.

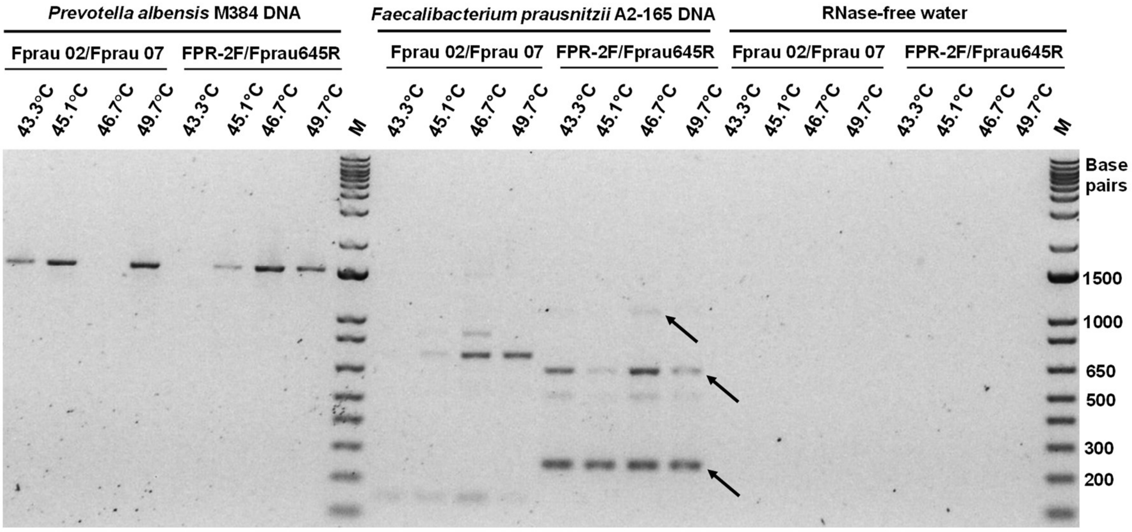


Figure 3-1. Agarose gel of PCR amplicons from multiplex PCR to determine the optimal *Faecalibacterium*-selective primer pair and thermocycling conditions to identify *Faecalibacterium*. A gradient PCR with annealing temperatures ranging from 43.3°C to 49.7°C was used and the two primer pairs were tested, Fprau 07 and Fprau 02, and FPR-2F and Fprau645R, together with universal bacterial primers, 27F and 1492R. Template DNA from reference strain *Faecalibacterium prausnitzii* A2-165 was used as a positive control, DNA from *Prevotella albensis* M384 was used as a positive control for Bacteria and negative control for *Faecalibacterium* and RNase-free water was used as a general PCR negative control. Arrows point to bands of approximately 248, 646 and 1,073 bp in size, consistent with expected amplicons generated by FPR-2F and Fprau645R multiplexed with 27F and 1492R, and also bands at approximately 129, 732 and 880 bp in size, consistent with amplicons generated by Fprau 07 and Fprau 02 multiplexed with 27F and 1492R. Bands at ~1,500 bp in length are consistent with full-length bacterial 16S rRNA genes amplified by 27F and 1492R. M: 1 Kb Plus DNA ladder, with selected band sizes indicated in bp.

The testing of bacterial colony and cell preparations as templates for PCR screening, instead of purified DNA, resulted in a higher number of amplicons using method 1, where colonies were resuspended in Milli-Q water (Method 1), compared to heating of the colony suspension at 100°C (Method 2) (**Figure 3-2**). The use of the InstaGene matrix kit-prepared template (Method 3), and colony cells directly resuspended in the PCR master mix (Method 4) provided variable results in the amplification of DNA (data not shown). Amplification of DNA from broth culture cells (Method 5) resulted in PCR fragments 1,471 bp in size (full-length 16S rRNA gene) in 60% (102 out of 170 broth cultures showing growth) of the isolates screened (data not shown). Following the development of the selective multiplex PCR screen and selection of a method to prepare DNA for PCR, calf samples were collected, cultured onto different media, and then PCR screened.

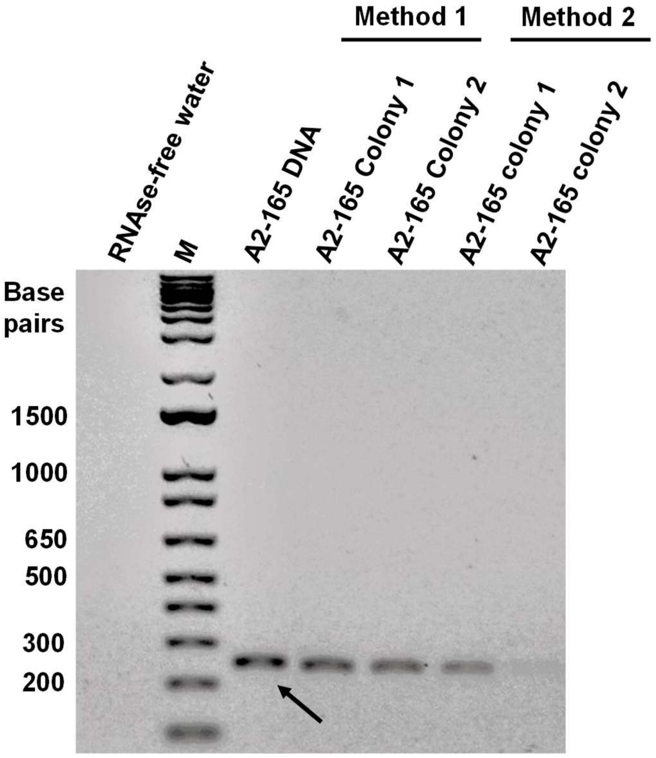


Figure 3-2. PCR of the strain A2-165 16S rRNA gene using methods (1) and (2), and *Faecalibacterium*-specific 16S rRNA gene primers. A2-165 DNA – pure DNA isolated with the NucleoSpin® Soil kit and amplified with *Faecalibacterium*-specific primers. Plate preparation Method 1 – the suspension of a fresh bacterial colony in water; Method 2 – the suspension of a fresh bacterial colony in water heated at 100°C for 5 minutes. The arrow indicates the expected DNA fragment size of ~250 bp. M: 1 Kb Plus DNA ladder.

3.2 Cultivation Trial One results

3.2.1 Isolation of calf faecal bacteria

In Cultivation Trial One, following the dilution and plating of pooled calf faecal samples, colonies appeared on YCFAG and BHI agar plates on days 2 and 3, respectively. The original faecal samples contained up to 3×10^{10} colony forming units (CFU) per gram of sample on YCFAG agar. BHI agar resulted in 1×10^{10} CFU per gram of sample.

Colonies of varying sizes, shapes and colours (ranging from white to yellow) grew on both BHI and YCFAG agar (**Figure 3-3**). In comparison, A2-165 grew faster on YCFAG than on BHI, with colonies up to ~2 mm in diameter on YCFAG by day 3 of incubation, while reaching 0.5–2 mm in diameter on BHI. A total of 33 colonies from YCFAG plates were picked for PCR screening based on the similarity of their colony morphologies to A2-165 on YCFAG (**Table 3-1**).

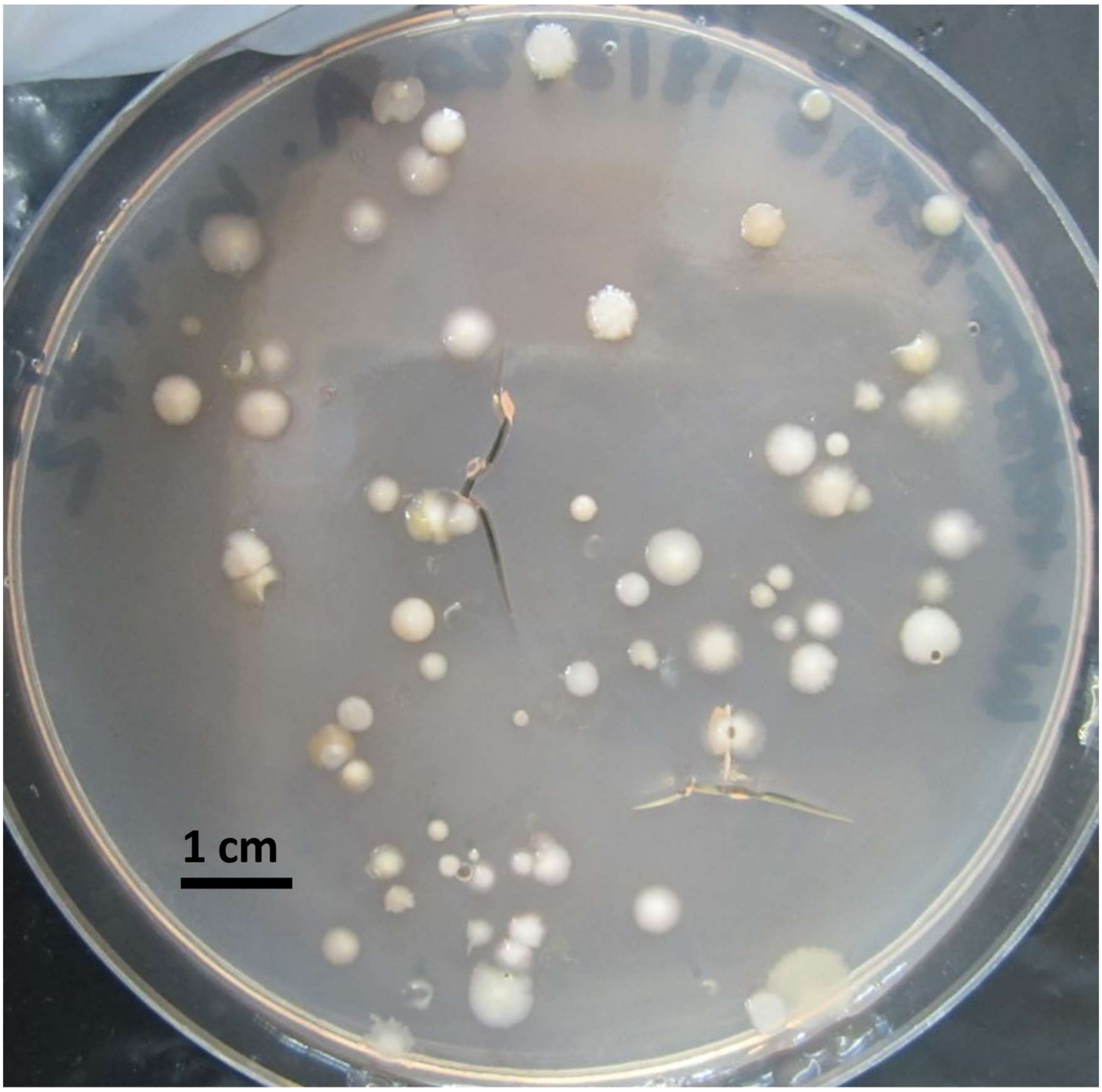


Figure 3-3. Calf faecal isolates from Pool A diluted to 10^{-7} CFU/ml and cultured anaerobically on YCFAG agar. This image was taken under aerobic conditions.

Table 3-1. Number of culturable *Faecalibacterium*-like colonies selected in Cultivation Trials One and Two.

Trial	Cultivation media	Number of colonies selected
Cultivation Trial One (Pooled calf sample isolations)	YCFAG	33 (18 from Farm 1, 15 from Farm 2).
Cultivation Trial One (Individual calf isolations)	YCFAG	50 (25 from Calf 1, 25 from Calf 6).
Cultivation Trial Two	YCFAG	222 (135 from Pool A, 87 from Pool B).
Cultivation Trial Two	Modified VTR2RF media	26 (18 from Pool A, eight from Pool B).
Total from Cultivation Trials One and Two		331

To assess whether isolates could be cultured from frozen faecal samples stored in glycerol at −80°C, faecal samples from Calf 1 from Farm 1 and Calf 6 from Farm 2 were diluted and plated onto YCFAG agar. After 3 days of incubation, colony counts indicated there were approximately 2×10^{10} CFU per gram of the original sample from Calf 1 and 1×10^{10} CFU per gram from Calf 6**.** A total of 50 colonies were picked (**Table 3-1**) to screen for *Faecalibacterium* (**Section 3.2.2**).

3.2.2 PCR screening of calf isolates and molecular identification

In Cultivation Trial One, colonies from the pooled faecal samples that amplified at least one of the three bands produced by either *Faecalibacterium*-specific 16S rRNA gene primers (248, 646 and/or 1,073 bp) were chosen for further analysis. Out of the 83 *Faecalibacterium*-like isolates selected for PCR screening, 16 gave positive PCR results using the *Faecalibacterium*-

specific primers. Five out of 16 were from the pooled Farm 1 sample (F1.004, F1.009, F1.010, F1.014 and F1.015), five from the pooled Farm 2 sample (F2.001, F2.002, F2.008, F2.013 and F2.015), four colonies from Calf 1 (C1.002, C1.021, C1.024 and C1.025) and two colonies from Calf 6 (C6.007 and C6.010). Ten isolates were purified by single colony streaking and sequencing the 16S rRNA-encoding gene. Following single colony streaking, some isolates still appeared to be contaminated due to the presence of different colony morphologies within the streaks. Each colony morphotype was single colony streaked again to obtain pure cultures, then prepared for 16S rRNA gene sequencing to determine their identities.

The top BLAST hits for all 16S rRNA gene sequences are shown in **Appendix 1**. Of the 10 faecal isolates from Cultivation Trial One sent for 16S rRNA gene sequencing, only C6.007 resulted in a top BLAST hit to a *Faecalibacterium* sequence, CLA-AA-H223 (98.55% ID) (**Table 3-2**). A few isolates returned a top hit to other bacterial genera that were closely related to *Faecalibacterium*, i.e., *Gemmiger* and *Fournierella*, which are of interest as they produce the anti-inflammatory compound butyrate (Gossling & Moore, 1975; Togo et al., 2017). *Bacteroides* and *Clostridium* were also commonly identified, which indicated that the *Faecalibacterium*-specific PCR was not stringent enough to exclude non-*Faecalibacterium* bacteria.

Few isolates with a top BLAST %identity to *Faecalibacterium*, following 16S rRNA gene sequencing, were isolated in Cultivation Trial One. If this hit rate was not representative of the true number of *Faecalibacterium* present in the faecal samples, it is likely that oxygen exposure upon sample collection may have prevented the growth of viable *Faecalibacterium* during culturing. Cultivation Trial Two (see **Section 3.3**) was conducted using stricter anaerobic conditions for sample collection and processing.

Table 3-2. 16S rRNA-encoding gene consensus sequence identities of putative *Faecalibacterium* isolates from Cultivation Trial One.

Isolate ID	Top BLAST hit[a] (based on %identity)	%identity to top BLAST hit	E-value	Query sequence length (bases)
C1.021	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.55%	0.00	1,396
C6.007	*Faecalibacterium* sp. strain CLA-AA-H223 (OK510340.1)	98.55%	0.00	1,381
C6.010	*Gemmiger formicilis* strain FAB_D4_2 (MT903192.1)	96.19%	0.00	1,409

[a] Against the non-redundant (nr/nt) database, excluding environmental ("uncultured") sample results. The GenBank accession number of each sequence is in parentheses.

3.3 Cultivation Trial Two results

3.3.1 Isolation of calf faecal bacteria

In Cultivation Trial Two, using stricter anaerobic sample collection and processing methods, including inoculating diluted samples in anaerobic agar roll tubes (**Section 2.3.3**), resulted in visible colonies after only one day of incubation. These methods yielded ~120 colonies at the 10^{-7} dilution after day one. This was equivalent to up to 4×10^9 CFU on YCFAG agar, per gram of the original faecal samples. Modified VTR2RF media roll tubes yielded an average of 25 colonies at the 10^{-7} dilution, equivalent to 8×10^8 CFU per gram of the faecal sample.

The colonies on both types of media were of varying shapes and colours (ranging from white to yellow), with the colonies on YCFAG being mostly ~1 mm in diameter (**Figure 3-4**) and those on modified VTR2RF media being generally smaller in comparison.

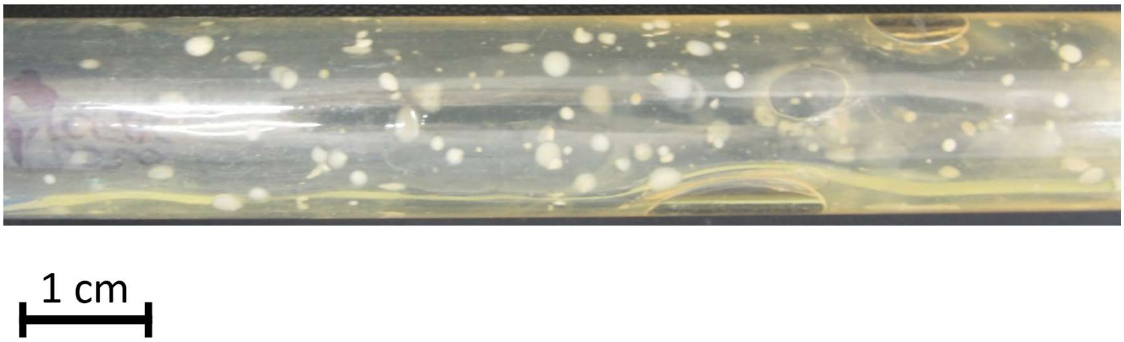


Figure 3-4. Anaerobic agar roll tube showing colony growth. This anaerobic YCFAG agar roll tube showed growth of calf faecal bacteria that had been diluted to 10^{-7} CFU/ml from the original faecal sample.

