final report

Project code: B.AHE.0248

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Date published: 31 May 2018

PUBLISHED BY
Meat and Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

Detoxification of fluoroacetate by naturally occurring rumen microorganisms

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Poisoning of cattle in northern Australia due to ingestion of plants containing fluoroacetate (FA) causes significant economic loss to producers. This project aimed to identify rumen bacteria capable of degrading FA and to determine whether rumen detoxification may be a viable strategy to protect animals from FA poisoning. We isolated two species of rumen bacteria (*Cloacibacillus* sp. and *Pyramidobacter* sp.) which degraded FA and showed their growth and ability to metabolize the toxin could be stimulated with a range of nutritional supplements (amino acids, citrate, sarcosine, and selenium) suitable for cattle. The genetic architecture that both these bacteria use to degrade FA is similar but unique and has never been described before in nature. A small survey of cattle in northern Australia showed that these bacteria were present in the rumen of most animals but at relatively low numbers (~10^5-10^6 per ml rumen fluid or 0.001% of the total bacterial population) and thus inoculation of animals with FA-degrading isolates would not be required. Future research needs to demonstrate that provision of nutritional supplements in cattle will stimulate these indigenous rumen bacteria to degrade FA and prevent poisoning.

Executive summary

Fluoroacetate (FA) is a compound found in over 40 plants species worldwide as a natural defense against grazing herbivores. Although not primary fodder, inadvertent ingestion of FA accumulating vegetation leads to the intoxication of many commercial livestock species. Consumption results in significant production losses and detrimental animal welfare issues (morbidity), and in some cases death of the animal. In Australia, such effects closely follow the geographic distribution of plants which accumulate FA. For instance, approximately 200,000 head of cattle are exposed to the tree Georgina gidyea (*Acacia georginae*) in the Georgina river basin of western Queensland and in the Northern Territory where many cattle are poisoned. Elsewhere the heartleaf tree (*Gastrolobium grandiflorum*) is more acutely toxic. Heartleaf poisoning of livestock arises predominantly across northern Queensland, in the north of the Northern Territory, the north of Western Australia, and in the southeast of Western Australia. Combined, the Australian economic impact of productivity losses directly attributable to FA intoxication were estimated to be over $45 million annually (Perkins et al, 2015).

Past strategies to overcome the problems associated with intoxication included the development of transgenic rumen bacteria containing FA dehalogenase genes (Gregg et al., 1994). These Genetically Modified Organisms (GMOs) successfully detoxified FA within the gastrointestinal tract of ruminant livestock (Gregg et al., 1998; Padmanabha et al., 2004). However, concerns over the release of transgenic bacteria and regulatory hurdles have necessitated the search and study of naturally-occurring anaerobic microorganisms that could reduce FA toxicity in ruminants. Despite this interest in the microbial degradation of fluorinated compounds, there are few reports of naturally occurring FA degradation under anaerobic conditions and a lack of information on FA-degrading rumen microbes in general. Therefore the aims of this project were to:

- Identify a consortium of FA-degrading rumen bacteria which can degrade FA under laboratory conditions
- Identify nutrients and compounds that stimulate growth and/or degradation of FA by these bacteria
- Develop molecular tools that can be used to measure the population abundance of these bacteria in the rumen

Firstly, the FA-degrading ability of herbivore gut contents collected from different animals (primarily ruminant), in varying areas and eating different diets were surveyed. Samples were cultured in enrichment media supplemented with FA and fluoride was detected in some of the mixed cultures, which provided an indication of microbial defluorination. Novel bacterial species responsible for such FA degradation were subsequently isolated, identified taxonomically and characterized in terms of nutrient requirements for optimal growth and degradation of FA. Subsequent 16S rRNA gene sequence analysis revealed that the best FA-degrading bacterial strains were from ruminant samples and belonged to the genera *Cloacibacillus* and *Pyramidobacter* within the phylum Synergistetes. Of these, *Cloacibacillus* sp. strain MFA1 and *Pyrimadobacter* sp. strain C12-8 degraded the most FA and became the primary focus of the project. Rumen bacteria of other genera with less ability to degrade FA were also identified, isolated and studied with the aim of potentially providing a cocktail of bacteria that could be dosed into ruminants. They belonged to the genera *Oribacterium* and *Eubacterium* within the phylum Firmicutes. Bacteria from all these genera are normal residents of the rumen but

vary in numbers depending on diet and ruminant species. The discovery of a diverse group of rumen bacteria capable of degrading FA under different physiological conditions provided the first opportunity to study possible microbial protection from FA poisoning using naturally-occurring bacteria.

Next, the nutrient requirements for optimal growth and degradation of FA were investigated in the *Cloacibacillus* and *Pyramidobacter* strains under different culture conditions. Both of these bacteria are asaccharolytic, amino acid-fermenting microorganisms. Whole genome analysis identified that these FA-degrading Synergistetes bacteria could also utilize betaine and sarcosine for growth which was verified in growth assays with these nutrients. In general, the growth of these bacteria was enhanced by peptide-rich protein hydrolysates (tryptone and yeast extract) compared to an amino acid-rich protein hydrolysate (casamino acid). Preference for arginine, asparagine, glutamate, glycine, and histidine as free amino acids was observed, while the utilisation of serine, threonine, and lysine in free form and peptide-bound glutamate was stimulated during growth on FA. Overall the greatest improvement in FA degradation occurred in response to supplementation with sarcosine, arginine, and histidine for the *Cloacibacillus* strain.

Transcriptomic analysis of MFA1 during growth in the presence of FA degradation revealed that several genes [Fe-S oxidoreductase and glycine reductase complex B (GrdB)] clustered in a glycine-like reductase operon were highly expressed and appear to be the molecular basis for reductive defluorination in these bacteria. Furthermore, the molecular process for reductive defluorination appears to specifically require selenium for selenoproteins involved in the glycine reductase system. As predicted from the gene analysis growth and degradation of FA by strains MFA1 and C12-8 were enhanced in the presence of different amounts of selenium with an optimal concentration of about 50 µM. Selenate as a supplement increased growth only in the presence of FA and degradation of FA was also increased.

Further tests were performed to determine whether expression of FA-degrading genes could be induced by compounds that may be fed as nutrients to the animal. This was achieved by constructing a gene expression reporter assay that was controlled by the cloned promoter of the FA-degrading gene sourced from MFA1 genomic DNA. It was discovered that the promoter was highly induced in response to the addition of arginine and citrate in the reporter assay.

To evaluate the ability of FA-degrading bacteria to persist and degrade FA in response to nutritional supplements, an *in vitro* rumen fermentation system was used with MFA1 as the model FA-degrader. It was observed that a combination of arginine and/or citrate as supplements effectively increased the percentage of MFA1 relative to rumen total bacteria as well as FA degradation. MFA1 was also able to persist and survive in the mixed rumen culture in the absence of FA. These results suggest that specific supplements may effectively increase the portion of FA-degrading bacteria and promote their FA-degrading ability in the rumen environment.

In order to track the presence of FA-degrading bacteria in the rumen, we developed an accurate detection method for monitoring the abundance of the two Synergistetes isolates (*Cloacibacillus* spp. and *Pyramidobacter* spp.) by designing primer sets targeting the 16S rDNA gene for quantitative real-time PCR (qPCR) assays using gDNA and RNA-derived cDNA templates. In addition, a qualitative method involving nested PCR for detection of the presence of both strains was used when their

numbers were below the limits of detection (LOD) of the qPCR assays. Here a larger genomic template was first amplified and a sub-sample of this reaction was used as a secondary PCR target. Specific amplification of the MFA1 and C12-8 genomic DNA targets as a qualitative measure using the nested PCR technique was effective in detecting the presence of the bacteria when they were below the LOD of the qPCR assay.

A survey was conducted of the prevalence of the lead FA-degrading isolates *Cloacibacillus* sp. strain MFA1 and *Pyramidobacter* sp. strain C12-8 in cattle throughout northern Australia. Rumen samples were collected from cattle in a range of areas where FA-containing vegetation was either present or absent. DNA based rather than RNA derived detection methods were used for the survey because the quality and stability of stored mRNA compromised the sensitivity of the assay which resulted in similar levels of detection for both methods. Both bacteria were present in all herds sampled but the numbers were relatively low (~ 10^5 - 10^6 per ml rumen fluid or 0.001% of the total bacterial population). Cattle in the Northern Territory and north-west Queensland appeared to have a greater abundance of these bacteria than animals sampled from coastal properties in Queensland but seasonal differences were not observed. Therefore these bacteria appear to be indigenous to cattle in northern Australia and thus inoculation of animals with FA-degrading isolates may not be required in a strategy to protect animals from FA poisoning.

In conclusion, peptide-rich protein hydrolysates are general nutritional stimulants for Synergistetes bacteria that degrade FA and specific nutrients such as arginine, histidine, sarcosine, and citrate may further enhance FA degradation. Selenium appears to be an essential co-factor that is required to degrade FA. Feed supplements enriched with these nutrients may optimise the ability of these bacteria to protect the animal from toxicity.

Future studies should focus on animal experiments to demonstrate the benefit that various supplements may provide in preventing FA poisoning in cattle. These experiments could be performed with small numbers of cattle fed differing supplements which are predicted to increase the population of FA degrading bacteria and stimulate FA degrading activity in each bacterium. Indicators of protection from FA intoxication could be measured from rumen samples of supplemented cattle without intoxicating the animal. Studies involving poisoning with FA are unlikely to gain ethical approval unless there is evidence that a treatment could be protective against poisoning.

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1 Background

Sodium monofluoroacetate FCH_2-COO-Na^+ (Fig. 1) (referred to as fluoroacetate or 'FA' hereafter) is a white, odourless and water-soluble compound that is described as tasteless or with a mild, salty or vinegar like taste (Atzert 1971). It is readily absorbed through the gastrointestinal tract or respiratory system or through mucous membranes and skin wounds or abrasions. It is not readily absorbed through intact skin. FA is a highly toxic compound to mammals causing sudden death (Fig. 7). First synthetically derived in the laboratory in 1896, it was only first purified from natural sources in 1943 from the South African plant "gifblaar" ("poison leaf") *Dichapetalum cymosum* (Marais, 1943). This toxin is found in vegetation throughout the world (Fig. 2) (Lee et al, 2014) where it likely naturally evolved as a defence mechanism against grazing herbivores. In ruminants, it has an LD_{50} of around 0.3 $mg.kg^{-1}$ of body weight (Annison et al, 1960; Padmanabha et al, 2004).

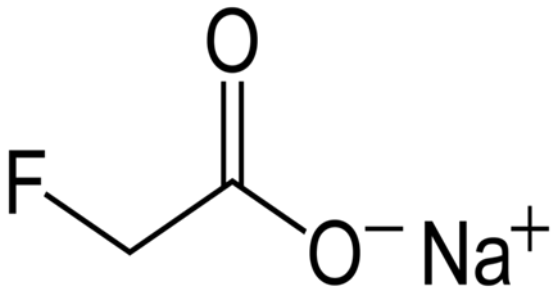

Fig. 1. Sodium monofluoroacetate (FCH_2-COO-Na+)

FA is reported as the toxic ingredient within a number of plants poisonous to livestock including *Dichapetalum spp* (1944, 1972), *Acacia georginae* (1961), *Palicourea marcgravii* (1963) and *Gastrolobium spp.* (1964) (Twigg and King, 1991; Weinstein and Davison, 2004). Unsurprisingly, poisonings in livestock are a major cause of significant economic loss to commercial enterprises worldwide, particularly in Australia, Brazil, and South Africa (Bell et al., 1995; Aplin, 1969; Nwude et al., 1977; Medeiros et al., 2002). Indeed it is estimated that 60 % of cattle losses in Brazil are due to ingestion of FA producing plants (Tokamia, 1990). In Australia, elevated fatality rates and associated productivity impacts due to increased morbidity as a result of intoxication are estimated to directly cost the Australian livestock industry ~$AUD45 million annually (Perkins et al., 2015). Significant further economic losses due to the impact of reduced carrying capacity and modified husbandry practices on properties affected by FA poisoning also indubitably occur.

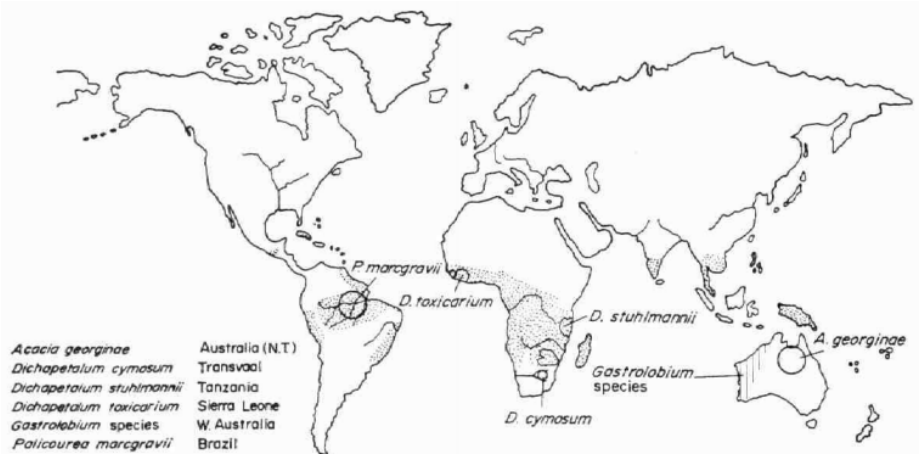

Fig. 2. The geographical distribution of plants containing organically-bound forms of fluorine.

* Mottled areas show the distribution of the whole Dichapetalaceae family (from Hall, 1972)

1.1 Fluoroacetate in the Australian environment

In Australia, FA is also found in about 40 species of plants, generally at low levels (Twigg and King, 1991), although some accumulate FA to levels of up to 5 g.kg(dry weight)$^{-1}$ (Hall, 1972). Grazing of these toxic plants largely follows a geographic distribution and is particularly a problem during the spring and episodes of drought (Kellerman, 2009) in the Northern Territory and Queensland (Fig. 3) (Leong et al., 2017), and after fire or rain when new growth is greatest.

Fig. 3. Distribution of fluoroacetate bearing plants in Australia.

*Black dots *Gastrlolobium* spp*.* Grey dots *Acacia georginae.*

Georgina Gidgea (Fig. 4) grows in nutritious soils and consumption of leaves is actually good for fattening cattle, particularly during the dry season. The widespread prevalence of these trees renders clearing impossible (current legislation often prevents this anyway) or uneconomical. Similarly, 'fencing off' is often not economically or physically practical. Death rates are predominantly dictated by the season and amount of other fodder as alternative feeds. In a 'typical' year fatalities occur through the latter months of the dry season until significant breaking wet-season rain is received (≥50 mm) when alternative vegetation readily grows. Smaller falls until such time, particularly in spring, increase new, palatable but toxic plant growth and there is often a corresponding increase in death rates after such rain events. One of the most toxic parts of the plant is the highly palatable flower pod and natural selective grazing on these can prove most fatal, often in late August. All cattle classes are affected and there appears no inherent immunity after time grazing or breeding on affected country. Current control strategies revolve around management and in particular low-stress handling techniques that do not cause stress to sub-clinically intoxicated animals (Perkins et al., 2015).

A)

B)

C)

D)

Fig. 4. (A) Georgina Gidgea, *Acacia georginae*, (B) *Gastrolobium bilobum* – Heartleaf poison, (C) *Gastrolobium grandiflorum* – Heartleaf poison, and D) *Gastrolobium* Heartleaf poison bush in dry season.

Gastrolobium Heartleaf Poison Bush (Fig. 4. (A) Georgina Gidgea*,* Acacia *georginae,* (B) Gastrolobium bilobum – Heartleaf poison, (C) *Gastrolobium grandiflorum*, and (D) heart leaf in the dry season) grows in the sweet country in an arid environment. Here poisoning is predominantly a problem after fire or rain, again during times of stress when the stock is handled, and with newly introduced cattle. Unlike Georgina Gidgee, Heartleaf is most probably grazed with low preference, only when there is no other feed available. There is some debate (and likely variation property to property) as to which parts of the plant are most toxic, which stages of plant growth, which time of year, and what other climatic or other events are worst. However, there is a predominant view amongst producers that fresh growth after rain at the end of a dry spell, or particularly after fire, presents the greatest risk of poisoning. After the latter Heartleaf is often the first plant to recover and if cattle are not removed from widely infested areas within about a fortnight after the first soft, green, highly palatable leaves shoot, then deaths frequently occur. Little grazing occurs during dry periods when leaves dry and harden, likely becoming unpalatable.

Again, deleterious effects are limited largely through management practices such as:

- Light stocking rates
- Low-stress handling techniques (including selective mustering if signs of poisoning are recognized)
- Fencing off areas where Heartleaf is highly prevalent
- Using infested grazing land only during the dry season
- Avoiding areas after fire until sufficient alternative edible plants have recovered (often 6-8 months)
- Quietly mustering cattle to ‘clean’ areas for 5-6 days before further processing and transport
- Increasing watering points to reduce the walking distance of cattle and associated physical stressors
- Consistent plant control and eradication in the selected country

Constant and immediate impacts of FA poisoning are increased morbidity and mortality rates and the necessity of reduced stocking rates. Restrictions on management practices including mustering and selling time options, costs of management strategies including plant eradication and stock handling, increased infrastructure costs (clearing, fencing and watering), diminished capacity to use fire as a management strategy, and reduced reproductive rates also contribute to production and ultimately economic impacts (Perkins et al., 2015).

1.2 Fluoroacetate toxicity mechanisms

Fluorocitrate inhibits the normal activity of the tricarboxylic acid (TCA) or Kreb’s cycle. There is some debate over the precise mechanisms of action for fluorocitrate but it appears to inhibit the activity of aconitate hydratase (Aconitase; EC 4.2.1.3) and also to inhibit citrate transport, two steps that are vital to normal processes within the Kreb’s cycle within mitochondria (Eason and Miller et al., 2011).

The Kreb’s cycle is a series of chemical reactions used by all cells to generate ATP (adenosine triphosphate). ATP is one of the major sources of energy within cells and the energy released when ATP is metabolized is the major driver of most of the energy-consuming reactions in cells including things such as the formation of other compounds, transmission of nerve impulses and muscle

contractions. Thus, the Kreb's cycle is a central cellular metabolic pathway that connects carbohydrate, fat and protein metabolism. In eukaryotes, eight enzymes within the mitochondria fully oxidize acetate into carbon dioxide and water as well as products which can further be converted into ATP energy through oxidative phosphorylation. It is one of these enzymes, aconitase, which is disrupted in FA poisoning (Lauble et al., 1996). In brief, upon absorption through the gut into the lumen, FA is converted to fluorocitrate by citrate synthase (EC 4.1.3.7) which in turn strongly binds to the essential enzyme (Fig. 5 and Fig. 6). A common hypothesis is a disruption to citrate transport and production has two main effects. One, within the mitochondria inside the cell it limits its supply and in turn prevents the conversion of citrate to succinate, as needed for the further metabolic conversion. Such effects can readily lead to termination of cellular respiration, often resulting in direct cell death (Eason et al., 2011). Secondly, in the body tissues and brain citrate concentrations can actually increase, in turn disturbing numerous other metabolic equilibria. Combined, these perturbations result in multiple morbidities with quick onset including hypocalcemia, heart failure, and acidosis (Shapira et al., 1980; Sherley, 2004; Goh et al., 2005; Proudfoot et al., 2006). Other non-specific biochemical imbalances resultant of poisoning include hypoglycaemia and hypokalaemia (Perkins, 2015).

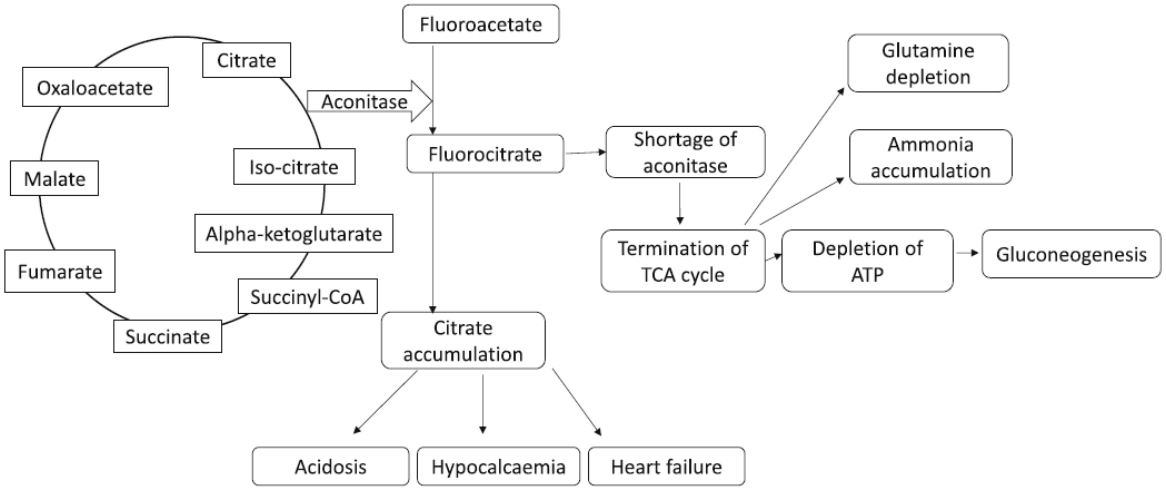

Fig. 5. Physiological responses to TCA cycle inhibition by fluoroacetate (Leong et al., 2017).

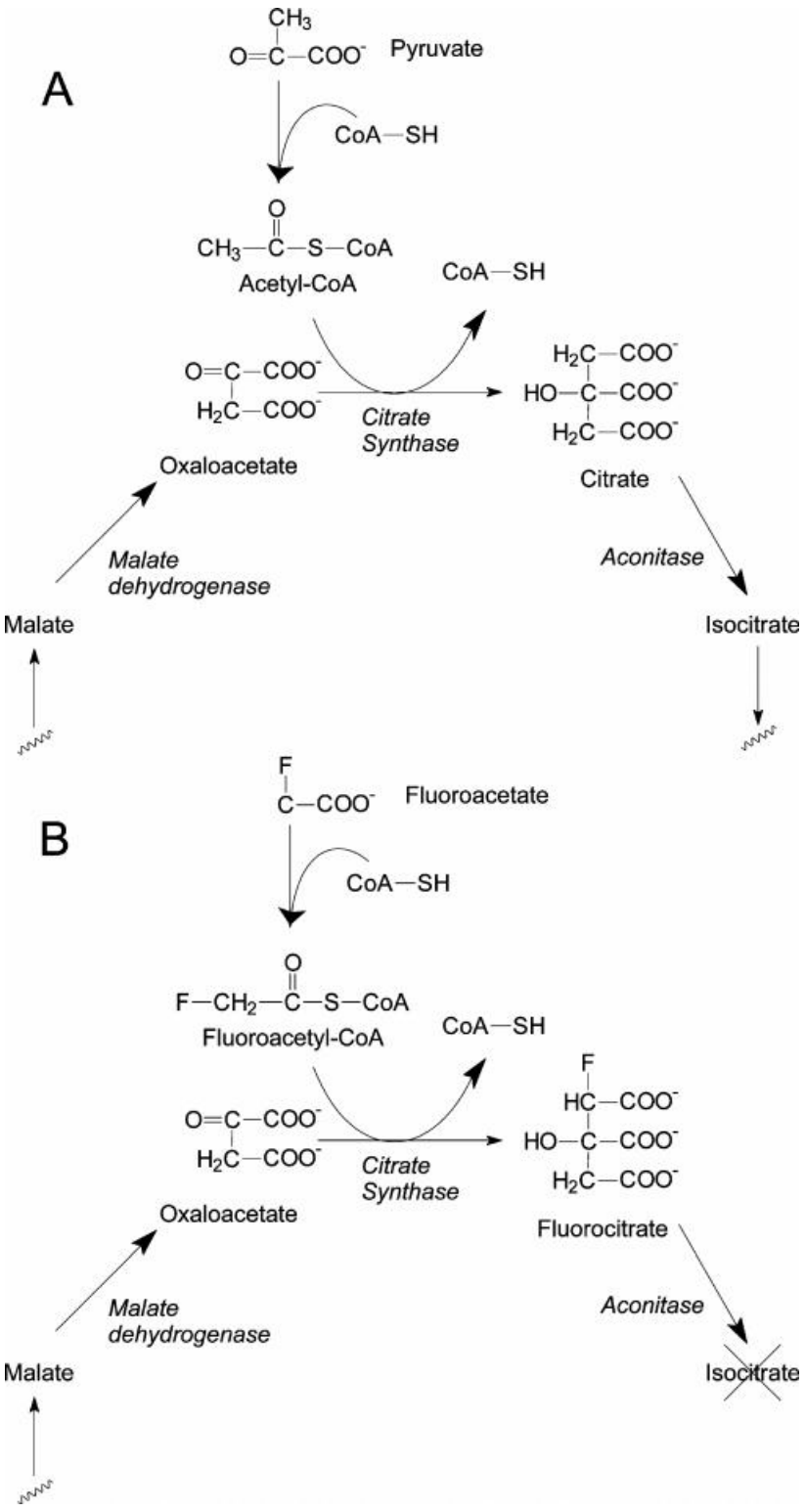

Fig. 6. (A) Partial tricarboxylic acid cycle illustrating the incorporation of acetyl-CoA into the cycle. (B) Partial tricarboxylic acid cycle illustrating the incorporation of fluoroacetyl-CoA into the cycle resulting in the formation of fluorocitrate and subsequent inhibition of aconitase. (Lee et al., 2014).

Clinical manifestations usually take 4-24 h to appear. This process is time-dependent due to the digestion of plant material in the rumen and absorption of FA into the bloodstream, subsequent transportation to target cells and conversion to toxic monofluorocitrate which disrupts cellular respiration (Lee et al., 2014). Common symptoms of FA poisoning in cattle can include balance loss, ataxia, tachycardia, laboured breathing, urinary incontinence, involuntary muscle contractions, convulsions, collapsing and in-place running lasting 3 to 20 minutes following repeated falling, often followed by death (Riet-Correa et al., 2009; Robinson, 1970). Sub-lethal intoxication usually manifests as a reluctance to walk or lethargy, frequent lying down, heart palpitations and observably pulsing jugular vein (Riet-Correa et al., 2009). Variation in the onset of these symptoms between individual beasts even of the same species is commonly observed (Marais, 1943). Indeed, animals can be asymptomatic up to 29 h following ingestion of a lethal dose of FA before a short period of acute illness and “sudden death” (Leong et al., 2017) (Fig. 7).

Fig. 7. A common scene of typical losses due to "sudden death" as a result of fluoroacetate poisoning.

1.3 Medicinal treatment FA poisoning

A number of strategies to treat the oft-fatal symptoms of FA toxicity have been investigated. Early studies researched administration of acetate to livestock immediately following ingestion with a view to ameliorating pathogenic acetate metabolism effects (Bartlett and Barron, 1947; Chenoweth, 1949). Similarly, citrate therapy has also been explored and researchers were able to successfully increase survival rates of poisoned livestock (Tourtellotte and Coon, 1951), though notably some of the studied animals died of other complications of poisoning. A more recent approach assessed the administration of activated charcoal or resins to absorb FA from the gastrointestinal fluid, and thus potentially prevent conversion into fluorocitrate and thus downstream pathogenesis (Norris et al., 2000).

Very recently, research from Brazil has pointed to the possible use of acetamide as an antidote to poisoning (Shokry et al., 2017). Cattle were administered sub-lethal doses of FA and in a novel use of a biological specimen, head-space gas of heated earwax samples were analyzed using an HS-GC/MS. The amount of FA detected in samples from animals was inversely proportional to the dose of acetamide administered with significant difference seen between groups receiving the lower dose compared with the higher dose.

Unfortunately, such treatments are impractical for a number of reasons. In each case there are one or more limiting realities: the dosages required to be therapeutically effective, timeframes following poisoning until treatment, the direct cost of such medicines and associated expenses of ongoing treatments, and logistical constraints on surveillance and administration in rangeland production systems make all such remedies fundamentally unfeasible. Indeed, the fact that they are treatments in themselves and not preventative is a significant limitation.

1.4 Fluoroacetate tolerance

Interestingly, mammalian herbivores are considered to be only intermediately sensitive to toxicity when compared to other carnivores (sensitive) or reptiles (even less sensitive). It has been postulated that this is due to a slower metabolic rate allowing more time for excretion and detoxification (King et al., 1978). This is supported by such facts as increased tolerance in the bandicoot, which has an uncommonly low metabolic rate compared to other similar sized rodents (Macmille and Nelson, 1969). Moreover, Australian native animals that live in areas where FA containing plants are endemic show increased tolerance to FA (Oliver et al., 1979; Twigg et al., 2003) when compared to the same species in areas without such FA-accumulating vegetation. This is especially true of Australian seed-eating birds, for example, the emu (Twigg et al., 1988 and 1989).

Various studies have looked at the biochemical mechanisms of FA tolerance and detoxification. Clearly, a number of key factors likely affect the rate of FA metabolism: (1) the conversion rate of fluoroacetate to fluorocitrate, (2) the sensitivity of aconitate hydratase (EC 4.2.1.3) to fluorocitrate, (3) citrate transport and FA-related metabolic processes within the mitochondria, (4) the ability of host microbes to detoxify FA, and (5) other host-specific differences. However, the nature of acquired tolerance is still not fully elucidated and further work is very much needed.

1.5 Detoxification of fluoroacetate

To date, studies looking to isolate, purify and characterize FA host detoxifying enzymes from tolerant animals have generally been unsuccessful and no scientific consensus has been reached. Although it is accepted that FA is largely defluorinated within the liver by an enzyme generically referred to as FA-specific defluorinase (Kostyniak et al., 1978; Mead et al., 1985), more recent research has yet to fully elucidate the mechanism of FA detoxification by the host itself.

1.6 Microbial degradation of fluoroacetate

The concomitant hypothesis suggests that microbes within the gut of tolerant animals are responsible for most FA detoxification. In this regard, much work has been done to document and characterize aerobic microbial FA degradation. Many such bacteria and fungi are found in soil and plants, and degrade FA, presumably using it as a carbon source. Conversely, isolation studies of anaerobic FA degrading microorganisms are in relative infancy and further study is needed. Accordingly, the body of work in this report focuses on a recently isolated native bacterium from the Australian bovine rumen using anaerobic agar plates (Davis et al., 2012). Unlike other microbial degraders, this bacterium appears to degrade FA via the reductive dehalogenation pathway, using it as a terminal electron acceptor rather than utilizing acetate from the degradation as a carbon source (Leong et al., 2017).

1.7 Biotechnological-derived methods for fluoroacetate degradation in cattle

To date, there have been a number of attempts to diminish the toxicity of FA and associated poisoning in livestock via biotechnological-derived methods. In a number of seminal studies by Gregg and colleagues, genetically modified bacteria were able to significantly diminish toxic effects. Briefly, the *dehalogenase H1* gene from *Delfitia acidovorans* strain B was incorporated into the pBHf plasmid and used to transfect the ruminant bacteria *Butyvibrio fibrisolvens* (Gregg et al., 1998). Not only were bacterial transfections very stable, maintaining the modified plasmids in 100 % of transformed bacteria over 500 generations without direct selection, but in separate trials, they were able to colonize the rumen of both sheep and cattle for an experimental period of 5 months, and 20 days respectively. Moreover, they were able to demonstrate fluorine production indicative of significant FA degradation *in vitro* and more importantly marked a reduction in FA poisoning in both sheep and cattle. Such studies show microbial protection in the ruminants is possible, but the technology has not been adopted in Australia due to government restrictions on the use of genetically modified organisms. Finally, a recent study in Brazil has shown administration of sub-toxic doses of the plant *Amoria pubiflora*, one of the chief culprits for livestock poisoning, to sheep is able to induce FA resistance. Furthermore, direct microbial transfer of rumen contents from FA resistant animals to naïve sheep conferred protection (Beckerl et al., 2016).

1.8 Conclusions

For the reasons discussed above, we advocate an alternative approach utilizing bacteria naturally found within the rumen of cattle exhibiting an innate ability to anaerobically degrade FA in the rumen.

2 Project objectives

- Identify a consortium of FA-degrading rumen bacteria which have been ‘trained’ in the laboratory to rapidly degrade FA and available for use in animals
- Identify FA analogues which are non-toxic and induce the enzyme that degrades FA as well as identifying nutrients that stimulate the growth of the bacteria
- Develop molecular tools that can be used to measure the population abundance of these bacteria in the rumen.
- Make recommendations on moving to an animal trial to evaluate the ability of the FA-degrading bacteria to counter a lethal dose of FA in the rumen

3 Methodology summary

3.1 FA degrading bacteria identification and characterisation

Samples were collected from the rumen of cattle and sheep in Australia including Belmont Research Station, Rockhampton; Lansdown Research Station, Townsville; and Floreat Park, Perth (Table 6, 8.2.1 Sample collection and isolation of FA degrading bacteria). FA-degrading bacteria were isolated (8.2.2 New isolations of FA degrading bacteria from rumen collections) and trained using selective media (8.1.1 Culture studies). FA degradation was assayed by measuring fluoride production in cultured media (8.1.1.2 Fluoride analysis from the culture supernatants). Microbial DNA from these presumptive degrading enrichments was extracted (8.2.3 DNA extraction of isolates) and isolated

strains were identified via 16S ribosomal DNA sequencing and subsequent taxonomic analysis (8.2.4 PCR amplicons of 16S rDNA genes and sequencing analysis).

FA degrading isolates from rumen and other herbivore gut were cultured and their growth and defluorinating ability measured (8.3.1 Ranking of bacterial growth and defluorination of isolates). A range of protein hydrolysates and other nutrients identified through genome analysis were tested in these bacteria in basal enrichment media to clearly elucidate the effect of each nutrient on the growth of the isolates and their FA degradation capability under anaerobic conditions (8.3.2 Growth on different sources of nitrogen of key FA degrading rumen isolates).