In parallel to inoculating agar roll tubes, samples were spread on YCFAG agar plates in the anaerobic chamber and visible colonies were observed by the end of day three. Here, estimates of the CFU concentrations in the original samples were approximately 2×10^9 CFU per gram from the original Pool A faecal material, and approximately 1×10^9 CFU per gram from Pool B**,** which is considerably lower than estimates based on the roll tube method.

A total of 248 colonies were picked from roll tubes over approximately 7 weeks for PCR screening (**Table 3-1**). The colony morphology criteria used for selection were broadened, to allow the possibility of capturing *Faecalibacterium* isolates that did not strictly fit the A2-165 colony morphology.

3.3.2 PCR screening of calf isolates and molecular identification

In Cultivation Trial Two, 248 colonies from both the YCFAG and modified VTR2RF media roll tubes (**Table 3-1**) were selected for PCR screening. As colonies were selected randomly compared to Cultivation Trial One, where only *Faecalibacterium*-like colonies were selected, this less stringent selection criterion resulted in many false positives (i.e., non-*Faecalibacterium* bacteria) following 16S rRNA gene sequencing. Only isolates that amplified the 248 bp band (produced from both *Faecalibacterium*-specific 16S rRNA gene primers) were selected for further analysis. In total, four out of 248 isolates yielded 248 bp bands (**Figure 3-5**). Three isolates were from Pool A (AY003, AY005 and AY052) and one from Pool B (BY057). During single-colony isolation of these isolates on agar to obtain pure cultures, three appeared to be mixed cultures, and individual colonies were assessed to determine their identities by 16S rRNA gene sequencing (AY003, AY003_3, AY003_7, AY005, AY005_A5, AY052, BY057 and BY057_B7).

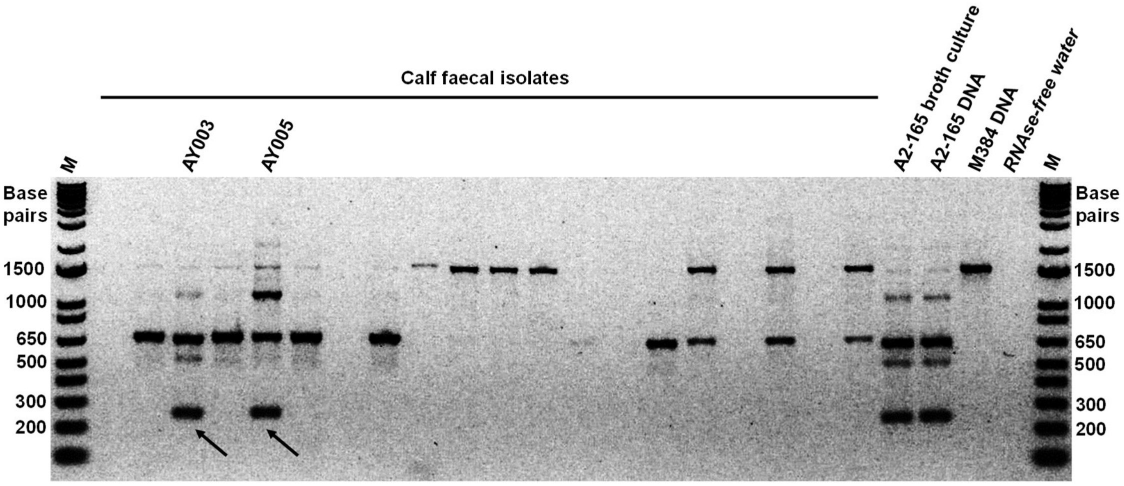


Figure 3-5. An example of an agarose gel with PCR amplicons (248 colonies) showing two faecal isolates from Cultivation Trial Two (named AY003 and AY005) that amplified the *Faecalibacterium*-specific fragment (arrows). Lanes: A2-165 DNA is *Faecalibacterium* PCR control, M384 DNA is non-*Faecalibacterium* control. M is 1 Kb Plus DNA ladder.

Sequencing of 16S rRNA-encoding gene amplicons generated from each isolate using universal and *Faecalibacterium*-specific primers gave ambiguous results for several isolates (AY003, AY005, AY052 and BY057) despite single colony purification of these isolates prior to amplification. Top BLAST hits aligned to *Faecalibacterium* or *Fournierella* sequences, as well as other organisms such as *Bacteroides* and *Clostridium* (**Appendix 1**), which suggested that the cultures were not pure. Gram staining (Sambrook & Russell, 2001) of AY003, AY005 and BY057 suggested these isolates were mixed cultures (data not shown). Therefore, these isolates were streaked to single colonies, and 15 well-separated colonies were checked by the multiplex PCR screen for *Faecalibacterium*. Two banding patterns were observed for the AY005 colonies, therefore AY003_A3 and AY003_A7 were selected as representatives for each banding pattern for AY003 (**Figure 3-6**). Additionally, AY005_A5 was selected as the representative colony for AY005 colonies, and BY057_B7 for BY057 colonies (data not shown). The 16S rRNA gene sequence identities of the putative *Faecalibacterium* and *Fournierella* isolates are shown in **Table 3-3**. Following confirmation of the 16S rRNA gene identities of the isolates, only those that had a top BLAST hit to *Faecalibacterium* or its close relatives were morphologically assessed.

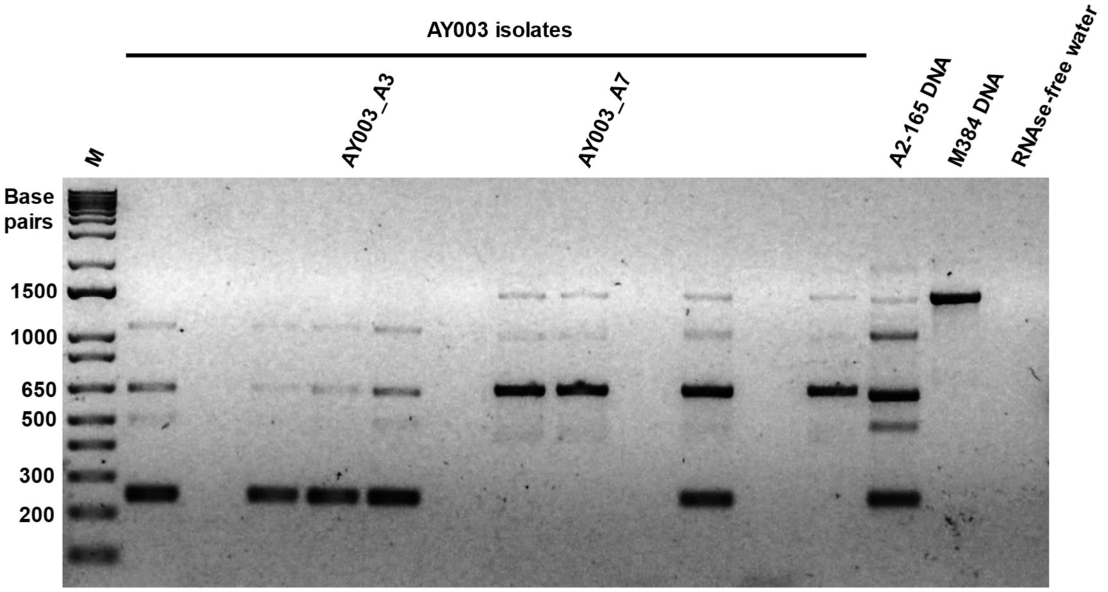


Figure 3-6. Agarose gel of PCR amplicons from single colonies that were selected from re-streaked isolate AY003. Lanes: A2-165 DNA is *Faecalibacterium* PCR control, M384 DNA is non-*Faecalibacterium* control. M is 1 Kb Plus DNA ladder.

Table 3-3. 16S rRNA-encoding gene sequence identities of putative *Faecalibacterium* isolates from Cultivation Trial Two.

Isolate ID	16S rRNA gene primer[a] or consensus sequence	Top BLAST hit[b] (based on %identity)	%identity to top BLAST hit	E-value	Query sequence length (bases)
AY003	Consensus sequence[c]	*Faecalibacterium prausnitzii* strain 02_10294632002__013 (KJ957842.1)	96.57%	0.00	525
AY005	FPR-2F	*Fournierella massiliensis* strain PCK025 (MN913794.1)	97.92%	0.00	694
AY005_A5	FPR-2F	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.25%	0.00	515
BY057	Consensus sequence[c]	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.33%	0.00	1,321

[a] 16S rRNA gene sequencing primer.

[b] Against the non-redundant (nr/nt) database, excluding environmental ("uncultured") sample results. The GenBank accession number of each isolate is in parentheses.

[c] Consensus sequence derived from the universal 16S rRNA gene forward primer 27F and internal primer FPR-2F.

3.4 Cell morphologies of calf *Faecalibacterium, Gemmiger formicilis* and *Fournierella massiliensis* isolates

To further characterise the putative *Faecalibacterium* calf isolate C6.007 and other closely related strains (C1.021, C6.010 and AY005_A5), their cell morphologies were assessed using light microscopy and Gram staining. The calf-derived isolates, AY005_A5, C1.021, C6.007 and C6.010 all stained as Gram-negative, chain-forming rods when grown in YCFAG broth (**Figure 3-7**).

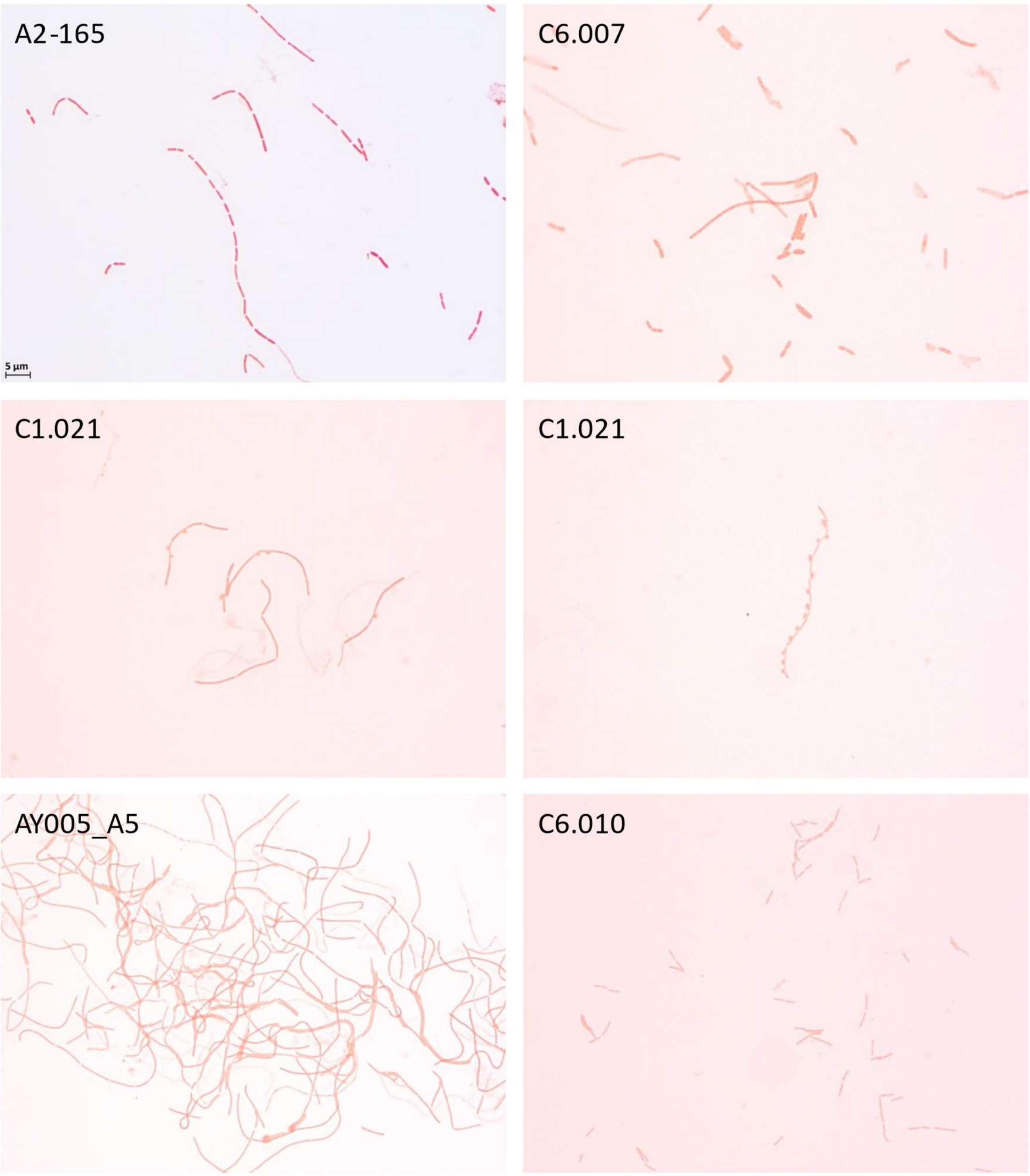


Figure 3-7. Gram-stained cultures of four *Faecalibacterium*-like isolates cultured in YCFAG media. The 5 µm scale bar applies to all panels.

Cells of the putative *Faecalibacterium prausnitzii* isolate, C6.007, and A2-165 were both approximately 2−6 µm × 1 µm. Some C6.007 cells appeared longer, though they may have been a chain of cells, possibly due to low resolution. Most AY005_A5 (putative *Fournierella massiliensis*) rods were approximately 2−10 µm × 0.5−1 µm, with some thicker rods that were approximately twice the width of the other rods. C1.021 (putative *Fournierella massiliensis*) rods were approximately 0.5 µm × 7 µm in length. In contrast to AY005_05, approximately half of the cells had bud-like protrusions extending from the lengths of the cells. C6.010 (putative *Gemmiger formicilis*) rods were approximately 0.5 µm × up to 5 µm.

Overall, all four isolates were Gram-negative and rod-shaped. *F. massiliensis* C1.021 unexpectedly displayed extracellular protrusions. To further characterise these strains *in vitro* growth dynamics experiments were performed.

3.5 Growth dynamics

The growth dynamics of the calf isolates, compared to A2-165, were determined in anaerobic YCFAG broth over 24 hours at 37°C. All isolates reached stationary phase at ~15 hours of incubation (**Figure 3-8**). Both *Faecalibacterium* isolates, A2-165 and C6.007, reached the maximum measurable OD_{600nm} 2.50 15 hours after inoculation. The *Fournierella* isolates, AY005_A5 and C1.021, reached maximum OD_{600nm} of 1.80 and 2.20, respectively, after 15 hours of incubation. The *Gemmiger* isolate, C6.010 grew more poorly than the other isolates under the growth conditions, reaching a maximal OD_{600nm} of 0.32 at 15 hours of incubation.

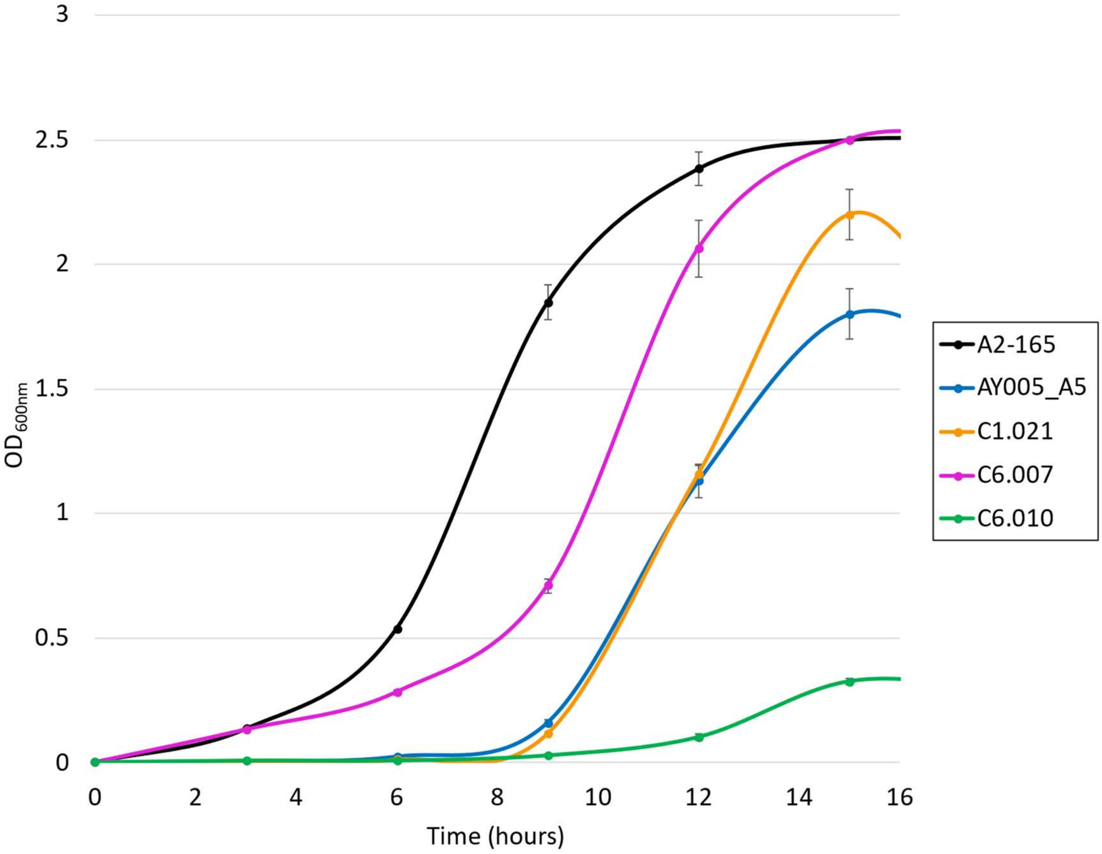


Figure 3-8. Growth of calf bacterial isolates and strain *F. prausnitzii* A2-165 in YCFAG media. Measurements were taken over 24 hours at 37°C. The average OD_{600} ± standard error of the mean (SEM) is shown.

The growth of C6.010 and C6.007 in BHI broth at 37°C was additionally assessed to attempt to promote greater biomass of C6.010 and identify active mid-log phase growth of C6.007 for high molecular weight DNA extractions for genome sequencing (**Section 3.8**). OD measurements were taken until stationary phase was reached (**Figure 3-9**). C6.010 began entering stationary phase after 15 hours of incubation, attaining a mean OD_{600nm} of 0.39, approximately 0.1 higher than when cultured in YCFAG. C6.007 reached mid-log phase after ~8 hours and stationary phase after ~13 hours, with a maximum OD_{600nm} of approximately 1.83, which was lower than the maximum OD_{600nm} reached when cultured in YCFAG.

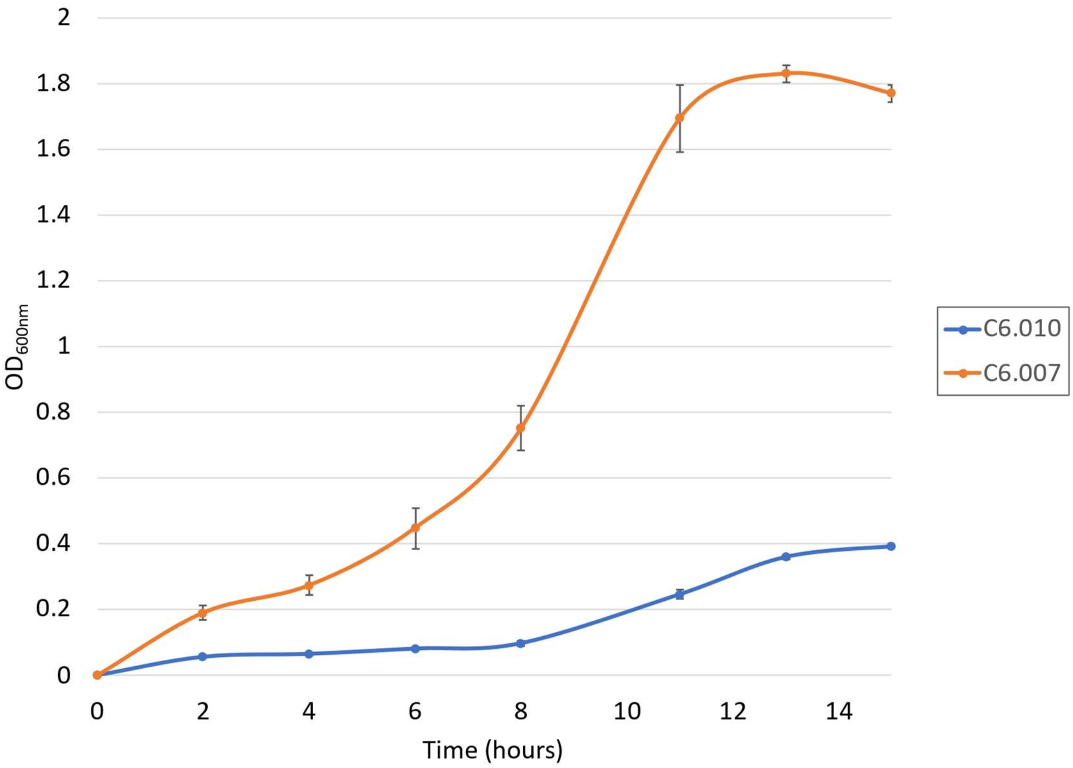


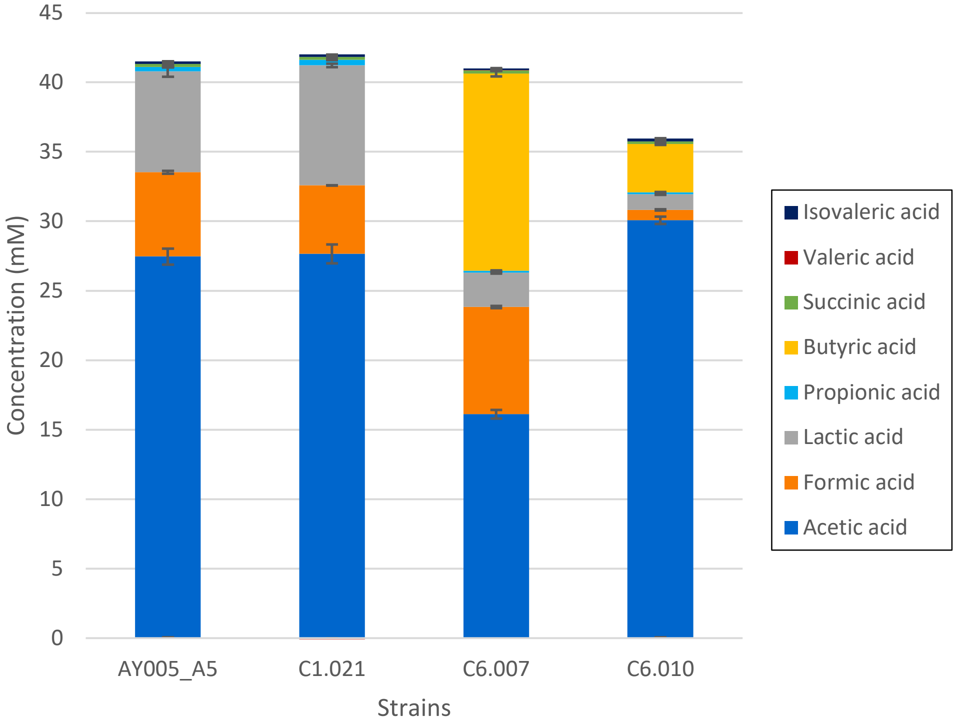
Figure 3-9. Growth of C6.010 and C6.007 in BHI. Performed over 24 hours at 37°C. The average OD_{600} ± SEM for each measurement is shown (n = 5).

Overall, the growth curves of all strains reached stationary phase after ~15 hours of incubation in YCFAG, and after ~13 hours in BHI. The biomass produced from strain C6.010 was significantly lower compared to all other strains, even in a general rich medium (BHI). In the next step of characterisation of all four strains production of butyrate, a major anti-inflammatory SCFA, and other SCFAs were assessed.

3.6 SCFA analysis

Analysis of SCFA profiles from stationary phase cultures of the faecal isolates were obtained by derivatised SCFA analysis. The results showed that C6.007 and C6.010 were the only isolates to produce butyric acid, with C6.007 producing 14.2 mM of butyric acid – approximately four times more than C6.010, which produced 3.46 mM (**Figure 3-10A**). All isolates produced formic, lactic, acetic, succinic and isovaleric acid. The production of acetic acid and butyric acid was increased to 0.95 mM in AY005_A5 and 1.61 mM in C6.007, respectively, between stationary phase and at 24 hours (**Appendix 2**). All isolates showed an insignificant difference in SCFA concentrations from stationary phase to 24 hours post-inoculation, with the largest differences being a reduction of acetic acid present by 2.93 mM for C6.010, and an increase in ethanol by 2.39 mM in AY005_A5 (**Figure 3-10** and **Appendix 2**). Isobutyric acid, caproic acid, methanol and propanol were not present above the limits of detection.

A



B

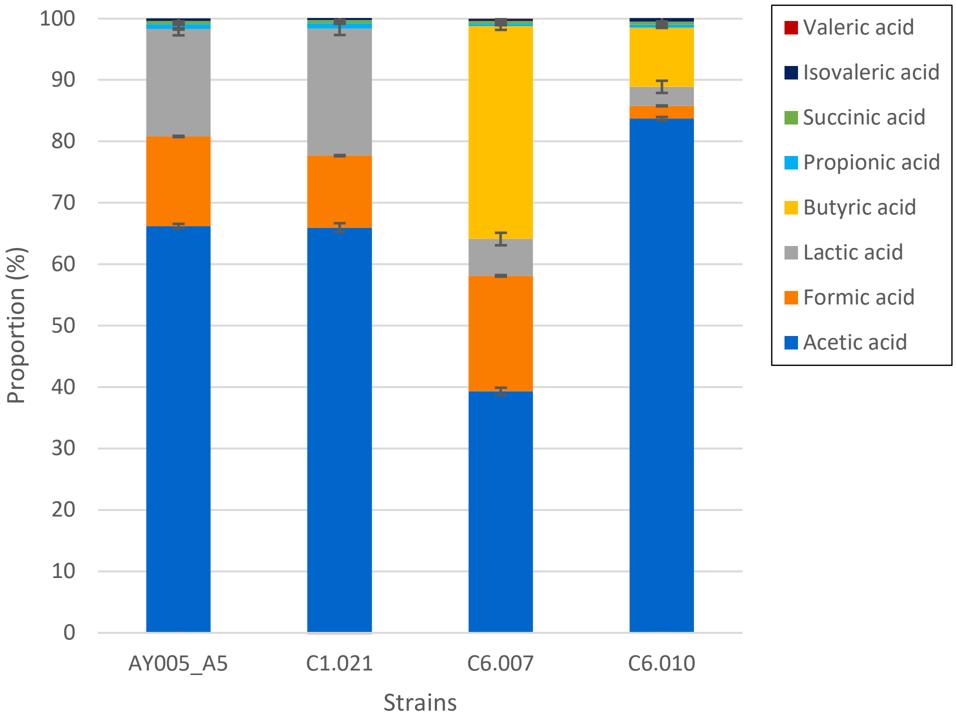


Figure 3-10. The mean concentration (A) and proportions (B) of SCFAs detected at stationary phase cultures of AY005_A5, C1.021, C6.007 and C6.010. Error bars show the upper and lower ranges of the SEM (n = 2 for AY005_A5, C1.021 and C6.010; n = 4 for C6.007).

High concentrations (>30 mM) of ethanol were produced by the putative *Fournierella* strains (C1.021 and AY005_A5), and ethanol was detected in the putative *Faecalibacterium* C6.007 culture, but not C6.010 isolate (**Figure 3-11**).

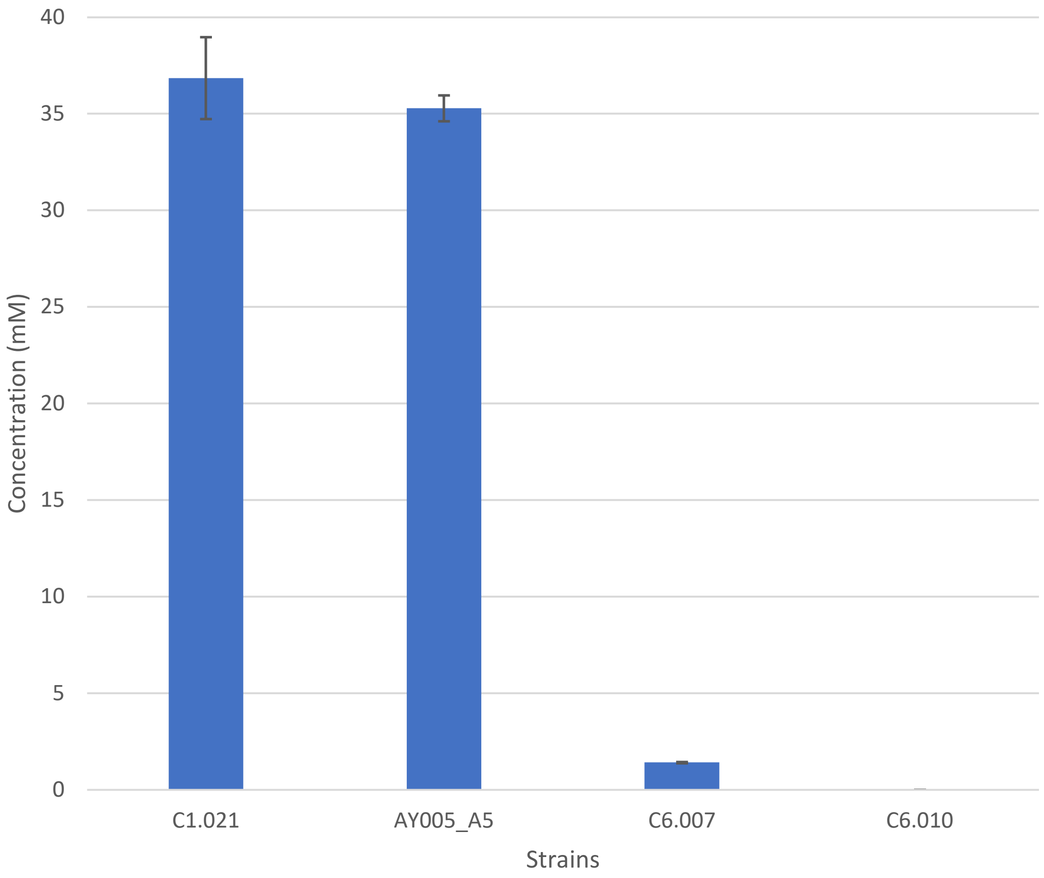


Figure 3-11. Ethanol concentrations in stationary phase cultures of AY005_A5, C1.021, C6.007 and C6.010 grown in YCFAG. Error bars show the upper and lower ranges of the SEM (n = 2 for AY005_A5, C1.021 and C6.010; n = 4 for C6.007).

We have confirmed the production of butyrate from our putative *Faecalibacterium* C6.007 and putative *Gemmiger formicilis* C6.010 strains, and also noted that high amounts of ethanol were produced by both putative *Fournierella massiliensis* strains C1.021 and AY005_A5.

3.7 Phylogenetic analysis of *Faecalibacterium* and other close relatives

The full-length 16S rRNA genes from the four isolates and other published calf-derived *Faecalibacterium* (Kumar et al., 2021) were used to reveal phylogenetic relationships. A neighbour-joining tree built using C6.007, A2-165 and other calf-derived *Faecalibacterium* showed that C6.007 was most closely related to A2-165 and was genetically distinct to other New Zealand calf-derived *Faecalibacterium* strains (**Figure 3-12**). C6.007 and A2-165 also appeared to belong to the same phylogroup. Overall, all isolates were closely related based on 16S rRNA gene analysis. Whole genome sequencing was then used to assess the similarities between the four strains at the genomic level and to confirm the presence of the butyrate synthesis pathway in the butyrate-producing isolates.

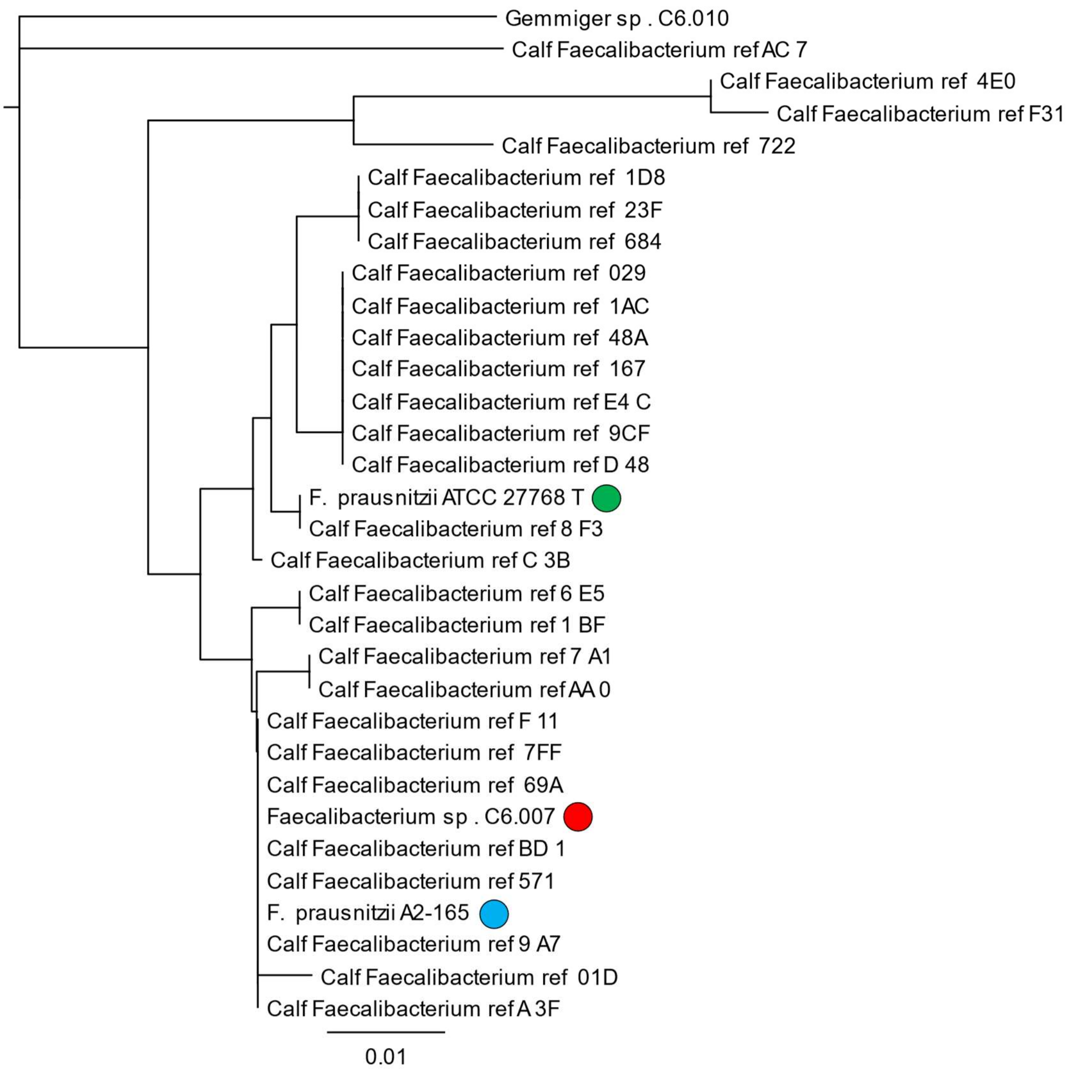


Figure 3-12. Phylogenetic tree of 16S rRNA gene sequences from *Faecalibacterium* sp. isolate C6.007 and other New Zealand calf-derived *Faecalibacterium*. Isolate C6.007 (red circle), reference *F. prausnitzii* strain A2-165 (blue circle), *F. prausnitzii* type strain ATCC 27768^T (green circle) and other New Zealand calf-derived *Faecalibacterium* strains are shown. Isolate C6.010 (*Gemmiger* sp.) was used as the outgroup.