3.2 Screening of compounds that up-regulate FA operon activity and their evaluation in pure culture studies

Genomic DNA of strain MFA1 was used as a template to clone the promoter region of the operon involved in FA degradation. The primers were designed (8.1.4.1 Design and validation of PCR primers) and used to target putative FA degrading functional genes (annotated as Major Facilitator Superfamily (MSF) transporter, Fe-S oxidoreductase, Glycine reductase complex B gamma subunit) from the genome sequence of MFA1 (Lex Leong PhD thesis, 2014) and the luciferase gene from the pGL4 vector for reporter assay (8.5.3 Luciferase reporter assay). A reporter assay utilized differential expression of the bioluminescent protein that fireflies use to emit light (8.5.3 Luciferase reporter assay). The promoter elements of MFA1 were analysed and cloned into a pGL4 vector encoding a luciferase protein (8.5.1 Cloning promoter region of strain MFA1 and 8.5.2 Construction of a plasmid for reporter assay). Next, healthy transformed *E. coli* bacterial monocultures were treated with a number of growth supplements and their effect on the reporter gene expression level (i.e. promoter function) was analyzed (8.1.3 RNA extraction and cDNA synthesis and 8.5.4 Transcriptional expression analysis by RT-qPCR). Based on these studies, the FA degrading isolate MFA1 was also grown in monoculture with the prime candidate supplements that upregulated promoter function. Thus, the effect of these supplements on target bacteria was analyzed (8.5.5 Evaluation of bacterial growth and FA degradation in pure culture for supplements identify through genome analysis).

A draft genomic analysis was performed on strains MFA1 and C12-8 to identify key metabolic pathways and nutrients involved in growth and metabolism which were evaluated *in vitro*. MFA1 and C12-8 were grown on a range of filter-sterilized supplements (Table 9) which were used to clearly elucidate the effect on the growth and FA degradation capability of MFA1 and C12-8. Growth and FA degradation were monitored by measuring optical density (OD) at 600 nm and fluoride production. In case of selenium (sodium selenate decahydrate), growth and FA degradation of both isolates were measured for investigating responses to different concentrations of selenium (8.5.5 Evaluation of bacterial growth and FA degradation in pure culture for supplements identify through genome analysis).

3.3 Molecular tools to measure population abundance

DNA and RNA were extracted from enrichment cultures or rumen fluids spiked with isolates using previously described methods (8.4 Monitoring FA degrading isolates using molecular tools). The limits of detection (LOD) were determined by qPCR methods (8.4.4 Evaluation of standard curves and limit of detection (LOD) of FA degrading isolates (strain MFA1 and C12-8) using qPCR).

The qPCR and nested PCR (8.1.4 PCR studies) were used to ascertain the prevalence of leading FA-degrading isolates from environmental rumen samples which were collected from grazing animals in northern Australia or directly from the rumen of slaughtered animals at Livingstone abattoir (Fig. 13, 8.4.5 Sampling for an environmental survey of FA degrading Synergistetes isolates in northern Australia).

3.4 The ability of MFA1 to persist and degrade FA with supplements applied to an *in vitro* rumen mixed fermentation

The persistence, growth, and FA degradation of strain MFA1 in mixed rumen bacteria, and its detection were also investigated using *in vitro* rumen mixed fermentation studies (8.6 Evaluation of ability for MFA1 to persist and degrade FA in an *in vitro* rumen mixed fermentation). In summary, five experiments were designed and combinations of arginine and/or citrate supplements were used to evaluate the performance of strain MFA1 in competition with a mixture of rumen bacteria (Table 10). The medium used to support the growth of a stable population of mixed rumen bacteria contained different sources of polysaccharides such as starch, xylan, and pectin (8.6.2 Preparation of medium, inoculum, and incubation). The percentages of strain MFA1 relative to rumen total bacteria (8.6.6 Quantitative PCR (qPCR)), FA degradation (8.6.3 Fluoride analysis from the culture supernatants), and SCFA (8.6.4 Short-chain fatty acids (SCFA) analysis) were analyzed from cultured media.

4 Results and discussion

4.1 FA degrading bacteria identification and characterisation

Enrichment cultures of gut contents from a range of herbivores revealed degradation of FA under anaerobic conditions. Subsequently, several bacteria capable of degrading FA were isolated (Table 1). The 16S rDNA gene sequence analysis revealed that all discovered FA degrading isolates could be classified into two phyla (Synergistetes and Firmicutes), and four genera: *Cloacibacillus*, *Pyramidobacter*, *Oribacterium,* and *Eubacterium* (Fig. 8).

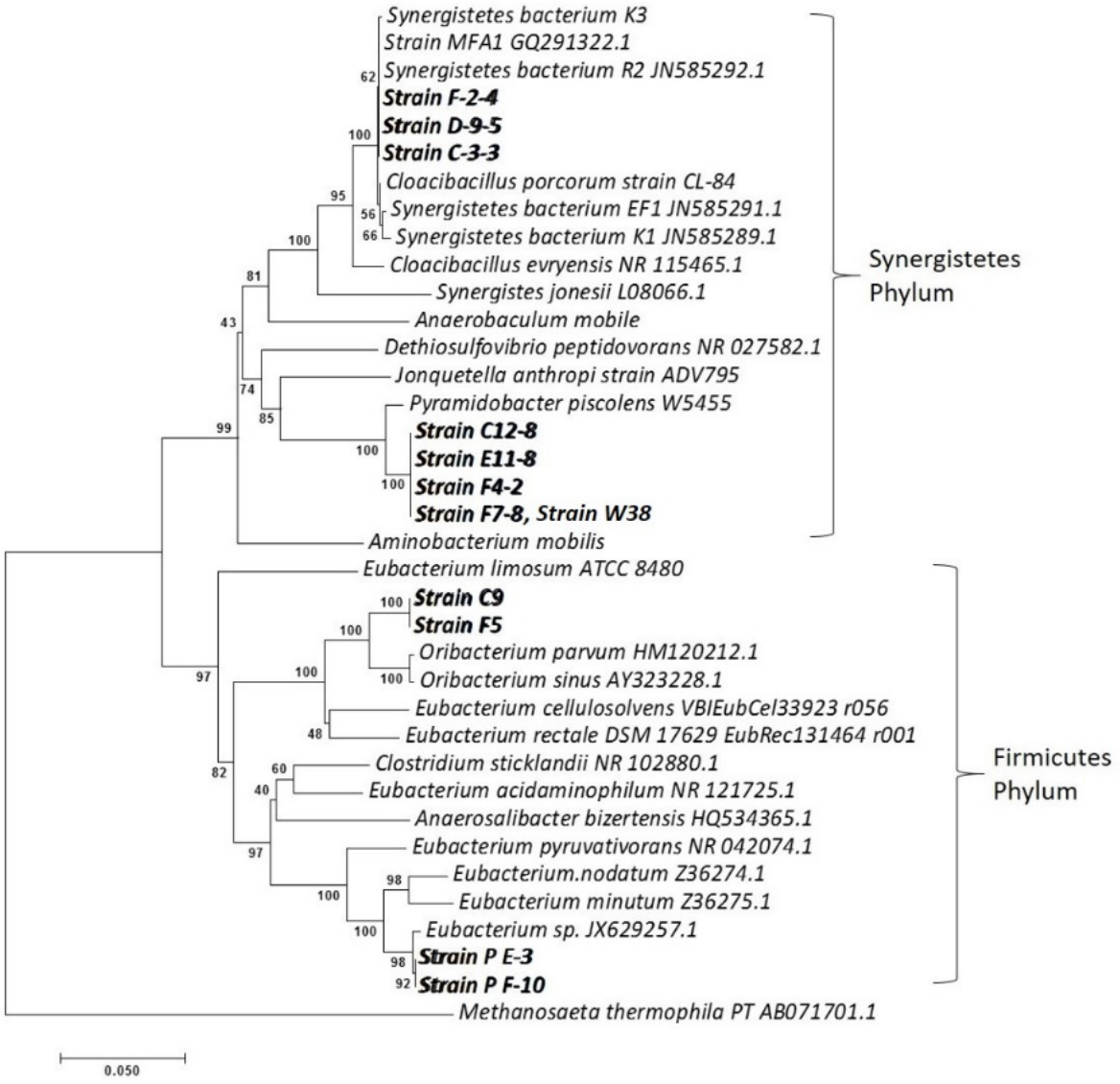

Fig. 8. Phylogenetic tree of isolates (bold) based on 16S rRNA gene sequences constructed using neighbour-joining methods. *Methanosaeta thermophila* is supplied as outgroup. The analysis involved 37 nucleotide sequences included in reference strains. The tree was constructed in MEGA6 program. The bootstrap values at nodes represent values for 1000 resampling's.

The isolates belonging to the Synergistetes phylum were affiliated with two genera; *Cloacibacillus* and *Pyramidobacter*. *Cloacibacillus* strains C-3-3, D-9-5, and F-2-4 had 99 % sequence identity with *Cloacibacillus* strain MFA1 and *Cloacibacillus porcorum, a* mucin-degrading bacterium from the swine intestinal tract (Looft et al., 2013). Previously the only FA-degrading bacterial isolates from the rumen and other herbivores gut systems belonged to the *Cloacibacillus* genus within the phylum Synergistetes (Davis et al., 2012). Until now, FA-degrading bacteria were mainly isolated from the soil around some plants which synthesize high concentrations of FA from fluoride in Australia, Africa and Central America (Liu et al., 1998; Camboim et al., 2012) and those soil microorganisms degrade FA aerobically. Synergistetes isolate strain MFA1 was the first bacterium identified that has an ability to degrade FA anaerobically in the rumen (Davis et al., 2012). Cells belonging to this phylum are mainly rods with various shapes and characterized by their ability to utilize amino acids as sources of energy (Jumas-Bilak and Marchandin, 2014). Currently, the phylum was classified as one class (Synergistia), one order (Synergistales), one family (Synergistaceae), and 20 species belonged to 12 genera (Jumas-Bilak and Marchandin, 2014). *Pyramidobacter* strains C12-8, E11-8, F4-2, F7-8, and W38 were 99 % identical to an uncultured bacterium *Synergistes* sp. L4M2 (clone 196.B09) from ovine rumen fluid, and 98 % identical to *Pyramidobacter piscolens* W5455 isolated from the human oral cavity (Downes et al., 2009). More investigations of this isolate including a morphology and metabolic characterisations are shown in section 9.1.4 (Appendix 2, 9.1.4) (Further investigations for *Pyramidobacter* strain C12-8).

The 16S rRNA gene sequence of the Firmicutes isolates from cattle (strains C9 and F5) in this study belong to *Oribacterium* genus and are 95 % similar to *Oribacterium sinus* isolated from sinus pus in a young child (Carlier et al., 2004) and 94 % similar to *O. parvum* ACB1T and *O. asaccharolyticum* ACB7T from human subgingival dental plaque (Sizova et al., 2014). *O. parvum* ACB1T and *O. asaccharolyticum* ACB7T were strictly anaerobic, gram-positive, non-spore-forming, rod-shaped, motile bacteria and they required yeast extract for growth (Sizova et al., 2014). Oribacteria previously studied were able to grow on yeast extract and Bacto proteose peptone No.3 but not on casaminoacids or trypticase alone. The strain C9 also grew well on the phytone peptone and yeast extract but not on the amine, casamino acids, and tryptone. The other isolates belonged to the phylum Firmicutes and within the *Eubacterium* genus (strain P-F-10 and strain P_E-3) and were derived from sheep rumen. Their 16S rRNA gene sequence is 95 % similar to *Eubacterium nodatum* isolated from the canine oral cavity (Dewhirst et al., 2012).

Table 1. Herbivore sources, taxonomy, growth and FA degradation of isolates. Cultures were grown to stationary growth phase (3-7 days depending on the strain) in PA10 media supplemented with 0.8% YE and 10 mM FA.

Animals (collection)	Phylum (Genus)	Isolates (strains)	Max OD (600nm)	FA degradation (%)
Cattle (rumen)	*Synergistetes (Cloacibacillus)*	MFA1, R2, C-3-3, D-9-5, F-2-4	0.4-0.6	100
	Synergistetes (Pyramidobacter)	C12-8, E11-5, F4-2, F7-8, W38	0.3-0.4	25-30
	Firmicutes (Oribacterium)	C9, F5	0.5–0.6	5-7
Kangaroo (foregut)	*Synergistetes (Cloacibacillus)*	K1, K3	0.4-0.6	100
Emu (faeces)	*Synergistetes (Cloacibacillus)*	EF1	0.4-0.6	100
Sheep (rumen)	*Firmicutes (Eubacterium)*	P_F-10, P_E-3	0.2–0.3	3

Underline: new isolates in this project

4.1.1 General ranking of FA degradation for bacterial isolates

The five Synergistetes isolates collected previously from the rumen and other herbivore gut systems (*Cloacibacillus* strains MFA1, K1, K3, EF1, and R2) degraded 100 % FA in PA10 media supplemented with 0.8% w/v YE and containing 10 mM FA (Appendix 2, Fig. 14). Similar degradation ability was shown in three other *Cloacibacillus* isolates (strain C-3-3, strain D-9-5, and strain F-2-4) from cattle rumen (Townsville). Four *Pyramidobacter* isolates (strains C12-8, E11-8, F4-2, and F7-8) from cattle rumen (Rockhampton) showed 30 % degradation of FA and *Oribacterium* isolates (strains C9, and F5) from cattle rumen (Rockhampton) showed 5 to 7 % degradation of FA in the same media but with only 0.4% w/v YE (Table 1). However, *Eubacterium* strain P_F-10 from sheep (Perth) showed no degradation activity of FA in PA10 media with 0.8% w/v YE but degraded FA slightly by 3 % when grown on BHIYE media (Table 1). Moreover, the rate of FA degradation of the five *Cloacibacilli* isolates was higher than the other taxa and generally related to their rate and extent of growth, suggesting that FA degradation is closely linked to bacterial cell density (Appendix 2, Fig. 14). This is in accordance with other Synergistetes bacteria, where the reduction of organic or inorganic compounds (e.g. crotonate, and elemental sulfur) and growth could be influenced by the type of nitrogen source (Baena et al., 2000; Zavarzina et al., 2000). This linked relationship between growth and total FA degradation was similarly seen with other *Cloacibacilli* strains, but interestingly not with *Pyramidobacter* isolates. *Pyramidobacter* showed quite good growth in yeast extract protein hydrolysates but FA degradation was lower than peptone sources, uncoupled to growth and occurred mainly during stationary phase (Appendix 2, Fig. 17).

In general, even after training for several sub cultures the isolates classified under the Firmicutes phylum showed very low ability to degrade FA in PA10 media supplemented with 0.8% w/v YE in FA containing media (FA degradation below ~3 %, data not shown). Unlike the Synergistetes isolates, the Firmicutes also use carbohydrate as a carbon source for growth which may influence their FA-degrading ability. These isolates require further study on their nutritional requirements which may further promote their ability to degrade FA.

4.1.2 Effect of different nitrogen sources on growth and FA degradation of key FA-degrading rumen isolates

Growth and FA degradation of leading isolates were elucidated and evaluated in media containing the FA with varying amounts of supplementary nutrients (Appendix 2, 9.1.3 Nutritional requirements of FA-degrading rumen bacteria). Work revealed that protein hydrolysates exert a stimulatory effect on the growth and FA degradation of the *Cloacibacillus* strain MFA1 and *Pyramidobacter* strain C12-8. The growth of both isolates was significantly higher in yeast extract, a protein hydrolysate from autolyzed yeast as compared to its growth in peptone, a peptic digest of meat protein (Appendix 2, Fig. 16, and Fig. 17). Recent study on the growth of strain MFA1 showed that amino acids such as arginine, asparagine, glutamic acid, glycine, and histidine regardless of the type of protein hydrolysate were the fundamental energy sources for growth and peptides containing these amino acids enhanced the growth more than free amino acids (Leong et al., 2015). It was noted that glutamate- and lysine-containing peptides were preferentially utilized by strain MFA1 from yeast extract in the presence of FA. Therefore, peptides, particularly the glutamate-containing peptides which are in higher abundance in yeast extract, seem to promote growth in both strain MFA1 and strain C12-8.

Bacterial growth and FA degradation ratios of strain MFA1 in the four different protein hydrolysates strongly correlated to its growth rate in the respective protein sources, suggesting that the FA degradation is linked to bacterial cell density (Appendix 2, Fig. 16). In accordance with previous culturing in PA10 medium, MFA1 was again able to completely degrade 10 mM FA in cultures containing either yeast extract or peptone after 2 days of incubation (Appendix 2, Fig. 18 B). Over a similar time strain C12-8 showed only 6 % FA degradation in both yeast extract and peptone (Appendix 2, Fig. 18 D). Interestingly, strain C12-8 degraded FA continuously during stationary phase and this effect was more pronounced with peptone compared to yeast extract (30 % and 10 %, respectively) (Appendix 2, Fig. 18 D). Moreover, a higher concentration of peptone (1.6 %) increased the FA degradation to 65 % in strain C12-8 (Appendix 2, Fig. 19). This suggests that amino acids or peptides derived from peptone hydrolysates may stimulate FA degradation rather than that of YE in the C12-8 strain. The SCFA profiles indicated the differences of metabolic end products under the same culturing conditions in these two isolates [Table 12, MFA1 (A) or C12-8 (B)]. When the SCFA profiles were analyzed after culturing in two different protein hydrolysates (yeast extract and peptone, Table 12), the acetate and propionate of strain MFA1 culture were increased significantly in the presence of FA compared to the absence of FA but those of C12-8 culture showed no effect regardless of FA additive. It has been reported MFA1 produced acetate as the primary end product of FA dehalogenation and the amount of acetate correlated with the stoichiometric release from FA degradation (Davis et al., 2012).

In general, the protein hydrolysates exert a stimulatory effect on MFA1 growth, regardless of the origins of the protein. Therefore, the use of specific growth-promoting substrates to enhance the

population of strain MFA1 may be a suitable approach towards protecting livestock from FA poisoning. Although the type of nitrogen source may have a stimulatory effect on its growth and FA degradation rate, FA itself results in a marked increase in growth of MFA1.

4.2 Screening of compounds that up-regulate FA operon activity and their evaluation in pure culture studies

4.2.1 Promoter region analysis and construction of the plasmid for reporter gene assay

The candidate promoter region for the FA degradation operon (Leong, 2014) (Fig. 9) was identified and used to study a rage of amino acids and organic / inorganic compounds (Table 2) which may up-regulate FA degradation in the host bacterium (Appendix 1, 8.5 Screening of compounds that up-regulate FA operon activity and their evaluation in pure culture studies).

Table 2. List of substrates for reporter assay.

Amino acids	Others sources
Alanine	*LB media
Arginine	Yeast extract
Glutamic acid	Casamino acid
Glycine	D-glucose
Histidine	Tryptone
Isoleucine	**SFA
Leucine	Citrate
Lysine	***EDTA
Ornithine	Succinate
Proline	Lactate
Serine	Fumarate
Threonine	Acetate
Valine	Canavanine

*LB, Luria-Bertani media; †SFA, Sodium monofluoroacetate; ‡EDTA, Ethylenediaminetetraacetic acid (pH 8.0)

The region identified contained strong promoter elements such as ribosomal binding site (RBS, Shine-Dalgarno sequence, AGGAGT), -10 and -35 promoter regions (TGTCAAAAT and TTTTCT, respectively) and transcription factor (TF) binding site (TTATATT) (Fig. 9). Interestingly, the TF binding site was related to glycine cleavage activator protein (GcvA) of gcv (glycine cleavage) operon. The operon contains four proteins, the leucine-responsive regulatory protein (Lrp), the purine repressor protein (PurR), the glycine cleavage activator protein (GcvA), and the glycine cleavage repressor protein (GcvR)

and those proteins have been shown to be involved in regulating expression of the gcv operon (Ghrist and Stauffer, 1998). The promoter (410 bp length) was cloned and fused with synthetic firefly luciferase (luc2, *Photinus pyralis*) gene in the 'pGL4, Luciferase Reporter Vector system' (Promega).

Fig. 9. Elements of promoter sequence region. Nucleic acid sequence from the promoter region of glycine like reductase operon (Leong, 2014) was obtained from the MFA1 genomic sequence. Promoter elements were detected by the BPROM program (www.softberry.com). The box indicates the transcription factor (TF) binding site, bold letters indicate -35 and -10 sequence elements, Shine-Dalgarno sequence for a ribosomal binding site (RBS) is underlined bold letters. Dash lines indicate the primers to clone the promoter region.

The luciferase activity of the *E.coli* revealed that arginine and citrate promoted gene expression (Appendix 2, Fig. 25 and Fig. 26 A), as supported further by transcriptional analysis using RT-qPCR with cDNA derived from RNA templates of genes of interest (Appendix 2, Fig. 26 B). Based on the results of the promotor-assay studies, arginine and citrate were added to cultures of strain MFA1 to ascertain the effect on FA degradation. Both compounds stimulated the growth of the bacterium as well as rate and extent of FA degradation (Appendix 2, Fig. 10).

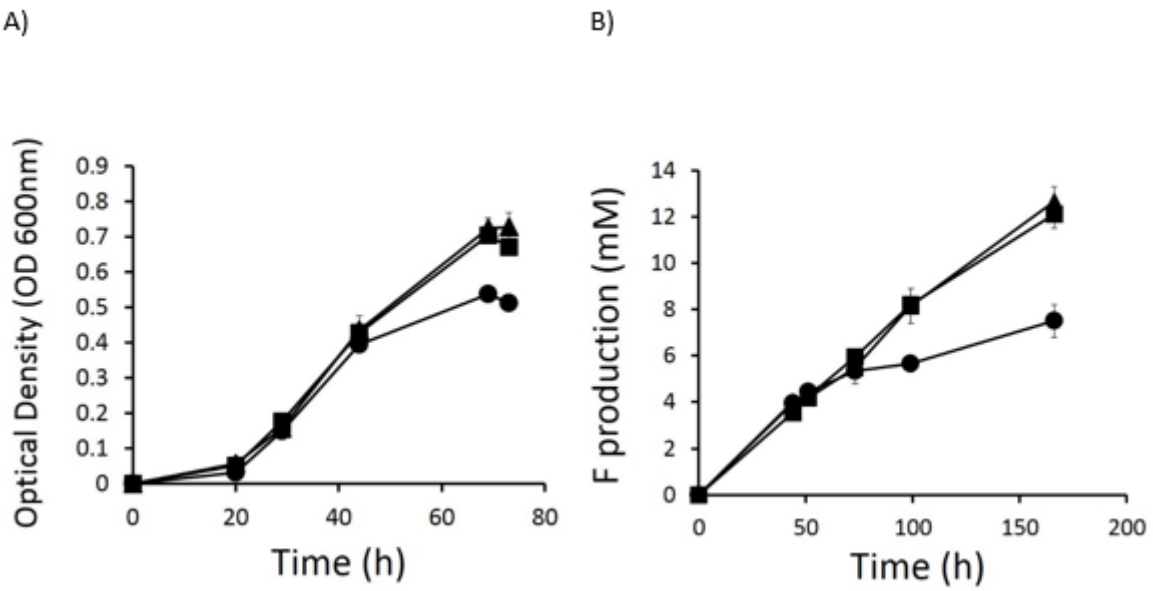

Fig. 10. Effect of different substrates on (A) the bacterial growth of strain MFA1; and (B) fluoride production following FA degradation.

* Markers represent media supplemented with 0.4 % yeast extract, 20 mM FA, and 20 mM of the respective amino acid supplements as indicated (●, control; ■, arginine; ▲, citrate).

In previous studies, arginine was identified as a major energy-yielding substrate for mimosine-degrading bacterium *Synergistes jonesii* which also belongs to the same phylum as MFA1 (McSweeney et al., 1993). To further examine the mode of action of arginine on promoting growth, the analogue canavanine was used as a competitive antagonist. Canavanine is a non-proteinogenic amino acid found in certain leguminous plants and is structurally related to the proteinogenic α-amino acid L-arginine. The sole difference being the replacement of a methylene bridge (-CH2- unit) in arginine with an oxa group (i.e. an oxygen atom) in canavanine. This antagonist interrupted the promoter activity induced by arginine or arginine related regulator proteins that may bind to the promoter (Appendix 2, Fig. 27).

Sodium citrate induced the promoter, and also increased the growth and FA degradation of strain MFA1 (Appendix 2, Fig. 10). The mode of action of citrate in the promoter assay is not clear but some organic acids such as citrate and amino acids are potentially used as the electron donors for the reduction of FA like compounds, which could account for their effect on pure cultures (Davis et al, 2012). Supplementation of citrate has also been shown to be beneficial by increasing ruminal microbial growth and feed digestion (Wang et al., 2009), so citrate could be considered as a feed additive to increase the amount of energy to the ruminant as well as potentially promoting the growth and FA degradation of strain MFA1.

4.2.2 Evaluation of bacterial growth and FA degradation in pure culture for supplements identified through genome analysis

A genomic analysis was performed on strains MFA1 and C12-8 to identify key metabolic pathways and nutrients (Table 3) involved in growth and metabolism which were evaluated *in vitro*. The effect of nutrients/supplements on bacterial growth and FA degradation were both evaluated separately by comparing each leading FA degrading Synergistetes isolate with controls containing no added supplements. A ratio '>1' indicated more growth/degradation than control cultures and '<1' indicated less growth/degradation. Both measures differed depending on which supplement was added to the culture media. This was completed for both strain MFA1 (Table 3) and strain C12-8 (Table 4). Strain MFA1 growth rates increased concomitantly with several supplements in the absence of FA in media except for creatine and carnitine supplementation. Sarcosine, arginine, histidine, glutamic acid, citrate, and formate promoted the growth as well as FA degradation as supplements (Table 3). Even though ethanolamine (6.1 ± 0.16), 1, 2 propanediol (6.2 ± 0.18), glycine (2.1 ± 0.09), and betaine (2.1 ± 0.09) were observed to promote growth, those supplements did not enhance the FA degradation (< 1).

Table 3. The ratio of growth and FA degradation compared with the control of strain MFA1 after 48 hours culture (except as noted).

Supplements	The ratio of Bacterial growth (S / C)*		The ratio of FA Degradation (S / C)**
	(-) FA	(+) FA	
Arginine	2 ± 0.05‡	2 ± 0.24‡	1.6 ± 0.09‡
Betaine (Bet)	2.1 ± 0.09‡	1 ± 0.02	0.7 ± 0.03
Bet+Formate	2.2 ± 0.07‡	1 ± 0.03	0.9 ± 0.05
L-Carnitine hydrochloride†	0.96 ± 0.01	0.96 ± 0.05	0.6‡
Choline dihydrogen phosphate†	1.05 ± 0.04	0.67 ± 0.02‡	0.6‡
Creatine	0.62 ± 0.07‡	1.0 ± 0.03	1.1 ± 0.07
Ethanolamine†	6.1 ± 0.16‡	2.2 ± 0.03‡	0.9 ± 0.02
Glutamic acid	1.6 ± 0.03‡	1.3 ± 0.1‡	1.2 ± 0.11
Glycine	2.1 ± 0.09‡	0.7 ± 0.12‡	0.7 ± 0.08‡
Histidine	1.7 ± 0.13‡	2 ± 0.04‡	1.3 ± 0.02‡
1,2 Propanediol†	6.2 ± 0.18‡	1.7 ± 0.06‡	0.87 ± 0.03
Sarcosine (Sar)	1.7 ± 0.05‡	1 ± 0.07	1.4 ± 0.01‡
Sar+Formate	1.5 ± 0.13	1.3 ± 0.12	1.9 ± 0.2‡
Sodium citrate	1.5 ± 0.02‡	1.7 ± 0.05‡	1.1 ± 0.05
Sodium formate	1 ± 0.08	1 ± 0.05	1.3 ± 0.2
Selenate (40µM) §	1	1.2	2.3‡

* Indicates the ratios of bacterial growth measured by optical density (OD 600 nm); S, Supplements; C, Control

** Indicates the ratios of fluoride production (mM); S, Supplements; C, Control

† Results of samples at 24 hours culture

‡ Results were significant to $P \leq 0.05$ as calculated using a two-tailed t-test between supplements and control

§ Further selenium concentration experiments (see below)

No growth effect observed on acetamide, trimethylamine, and proline.

C12-8 growth rates increased with supplements such as glycine, betaine, sarcosine, citrate, and formate regardless of presence and absence of FA in media. However, the FA degradation did not improve (Table 4). Arginine, carnitine, choline, creatine, acetamide, trimethylamine, proline, ethanolamine, and 1, 2 propanediol had no effect on growth and degradation.

Table 4. The ratio of growth and FA degradation compared with the control of C12-8 at 24 - 48 hours culture.

Supplements	The ratio of Bacterial growth (S / C)*		The ratio of FA Degradation (S / C)**
	(-) FA	(+) FA	
Betaine (Bet)	3.4 ± 0.11‡	2.6 ± 0.11‡	0.3 ± 0.03‡
Bet+Formate	3.7 ± 0.24‡	2.6 ± 0.05‡	0.4 ± 0.03‡
Glycine	2.5 ± 0.04‡	1.5 ± 0.03‡	0.9 ± 0.03
Sarcosine (Sar)	2.8 ± 0.03‡	1.8 ± 0.12‡	0.3 ± 0.05‡
Sar+Formate	3.0 ± 0.11‡	2.1 ± 0.18‡	0.2 ± 0.05‡
Sodium citrate	2.2 ± 0.02‡	1.4 ± 0.03‡	0.4 ± 0.03‡
Sodium formate	2.3 ± 0.17‡	1.6 ± 0.15	0.4 ± 0‡

* Indicates the ratios of bacterial growth were measured by optical density (OD 600 nm); S, Supplements; C, Control

** Indicates the ratios of fluoride production (mM); S, Supplements; C, Control

‡ Results were significant to $P \leq 0.05$ as calculated using a two-tailed *t-test* between supplements and control

Supplements were selected based on nutrients involved in metabolic pathways of MFA1 and C12-8. Regulatory genes for the ethanolamine and 1, 2 propanediol utilisation (*eut and pdu,* respectively) operons were found in the draft genome of MFA1 but not observed in C12-8. As expected, these two nutrients had no effect on C12-8 because the genes involved in their metabolism were not present in the genome. Those substrates have been reported as producing acetyl-CoA and propionyl-CoA as intermediates in catabolic pathways of ethanolamine and 1,2 propanediol, subsequently producing acetate and propionate end-products, respectively (Palacios et al., 2003, Garsin, 2010).

Genome analysis of MFA1 and C12-8 confirmed the presence of the glycine reductase (GR) system in both bacteria. *Treponema denticola* was the first Gram-negative bacterium known to contain a GR system (Rother et al., 2001), and the energy conservation that occurs by reducing glycine to acetyl-phosphate have been studied for about a half century (Andreesen, 2004). In addition to glycine, its N-methylated derivatives sarcosine and betaine can also act as an electron acceptor to form acetyl phosphate (Andreesen, 1994). According to the derived amino acid sequences of GR related proteins from MFA1 and C12-8 genome, glycine, sarcosine, and betaine reductase genes were highly conserved and involved in acetyl phosphate formation from these substrates. Both of MFA1 and C12-8 grew better with supplements of glycine, betaine, and sarcosine but the degradation of FA was lower than

that of control except for the sarcosine supplement in MFA1. Unlike C12-8, the growth and FA degradation of MFA1 were increased in supplements of arginine, histidine, glutamic acid, sarcosine, sodium citrate, and sodium formate regardless of presence or absence of FA (Table 3). For both Synergistetes isolates where there was a marked increase in growth but no commensurate increase in FA degradation, it is likely that bacteria are competitively utilizing supplements in preference to FA. However, after the primary growth-promoting supplement become limiting, it is likely that those isolates would utilize FA as an alternative energy source.

4.2.3 Effect of selenium on growth and FA degradation

For MFA1, bacterial growth and FA degradation were investigated on different amounts of selenium (0 to 40 µM). Growth was enhanced by increasing concentrations of selenium in media containing FA, and FA degradation showed a positive correlation with the concentration of selenium. However, the growth of MFA1 was not affected by selenium when FA was absent from the media (Fig. 11 A).

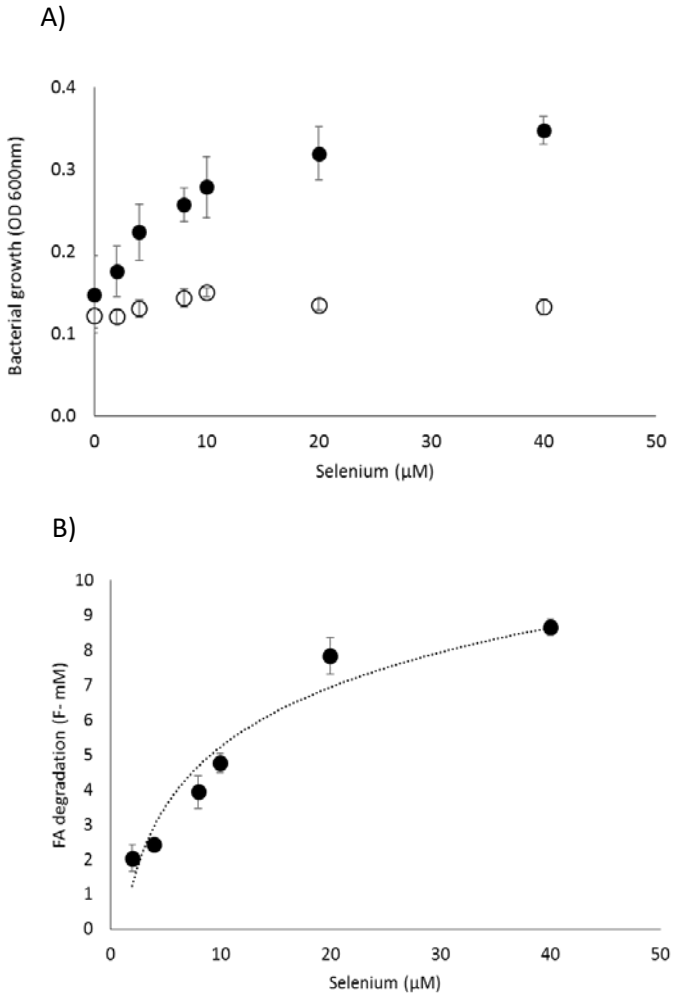

Fig. 11. Effect of growth (A) and FA degradation (B) of strain MFA1 in PA-10 media with 0.2 % YE, 10 mM FA and various concentrations of supplemented selenium.

* ●, presence of FA; ○ absence of FA) (A)

† dotted trend line indicated logarithmic trend line (B)

For C12-8, bacterial growth and FA degradation were investigated on different amount of selenium (0 to 100 µM). Like MFA1, the growth of C12-8 was not affected by selenium in the absence of 10 mM FA (see △, 0 µM selenium (no FA) *vs.* ○, 100 µM selenium (no FA) in Fig. 12 (A)). But, growth and FA degradation increased as selenium concentration increased (Fig. 12).