3.8 Genome sequence analysis

Genome sequence analysis was used to determine the similarities between the four isolates (C6.007, C1.021, C6.010 and AY005_A5) at the whole genome level and to determine the butyrate synthesis pathways used by the two butyrate-producing isolates C6.007 and C6.010. Quality checks of the DNA samples are shown in **Appendix 3**. The genomes of C6.007, C1.021, C6.010 and AY005_A5 were closed after whole genome sequencing and a summary of the genome details is given in **Table 3-4**. The isolate identities, as determined by the top genome sequence highest average nucleotide identities (ANIs) to the Genome Taxonomy Database (GTDB), were: C6.007 to *Faecalibacterium prausnitzii*, AY005_A5 to *Fournierella massiliensis*, C1.021 to *F. massiliensis* and C6.010 to *Gemmiger* sp004555405, suggesting that the C6.010 was most closely matched to a currently undescribed species (**Figure 3-13** and **Table 3-4**). ANI analysis using the NCBI top BLAST hit showed that strain C6.007 was more closely related to *F. prausnitzii* A2-165 than to *F. prausnitzii* ATCC 27768^T, and there were small differences in the ANIs of C1.021 and AY005_A5 to each other and to *F. massiliensis* AT2^T. There were no obvious plasmid sequences found in any of the isolates *via* BLASTN comparison to the plasmid database, though additional closed contigs were assembled in AY005_05 and C6.010 that displayed average sequencing depths that differed to the main chromosome (**Table 3-4**). C6.010 also had the greatest number of contigs and the highest proportion of genes assigned to the COG and KEGG databases. Out of all four strains, C6.010 had the highest ANI% to a GTDB reference genome, while C6.007 had the lowest, which was unexpected – as there are more *Faecalibacterium* strains publicly available than *Gemmiger*, therefore there would have been a better chance of C6.007 having a high ANI% to a known strain.

Table 3-4. Genome sequencing details for C6.007, C1.021, AY005_A5 and C6.010.

	Isolate			
Parameter	**C6.007**	**C1.021**	**AY005_A5**	**C6.010**
GTDB classification *via* FastANI	*Faecalibacterium prausnitzii* C	*Fournierella massiliensis*	*Fournierella massiliensis*	*Gemmiger* sp004555405
GTDB ID[a] of closest reference genome	GCF_010509575.1	GCF_004345265.1	GCF_004345265.1	GCA_004555405.1
ANI (%) to closest reference genome	96.05	97.00	96.82	98.93
NCBI top BLAST hit 10[b]	*Faecalibacterium prausnitzii* A2-165 (JCM 31915)	*Fournierella massiliensis* (DSM 100451)	*Fournierella massiliensis* (DSM 100451)	*Subdoligranulum* sp. (W1P18.021)
No. of raw reads	641,164	136,698	121,982	810,295
No. of filtered reads	496,753	82,830	86,851	610,775
N_{50} of filtered reads (bp)	4,115	9,624	6,555	4,111
Genome size (Mb)	2.877	3.487	3.731	2.552
No. of contigs	1	1	2	3
Contig sizes (Mb)	2.877 (tig00001)	3.487 (tig00001)	3.686 (tig00001) 0.045 (tig00002)	2.256 (tig00001) 0.184 (tig00002) 0.111 (tig00003)

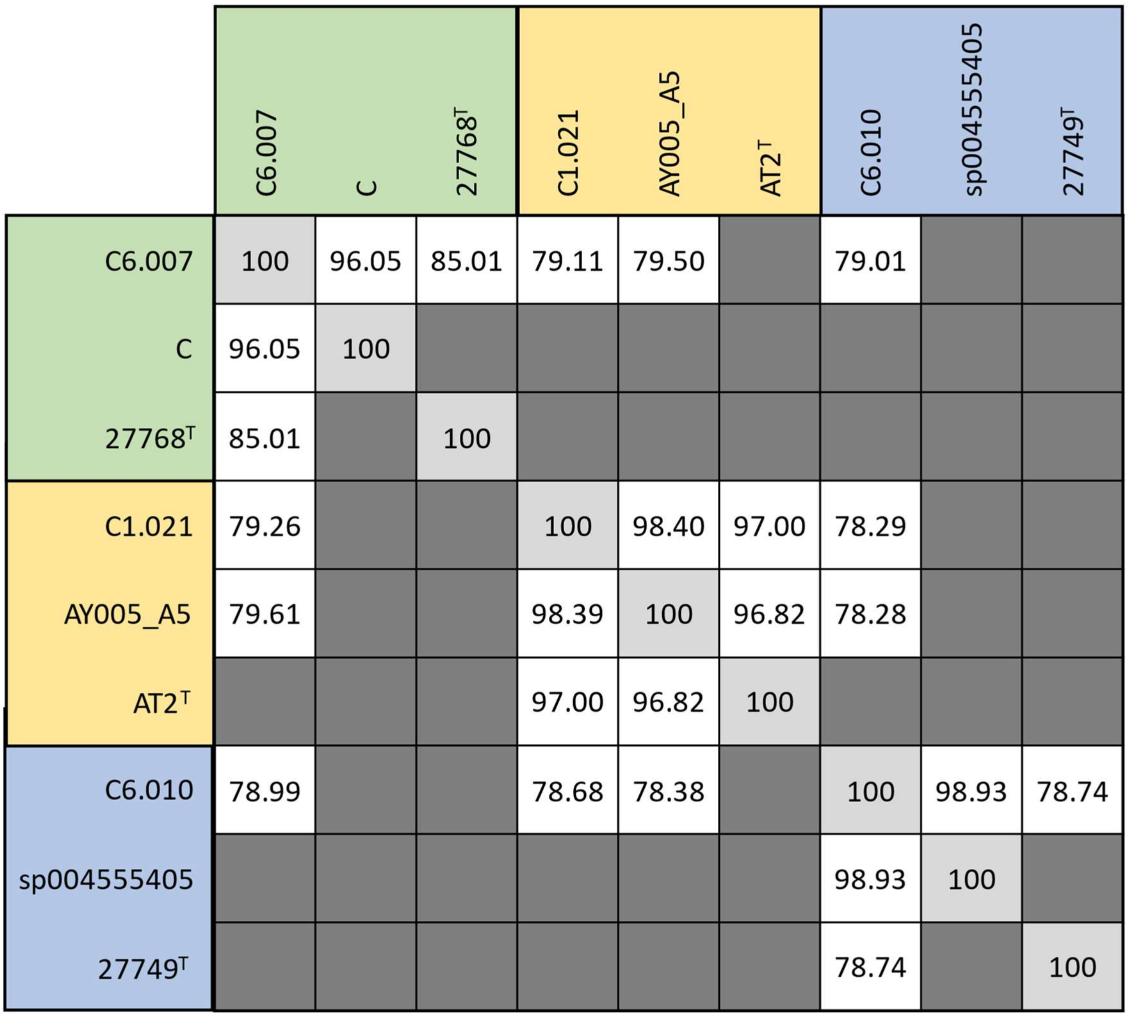
	Isolate			
Parameter	**C6.007**	**C1.021**	**AY005_A5**	**C6.010**
Sequencing coverage, average (×)	399.18	118.22	90.59 (tig00001) 73.71 (tig00002)	492.23 (tig00001) 1092.53 (tig00002) 721.05 (tig00003)
GC content (%)	56.60	57.39	57.10	54.91
CDS[c]	2,810	3,206	3,508	2,328
rRNAs (5S, 16S, 23S)	6 (5S), 6 (16S), 6 (23S)	7 (5S), 5 (16S), 5 (23S)	6 (5S), 5 (16S), 5 (23S)	5 (5S), 5 (16S), 5 (23S)
tRNAs	67	82	80	72
RNA[d] other	14	10	14	8
Genes assigned to COG database (%)	67.86	70.96	67.39	71.31
Genes assigned to KEGG database (%)	43.52	43.54	41.11	48.15

[a] ID: identity.

[b] NCBI strain identifier in parentheses.

[c] CDS: coding sequence.

[d] RNA: ncRNAs including snRNA, siRNA, etc.



	C6.007	C	27768^{T}	C1.021	AY005_A5	AT2^{T}	C6.010	sp004555405	27749^{T}
C6.007	100	96.05	85.01	79.11	79.50		79.01		
C	96.05	100							
27768^{T}	85.01		100						
C1.021	79.26			100	98.40	97.00	78.29		
AY005_A5	79.61			98.39	100	96.82	78.28		
AT2^{T}				97.00	96.82	100			
C6.010	78.99			78.68	78.38		100	98.93	78.74
sp004555405							98.93	100	
27749^{T}							78.74		100

Figure 3-13. ANI pairwise comparisons of genomes. The *Faecalibacterium*, *Fournierella* and *Gemmiger* isolate genomes were compared to each other and their top ANI matches in GTDB, and most closely related strains, as determined by FastANI analysis (Jain et al., 2018). C6.007 was compared to *Faecalibacterium prausnitzii* C (C) and *F. prausnitzii* ATCC 27768^{T} (27768^{T}); C1.021 and AY005_A5 were both compared to *Fournierella massiliensis* AT2^{T} (AT2^{T}); and C6.010 to *Gemmiger* sp004555405 (sp004555405) and *Gemmiger formicilis* ATCC 27749^{T} (27749^{T}).

Genome maps of the main chromosomes of each isolate are shown in **Figure 3-14.** There were also two CRISPR sequences identified in C1.021, and one in each of both C6.007 and C6.010 (**Table 3-5**). Summaries of COG and KEGG classification of genes are shown in **Appendix 4**. COG classification profiles were similar among isolates and identified the most genes under the “Translation, ribosomal structure and biogenesis” group for C6.007 and C6.010, and the

“Transcription” category for AY005_A5 and C1.021. KEGG classifications showed that all isolates had the most genes (>400) in the “Metabolism” group. Additionally, the isolates had similar numbers of genes allocated to the “Genetic Information Processing” group as the “Environmental Information Processing” group, except C6.010, which had approximately half the number of “Environmental Information Processing” genes as “Genetic Information Processing” genes. Overall, the GTDB classifications of each isolate were similar to the top BLAST identities from 16S rRNA gene sequences (**Table 3-2** and **Table 3-3**).

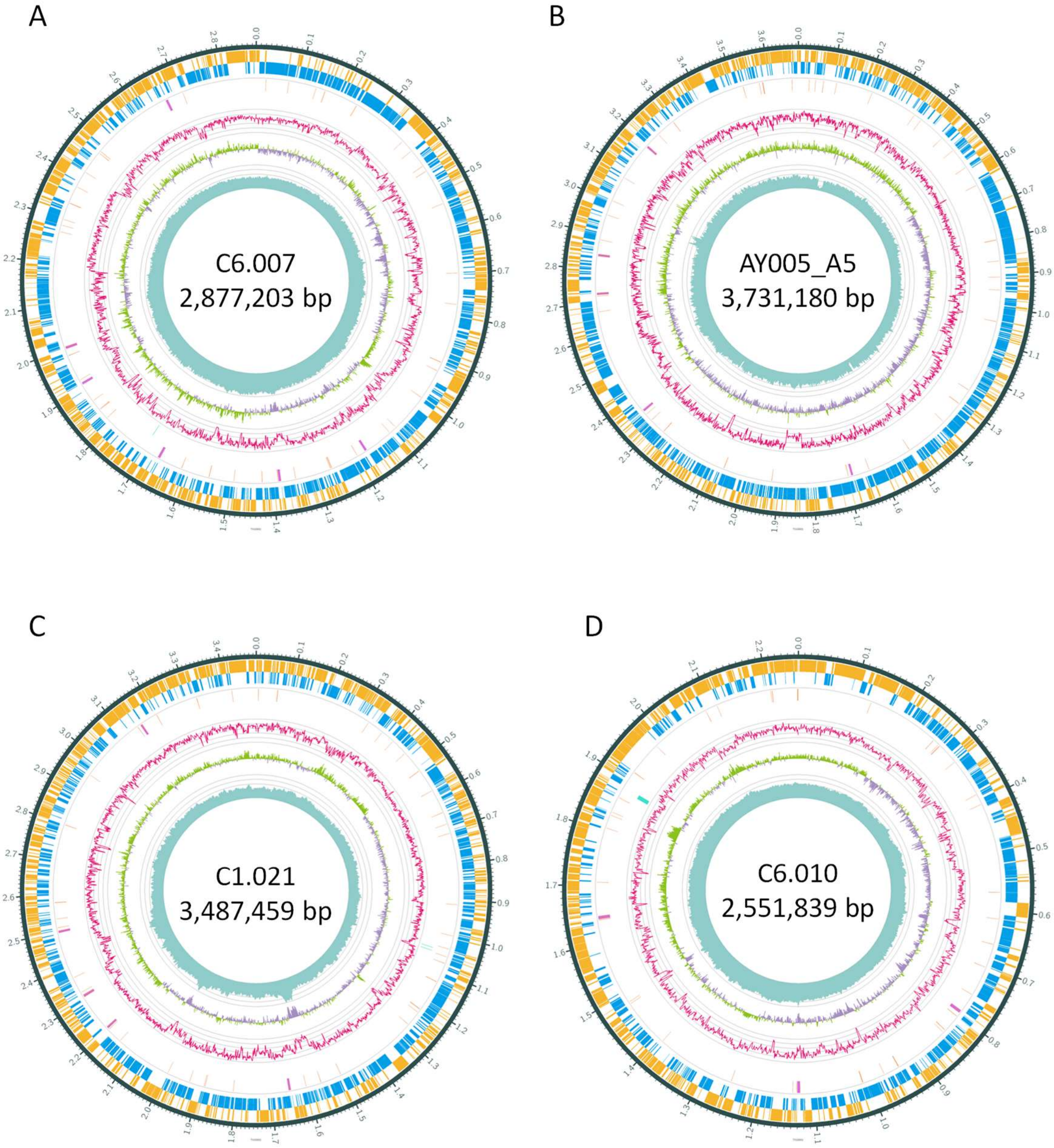


Figure 3-14. Genomic maps of the main chromosomes of calf faecal isolates (A) C6.007, (B) AY005_A5, (C) C1.021 and (D) C6.010. From the outermost ring to the innermost ring: coding genes on the sense strand (orange); coding genes on the antisense strand (blue); tRNA genes (orange) and rRNA genes (purple); clustered regularly interspaced short palindromic repeats (CRISPR) (pale blue); guanine-cytosine (GC) ratio (pink); GC-skew (green and purple); and sequencing depth.

3.8.1 Butyrate pathway gene analysis

The six genes within the pathway for butyrate production from acetyl-CoA *via* butyrate kinase removal of CoA from butyryl-CoA (*thl, hbd, cro, bcd* and *but*) were found in the butyrate-producing strains, C6.007 and C6.010. The presence of these genes suggested that butyrate was produced *via* the acetyl-CoA to butyrate pathway, and butanoyl phosphate was not produced as an intermediate (**Figure 3-15**). Identifying the genetic potential to form butyrate was consistent with the observations of high butyrate production in C6.007 and C6.010 from SCFA analysis. There was one copy of each gene from the pathway present in both C6.007 and C6.010, except two copies of the *hbd* gene were found in C6.010. In contrast, none of the copies of any of the seven genes in the acetyl-CoA to butyrate pathway were found in the genomes of C1.021 and AY005_A5 following both KEGG assessment by GrandOmics, and additional searches for the genes using the NCBI Gene database or Integrated Microbial Genomes & Microbiomes (IMG/M) system.