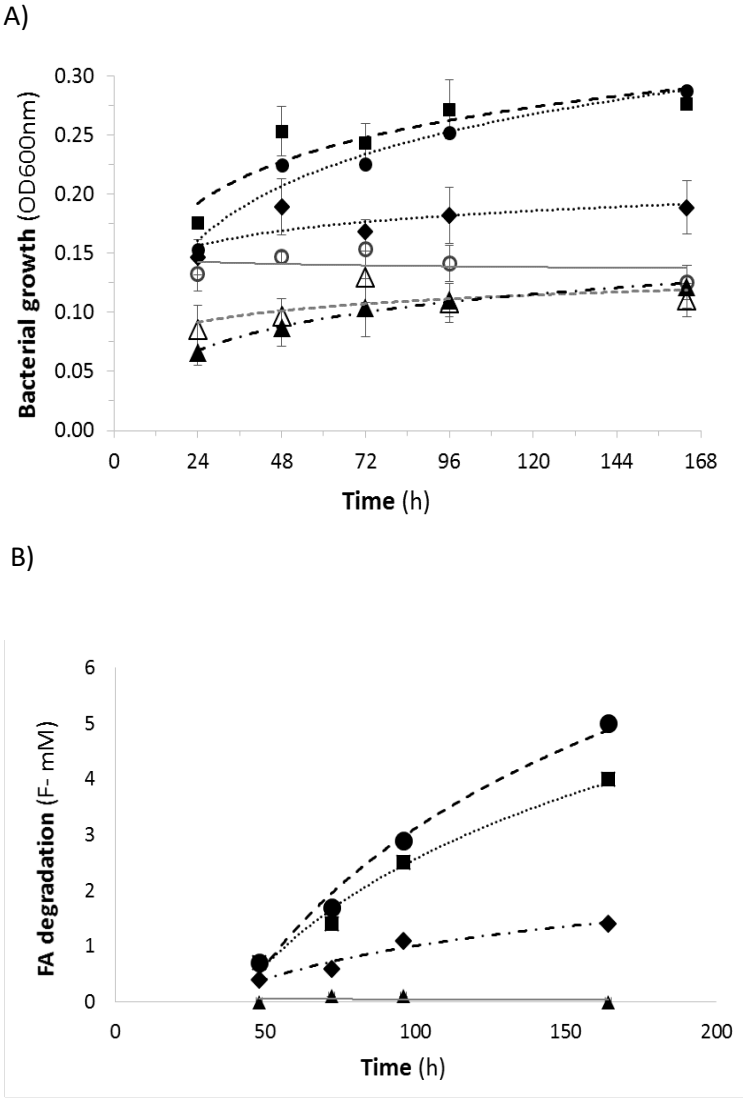

Fig. 12. Effect on growth (A) and FA degradation (B) of C12-8 in PA10 media with 0.4 % YE and various concentrations of supplemented selenium (in presence and absence of 10 mM FA).

△, 0 µM Selenium (no FA); ○, 100 µM Selenium (no FA); ▲, 0 µM Selenium (FA); ♦, 5 µM Selenium (FA); ■, 20 µM Selenium (FA); ●, 100 µM Selenium (FA); dotted lines indicated trend line

In case of selenate supplementation, the growth and degradation in both isolates were positively correlated with increasing concentrations of selenate in the presence of FA (Fig. 11 and Fig. 12). Because the growth of both isolates was unaffected by selenium in absence of FA, it suggests that this trace element is directly involved in FA degradation which generates energy for growth. Rother M. et al., (Rother et al., 2001) reported that the amino acid fermenting bacterium *T. denticola* has a selenium requirement for selenoproteins involved in the GR system.

4.3 Molecular tools to measure population abundance

The FA degrading Synergistetes strains need to be studied *in situ* because of the role they may play in detoxification of FA in the rumen. Detection and quantification of microorganisms by a qPCR approach has proven to be a powerful tool for monitoring microbial populations in the complex ruminal ecological environment (Tajima et al., 2001; Klieve et al., 2003; Koike et al., 2007).

To this end, qPCR assays were developed for the detection of strain MFA1 and C12-8 using the DNA and RNA-based approaches (9.2 Monitoring abundance FA degrading bacteria using molecular tools). Strain MFA1 and C12-8 specific primer sets were designed (Table 6 and Appendix 2, 9.2.1 Design and validation of qPCR primers) and their specificity and sensitivity evaluated using sequencing analysis and qPCR assays (Appendix 2, 9.2.2 Specificity and sensitivity of qPCR primers for strain MFA1).

4.3.1 Detection of leading FA degrading isolates by qPCR using either DNA and RNA (cDNA) based molecules

In the first instance, PCR primer sets specific to 16S rRNA gene targets in target strains were designed and validated using gDNA and RNA-derived cDNA templates from cells grown in pure culture. Extracted nucleic acids were directly diluted in rumen fluid. The qPCR assay using RNA-based molecules for detection of MFA1 was shown to be a 100-fold and 10-fold more sensitive than corresponding DNA-based detection in the enrichment mixed cultures and rumen fluid, respectively. This corresponded to a limit of detection (LOD) of 2.2×10^5 cells / ml for DNA, and 2.2×10^4 cells / ml for RNA (cDNA) based analyses in rumen fluid (9.2.3 Detection of spiked FA degrading isolates (MFA1 and C12-8) using DNA or RNA from rumen enrichment culture and rumen fluids) (Appendix 2, Table 17 and Table 18). This was in line with similar research elsewhere (Backstedt et al., 2015). In the case of MFA1, a nested PCR assay was also developed to detect the presence of the bacterium in the rumen because the LOD by qPCR prevented detection in some animals even though the bacterium was present. Nested PCR (Appendix 1, 8.1.4.3 Nested qPCR) is more sensitive than qPCR and is able to detect the presence of the bacterium but is not quantitative for determining the number of organisms. These initial experiments demonstrated that the validity of a PCR-based approach for a survey of these two biologically important species, and for quantifying growth using *in vitro* supplementation experiments.

4.3.2 Detection of FA-degrading Synergistetes isolates in grazing cattle in northern Australia

A survey was conducted of the prevalence of the lead FA-degrading isolates strain MFA1 and strain C12-8 throughout northern Australia from environmental ruminal samples. These samples were collected from a range of areas where FA-containing vegetation was either present or absent (Fig. 3 and Fig. 13). DNA based rather than RNA derived detection methods were used for the survey because

the quality and stability of stored bacterial RNA compromised the sensitivity of the assay which resulted in similar levels of detection for both methods.

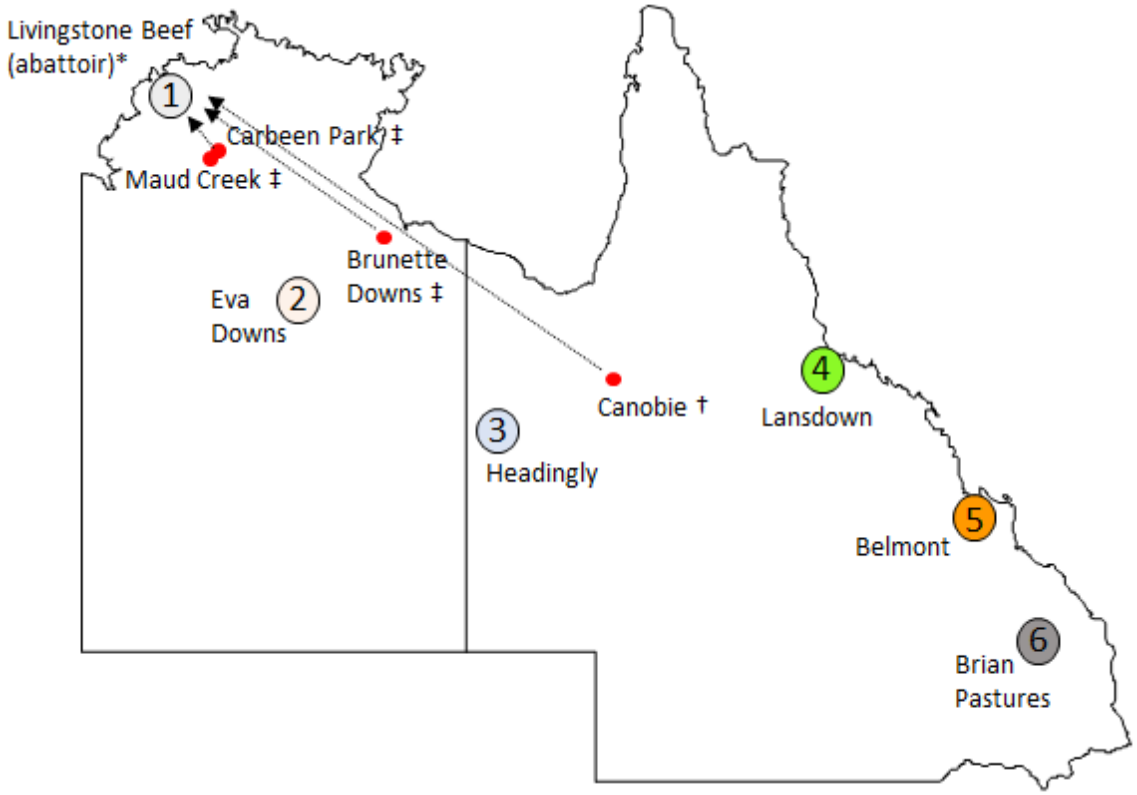

Fig. 13 – Environmental rumen fluid sample collection sites of various areas across northern Australia.

* Livingstone Beef abattoir samples were collected from cattle transported to Darwin primarily from surrounding AACo Stations
† All 52 samples analysed in the first sampling represented cattle from Canobie Station
‡ The remaining samples analysed in the second sampling represented cattle from three other properties

In general, it was found that strain MFA1 was not as prevalent in samples from the eastern seaboard as in those from the central northern Australia where FA-containing plants are more prevalent (Table 5).

Table 5. Detection of FA-degrading rumen bacteria strain MFA1 and strain C12-8 via qPCR analysis of DNA, collected from various sites in northern Australia.

Site No.	Collection	Sampling time	MFA1 %, (n)	C12-8 %, (n)	C12-8 cells.mL^{-1} (± SEM)
1	Livingstone Beef, Darwin, NT (Abattoir)* Samples from: - Canobie Station, Julia Creek, QLD (n=52)† - Carbeen Park, Katherine, NT (n=24)‡ - Maud Creek, Katherine, NT (n=5)‡ - Brunette Downs, Barkly Tablelands, NT (n=10) ‡	Jun	87, (52)† 38, (39)‡	42, (52)† 38, (39)‡	7.36 (± 1.93) x 10^5 3.10 (± 0.67) x 10^6
2	Eva downs, Creswell, NT	Feb	26, (98)	26, (98)	6.54 (± 0.61) x 10^6
		Aug	33, (103)	30, (103)	2.20 (± 0.09) x 10^6
		Oct	32, (98)	34, (98)	1.93 (± 0.12) x 10^6
3	Headingly, Urandangie (SW Mt Isa) QLD	Mar	20, (29)	41, (29)	7.97 (± 9.16) x 10^5
		Aug	100, (30)	40, (30)	2.36 (± 1.08) x 10^6
4	Lansdown, Woodstock, (Townsville), QLD	Feb	0, (88)	22, (88)	1.56 (± 0.1) x 10^6
		May	0, (88)	11, (88)	1.69 (± 1) x 10^6
		Aug	5, (88)	28, (88)	2.13 (± 0.1) x 10^6
		Dec	8, (88)	18, (88)	1.06 (± 0.1) x 10^6
5	Belmont, (Rockhampton), QLD	Jun	0, (35)	50-73 (35)	3.20 (± 1.04) x 10^4
6	Brian Pastures, Gayndah, QLD	Aug	0, (40)	55, (40)	5.64 (± 0.74) x 10^4

* Livingstone Beef abattoir samples were collected from cattle sourced from surrounding AACo. Stations and transported to Darwin.

† All 52 samples analyzed in the first sampling represented cattle from Canobie Station

‡ The remaining samples analyzed in the second sampling represented cattle from three other properties.

RNA is inherently more unstable than DNA and prone to degradation. As such, despite its prior empirical superiority for sensitivity in pure culture, RNA quantification was not used for survey purposes as it did not improve the LOD compared with DNA based methods when applied to environmental samples. Survey results for both Synergistetes bacteria are likely to under-represent the actual prevalence due to the limitations of PCR with environmental samples. It is highly likely that both species are endemic to cattle in the northern beef herd. Arguably, re-survey after their growth was promoted, through supplementation or other means, above experimental threshold levels, would prove this to be fact.

4.4 The ability of MFA1 to persist and degrade FA with supplements applied to an *in vitro* rumen mixed fermentation

It was demonstrated previously that arginine and citrate enhanced the growth of strain MFA1 and associated FA degradation in pure culture. In this round of experiments, a better understanding of the ability of strain MFA1 to persist and degrade FA in competition with other rumen bacteria was gained (Appendix 2, 9.4 Evaluation of ability for MFA1 to persist and degrade FA using rumen *in vitro* fermentation). Five experiments were designed and combinations of arginine and/or citrate supplements were used to evaluate the performance of strain MFA1 in competition with a mixture of rumen bacteria (Table 9, 9.4.2 Effect of different supplements on FA degradation by strain MFA1 in mixed rumen bacteria *in vitro* fermentations). The medium used to support the growth of a stable population of rumen bacteria contained different sources of polysaccharides such as starch, xylan, and pectin (Appendix 1, 8.6.2 Preparation of medium, inoculum, and incubation). The percentages of strain MFA1 relative to rumen total bacteria and FA degradation were analyzed using qPCR (8.6.6 Quantitative PCR (qPCR)) and fluoride ion production, respectively.

The abundance of strain MFA1 relative to total rumen bacteria, and FA degradation were markedly increased by arginine supplementation in mixed rumen cultures. Moreover, arginine (10 mM) and citrate (5 mM) combined supplements showed a synergistic effect on growth and FA degradation with almost 100 % degradation of FA (10 mM) in less than 2 days (Fig. 32). Arginine is one of the most versatile amino acids, which serves as a precursor for the synthesis of urea, nitric oxide and polyamines and regulates key metabolic pathways that are critical to health, growth, reproduction, and homeostasis of the animals (Chacher et al., 2013). Arginine supplementation increases milk production in cattle and growth hormone in sheep (Chacher et al., 2016). In rumen bacteria, arginine was reported as a major energy-yielding substrate for *Synergistes jonesii* which belongs to the same phylum as strain MFA1 (McSweeney et al., 1993). *Synergistes jonesii* (strain 78-1) degrades arginine via the arginine deiminase (ADI; EC 3.5.3.6) pathway, and the ADI gene was observed in the draft genome sequence of strain MFA1 along with two other genes related to metabolism via this pathway; ornithine carbamoyltransferase (OCTase; EC 2.1.3.3) and carbamate kinase (EC 2.7.2.2). One mole of arginine metabolized via the ADI pathway produces two-mole ammonia and one mole ATP. So, during the catabolism of arginine in strain MFA1, the growth and degradation of FA may be improved particularly if nitrogen is deficient in the diet.

Anaerobes cannot take advantage of oxidizing citrate through the reactions of the tricarboxylic acid cycle (TCA), because they usually have an incomplete TCA (Antranikian and Giffhorn, 1987). Most of the anaerobes, therefore, have developed a unique pathway, "citrate fermentation pathway", which results in the formation of pyruvate, acetate and carbon dioxide; the enzymes involved in these

reactions are citrate lyase and oxaloacetate decarboxylase. Those two key enzymes, as well as other enzymes related to citrate fermentation pathway, were annotated in the genome sequence of MFA1 (data not shown). Through this pathway, some rumen bacteria including strain MFA1 can make two moles of acetate using citrate lyase and acetate kinase enzymes from one mole of citrate (Dimroth, 2004). In our experiments (Appendix 2, 9.4.2.3 Effect of citrate on strain MFA1 grown with mixed rumen bacteria, Table 21), we showed that acetate was increased around 15 to 19 mM in the culture media with 10 mM citrate supplementation compared to un-supplemented culture. Through this pathway, the acetate kinase enzyme generates ATP and it is possible to increase the percentage of strain MFA1 relative to rumen total bacteria and FA degradation by adding citrate supplement (Appendix 2, Fig. 32).

4.4.1 Other candidate nutrients and compounds may effect growth and FA degradation of isolates

Other compounds that may stimulate growth and FA degradation of strain MFA1 have also been identified from recent publications. Extracts from the Ginkgo plant (*Ginkgo biloba*) have been fed to ruminants to increase propionate production and reduce methane emissions and as part of that study, it was observed that the extracts also stimulated the abundance of members of the Synegistetes phylum like *Cloacibacillus* strain MFA1 or *Pyramidobacter* strain C12-8 (Oh et al., 2017). Also, marine algae produce a cocktail of halogenated metabolites including fluorinated compounds (Cabrita et al., 2010) which may also induce the expression of the operon responsible for FA degradation. In future studies, those two feedstuffs could be tested for their ability to promote growth and FA degradation possibly in comparison with supplements which showed the greatest benefit in stimulating growth only when FA was present.

5 Conclusions/recommendations

Fluoroacetate poisoning of livestock in northern Australia is caused by ingestion of gidyea and heartleaf trees and causes significant financial losses due to mortality and morbidity of affected stock and restricted utilisation of grazing land where these plant are prevalent. To mitigate this problem our project sought to isolate rumen microorganisms capable of degrading this naturally occurring toxic compound and characterise the nutritional requirements so that a management strategy could be developed to promote these organisms in grazing cattle that was protective from FA poisoning. Molecular tools were also developed to detect the presence of these microorganisms in cattle and predict whether their populations could be enhanced through nutritional supplements.

Initial work focused on isolation and classification of FA degrading bacteria from cattle rumen samples from different geographical locations and diets across northern Australia. Genetic analysis revealed that bacteria responsible for FA degradation were primarily from two phyla, Synergistetes and Firmicutes. Further testing demonstrated that the bacteria with greatest FA detoxifying ability were the Synergistetes isolates, one from *Cloacibacillus* genus (strain MFA1), and the other from *Pyramidobacter* genus (strain C12-8). These bacteria are asaccharolytic and primarily gain energy for growth by fermenting some of the amino acids and peptides from protein. They are also able to gain energy from some methylated nitrogenous compounds such as sarcosine and betaine. Sarcosine, arginine, histidine, glutamic acid, citrate, and formate promoted the growth as well as FA degradation as supplements in the *Cloacibacillus* isolate.

These results coupled with further genome analysis revealed possible genes involved in FA degradation which also provide a concomitant energetic advantage. Specifically, transcriptomic analysis of MFA1 cultures exhibiting elevated growth in the presence of FA showed several genes clustering in a glycine-like reductase operon [Fe-S oxidoreductase and glycine reductase complex B (GrdB)] were highly expressed. This is likely the molecular basis for anaerobic reductive defluorination in these bacteria. In depth analysis of the function of this operon identified that selenium is likely essential to several proteins within this complex and this was partially substantiated in other tests that showed increased growth and degradation when this element was added as a further supplement. To facilitate greater understanding of the regulation of these genes, a promoter assay was devised. In summary, it was found that both arginine and citrate had a stimulating effect and induced promoter activity that regulates the FA degrading genes.

To further evaluate the ability of FA degrading bacteria to persist and degrade FA in response to nutritional supplements, an *in vitro* rumen fermentation system was used with MFA1 as the model FA-degrader grown in a stable background of environmental rumen bacteria. This bacterium appeared to compete, persist and degrade FA once established in a simulated rumen mixed culture fermentation. It was concluded that peptide-rich protein hydrolysates are general nutritional stimulants for MFA1 and that arginine and citrate may specifically enhance cell growth and FA degradation when selenium is not deficient. Thus feed supplements enriched with these nutrients may enhance the ability of this bacterium to protect the animal from toxicity.

Our survey of rumen bacteria in grazing cattle indicates that FA detoxifying rumen bacteria are endemic to cattle in the northern beef herd but at relatively low numbers in the rumen. Also, the number of those rumen bacteria would be dependent on nutritional conditions such as nitrogen sources. Some of the nutrient supplements tested significantly enhanced the growth and FA detoxifying ability of rumen bacteria and these nutrients are also likely to improve the growth of cattle especially in the dry season when nutrition is limiting. FA degradation appears to depend upon selenium which is likely to be deficient in the diet of cattle in parts of northern Australia and these animals would benefit from selenium supplementation.

FA poisoning usually occurs under grazing conditions where nutrients are in short supply, it would appear that providing organic nitrogen would be of practical value nutritionally, but might also contribute to a larger and more active population of FA-degrading bacteria. Additionally, provision of other supplements including selenium could also be beneficial in areas of mineral deficiency.

Future studies should focus on demonstrating the benefit that various supplements may provide in preventing FA poisoning in cattle. These experiments could be performed with small numbers of cattle fed differing supplements which are predicted to increase the population of FA degrading bacteria and stimulate FA degrading activity in each bacterium. These indicators of protection from FA intoxication could be measured from rumen samples of supplemented cattle without intoxicating the animal. Studies involving poisoning with FA are unlikely to gain ethical approval unless there is evidence that a treatment could be protective against poisoning.

6 Key messages

The project has confirmed that specific rumen bacteria are capable of detoxifying FA which had not been recognised by a previous project that sought to construct genetically modified rumen bacteria for this purpose. The naturally occurring FA degraders in the rumen belong to the genera *Cloacibacillus* and *Pyrimidobacter*. These bacteria are related to the rumen bacterium *Synergistes jonesii* which detoxifies leucaena, and all three genera are members of the *Synergistetes* family of bacteria which are normal residents of the rumen. Bacteria from this family have similar nutritional requirements in that that they primarily ferment protein rather than carbohydrate to gain energy which means their numbers are relatively low in the rumen.

Our small survey of cattle in northern Australia indicated that the bacteria capable of degrading FA appear to be present in cattle from this geographic region which means there should not be a need to inoculate animals with FA degrading microorganisms. However the abundance of those bacteria in the rumen would be dependent on nutritional conditions such as nitrogen availability. Some other nutrient supplements tested, significantly enhanced the growth and FA detoxifying ability of rumen bacteria and these nutrients are also likely to improve the growth of cattle especially in the dry season when nutrition is limiting. FA degradation also appears to depend upon selenium which is likely to be deficient in the diet of cattle in parts of northern Australia and these animals would benefit from selenium supplementation. In summary, it would appear that providing organic nitrogen would be of practical value nutritionally, but might also contribute to a larger and more active population of FA-degrading bacteria.

Future studies should focus on animal experiments to demonstrate the benefit that various supplements may provide in preventing FA poisoning in cattle. These experiments could be performed with small numbers of cattle fed differing supplements which are predicted to increase the population of FA degrading bacteria and stimulate FA degrading activity in each bacterium. These indicators of protection from FA intoxication could be measured from rumen samples of supplemented cattle without intoxicating the animal. Studies involving poisoning with FA are unlikely to gain ethical approval unless there is evidence that a treatment could be protective against poisoning.

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8 Appendix 1 – Full Methodology

8.1 General methodology

8.1.1 Culture studies

8.1.1.1 General culture media

Anaerobic PA10 basal medium was adapted as previously described by Davis et al., (2012) with the addition of yeast extract (YE). One litre of PA10 basal medium was prepared by adding 100 ml of clarified rumen fluid (final concentration of 10 %, v/v), 100 ml of 100 mM PIPES buffer[1], 100 ml mineral solution 3, 50 mL 10 × VFA mix, 1 ml trace element solution, 1 ml haemin solution, 1 ml resazurin indicator solution (0.1 %, w/v, in water), 1.64 g sodium acetate and 0.2 g of K_2HPO_4 to distilled water. 5 g of Na_2CO_3 was added to the cooled solution, and pH of the solution was adjusted to 6.8. After 30 min further gassing with CO_2 0.25 g of L-cysteine hydrochloride was added to the solution before immediately closing the vessel and moving to an anaerobic chamber with a 95:5 (CO_2 : H_2) atmosphere. Generally, this base PA10 medium was supplemented as indicated with either just 0.8 % w/v YE, or a combination and 0.8 % w/v YE and sodium monofluoroacetate (ABCR GmbH & Co. KG, Karlsruhe, Germany) (FA) (either 10 mM or 20 mM). Other growth promoting supplements were also trialed as indicated in specific experiments (8.5.5 Evaluation of bacterial growth and FA degradation in pure culture for supplements identify through genome analysis). Latter experiments were conducted as per basal PA10 but supplemented with only 0.4 % or 0.2 % (w/v) YE, thus enabling growth and FA degradation dynamics to be better observed during experimental timeframes.

Brain Heart Infusion Broth (BHI, 37 g / L) (Calf Brain Infusion, 7.7 g; Beef Heart Infusion, 9.8 g; Protease Peptone, 10.0 g; Dextrose, 2.0 g; Sodium Chloride, 5.0 g; Disodium Phosphate, 2.5 g) with 0.8 % yeast extract per litre of boiling distilled water (BHYE) was used as an enriched non-selective medium intended for the cultivation of most anaerobic bacteria and other fastidious microorganisms. The solution was boiled to remove dissolved oxygen and cooled to room temperature by gassing the solution with oxygen-free carbon dioxide gas for approximately 1 h.

- **Clarified rumen fluid** was prepared by centrifugation at 12,000 × *g* for 15 min.
- **Trace element solution** as prepared by McSweeney et al., (2005) contained ($g.L^{-1}$): 1.5 of nitrilotriacetate, 3.0 of $MgSO_4.7H_2O$, 1.0 of NaCl, 0.5 of $MnSO_4.7H_2O$, 0.1 of $FeSO_4.7H_2O$, 0.1 of $CaCl_2$, 0.03 of $NiSO_4.6H_2O$, 0.1 of $CoCl_2.6H_2O$, 0.01 of $Na_2MoO_4.2H_2O$, 0.1 of $ZnSO_4.7H_2O$, 0.01 of $AlK(SO_4)_2.12H_2O$, 0.01 H_3BO_3, 0.02 of Na_2SeO_3, 0.02 of $Na_2WO_4.2H_2O$ and 0.01 of $CuSO_4.5H_2O$.
- **Mineral solution 3** prepared by McSweeney et al., (2005), containing ($g.L^{-1}$): 6.0 of KH_2PO4, 12.0 of NaCl, 6.0 of $(NH_4)^2SO_4$, 2.5 of $MgSO_4.7H2O$ and 1.6 of $CaCl_2.2H_2O$ in distilled water.
- **10 × VFA mix** was prepared (L^{-1}) by mixing 17 ml of glacial acetic acid, 6 ml of propionic acid, 4 ml of *n* butyric acid and 1 ml of each *n*-valeric acid, isovaleric acid, isobutyric acid and 2-methylbutyric acid,

In general 10 ml, aliquots of each respective media were dispensed into 22 ml Hungate tubes. The tubes were then sterilized through autoclaving at 121 °C for 20 min at 172 kPa pressure and then

[1] In some latter experiments also had a slightly modified recipe as indicated of basal PA10 but without PIPES.

stored at room temperature. Other culture studies investigating FA degrading isolates in a mixed rumen culture background were conducted using larger volumes in the Ankom RS Gas Production System (Ankom, USA) (8.6 Evaluation of ability for MFA1 to persist and degrade FA in an *in vitro* rumen mixed fermentation).

8.1.1.2 Fluoride analysis from the culture supernatants

The rate of FA degradation was determined by detecting fluoride ions in the medium using a fluoride ion selective electrode (ISE) (Cole Parmer, USA). A fluoride analysis buffer, total ionic strength adjustment buffer (TISAB, Diaminocyclo- hexane, sodium chloride, and glacial acetic acid, pH 5.5), was prepared by dissolving 57 ml of glacial acetic acid, 58 g of NaCl and 4 g of 1,2-cyclohexylene-diamine-tetra-acetic acid (CTAB) in 500 ml distilled water. The pH was adjusted to 5.5 with 6 M NaOH with continuous stirring in an ice bath. The solution was made up to a final volume of 1 L. A 100 mM sodium fluoride stock solution was prepared by dissolving 0.4199 g of anhydrous analytical grade sodium fluoride in 100 ml distilled water. Calibration standards were prepared by serial dilution of the stock solution, and diluted 1:1 with TISAB (final concentration of 0.1, 1, 5, 10, and 30 mM sodium fluoride). Fluoride ions were quantified using a fluoride ISE connected to an Orion 4-Star pH/ISE benchtop ion meter (Thermo Fisher Scientific, USA). Samples were also diluted 1:1 with TISAB prior to analysis. All samples and standards were maintained at room temperature.

8.1.1.3 Short-chain fatty acids (SCFA) analysis

Concentrations of the SCFAs - acetic, propionic, n-butyric, isobutyric, isovaleric and n-valeric acid were determined by flame ionization detection on a Shimadzu GC-2014 with a Zebron™ ZB-FFAP column (30 m x 0.53 mm ID, Phenomenex, Torrance, CA). Samples of culture supernatant were collected by centrifuging 1 ml of cultured media at 14,000x g for 5 min, followed by acidification with ortho-phosphoric acid and inclusion of 4 methyl valeric acid at a final concentration of 1 mM as an internal standard. A 0.5 µl aliquot of each sample was injected; the carrier gas was hydrogen at 5 $ml.min^{-1}$ and separation of the acids were achieved over 13 minutes. The injector and detector temperature were 200°C and 230°C respectively; the column temperature was initially set at 100 °C for 2 minutes followed by an increasing temperature gradient of 15 °C min^{-1} to 230 °C with a final 2 minutes hold. Peak detection and chromatogram integration were performed using GC solution v 3.30.00 (Shimadzu).

8.1.2 DNA extraction from culture media and rumen samples

8.1.2.1 DNA extractions from pure or enrichment culture

Microbial DNA extractions from pure isolates or *in vitro* cultures were performed using a modified RBB+C method (Yu and Morrison, 2004) followed by bead-beater and CTAB buffer was used for genomic DNA extraction from rumen samples. One milliliter of culture media was centrifuged at 10,000 × g for 2 min and the harvested cells were washed with 0.3 ml PBS (Phosphate buffered saline). The suspended cells were transferred to 2 ml screw cap tube with lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 8.0 50 mM EDTA, and 4 % sodium dodecyl sulfate (SDS)] and ~250 mg of 50:50 Ø 0.1mm-1mm zirconium beads. The cells were homogenized for 3 min at maximum speed on a Bead-beater (FastPrep-24, MP, USA) and incubated at 70 °C for 15 min. After incubation 260 µl of 10 M ammonium acetate was added to each lysate tube, mixed well and incubated on ice for 5 min. Samples were then centrifuged at 4 °C for 10 min at maximum speed (14,000 × g), the supernatants were precipitated by

alcohol. After resuspending the pellet, DNA concentration was measured using NanoDrop 8000 spectrophotometer (Thermo Scientific, USA).

8.1.2.2 DNA extractions from rumen samples

Genomic DNA was extracted from rumen samples by bead-beating followed by phenol-chloroform extraction. 2 ml of rumen fluid samples were added to a 2 ml screw-cap tube. Samples were centrifuged at 14,000 rpm for 15mins at 4°C. Rest of supernatants were removed, and 1 scoop (~250 mg) of silica/zirconium beads were added to each sample. 800 µl CTAB isolation buffer was added to each sample and bead-beating was done at 6.5m/s (FastPrep-24, MP, USA) for two minutes. The sample was incubated at 70°C for 20 mins, then centrifuged at 14,000g (13000 rpm) for 12 mins at room temperature. Around 800 µl supernatant was removed to a new 1.5 ml Eppendorf tube. 10 µl of (DNase-Free) RNase Mix was added and incubated at 37°C for 20 mins. An equal amount (~800 µl) of Chloroform : Isoamyl alcohol (24:1) was added (Amresco Cat no. X205) and mixed vigorously for the 30s to form a white emulsion. Centrifugation did at 14000 rpm for 12 mins and then 600 µl of upper aqueous phase supernatant was transferred to a new Eppendorf tube. An equal volume of Phenol : Chloroform : Isoamyl alcohol (25:24:1) (Amresco Cat no. 0883) was added and mixed vigorously to form a white emulsion. After centrifuging at 14000 rpm for 12 mins, 500 µl of upper aqueous phase supernatant was transferred to a new Eppendorf tube. ~400 µl of isopropanol was added and mixed by gently inverting the tubes several times and then incubated at -20°C overnight. After alcohol precipitation, the pellet was resuspended in 200 µl of EB buffer (Qiagen) and dissolved by warming at 70°C for 10 mins. The extracted gDNA was stored at 4°C. The quality and quantity of DNA from the samples were determined using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific).

8.1.3 RNA extraction and cDNA synthesis

Total RNA was extracted from either environmental samples or pure MFA1 enrichment cultures. Briefly, after cells were resuspended in RNAlater solution (Ambion) overnight, and the cells were transferred to 2 ml screw cap tube with lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 8.0 50 mM EDTA, and 4 % sodium dodecyl sulphate (SDS)] and ~250 mg of 50:50 Ø 0.1mm-1mm zirconium beads. The cells were then homogenized for 3 min at maximum speed 6.5 m/S using a bead beater cell disrupter (FastPrep-24, MP, USA). RNA extracted from lysates via a spin column using an ISOLATE II RNA Mini kit (Bioline, UK) according to manufacturer's instructions. The quantity and quality of extracted RNA were determined via a Bioanalyzer RNA kit (Agilent Technologies) using manufacturer protocols. cDNA was synthesized by random hexa-primers using either SuperScript III Reverse Transcriptase (Invitrogen) or SensiFAST cDNA Synthesis kit (Bioline) according to the manufacturer protocols ready for quantitative reverse transcription PCR (RT-qPCR) reactions.

8.1.4 PCR studies

8.1.4.1 Design and validation of PCR primers

The primers used to target the 16S ribosomal DNA (rDNA) genes and putative FA degrading functional genes annotated as (Major Facilitator Superfamily (MSF) transporter, Fe-S oxidoreductase, Glycine reductase complex B gamma subunit) from genome sequence of MFA1 (Lex Leong Ph.D. thesis, 2014) and luciferase genes from pGL4 vector for reporter assay studies are shown in Table 6. Target gene-specific primer pairs were designed by NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or PRISE program (Fu et al., 2008). The specificity of the primers was tested

through PCR amplification using genomic DNA extracts from a pure culture of *Cloacibacillus* strain MFA1, *C. everyensis,* and *Pyramidobacter* strain C12-8 or rumen samples. The PCR conditions both of 16S rDNA genes and functional genes were as follows: initial denaturation for 5 min at 94 °C, 35 cycles of amplification (30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C) and a final elongation cycle at 72 °C for 5 min. To validate the specificity of the primers, the PCR amplicons from rumen DNA samples were cloned in a pGEM-T easy vector (Promega, USA) and the PCR products were sequenced with an ABI3130xl automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned and analyzed using AlignX software (Vector NTI software, Invitrogen).

Table 6. Sequences of primers used in this study.