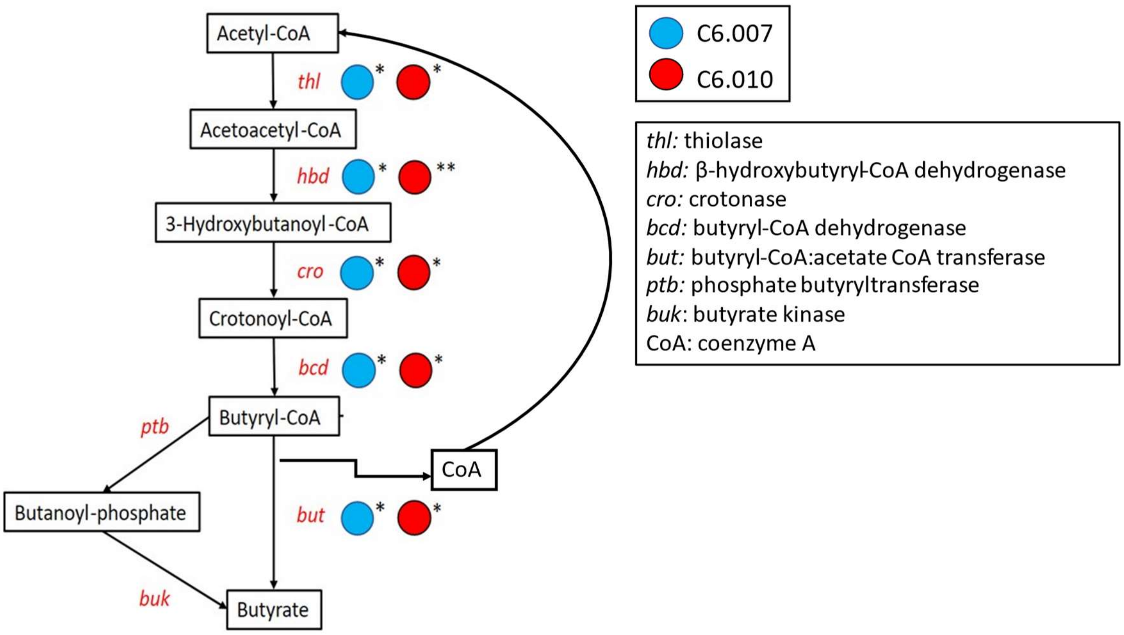


Figure 3-15. The acetyl-CoA to butyrate pathway. Genes that are present in C6.007 (blue circle) and C6.010 (red circle). Asterisks (*) indicate the number of copies of the gene present. Image modified with permission from Zou et al. (2021).

3.8.2 Ethanol pathway gene analysis

As ethanol was detected by gas chromatography, the ethanol production pathway genes were queried against those assigned to the KEGG database by GrandOmics. The three genes of the Por-AldH-Adh pathway were found in the ethanol-producing strains C1.021 and AY005_A5 (**Figure 3-16)**. In addition, genes encoding for acetate kinase (*ack*) and the bifunctional enzyme aldehyde-alcohol dehydrogenase (*adhE*) were also detected in both strains. Isolate C6.007 produced ~30× less ethanol in comparison to C1.021 and AY005_A5 and *adhE*, *por,* phosphotransacetylase (*pta*), *ack* and aldehyde:ferredoxin oxidoreductase (*aor*) were detected (the *por*, *pta*, *ack* and *aor* genes are all part of one of the three ethanol synthesis pathways). The full gene complements for the enzymes in the other ethanol synthesis pathways were either not present in the *F. prausnitzii* and *F. massiliensis* reference strain from the NCBI Gene database (Sayers et al., 2022) or Integrated Microbial Genomes & Microbiomes (IMG/M) system (Chen et al., 2021), and were not further pursued within C1.021 and AY005_A5 or showed no significant similarity to the C1.021 and AY005_A5 following BLASTP analysis.

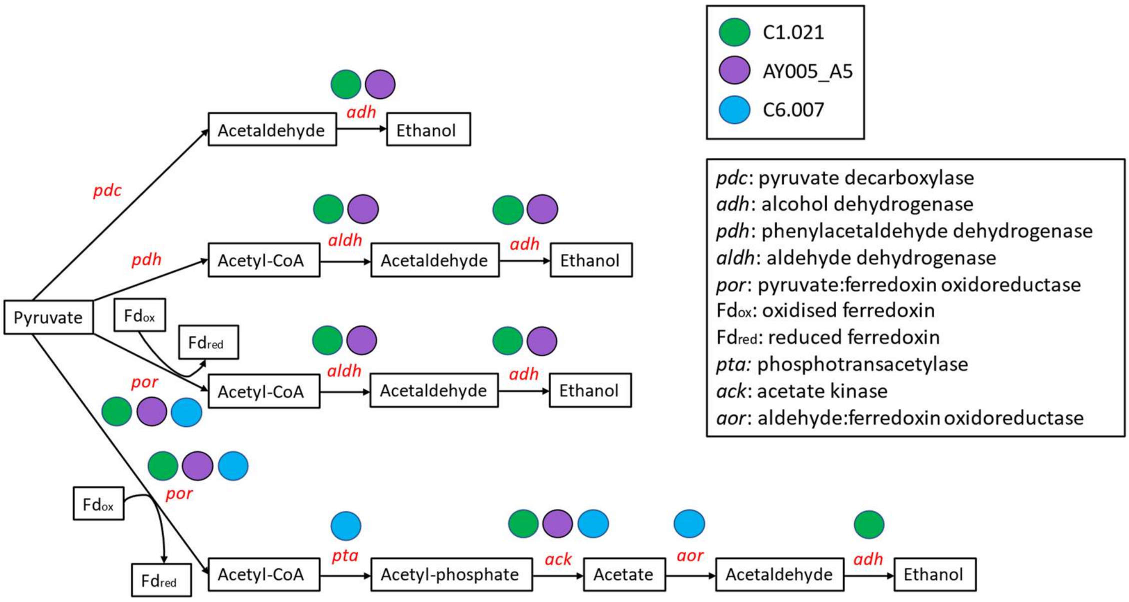


Figure 3-16. The pyruvate to ethanol metabolic pathways. Genes that are present in C1.021 (green circle), AY005_A5 (purple circle) and C6.007 (blue circle). Image adopted from Nissen and Basen (2019) with permission.

Chapter Four. Discussion

The aim of this study was to isolate and characterise *Faecalibacterium* spp. from New Zealand calves. Out of 331 *Faecalibacterium*-like colonies screened by using a newly developed PCR assay to screen for *Faecalibacterium*, only one *Faecalibacterium* strain, and several relatives of interest, were identified. The number of *Faecalibacterium* isolates cultured was much lower than expected, given that *Faecalibacterium* has been previously reported to be one of the most abundant bacteria in whole milk-fed calves (Maclean et al., 2018; Malmuthuge et al., 2014). Potential reasons for the low number of *Faecalibacterium* isolates obtained could be that the *Faecalibacterium*-specific 16S rRNA gene primers used in the PCR screen to identify *Faecalibacterium* were not specific enough, as non-*Faecalibacterium* bacteria, including *Bacteroides* and *Clostridium,* did amplify using these primers. It is not known whether the calf *Faecalibacterium* in the samples were not being amplified or its abundance in faecal samples was low. It is also possible that the growth conditions in this study were unfavourable for the growth of the *Faecalibacterium* from the New Zealand calf samples examined, and a factor required for their growth was missing, despite A2-165 being able to grow. Potentially a calf digesta or faecal extract added to the growth medium may supply such a growth substrate to enable the calf-derived *Faecalibacterium* to grow. It is possible that *Faecalibacterium* was absent or lowly abundant in the calves that were sampled in this study. Faecal microbiome community profiling, or qPCR to estimate the amount of *Faecalibacterium* in the samples would allow us to assess whether the number of isolates obtained in this study was expected for the abundance in the sample. Nonetheless, the recovery of one *Faecalibacterium* isolate and a number of close relatives (*Fournierella* and *Gemmiger*) in this study has allowed us to gain more insight into the biology of these species from New Zealand calves.

4.1 Cultivation of *Faecalibacterium*

The 16S rRNA-encoding gene-based multiplex PCR screening that was developed in this study to identify *Faecalibacterium* isolates was not as specific for *Faecalibacterium* as intended. Only ca. 3.6% of isolates that yielded PCR products indicative of amplification with the *Faecalibacterium* specific primers identified as *Faecalibacterium* sp. across the two cultivation trials conducted. It is not currently known what the original abundance of *Faecalibacterium* was in the original faecal samples, but on the assumption that 0.03% (1 out of 331) was representative of the abundance of *Faecalibacterium* in our samples, this number was substantially lower than what had been previously observed, such as ~8% (Maclean et al., 2018) to ~15% (Malmuthuge et al., 2014) in whole milk-fed calves (the latter being seen in the colon tissue of 3-week-old calves). However, the calves that were sampled in this project were 5-6-weeks old, and it had been previously reported that *Faecalibacterium* abundance decreases with age following a peak (~20% of the total 16S rRNA sequences) in 1-week-old calves fed milk replacer, to ~5% of the total 16S rRNA sequences when the calves were 5-weeks-old (Uyeno et al., 2010). Therefore, it is possible that *Faecalibacterium* abundance in the 5-6-week-old calves may have already dropped significantly from the 3.6-15% abundance range.

From Cultivation Trial One, only one isolate that amplified with at least one of the pair of *Faecalibacterium*-specific primers appeared to be closely related to the *Faecalibacterium prausnitzii* strain A2-165 by both 16S rRNA gene and whole genome sequences (**Section 3.8**). This showed that A2-165-like *Faecalibacterium* strains can be successfully isolated from New Zealand dairy calves, although not easily, with the current methodology. In Cultivation Trial Two, anaerobic conditions were maintained more stringently during sample collection and inoculation, using agar roll tubes, as compared to anaerobic agar plates in the anaerobic chamber. These changes were made to support *Faecalibacterium* viability. Unexpectedly, no *Faecalibacterium* were identified among the isolates screened, which imposed more stringent amplification by both *Faecalibacterium*-specific primers, prior to identification *via* 16S rRNA-encoding gene sequencing. It is possible that using human-derived *F. prausnitzii* A2-165 as the reference isolate upon which colony selections were based, could have had a negative impact on the isolation of *Faecalibacterium* from calf faecal samples, especially if the colony morphology of calf-derived strains differed significantly from this human-derived reference isolate. It would have been more appropriate to use a calf-derived strain for morphological comparisons, which is recommended for future studies. A few close relatives of *Faecalibacterium* were also identified – *Fournierella* and *Gemmiger* (members of the *Ruminococcaceae*), which were of interest as these genera also contain butyrate producers (Gossling & Moore, 1975; Togo et al., 2017). However, more distantly related bacterial genera, including *Bacteroides*, *Prevotella*, *Streptococcus* and *Clostridium* were also identified among the isolates that yielded amplicons using the *Faecalibacterium*-specific primers, indicating that the multiplex PCR screening assay was not stringent enough to exclude non-*Faecalibacterium* spp. Other primer pairs could be trialled for improving the specificity of the PCR screen, such as GC-357F/907R (Lopez-Siles et al., 2012), Fprau223F/Fprau420R (Bartosch et al., 2004), FPR1/FPR2 (Wang et al., 1996), or to design new primers. Zheng et al. (2009) found that *Faecalibacterium* 16S rRNA gene sequences differed across different animal species (e.g., human, dog, chicken). A very recent study by Tanno et al. (2022) of 10 commonly used *Faecalibacterium*-specific primer pairs for qPCR found that some *Faecalibacterium*-specific 16S rRNA gene primers used for PCR did not capture all *Faecalibacterium prausnitzii* strains. These included FPR-2F (Wang et al., 1996)/Fprau645R (Ramirez-Farias et al., 2009), which was used for the selective screen in this project, and Fprau 07/Fprau 02 (Sokol et al., 2009), which was also tested during the selective screening. Lack of amplification was due to variations in the 16S rRNA genes of *Faecalibacterium* strains, and even within copies of the gene within members of the same strain. Further, qPCR with Fprau 07/Fprau 02 pair did not result in amplification of all 11 *F. prausnitzii* strains (representative of eight genomogroups proposed by Fitzgerald et al. (2018)), while the FPR-2F/Fprau645R pair did, and with high copy numbers of the 16S rRNA gene across all 11 strains with no sequence mismatches. While the primer pair used in our study should have amplified a diverse range of *F. prausnitzii*, it is still not known if they would work against all *Faecalibacterium* in the calf samples examined. Two other primer pairs, Fprau223F/Fprau420R and FPR1/FPR2 were also shown to result in the amplification of high copy numbers of the 16S rRNA gene with no mismatches in the primers. These two primer pairs targeted an area of the gene between the V2 and V3 regions (Tanno et al., 2022), and may be worthwhile testing on calf faecal samples to assess levels of amplification.

It was challenging to determine if the different anaerobic collection and cultivation methods improved the chances of isolating *Faecalibacterium* from fresh calf faeces, although calf-derived *Faecalibacterium* had previously been isolated from modified VTR2RF and cultured in an anaerobic chamber (Foditsch et al., 2014). Compared to plating samples on anaerobic agar plates, the use of roll tubes (Hungate, 1950), together with the collection of fresh faecal samples in anaerobically prepared PBS-filled bottles, was expected to have a higher chance of isolating the strict anaerobe like *Faecalibacterium.* However, *Faecalibacterium* was not isolated *via* this method. It is recommended that future isolations are undertaken alongside efforts to determine abundances of *Faecalibacterium via* culture-independent methods (e.g., 16S rRNA gene community profiling or qPCR) to confirm the presence and abundance of *Faecalibacterium*, and therefore, whether the cultivation and screening conditions used had been effective. Moreover, two different incubation temperatures were used also between the trials: in Cultivation Trial One, 37°C, which was the optimal growth temperature for human strains of *Faecalibacterium* (Cato et al., 1974), and 39°C in Cultivation Trial Two, which is more similar to the body temperature of cattle (New Zealand Government, 2019). The higher temperature resulted in faster growth of A2-165, but no *Faecalibacterium* were recovered from this trial.

The type of isolation media used in this study affected the number of colony-forming units, per gram of sample, that grew. YCFAG medium, which enriches for *Faecalibacterium* (Duncan, 2002; Lopez-Siles et al., 2012; Maier, 2017), resulted in the highest numbers of CFUs per gram of faecal sample, when compared to BHI and modified VTR2RF. YCFAG also supported faster growth of A2-165 compared to BHI and was the medium from which all four isolates (C6.007, C6.010, C1.021 and AY005_A5) were isolated. However, the YCFAG used for this study contained only one SCFA (sodium acetate), in comparison to the SCFA mix used by other studies (Duncan, 2002; Lopez-Siles et al., 2012), which may have prevented the appearance of more *Faecalibacterium* colonies if it had required more than one fatty acid for growth. The closely related YCFA broth was also found to be one of the most successful culture media that isolated among the highest number of bacterial species in an assessment of culture media and growth conditions for culturomics studies, and this was the only culture medium tested that was able to recover *F. prausnitzii* from faeces (Diakite et al., 2020). M2GSC and (L)YBHI media with 20% rumen fluid have also previously been successfully used to recover *F. prausnitzii* from faecal samples (Duncan, 2002; Martín et al., 2017).

4.2 Characterisation of putative *Faecalibacterium* isolates

The *Faecalibacterium*, *Fournierella* and *Gemmiger* isolates identified in this study were cultured and characterised by Gram staining, *in vitro* growth and SCFA analysis. All isolates stained Gram-negative and had rod-shaped morphologies. The *Faecalibacterium* C6.007 cell morphologies were near-identical to those of the A2-165 model strain, and to the original morphological description of *Faecalibacterium* (Duncan, 2002), although a few longer rods were not as visibly segmented as in A2-165. Interestingly, *F. massiliensis* C1.021 cells displayed bud-like protrusions, along the lengths of approximately half of the visible cells (**Figure 3-7**). To the best of our knowledge, this is the first instance of such extracellular structures being seen in *Fournierella,* which was originally described as rods that were 0.5 µm × 2 µm (Togo et al., 2017). In contrast, bud-like protrusions, visible under electron microscopy, were previously reported in some *Faecalibacterium prausnitzii* strains, including A2-165 (Hu et al., 2022; Martín et al., 2017; Miquel et al., 2013), and in *Gemmiger formicilis* by Gossling and Moore (1975) and (Salanitro et al., 1976). The *Gemmiger* buds occurred in pairs or chains of spherical or tear-drop-shaped cells, or as bowling pin cells, though the C1.021 protrusions only appeared on rod-shaped cells in chains. Electron microscopy of C1.021 cells would need to be conducted to gain a better understanding of the ultrastructure of these protrusions. The *Gemmiger* isolate C6.010, in contrast, did not appear to produce any buds in the conditions of the present study, but it is possible that they may do so under different growth conditions.

From examination of the growth dynamics in batch culture, all four isolates reached their stationary phase of growth after ~15 hours of incubation in YCFAG at 37°C, with C6.007 displaying a similar growth profile as the A2-165 control. In Maier (2017), A2-165 (when inoculated in approximately half the volume of BHI) reached stationary phase approximately one hour later than C6.007. In contrast, inoculation of A2-165 in half the volume of medium, YCFAG with supplemented with a fatty acid mixture (Lopez-Siles et al., 2012), A2-165 reached stationary phase approximately one hour earlier than C6.007 (Cheng et al., 2020). Overall, the growth profile of C6.007 was similar to what has been previously reported.