Target strains or vector	Primer names	Primer sequences	Annealing temperature °C	Product size (bp)	Target genes
Total bacteria	TB_F TB_R	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	60 58	141	16S rRNA gene
Cloacibacillus strain MFA1, C. everyensis, and C. pocorum	MFA_181_F MFA_181_R	CCCTATGTCCAGTTGCTAACAA GGCTTTTAAGGATTCGCCAACT	57 61	181	
C. strain MFA1	MFA_965_F MFA_1256_R	TGAGATGAAAGTTGAGGGAC GGCTTTTAAGGATTCGCCAACT	53 61	292	
C. strain MFA1	MFA_973_F MFA_1125_R	AAGTTGAGGGACTGTAGCTTGC GCAGTCTCTCCAGAGTGCTCA	58 59	153	
Pyramidobacter strain C12-8	C12-8_256F C12-8_488R	GGTGAAAGCTCACCAAACCG TCTCAGTTCCAATGTGGCCG	58 58	229	
C. strain MFA1	MFA_31400_F MFA_31400_R	CGTGAGTCAGTGGCTGAAAAA CGCTTACCTCATACTGCTTCTT	59 56	179	Putative FA degrading operon genes*
C. strain MFA1	MFA_31410_F MFA_31410_R	CAACGTCCTGGCCTTCAAAA AATCTGCGCGTTTCCGGATT	61 64	200	
C. strain MFA1	MFA_31430_F MFA_31430_R	CCAGGCACGGATCTTTACAAAA CGAAGCCGGCAATCTGATTT	61 62	188	
pGL4.10[luc2] vector	Luc_8F Luc_8R	GTGTAGGTCGTTCGCTCCAA AAGAACTCTGTAGCACCGCC	60 60	185	Luciferase_1 (*Luc2*)

* Targeted functional genes of MFA1 (31400F/R, Major Facilitator Superfamily (MSF) transporter; 31410F/R, Fe-S oxidoreductase; 31430F/R, Glycine reductase complex B gamma subunit)

8.1.4.2 Quantitative real-time PCR (qPCR)

Genomic DNA of MFA1 and C12-8 were used as a standard curve. The genomic DNA was subjected to eight sequential tenfold dilutions and the different specific primer sets were used in each qPCR assay (Table 6). To examine the amplification efficiencies and sensitivities for each primer pair, a tenfold dilution series of genomic DNA standard was run as triplicate samples. The PCR efficiencies were calculated using the formula E = -1+10 (-1/slop), and respective cell numbers in the samples were quantified. The qPCR was performed using ViiA 7 Real-Time PCR system (Life Technologies) according to SensiFAST SYBR Lo-ROX kit (BIOLINE). The PCR mixture contained 20 µL of 2X SYBR Lo-ROX mix, 2 µl of template DNA, and 1.5 µl 10 µM each primer in a total volume of 44 µl. The amplification procedure consisted of one cycle of 50°C for 2 min and 95°C for 2 min 30 s for initial denaturation, followed by 40 cycles of 95°C for 15 s, and annealing and extension at 60 °C for 1 min. Melting curve analysis was performed after amplification to verify the specificity of the qPCR.

8.1.4.3 Nested qPCR

In case of MFA1, a nested PCR assay was also developed to detect the presence of the bacterium in the rumen because the LOD by qPCR prevented detection in some animals even though the bacterium was present. Genomic DNA was PCR amplified in 20 µl reactions containing 1 or 2 µl DNA template

(concentration undetermined), 10 µl GoTaq PCR master mix (Promega, Wisconsin, USA), each 1µl of forward and reverse primer, and 7 µl of water. Reactions were followed by 15 cycles of denaturation at 95°C for the 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s with a final extension of 7 min at 72°C. After PCR amplification, 0.25 µl EXO I (Exonuclease I, NEB), 0.25 µl CIP (Alkaline phosphate, Calf Intestinal, NEB) and 0.5 µl of water (total volume 1 µl) were added and incubated at 37°C for 20 minutes. The enzyme reactions were inactivated at 80°C for 20 minutes. After cleaning, qPCR was carried out using the Applied Biosystems Real-Time PCR system (Applied Biosystems, Foster City, CA).

8.1.4.4 Transcriptional analysis by reverse transcriptase PCR (RT-qPCR)

Total RNA was extracted and cDNA synthesized as described previously in 8.1.3 RNA extraction and cDNA synthesis. The RT-qPCR reaction from synthesized cDNA was performed as described directly above in 8.1.4.2 Quantitative real-time PCR (qPCR). Primers used in the RT-qPCR reactions are listed in Table 6. Each cDNA sample was run in quadruplicate. The correct size of PCR product and the specificity of each primer pair was confirmed by examination of PCR products on an agarose gel. To relatively quantify gene transcription in both treatments against each other, the transcription of 16S rDNA was used as a reference. Levels were normalized using the 2ΔΔCT method (Livak and Schmittgen, 2001).

8.2 Isolation and identification of FA degrading bacteria

8.2.1 Sample collection and isolation of FA degrading bacteria

Digesta were collected from the rumen of cattle and sheep in Australia (Belmont Research Station, Rockhampton; Lansdown Research Station, Townsville; and Floreat Park, Perth) (Table 7). Digesta was passed through sterile nylon mesh (1 × 1 mm) to remove coarse material and inoculated into an equal volume containing 30 % anaerobic glycerol solution for preservation. Samples were frozen immediately on dry ice. A collection of FA-degrading bacteria which had previously been isolated from the rumen and gut of native herbivores were also tested further for differences in FA-degrading ability and growth (Table 8).

Table 7. Collected rumen samples and culture media.

Region	Animals #	Diets	Culture Media
Rockhampton	3 Cattle (# 393, #411 and # 500)	Rhodes grass	PA10 (0.8 % YE + 10 mM FA)
	3 Cattle (#177, #480 and #498)	Leucaena	PA10 (0.8 % YE + 10 mM FA)
Townsville	8 Steers (#3354, #3347, #3323, #3288, #3385,#3389, #3361, #3415)	60 % Rhodes grass Hay + 40 % Conc.	PA10 (0.8 % YE + 10 mM FA) BHIYE (0.8 % YE + 10 mM FA)
Perth	7 Sheep (#634, #656, #676, #681, #1139, #114,1#1149)	Pelleted commercial roughage with 0.5 ~ 3 % Algae treatment	PA10 (0.8 % YE + 10 mM FA) BHIYE (0.8 % YE + 10 mM FA)

Table 8. Previous isolated FA-degrading bacteria.

Region	Animals (collection)	Name/Genus	Culture Media
Queensland (Mt. Cotton)	Cattle (fistulated rumen)	MFA1/*Cloacibacillus*	PA10 (0.8 % YE + 10 mM FA)
Queensland (Aramac)	Red Kangaroo (foregut)	K1/*Cloacibacillus*	PA10 (0.8 % YE + 10 mM FA)
Queensland (Aramac)	Grey Kangaroo (foregut)	K3/*Cloacibacillus*	PA10 (0.8 % YE + 10 mM FA)
Queensland (Marburg)	Emu (faeces)	E1/*Cloacibacillus*	PA10 (0.8 % YE + 10 mM FA)
Queensland (Mt. Cotton)	Cattle (rumen)	R2/*Cloacibacillus*	PA10 (0.8 % YE + 10 mM FA)

8.2.2 New isolations of FA degrading bacteria from rumen collections

Bacterial isolation was performed by the inoculation of 100 µl of rumen fluid in tubes containing 5 ml of PA10 medium with 0.8 % yeast extract and 10 mM FA. Samples were incubated at 39 °C in an orbital shaker (40 rpm, dark). After 72 hours, 100 µl of culture growth was transferred to fresh Hungate tubes containing 5 ml of PA10 medium with 0.8 % w/v YE and 10 mM FA and incubated under the same conditions described above. The FA defluorination was measured with a fluoride selective electrode (Thermo Fisher Scientific, USA) in 24-well plates containing 200 µl of culture and 200 µl of TISAB (8.1.1.2 Fluoride analysis from the culture supernatants). The fluoride ion released by the microbial FA degradation was expressed in millimoles. Samples presenting defluorinating ability were cultivated again in fresh PA10 medium with 0.8 % w/v YE media and 10 mM FA. To obtain pure colonies dilutions to 10^{-4} to 10^{-6} were performed and cultures that produced FA defluorination were plated onto the PA10 medium with 0.8 % w/v YE agar (1.5 %) plates and incubated 39 °C for 96 h in the anaerobic chamber. Subsequently, single colonies were picked and cultured in 96 well culture plates containing 500 µl PA10 medium with 0.8 % w/v YE and 10 mM FA for further selection.

8.2.3 DNA extraction of isolates

Microbial DNA extraction from pure isolates was performed using a modified version of the RBB+C method as described above (8.1.2.1 DNA extractions from pure or enrichment culture).

8.2.4 PCR amplicons of 16S rDNA genes and sequencing analysis

The 16S rDNA gene was amplified from DNA extractions by PCR using universal bacterial 16S rDNA gene primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and a 1492R primer (5'-GGYTACCTTGTTACGACTT-3') using the following the PCR conditions: initial denaturation for 5 min at 95 °C, 30 cycles of amplification (30 s at 94 °C, 30 s at 58 °C and 90 s at 72 °C) and a final elongation cycle at 72 °C for 5 min. After confirming the PCR amplicons by running samples on a 1 % agarose gel, the amplicon product was sequenced with an ABI3130xl automatic sequencer (Applied Biosystems, USA). Sequences of sufficient quality were classified taxonomically using the Ribosomal Database Project (RDP) classifier program. Sequences were aligned to related bacterial sequences to construct

a phylogenetic tree with 1000 bootstrap re-samplings to test the robustness of the branching order using MEGA6 (Tamura *et al*., 2013).

8.3 Effect of different nitrogen sources on growth and FA degradation of key FA-degrading rumen isolates

8.3.1 Ranking of bacterial growth and defluorination of isolates

Five isolates collected previously from rumen and other herbivore gut systems (*Cloacibacillus* strain MFA1, cattle rumen; K1, Red Kangaroo; K3, Gray Kangaroo; E1, Emu; and R2, cattle rumen 2) were cultured in 5 ml PA10 medium with 0.8% w/v YE and 10 mM FA from 30 % glycerol stocks. After sub-culturing three times with 10 mM FA, the growth (OD 600nm) and the defluorinating ability of cultures were measured.

8.3.2 Growth on different sources of nitrogen of key FA degrading rumen isolates

A range of protein hydrolysates at 0.8 % (w/v) were used to clearly elucidate the effect of each hydrolysate on the growth of the isolates and their FA degradation capability. Filter-sterilized protein hydrolysates (casamino acids, acid hydrolysate of casein; amine, organic compounds and functional groups that contain a basic nitrogen atom; phytone peptone, enzymatic digest of soy peptone; tryptone, enzymatic digest of casein; peptone P, a peptic digest of meat protein; and yeast extract, water soluble portion of autolyzed *Saccharomyces cerevisiae* cells) (BD Biosciences, CA, USA) were added separately to a final concentration of 0.8 % (w/v) in basal PA10 medium with 0.8% w/v YE (8.1.1.1 General culture media). Similar concentrations of the protein hydrolysates were also added separately into a medium containing 20 mM or 10 mM FA. All experiments were performed in triplicate in anaerobic 27 ml Hungate tubes with a headspace of 17 ml that consisted of 95 % CO_2 and 5 % H_2 incubated at 39 °C for 140 h. Growth and FA degradation by isolates were monitored by measuring optical density (OD) at 600 nm and fluoride production using a Thermo Scientific fluoride ion-selective electrode (Thermo Fisher Scientific, MA, USA), respectively, as described by Davis *et al*. (2012). Concentrations of the SCFAs were analyzed as described previously in 8.1.1.3 (Short-chain fatty acids (SCFA) analysis).

8.4 Monitoring FA degrading isolates using molecular tools

8.4.1 Isolates cultures, cell counts and spiked with rumen enrichment culture

Two pure strain MFA1 or C12-8 have grown anaerobically in 5 ml artificial rumen PA10 medium with 0.8% w/v YE (Appendix 1, 8.1.1.1) and 10 mM FA at 39 °C for 48 hours. The number of each isolate per milliliter were counted using a Thoma cell counting chamber. Rumen enrichment cultures were grown in 50 ml Brain Heart Infusion media (BHI, 37 $g.l^{-1}$, BD, USA) with 0.8 % yeast extract at 39 °C for 30 hours. The fresh rumen fluid was collected from a fistulated beef cow at Gatton (collected at March 2016). 100 µl of each isolates culture (cell numbers were 2.26×10^8 $cells.ml^{-1}$ and 1.2×10^9 $cells.ml^{-1}$, MFA1 and C12-8, respectively) was added to 900 µl of enrichment rumen culture or fresh rumen fluid and then those were diluted as ten-fold serial dilutions.

8.4.2 DNA extractions from enrichment cultures or rumen fluids spiked with isolates

Total genomic DNA extraction was performed using a modified version of the RBB+C or phenol-chloroform methods as described previously (8.1.2.1 DNA extractions from pure or enrichment culture or 8.1.2.2 DNA extractions from rumen samples). Genomic DNA from 1 ml of neat enrichment cultures, or 350 µl of rumen fluid containing serially diluted (10 fold) pure culture of isolates as above were each extracted as triplicates.

8.4.3 RNA extractions from enrichment cultures or rumen fluids spiked with isolates

Total RNA was extracted from triplicate enrichment cultures or rumen fluids containing serially diluted (10 fold) pure cultures of isolates (MFA1 or C12-8) as described above (8.1.3 RNA extraction and cDNA synthesis).

8.4.4 Evaluation of standard curves and limit of detection (LOD) of FA degrading isolates (strain MFA1 and C12-8) using qPCR

Standard curves for the absolute quantification of strain MFA1 and C12-8 were obtained using DNA from pure bacteria grown overnight in the culture at 39 °C. Cultures were centrifuged and the cells were resuspended in clarified rumen fluid. The numbers of the bacterial cell were determined using Thoma cell counting chamber. The genomic DNA was subjected to six or seven sequential tenfold serial dilutions and designed specific primer sets (Table 6) were used in each qPCR assay (8.1.4.2 Quantitative real-time PCR (qPCR)). The bacterial universal primer set TB_F and TB_R was used to estimate the relative abundance of the isolates against total bacteria by the 2^-(Specific Primer (SP) – Total Bacteria (TB)) method. Standard curves were generated for each bacteria separately. PCR amplification efficiencies were calculated using the formula E = -1 + 10 (-1/slope), and respective cell numbers in the samples were quantified. The qPCR method was described above (8.1.4.2 Quantitative real-time PCR (qPCR)).

8.4.5 Sampling for an environmental survey of FA degrading Synergistetes isolates in northern Australia

Rumen samples were collected using an intra-gastric tube from grazing animals under standard commercial practice for cattle properties in northern Australia or directly from the rumen of slaughtered animals at Livingstone abattoir (Fig. 13). The qPCR and nested PCR methods (8.1.4 PCR studies) were conducted of the prevalence of leading FA degrading isolates, strain MFA1 and C12-8 from the environmental samples.

8.5 Screening of compounds that up-regulate FA operon activity and their evaluation in pure culture studies

8.5.1 Cloning promoter region of strain MFA1

Genomic DNA was extracted from an overnight culture of strain MFA1. To clone the promoter region from the operon which is putative FA degrading cluster up-regulated in the presence of FA (Leong et al., 2014), primers were designed by NCBI designing tool (http://www.ncbi.nlm.nih.gov/ tools/primer-blast/) to amplify the region. The bacterial promoter elements were predicted by BPROM [Online

search program, www.softberry.com, (Solovyev and Salamov, 2011). Amplification of the promoter region (401 bp) flanked by *Xho*I and *Hind*III sites was amplified from genomic DNA of strain MFA1 by PCR using the forward sequence of 5'- TTCTCGAGTAGCCTGGCCGGGAAGTTTT-3' and a reverse sequence of 5'- TTAAGCTTGGCTGGAAGAGGCTGTAT-GT-3'. Underlined letters indicate the restriction sites for *Xho*I and *Hind*III in the forward and reverse primers, respectively. The PCR condition was as follows: initial denaturation for 5 min at 94 °C, 35 cycles of amplification (30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C) and a final elongation cycle at 72 °C for 5 min. A PCR clean-up was firstly performed for amplified DNA by following the manufacturer's instructions for a QIAquick PCR Purification kit (Qiagen, Germany). The cleaned PCR product was then ligated to a vector in a 10 µl reaction using T4 DNA Ligase kit (Promega, USA) that consisted of 5 µl of 2 × Rapid Ligation Buffer, 1 µl of T4 DNA Ligase and volumes of pGEM-T Easy vector (Promega, USA) and insert for molar ratio of 1:3 (vector: insert) incubated at room temperature (RT) for 3 hour. Then, 2 µl of the ligation mix was used to transform One Shot TOP10 Electrocompetent *E. coli* cells (Invitrogen), using the GenePulser II Electroporation system (Bio-Rad) with electroporation conditions at 2.5 kV, 200 Ω, and 25 µF. After transformation in a 2 mm cuvette (Bio-Rad), 1 ml of SOC medium was added to the cuvette, and resuspended cells were immediately transferred to 1.5 ml tubes. This culture containing the transformed cells was allowed to recover at 37 °C with shaking at 225 rpm for 1 h. Either 100 µl or 200 µl of the transformation culture was then spread onto an LB agar plate with 100 µg / ml of ampicillin (Amp) and 20 mg / ml of 5-bromo-4-chrolo-3-indoly- β-D-galactopyranoside (X-gal) and incubated overnight at 37 °C. Well separated white colonies were picked from the plates and used as DNA template for the screening of DNA inserts using a colony PCR protocol with reaction conditions as described above.