SCFA analysis was also performed, with a particular interest in the production of butyrate, an important short-chain fatty acid that provides energy to enterocytes (Bergman, 1990) and is thought to have an anti-inflammatory effect in the GIT (Plöger et al., 2012). Anti-inflammatory property is a desirable trait in preventing or reducing the effects of scours in calves. Supplementation of butyrate in milk replacer and calf starter for young calves has been previously correlated with increased weight gain, and health benefits such as a decrease in the incidence of scours, promotion of rumen development, enhanced absorptive function of the GIT and increased expression of heat shock proteins HSP27 and HSP20 (Górka et al., 2011; Gorka et al., 2009; Guilloteau et al., 2009), thus making it a potentially useful additive that can be used in the calf diet to improve their wellbeing. Butyrate was produced by C6.007 and C6.010 under the growth conditions used in this study and consistent with this, all genes in the butyrate synthesis pathway from pyruvate were found in the C6.007 and C6.010 genomes. There are two main pathways to butyrate production from pyruvate (Louis & Flint, 2017), that can begin from the breakdown of a carbohydrate, such as glucose, or fatty acid, such as acetate, that split into the two pathways when butyryl-CoA is converted into butanoyl-phosphate by phosphate butyryltransferase and then into butyrate by butyrate kinase, or butyryl-CoA is converted into butyrate by butyryl-CoA:acetate CoA transferase – the latter pathway was identified in C6.007 and C6.010. This was expected as the isolates are members of the *Faecalibacterium* (Duncan, 2002) and *Gemmiger* (Gossling & Moore, 1975), respectively, which are known to produce butyrate. This same pathway has also been observed in human-derived *Faecalibacterium* (Anand et al., 2016; Heinken et al., 2014) and is one of four separate pathways used by butyrate-producing bacteria to synthesise butyrate (Anand et al., 2016), showing that this particular pathway used by C6.007 and C6.010 is conserved amongst some groups of butyrate-producing bacteria. However, AY005_A5 and C1.021 (both *Fournierella massiliensis*), did not produce detectable levels of butyrate, despite the type strain, *F. massiliensis* AT2^T, having previously been described to do so in minor amounts (Togo et al., 2017). In addition, AY005_A5 and C1.021 did not contain any of the genes in the acetyl-CoA to butyrate pathway, indicating that the complete absence of the butyrate pathway, rather than, for example, deleterious mutations in the pathway genes, or downregulation of the pathway, appears to be the reason why butyrate production was not observed in the *Fournierella* strains. Due to the anti-inflammatory properties of butyrate, C6.007 and C6.010 may therefore be beneficial for improving calf health as alternate probiotic candidates that can prevent or reduce the incidence of scours in artificially reared calves – further research will need to be performed on these two isolates in the future to assess their potential as probiotics.

With regard to other fermentation end-products, *F. prausnitzii* A2-165 produces formate and D-lactate when cultured in M2GSC medium (Duncan, 2002). Both were also produced by C6.007 in addition to acetic, succinic and isovaleric acids. The production of lactic acid by C6.007 may be advantageous for potential use as a probiotic as a decrease of pH by lactic acid can make the local environment unfavourable for many bacteria and fungi, including pathogens. *Fournierella massiliensis* AT2^T has been shown to produce propionic and acetic acids (which is the major SCFA produced by this strain) in Wilkins-Chalgren-Anaerobe broth supplemented with cysteine and dithiothreitol (Togo et al., 2017). These SCFAs were also produced by C1.021 and AY005_A5, which additionally produced formic, lactic, succinic and isovaleric acids. Lastly, *Gemmiger formicilis* has previously been described to produce formic, lactic, pyruvic, acetic, succinic and malonic acids (Gossling & Moore, 1975) when cultured in peptone yeast extract glucose medium. These SCFAs were also produced by C6.010. However, C6.010 also produced propionic and isovaleric acids, which may have been due to the different growth medium used or inherent differences between *G. formicilis* and *Gemmiger* sp004555405 (of which C6.010 is a member). Interestingly, ethanol was the only alcohol detected and was produced by C6.007, C1.021 and AY005_A5, with concentrations from C1.021 and AY005_A5 being ~30× greater than those from C6.007. Detection of the complete set of genes from the Por-AldH-Adh pathway of ethanol synthesis in C1.021 and AY005_A5 suggests that this pathway was used by these strains to produce ethanol (Nissen & Basen, 2019). The Por-AldH-Adh pathway is commonly used by anaerobic bacteria, some anaerobic protozoa, archaea and other microaerophilic microorganisms, in carbohydrate metabolism (Eram & Ma, 2013). These include *Thermoanaerobacterium saccharolyticum* (Cui et al., 2019) and *Entamoeba histolytica* (Pineda et al., 2010). Almost all of the genes, except *adh*, in the Por-Pta-Ack-Aor-Adh pathway were found in C6.007 to produce ethanol. Aor is used by strict anaerobic microorganisms such as the archaeon *Pyrococcus furiosus*, and recently this pathway has also been determined to occur in multiple *Clostridium* species, such as *Clostridium ljungdahlii* and *Clostridium ragsdalei* (Nissen & Basen, 2019). Further research would be needed to confirm the ethanol production pathway in these isolates, which may have potential as ethanol-producing bacteria for future applications.

4.3 Genomic analyses and genetic comparison to human and other calf strains

Phylogenetic and genomic analyses were performed on the *Faecalibacterium*, *Fournierella* and *Gemmiger* isolates to determine their relatedness to each other and reference strains. Analyses of 16S rRNA-encoding gene sequences showed that all four isolates were closely-related, with C6.007 sharing a clade with A2-165, and were genetically distinct to many of the New Zealand calf-derived *Faecalibacterium* sequences from the recent study by (Kumar et al., 2021). However, whole genome sequencing revealed that C6.007 was classified as *Faecalibacterium prausnitzii* C, C6.010 as *Gemmiger* sp004555405 and both C1.021 and AY005_A5 as *Fournierella massiliensis* in the GTDB taxonomic framework. In addition, C6.007 had a closest ANI match of 96.05% to strain A2-165 in the NCBI database. Given the species-level cut-off of 96% ANI for bacteria (Ciufo et al., 2018), at the genomic level, our calf-derived *F. prausnitzii* isolate appeared distinct to the closest sequenced human-derived strain of *F. prausnitzii*, and furthermore, was at the very cusp of being considered the same species. Given the large differences between the human and calf GIT environments, it is not unexpected that the human and calf-derived isolates differ (Ciufo et al., 2018). *Faecalibacterium* is a diverse genus – there are at least two phylogroups of *Faecalibacterium* as seen in a human study (Fitzgerald et al., 2018), multiple phylotypes within individual cows and potentially two phylotypes of *Faecalibacterium* with less than 96% 16S rRNA gene sequence identity to the *F. prausnitzii* type strain ATCC 27768^T (Savin et al., 2019). In future studies, such genomic comparisons could be expanded to more isolates to more generally compare the relatedness of *Faecalibacterium* from the two different hosts. The genome size and GC% of C6.007 was within the expected ranges of 2.68 to 3.42 Mb and 47 to 63%, respectively, for *Faecalibacterium* (Cato et al., 1974; Fitzgerald et al., 2018; Zou et al., 2021). Closed circular extrachromosomal elements, that were not deemed to be plasmids by comparison against the plasmid database using BLASTN analysis, were found in AY005_A5, which resulted in two contigs upon genome assembly, and C6.010, which resulted in three contigs. Extrachromosomal elements have not been previously observed in *Fournierella massiliensis* or *Gemmiger* sp. Additional analyses to determine the nature of these elements, and the functions and attributes they confer, will be of interest in downstream studies.

Chapter Five. Conclusions and Future Directions

5.1 Conclusions

In conclusion, one *Faecalibacterium prausnitzii* strain, C6.007, has been isolated from New Zealand calves and by 16S rRNA gene sequence data it appears to be related to *Faecalibacterium* strains of calf and human origin. In contrast, at the genomic level, C6.007 may represent a new *Faecalibacterium* species, given the extent of the genome sequence difference between it and existing reference genomes from human sources. These results support our hypothesis that calf-derived *Faecalibacterium* spp. can significantly differ genetically from human-derived *Faecalibacterium.* We observed C6.007 to produce butyrate, acetate, formate and lactic acid upon fermentation of glucose. While only one *Faecalibacterium* had been isolated, lesser-studied butyrate-producing bacteria, *Gemmiger* sp004555405 and *Fournierella massiliensis*, were also isolated and characterised. Genes in the butyrate synthesis pathway were identified in the *Faecalibacterium* and *Gemmiger* isolates and were consistent with their observed production of butyrate. However, the *Fournierella* isolates appeared to produce high concentrations of ethanol from pyruvate *via* acetyl-CoA. One *Fournierella* isolate cell morphology showed bud-like protrusions which may be the first such report of this morphology in *Fournierella.* These new butyrate-producing bacterial isolates from the New Zealand calves may have potential as probiotic candidates for calves.

5.2 Future Directions

This study has resulted in the isolation and characterisation of *Faecalibacterium* and close relatives from New Zealand whole milk-fed calves and several areas that could be followed up from this work are identified. To assess the potential use of the isolates characterised in this study as probiotic candidates or direct-fed microbials, these could be further tested for their resistance to bile salts and low pHs, which are important features for their survival through the GIT as oral probiotics, and antibiotic resistance. Future experiments may also include calf trials in which the isolates are fed to young calves and their effect on calf health assessed.

The original microbial community profiles of the original calf faecal samples, from which the isolates were derived, should be determined (e.g. *via* 16S rRNA community profiling) to determine if the sole *Faecalibacterium* isolate identified was representative of the *Faecalibacterium* population in these calves. This analysis would provide further insight into the diversity and abundance of *Faecalibacterium* and other microbiota in the hindgut microbiomes of calves, and the culturability of *Faecalibacterium* in this study.

A collection of additional calf faecal isolates was also stored during the isolation experiments and these could be further explored for their diversity, functions and potential as calf beneficial microorganisms, especially butyrate-producing isolates. Additional *Faecalibacterium* isolates might also be identified from this collection and their characterisation would expand our knowledge of calf-derived *Faecalibacterium*. Further optimisation of PCR or other screens, such as NanoString nCounter technology (Geiss et al., 2008) or MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight) (Hillenkamp et al., 1991), to identify *Faecalibacterium* may be undertaken to better facilitate their isolation. Further morphological characterisation of C1.021, which showed bud-like extracellular protrusions that have not been previously described in *Fournierella* (Togo et al., 2017), and C6.007, as some *Faecalibacterium* strains have previously been shown to have extracellular protrusions visible under electron microscopy (Hu et al., 2022; Martín et al., 2017; Miquel et al., 2013), could be examined using electron microscopy to gain a better understanding of their composition and functions. Growth conditions may also be assessed to understand the conditions by which this phenotype is expressed.

Isolates C1.021 and AY005_A5 produced relatively high amounts of ethanol and may have commercial potential. Ethanol can have many industrial uses, such as in disinfectants. The regulation of metabolic pathways of C1.021 and AY005_A5 may be further studied and assessed for their potential for commercial ethanol production.

The sampling of other dairy and beef cattle breeds in New Zealand, for example, Ayrshires and Wagyu, on different diets (e.g. a variety of whole milks *vs.* a variety of milk replacers, or different solid diets such as hay, pellets with different nutritional qualities, red clover pasture and ryegrass pasture), at different ages, or artificially reared *vs.* reared with their dam, could also be performed to see if these factors have an effect on *Faecalibacterium* abundance and diversity. In this project, sampling was performed on Friesian × Angus cross calves and KiwiCross (Holstein-Friesian x Jersey) calves. If *Faecalibacterium* from less common cattle breeds were to be isolated and studied as potential probiotics for calves, the relationship between the breed from which *Faecalibacterium* was isolated and its differences in probiotic effects (if any) could be examined.

Appendix 1. BLASTN results following 16S rRNA gene sequencing

Table A-1. Top BLASTN identity of all faecal isolates following Sanger 16S rRNA gene sequencing.

Isolate ID	16S rRNA gene primer[a] or consensus sequence	Top BLAST hit[b] (based on %identity)	%identity to top BLAST hit	E-value	Query sequence length (bases)
F1.004	Consensus sequence [c]	*Prevotellaceae* strain AGP2-02-00-08 (MH699326.1)	99.49%	0.00	1,377
F1.015	Consensus sequence [c]	*Streptococcus lutetiensis* strain 2709 (MT611722.1)	100%	0.00	1,327
F2.001	27F	*Bacteroides xylanisolvens* strain A3 (KX658684.1)	99.23%	0.00	651
F2.001	1492R	*Bacteroides* sp. JJM0207-12 (KR364742.1)	100%	0.00	694
F2.002	Consensus sequence [c]	*Bacteroides* sp. Smarlab 3302996 (AY643081.1)	99.56%	0.00	1,377
F2.015	1492R	*Lachnospiraceae* bacterium strain AGP1-03-21-03 (MH699297.1)	100%	0.00	802
C1.021	Consensus sequence [d]	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.55%	0.00	1,396
C6.007	Consensus sequence [e]	*Faecalibacterium* sp. strain CLA-AA-H223 (OK510340.1)	98.55%	0.00	1,381

Isolate ID	16S rRNA gene primer[a] or consensus sequence	Top BLAST hit[b] (based on %identity)	%identity to top BLAST hit	E-value	Query sequence length (bases)
C6.010	Consensus sequence[d]	*Gemmiger formicilis* strain FAB_D4_2 (MT903192.1)	96.19%	0.00	1409
AY003 (mixed culture)	Consensus sequence[f]	*Faecalibacterium prausnitzii* strain 02_10294632002__013 (KJ957842.1)	96.57%	0.00	525
AY003_3	27F	*Phocaeicola* sp. strain W1-Q-100 (OK326515.1)	99.75%	0.00	399
AY003_3	514R	*Bacteroides vulgatus* (EU728705.1)	99.57%	0.00	460
AY003_7	Consensus sequence[f]	*Bacteroides vulgatus* strain DSM 107446 (MN537534.1)	99.57%	0.00	928
AY005 (mixed culture)	FPR-2F	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.00%	0.00	694
AY005_A5	27F	*Fournierella massiliensis* strain AT2 (NR_156911.1)	99.24%	0.00	661
AY005_A5	FPR-2F	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.25%	0.00	515
AY052	27F	*Clostridium* sp. strain B2-J-110 (OK325753.1)	99.89%	0.00	886

Isolate ID	16S rRNA gene primer[a] or consensus sequence	Top BLAST hit[b] (based on %identity)	%identity to top BLAST hit	E-value	Query sequence length (bases)
BY057 (mixed culture)	27F	*Fournierella massiliensis* strain AT2 (NR_156911.1)	98.97%	0.00	778
BY057 (mixed culture)	FPR-2F	*Fournierella massiliensis* strain PCK025 (MN913794.1)	98.49%	0.00	917
BY057_B7	27F	*Bacteroides vulgatus* strain 6658 (MT515977.1)	100%	0.00	455

[a] 16S rRNA gene sequencing primer.

[b] Against the non-redundant (nr/nt) database, excluding environmental ("uncultured") sample results. The GenBank accession number of each isolate is in parentheses.

[c] Consensus sequence derived from the universal 16S rRNA gene primer pair 27F/1492R.

[d] Consensus sequence derived from the universal 16S rRNA gene primer pair 27F/1492R and internal 16S rRNA gene primers FPR-2F, 514R, 1100R and 1382R.

[e] Consensus sequence derived from the universal 16S rRNA gene reverse primer 1492R and internal 16S rRNA gene primers FPR-2F, 514R, 1100R and 1382R.

[f] Consensus sequence derived from the universal 16S rRNA gene forward primer 27F and internal 16S rRNA gene primer FPR-2F.

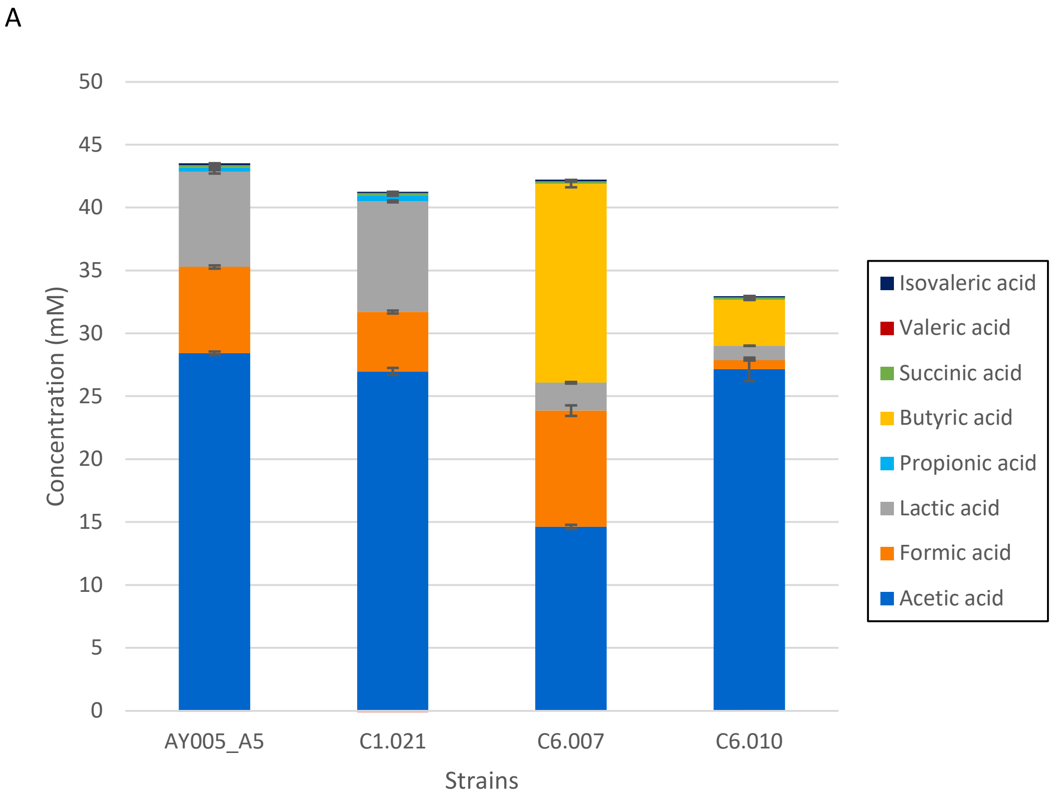
Appendix 2. Concentration of SCFAs and alcohols from derivatised SCFA analysis.

Table A-2. Mean concentration of fermentation products at stationary phase.

SCFAs and alcohols		Concentration (mM)								
		Formic acid	**Acetic acid**	**Lactic acid**	**Propionic acid**	**Butyric acid**	**Succinic acid**	**Valeric acid**	**Isovaleric acid**	**Ethanol**
Isolates										
Stationary phase										
AY005_A5	Mean	6.06	27.47	7.26	0.31	0.01	0.21	0.00	0.19	35.28
	SEM	0.11	0.58	0.38	0.01	0.04	0.00	0.00	0.01	0.67
C1.021	Mean	4.92	27.66	8.65	0.39	-0.05	0.21	0.00	0.18	36.85
	SEM	0.02	0.67	0.13	0.02	0.00	0.00	0.00	0.01	2.12
C6.007	Mean	7.72	16.12	2.46	0.13	14.20	0.22	0.02	0.13	1.41
	SEM	0.08	0.30	0.06	0.04	0.20	0.00	0.01	0.02	0.04
C6.010	Mean	0.75	30.08	1.11	0.16	3.46	0.19	0.00	0.21	0.00
	SEM	0.04	0.26	0.02	0.03	0.06	0.00	0.00	0.01	0.00

Table A-3. Mean concentration of fermentation products at 24 hours post-inoculation.

SCFAs and alcohols		**Concentration (mM)**								
		Formic acid	**Acetic acid**	**Lactic acid**	**Propionic acid**	**Butyric acid**	**Succinic acid**	**Valeric acid**	**Isovaleric acid**	**Ethanol**
Isolates *24 h post-inoculation*										
AY005_A5	Mean	6.87	28.42	7.56	0.33	-0.06	0.19	0.00	0.16	37.67
	SEM	0.13	0.15	0.14	0.01	0.01	0.00	0.00	0.01	0.50
C1.021	Mean	4.72	26.98	8.80	0.46	-0.08	0.19	0.00	0.10	36.52
	SEM	0.12	0.29	0.08	0.02	0.00	0.01	0.00	0.01	0.69
C6.007	Mean	9.23	14.63	2.21	0.02	15.81	0.21	0.00	0.11	1.97
	SEM	0.41	0.16	0.07	0.00	0.28	0.01	0.01	0.00	0.07
C6.010	Mean	0.72	27.15	1.14	-0.01	3.66	0.18	0.00	0.11	0.00
	SEM	0.02	0.92	0.01	0.00	0.03	0.01	0.00	0.01	0.00



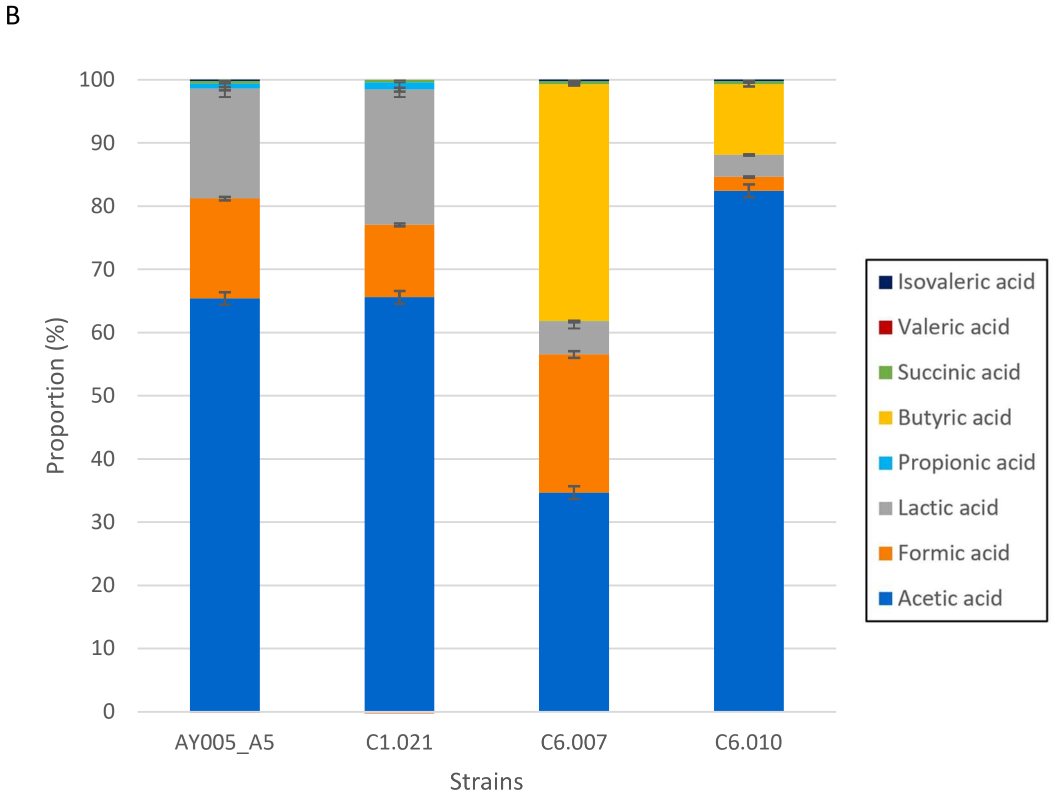


Figure A-1. The mean concentration (A) and proportions (B) of SCFAs detected in cultures of AY005_A5, C1.021, C6.007 and C6.010 at 24 hours post-inoculation. Error bars show the upper and lower ranges of the standard error of the means (n = 4 for all strains).

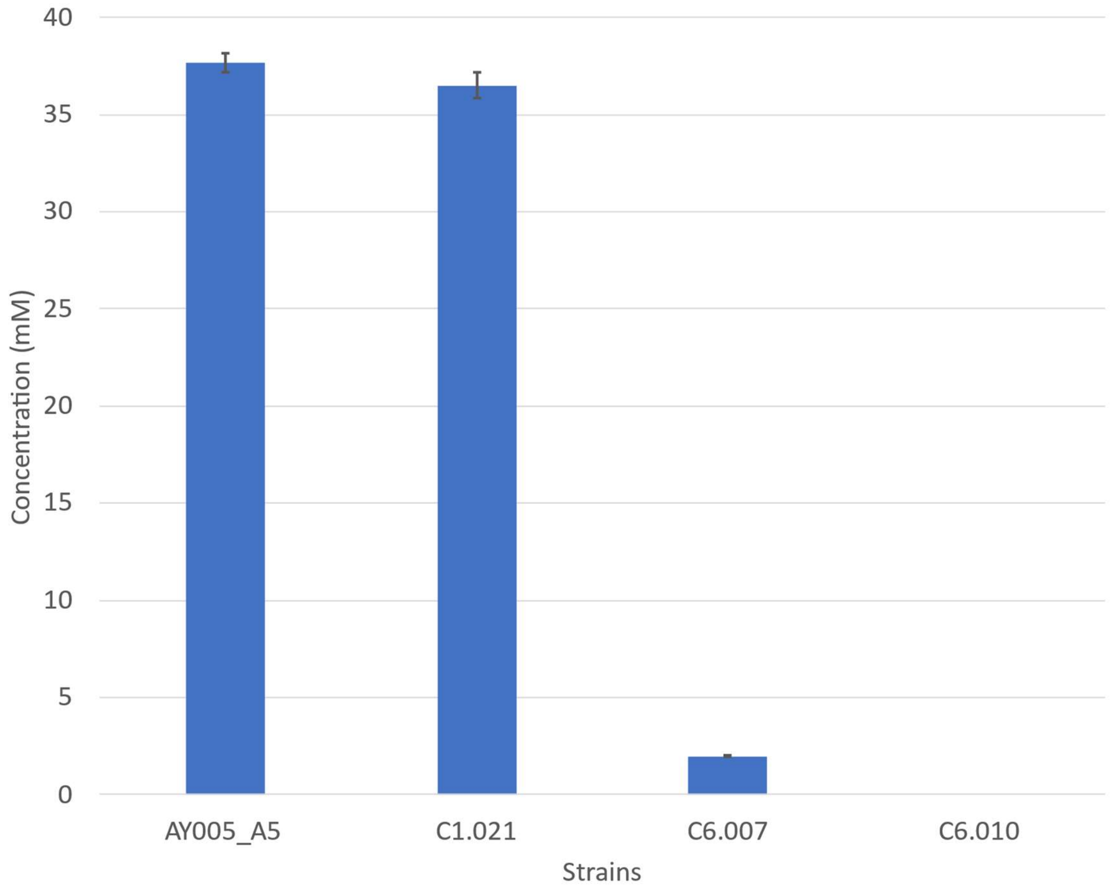
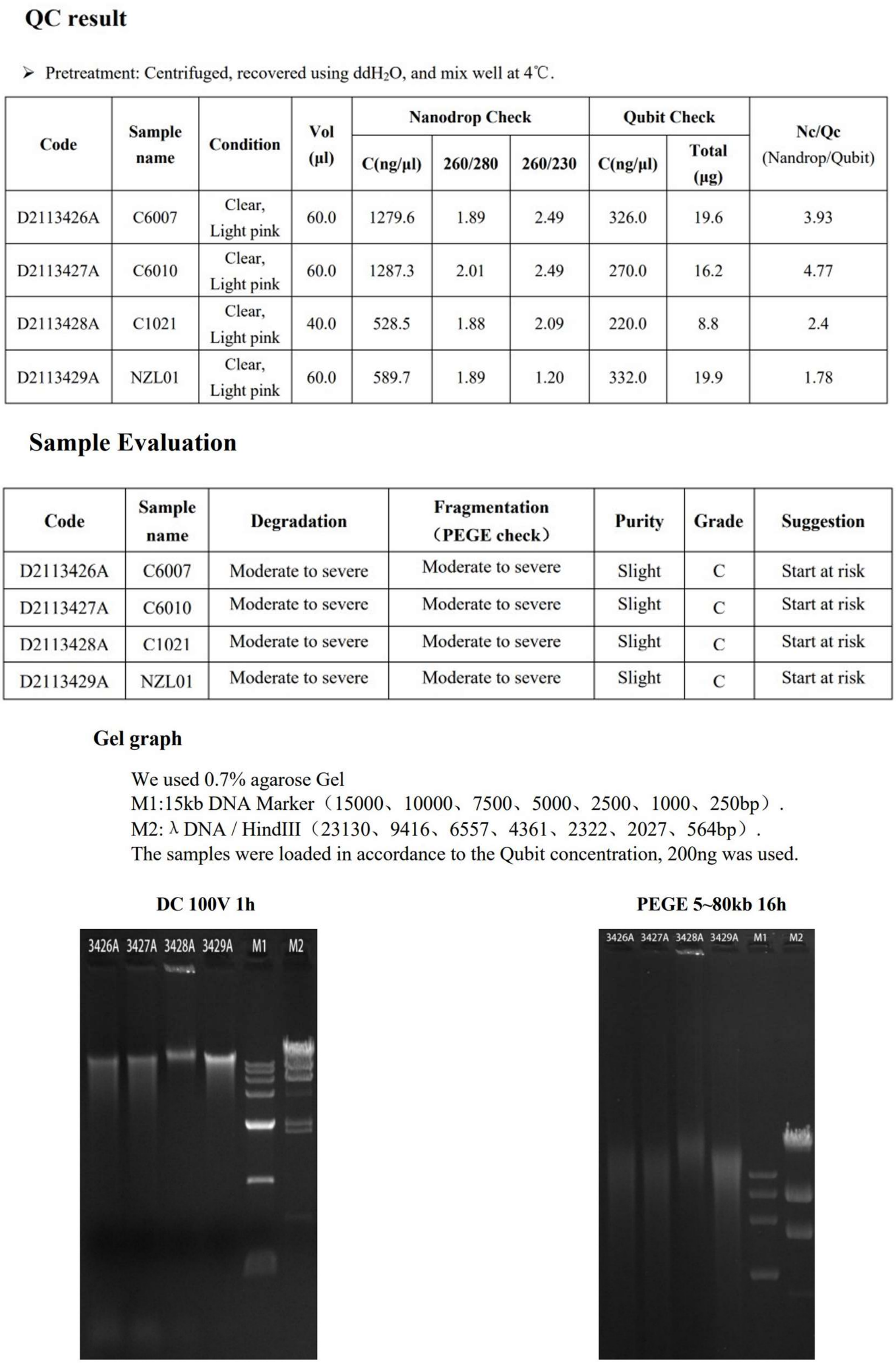


Figure A-2. Ethanol concentrations in cultures of AY005_A5, C1.021, C6.007 and C6.010 grown in YCFAG after 24 hours post-inoculation. Error bars show the upper and lower ranges of the standard error of the means (n = 4 for all strains).

Appendix 3. DNA quality check report prior to genome sequencing.



QC result

- Pretreatment: Centrifuged, recovered using ddH$_2$O, and mix well at 4℃.

Code	Sample name	Condition	Vol (μl)	Nanodrop Check			Qubit Check		Nc/Qc (Nandrop/Qubit)
				C(ng/μl)	260/280	260/230	C(ng/μl)	Total (μg)	
D2113426A	C6007	Clear, Light pink	60.0	1279.6	1.89	2.49	326.0	19.6	3.93
D2113427A	C6010	Clear, Light pink	60.0	1287.3	2.01	2.49	270.0	16.2	4.77
D2113428A	C1021	Clear, Light pink	40.0	528.5	1.88	2.09	220.0	8.8	2.4
D2113429A	NZL01	Clear, Light pink	60.0	589.7	1.89	1.20	332.0	19.9	1.78

Sample Evaluation

Code	Sample name	Degradation	Fragmentation （PEGE check）	Purity	Grade	Suggestion
D2113426A	C6007	Moderate to severe	Moderate to severe	Slight	C	Start at risk
D2113427A	C6010	Moderate to severe	Moderate to severe	Slight	C	Start at risk
D2113428A	C1021	Moderate to severe	Moderate to severe	Slight	C	Start at risk
D2113429A	NZL01	Moderate to severe	Moderate to severe	Slight	C	Start at risk

Gel graph

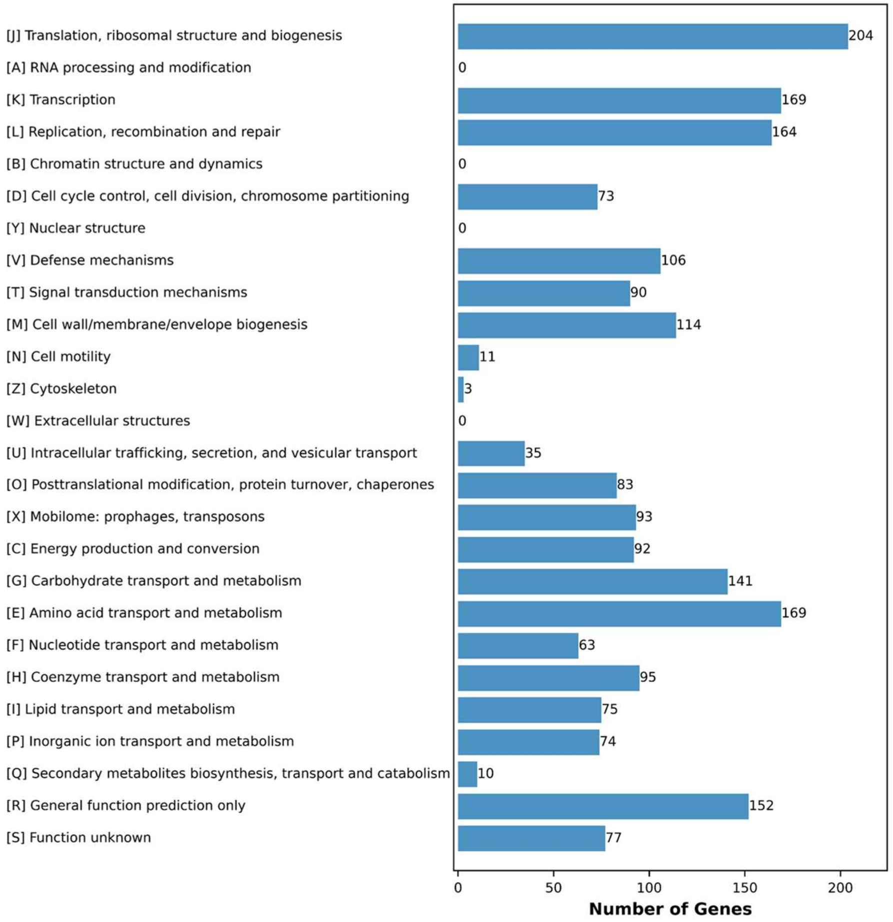
We used 0.7% agarose Gel
M1:15kb DNA Marker（15000、10000、7500、5000、2500、1000、250bp）.
M2: λ DNA / HindIII（23130、9416、6557、4361、2322、2027、564bp）.
The samples were loaded in accordance to the Qubit concentration, 200ng was used.

Figure A-3. DNA quality check performed by GrandOmics prior to genome sequencing. Sample NZL01: AY005_A5.

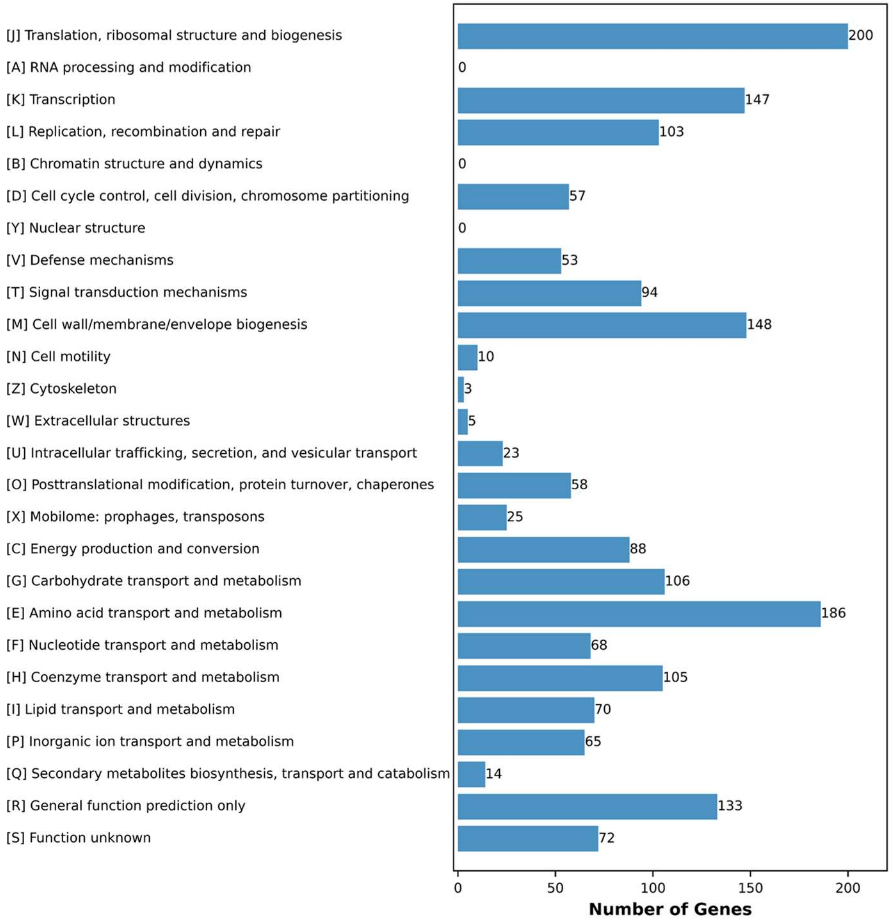
Appendix 4. Gene matches to the COG and KEGG databases following genome sequencing.

A

C6.007



C6.010



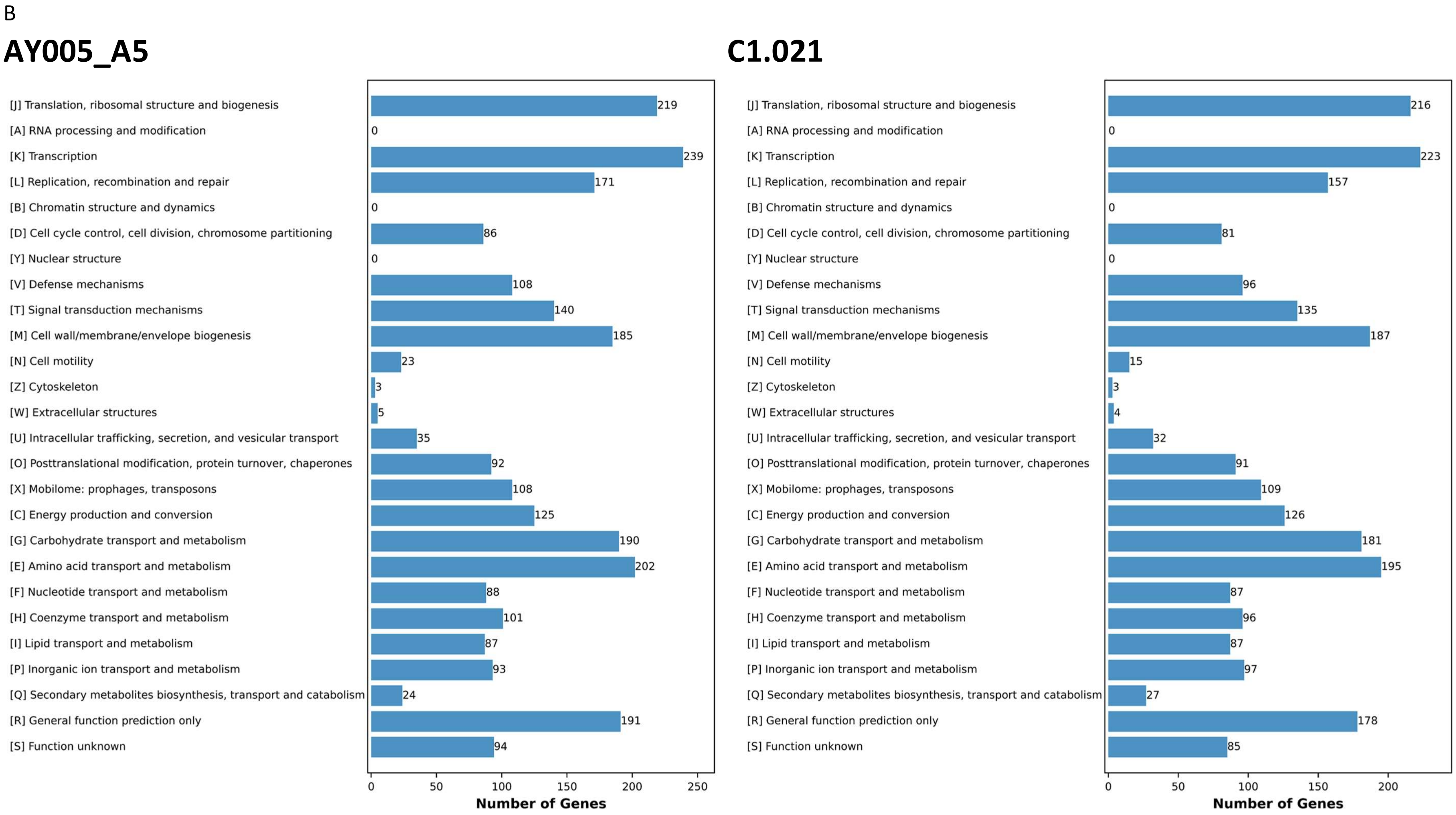
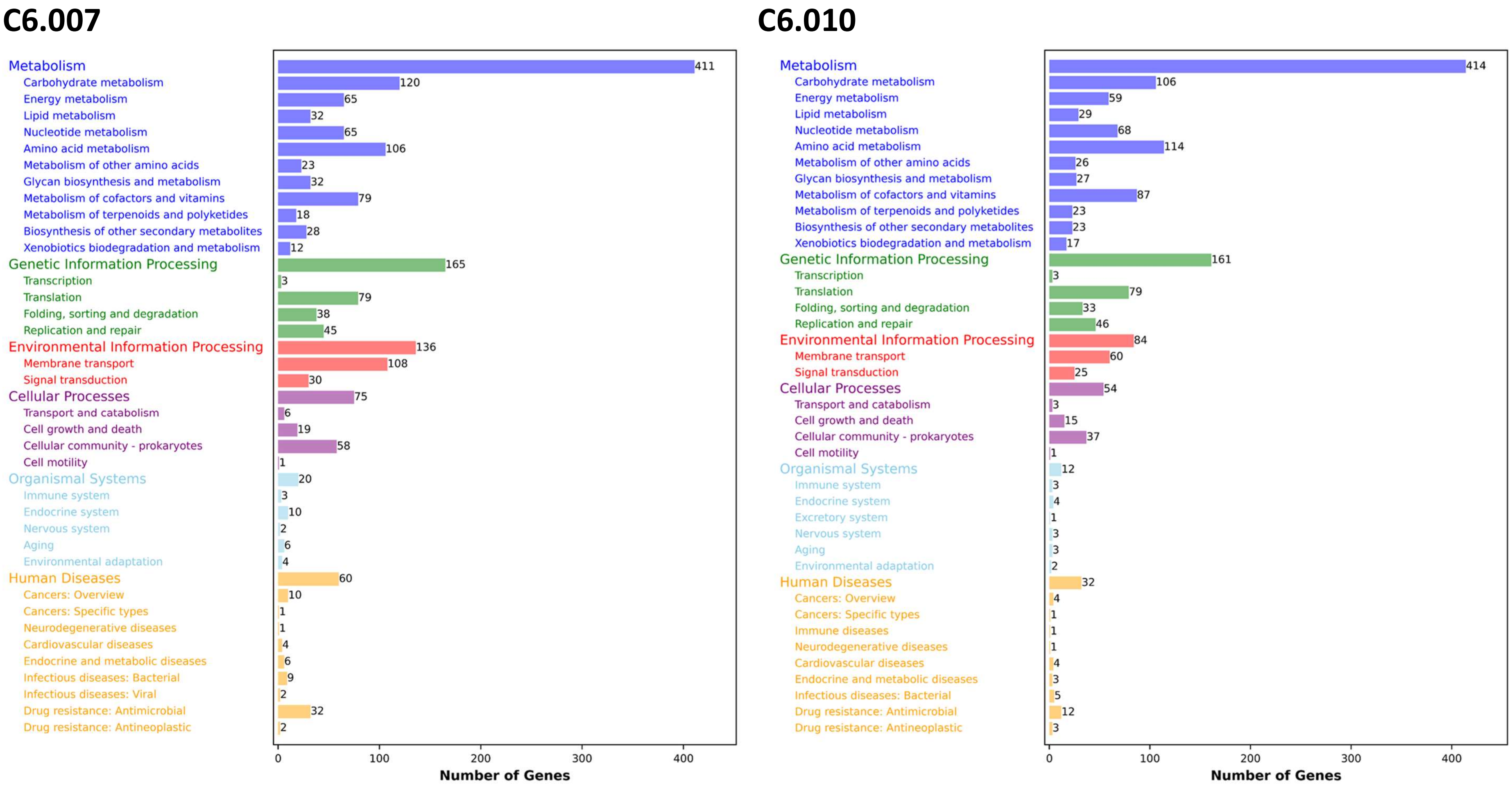


Figure A-4. Genes from faecal isolates (A) C6.007 and C6.010, and (B) AY005_A5 and C1.021, following genome sequencing, that matched the COG database results.

A


C6.007
Metabolism 411
Carbohydrate metabolism 120
Energy metabolism 65
Lipid metabolism 32
Nucleotide metabolism 65
Amino acid metabolism 106
Metabolism of other amino acids 23
Glycan biosynthesis and metabolism 32
Metabolism of cofactors and vitamins 79
Metabolism of terpenoids and polyketides 18
Biosynthesis of other secondary metabolites 28
Xenobiotics biodegradation and metabolism 12
Genetic Information Processing 165
Transcription 3
Translation 79
Folding, sorting and degradation 38
Replication and repair 45
Environmental Information Processing 136
Membrane transport 108
Signal transduction 30
Cellular Processes 75
Transport and catabolism 6
Cell growth and death 19
Cellular community - prokaryotes 58
Cell motility 1
Organismal Systems 20
Immune system 3
Endocrine system 10
Nervous system 2
Aging 6
Environmental adaptation 4
Human Diseases 60
Cancers: Overview 10
Cancers: Specific types 1
Neurodegenerative diseases 1
Cardiovascular diseases 4
Endocrine and metabolic diseases 6
Infectious diseases: Bacterial 9
Infectious diseases: Viral 2
Drug resistance: Antimicrobial 32
Drug resistance: Antineoplastic 2
0
100
200
300
400
Number of Genes
C6.010
Metabolism 414
Carbohydrate metabolism 106
Energy metabolism 59
Lipid metabolism 29
Nucleotide metabolism 68
Amino acid metabolism 114
Metabolism of other amino acids 26
Glycan biosynthesis and metabolism 27
Metabolism of cofactors and vitamins 87
Metabolism of terpenoids and polyketides 23
Biosynthesis of other secondary metabolites 23
Xenobiotics biodegradation and metabolism 17
Genetic Information Processing 161
Transcription 3
Translation 79
Folding, sorting and degradation 33
Replication and repair 46
Environmental Information Processing 84
Membrane transport 60
Signal transduction 25
Cellular Processes 54
Transport and catabolism 3
Cell growth and death 15
Cellular community - prokaryotes 37
Cell motility 1
Organismal Systems 12
Immune system 3
Endocrine system 4
Excretory system 1
Nervous system 3
Aging 3
Environmental adaptation 2
Human Diseases 32
Cancers: Overview 4
Cancers: Specific types 1
Immune diseases 1
Neurodegenerative diseases 1
Cardiovascular diseases 4
Endocrine and metabolic diseases 3
Infectious diseases: Bacterial 5
Drug resistance: Antimicrobial 12
Drug resistance: Antineoplastic 3
0
100
200
300
400
Number of Genes

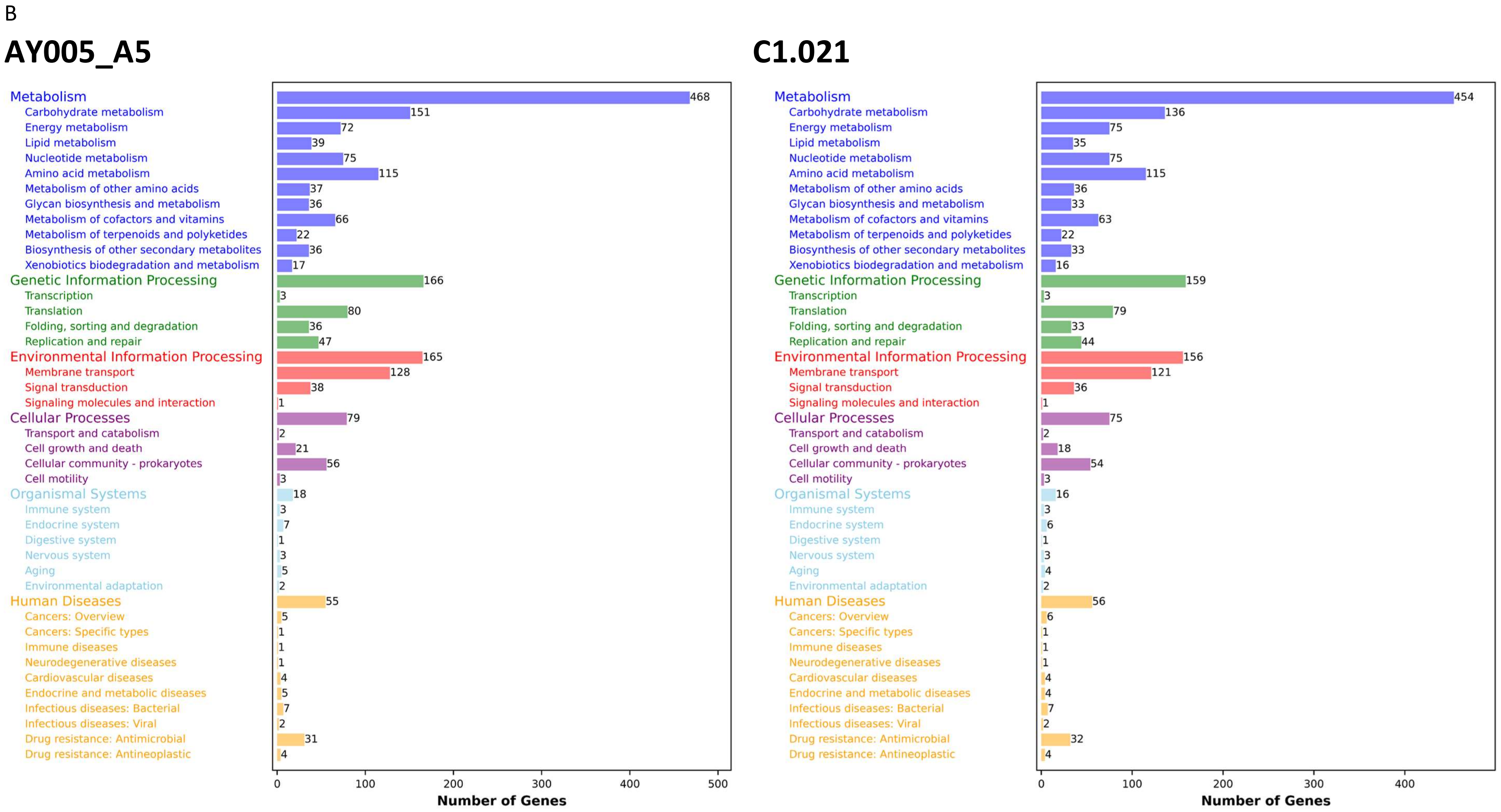


Figure A-5. Genes from faecal isolates (A) C6.007 and C6.010, and (B) AY005_A5 and C1.021, following genome sequencing, that matched the KEGG database results.

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