8.5.2 Construction of a plasmid for reporter assay

The plasmid pGL4.10 [luc2] vector (no promoter) encoding the luciferase reporter gene *luc2* (*Photius pyralis*) (Promega, WI, USA) was used for the promoter assay. The 401 bp promoter fragment was released from the pGEM-T Easy vector by digesting with *Xho*I and *Hind*III and was subsequently inserted into pGL4.10 vector in the multiple cloning sites via ligation as described above to yield plasmid pGL4-promo vector. The *E.coli* transformants containing control pGL4 (vector only, no promoter) and pGL4-promo plasmid were grown in LB / Amp broth media and the plasmid DNA was extracted by Plasmid Miniprep Kit (Qiagen) to confirm the 401 bp promoter fragment.

8.5.3 Luciferase reporter assay

A luciferase reporter assay was performed using a modified version of the Luciferase Assay System kit (Promega, USA) protocol. Positive transformants containing pGL4 (as control) or pGL4-promo vector were separately grown for 6 to 8 hours (exponential phase of growth, OD 600 nm, 0.4-0.5) in LB / Amp broth media. Cultures were placed on ice for 10 min and 20 µl cells were transferred to an opaque 96 well plate and then incubated with substrates (~10 mM concentrations) (Table 2) for 10 min at RT. To lyse the cells, 100 µl of freshly prepared 1 × lysis buffer (Cell Culture Lysis Reagent (CCLR), Promega) was added to the wells and the plates were incubated for 10 min. After lysis, 20 µl of cell lysates per well were dispensed into a new well, 50 µl of Luciferase Assay Reagent (Promega, USA), was added to each well. Plates were read immediately using multimode microplate reader (SpectraMax M3, Molecular Devices, USA). The relative percentage of luciferase activity was defined by (100 × Luminescence (Lumi) of substrate / Lumi of no substrate). All assays were performed at least twice

and the activity of each sample was determined in triplicate. Errors bars shown in all figures represent the standard error of the mean of triplicate measurements.

8.5.4 Transcriptional expression analysis by RT-qPCR

Total RNA extraction and cDNA synthesis from transformed *E. coil* or MFA1 were described in 8.1.3 (RNA extraction and cDNA synthesis) and the RT-qPCR was performed as described previously in 8.1.4.2 (Quantitative real-time PCR (qPCR)).

8.5.5 Evaluation of bacterial growth and FA degradation in pure culture for supplements identify through genome analysis

MFA1 and C12-8 have grown anaerobically in 5 ml artificial rumen basal media PA10 (Appendix1, 8.1.1.1) with 0.2% and 0.4% yeast extract, respectively and 10 mM FA at 39 °C. A range of filter-sterilized supplements (Table 9) was added separately to a final concentration of 10 mM in the culture media and those were used to clearly elucidate the effect on the growth and FA degradation capability of MFA1 and C12-8. All experiments were performed in triplicate in anaerobic Hungate tubes that consisted of 95 % CO_2 and 5 % H_2. Tubes were incubated at 39 °C for 48 h. Growth and FA degradation were monitored by measuring optical density (OD) at 600 nm and fluoride production using a Thermo Scientific fluoride ion-selective electrode (Thermo Fisher Scientific, MA, USA) (Appendix 1, 8.1.1.2). In case of selenium (sodium selenate decahydrate) supplement, growth and FA degradation of both isolates were measured for investigating responses to different concentrations of selenium.

Table 9. Growth supplements for strain MFA1 and strain C12-8.

Supplement	Chemical formula	g/mol	Natural sources in high level
Acetamide	C_2H_5NO	59.07	non-toxic organic amides
Arginine	$C_6H_{14}N_4O_2$	174.2	Seeds, soy protein, Nut, legumes
Betaine	$C_5H_{11}NO_2$	117.15	Quinoa, spinach, Beets, rye
Choline dihydrogen phosphate	$C_5H_{16}F_6NPO_5$	201.16	Egg, peanuts, fish, milk, soybeans
Creatine	$C_4H_9N_3O_2$	131.13	Meat, Fish
L-carnitine hydrochloride	$(CH_3)_3N+CH_2CH(OH)CH_2CO_2HCl^-$	197.66	Nuts, seeds, asparagus, beet green
Ethanolamine	C_2H_7NO	61.08	a breakdown product of cell membrane
Glutamic acid	$C_5H_9NO_4$	147.13	Soy protein
Glycine	$C_2H_5NO_2$	75.07	beans, soybean, spinach, pumpkin, kale
Histidine	$C_6H_9N_3O_2$	155.15	rice, green vegetables, bananas, wheat, and rye, meat, dairy products
1,2-propanediol (Propylene glycol)	$C_3H_8O_2$	76.09	Synthetic organic compound
Proline	$C_5H_9NO_2$	115.13	Soy protein, Cabbage, bamboo shoot
Sarcosine	$C_3H_7NO_2$	89.1	Egg York, ham, legumes
Sodium Citrate	$Na_3C_6H_5O_7$	258.06	Fruits, orange, vegetables
Sodium formate	HCOONa	68.01	a major intermediary metabolite, silage additives
Sodium selenate decahydrate	$Na_2SeO_410.H_2O$	369.09	Nuts, seeds, green vegetable
Trimethylamine (TMA)	C_3H_9N	59.11	a product of decomposition of plants and animals, synthesized from dietary nutrients such as choline and carnitine

8.6 Evaluation of ability for MFA1 to persist and degrade FA in an *in vitro* rumen mixed fermentation

8.6.1 Experimental design

All experiments were performed in triplicate in anaerobic 250 ml Ankom RF Gas Production system bottles (ANKOM Technology, USA) with a headspace of 220 ml that consisted of 95 % CO_2 and 5 % H_2. The *in vitro* fermentation was carried out in 30 ml media and incubated at 39 °C. FA degradation was monitored by fluoride productions using a Thermo Scientific fluoride ion-selective electrode (Thermo Fisher Scientific, USA), as described by previous milestones. Rumen total bacterial inocula (RM) were prepared as an enrichment culture from fresh rumen fluid which was validated as a free of strain MFA1 (8.4.1 Isolates cultures, cell counts and spiked with rumen enrichment culture). Strain MFA1 cells (M), L-Arginine (Arg) (Sigma-Aldrich, USA) and sodium citrate (Cit) (Sigma-Aldrich, USA) were used separately or in binary and tertiary combinations; control rumen bacterial mixture (RM), RM+MFA1 (RM+M), RM+M+Arg, RM+M+Cit, RM+M+Arg+Cit (RM+M+AC). Those combinations included or excluded the 10mM FA (ABCR GmbH & Co. KG, Germany). Experimental designs are shown in Table 10.

Table 10. Experimental designs of rumen in vitro fermentation.

Experiments	**Treatment combinations**	**Sample collections and analysis**
Pre-experiment	RM	Day 1,2,3,4,5, and 6
1st experiment	RM, RM+FA, M, M+FA, RM+M, RM+M+FA	24h (day1) and 43h (day 2) collection
2nd experiment	RM, RM+FA, RM+M, RM+M+FA, RM+M+Arg, and RM+M+Arg+FA	24h (day 1) and 43h (day 2) collection
3rd experiment	RM, RM+FA, RM+M, RM+M+FA, RM+M+Cit, and RM+M+Cit+FA	24h (day 1) and 43h (day 2) collection
4th experiment	RM, RM+FA, RM+M, RM+M+FA, RM+M+AC, and RM+M+AC+FA	24h (day 1) and 43h (day 2) collection
5th experiment	RM+M, RM+M+FA	Day 2, 4, 6, 7, and 8

*RM, Rumen mixture; RM+FA, Rumen mixture + FA; RM+M, Rumen mixture + MFA1; Arg, Arginine; Cit, Citrate; AC, Arginine + Citrate

8.6.2 Preparation of medium, inoculum, and incubation

The medium for mixed rumen *in vitro* fermentation was prepared as described previously (McSweeney et al., 2001) using the Ankom bottles. One litre of medium was prepared by adding 300 ml clarified rumen fluid (centrifuged at 12,000 × g for 15 min), 100 ml mineral solution 3 (Appendix 1, 8.1.1), 50 ml 10X VFA mix (Appendix 1, 8.1.1), 1 ml trace element solution (Appendix 1, 8.1.1), 1 ml haemin solution, 1 ml resazurin indicator solution (0.1 %, w/v, in water), 1.64 g sodium acetate, 0.2 g of K_2HPO_4, 0.7 g yeast extract, 0.7g Tryptone soya, 0.7 g casamino acids, 0.7 g soluble starch, 0.7 g cellobiose, 0.4 g xylan (birchwood, Sigma Aldrich, US), 0.4 g xylan (oat spelt, Sigma Aldrich, US) and 0.7 g pectin (from apple, Sigma-Aldrich, US) to distilled water. The solution was boiled to remove dissolved oxygen and cooled to room temperature by gassing the solution with oxygen-free carbon dioxide gas. 5 g of Na_2CO_3 was added to the cooled solution, and pH of the solution was adjusted to 6.8. L-cysteine hydrochloride (0.25 g) was added to the solution before dispensing 30 ml aliquots of the solution into 100 ml serum bottles in an anaerobic chamber with a 95:5 (CO_2 : H_2) atmosphere. The serum bottles were then sterilized through autoclaving at 121 °C for 20 min under 172 kPa pressures and stored at room temperature. Ankom bottles were sterilized by 70 % alcohol in the anaerobic chamber and the autoclaved 30 ml media of the serum bottle poured into the bottle. The Ankom RF Gas Production System is different from the batch culture serum bottle system because it is possible to control pressure release remotely, collect culture sub-samples and transfer of new media easily compared to serum bottle (Castro-Montoya et al., 2017). Gas accumulated in the headspace of the flasks was automatically released when the pressure reached 0.5 psi (Pounds per square inch) via ANKOM sensor module. 300 µl of RM which was validated as a free of MFA1 strains were inoculated into the 30 ml medium with combinations as above (Table 10) and incubated at 39 °C. Based on the experimental designs, 15 ml cultured media were collected for analysis and then added to same volume fresh media (15 ml) with each supplements.

8.6.3 Fluoride analysis from the culture supernatants

The rate of FA degradation was determined by detecting fluoride ions in the medium as described previously in 8.1.1.2 Fluoride analysis from the culture supernatants.

8.6.4 Short-chain fatty acids (SCFA) analysis

Concentrations of the SCFAs were analyzed as described previously in 8.1.1.3 Short-chain fatty acids (SCFA) analysis.

8.6.5 DNA extraction

Genomic DNAs from 1 ml of cultures as described previously in 8.1.2 DNA extraction from culture media and rumen samples

8.6.6 Quantitative PCR (qPCR)

The qPCR was again performed as described previously. The primer pairs used in the qPCR are listed in Table 6. Each DNA sample was run in quadruplicate. The percentages of strain MFA1 relative to total rumen bacteria were normalized as before using the 2ΔΔCT method (Livak and Schmittgen, 2001).

8.6.7 Statistical analysis

SCFA data from *in vitro* fermentation studies were analysed by one-way ANOVA using the General Linear Model (GLM) procedure of SPSS version 23 (IBM, US) except where otherwise stipulated. Tukey's HSD (honestly significant different) method was employed for multiple comparisons. Between class correspondence analysis (BCA) obtained after a correspondence analysis (CA) was performed with R package 'ade4' with a permutation test (Monte-Carlo test) to assess the statistical significance of between-class analysis. Co-inertia analysis (CIA) were used to test the relationship between VFA profiles and supplements. The data on VFA and fluoride productions between two groups were averaged and compared by *t-test*. Statistical significance was considered at $P < 0.05$.

9 Appendix 2 - Supplementary data

9.1 Isolation and characterisation of FA-degrading bacteria

9.1.1 Sample collection for FA degrading isolates

Rumen digesta was collected from cattle and sheep in Australia (Belmont Research Station, Rockhampton; Lansdown Research Station, Townsville; and Floreat Park, Perth) to survey and isolate FA degrading bacteria (Table 7). Five isolates were collected from the rumen and other herbivore gut systems (strain MFA1, cattle; strain K1, Red Kangaroo; strain K3, Grey Kangaroo; strain EF1, Emu; and strain R2, cattle) (Table 8). The collected samples were cultured anaerobically in 5 ml PA10 medium with 0.8% w/v YE and 10 mM FA. Enrichment cultures that defluorinated FA was initially established using cattle or sheep rumen digesta inoculum. Initial enrichments degraded FA slowly (0.5 or 5 mM) after 5 to 10 days of growth. Subsequent transfers over a period of 10 or 15 days yielded enrichments with the capacity to completely or partially (~30 %) degrade the amount of FA provided (10 mM FA). When transfers into a fresh PA10 medium with 0.8% w/v YE and 10 mM FA resulted in over 50 % degradation of FA, the 10^{-4} to 10^{-6} dilutions were plated on PA10 medium with 0.8% w/v YE agar and incubated at 39 °C for 96 hours to obtain pure colonies. Repeated streaking on agar plates from the 96 well cultures led to the isolation of one predominant morphology using phase contrast microscopy. Seventeen isolates from cattle and sheep rumen digesta exhibited varying ability to degrade FA (Table 11).

Table 11. Collection sites and identified isolates.

Region	Herbivore (collection)	Phylum	Genus and strains
QLD (Mt. Cotton)	Cattle (rumen)	*Synergistetes*	*Cloacibacillus* MFA1
QLD (Aramac)	Red Kangaroo (foregut)	*Synergistetes*	*Cloacibacillus* K1
QLD (Aramac)	Grey Kangaroo (foregut)	*Synergistetes*	*Cloacibacillus* K3
QLD (Marburg)	Emu (faeces)	*Synergistetes*	*Cloacibacillus* EF1
QLD (Mt. Cotton)	Cattle (rumen)	*Synergistetes*	*Cloacibacillus* R2
QLD (Rockhampton)	Cattle (rumen)	*Synergistetes*	Pyramidobacter C12-8, E11-8, F4-2, and F7-8
QLD (Rockhampton)	Cattle (rumen)	*Firmicutes*	*Oribacterium* C9, and F5
QLD (Townsville)	Cattle (rumen)	*Synergistetes*	*Cloacibacillus* C-3-3, D-9-5, and F-2-4
QLD (Townsville)	Cattle (rumen)	*Synergistetes*	*Pyramidobacter* W38
WA (Perth)	Sheep (rumen)	*Firmicutes*	*Eubacterium* P_F-10, and P_E-3

9.1.2 General ranking of FA degrading abilities of *Cloacibacillus* isolates and other genera

A comparison between five isolates (*Cloacibacillus* strains MFA1, K1, K3, EF1, and R2) was performed in PA10 medium with 0.8% w/v YE and 10mM FA (8.1.1.1 General culture media) to rank their ability

to degrade FA (Fig. 14). The highest growth yields (OD_{600} = 0.7) occurred in strain K1. The ability to degrade FA was presumptively correlated with bacterial growth for *Cloacibacillus* isolates. In accordance with the highest growth rates, strain K1 also showed the highest FA degradation in 10 mM FA. Conversely, strain K3 had the lowest growth and extent of FA degradation (Fig. 14).

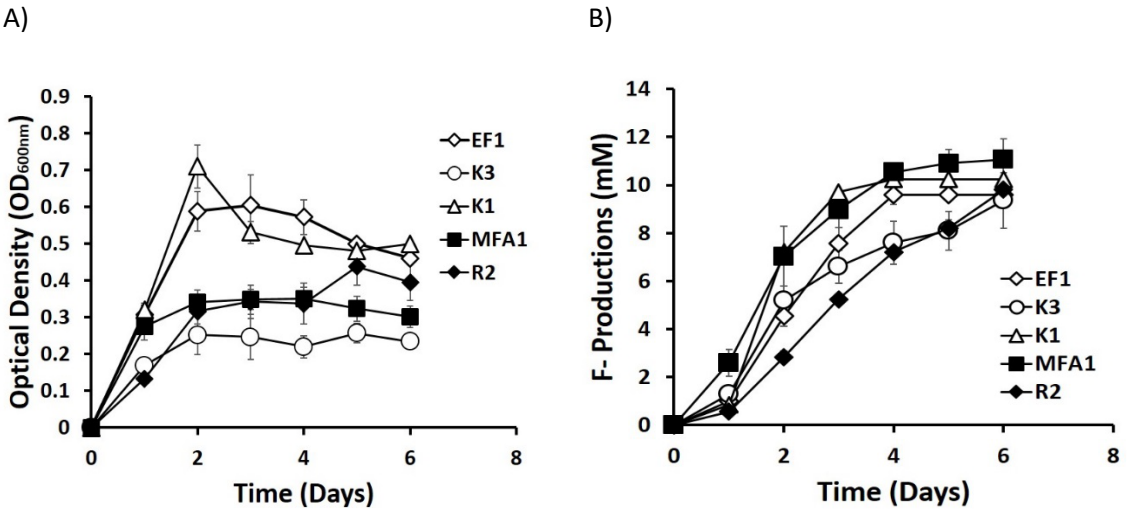


Fig. 14. Growth (A) and defluorination (B) of *Cloacibacillus* isolates from rumen and other herbivore gut systems in PA10 media supplemented with 0.8 % w/v YE and 10 mM FA.

* (*Cloacibacillus* strains (■, MFA1; Δ K1; ○, K3; ♦, R2; and ◊, EF1).

† Growth in media supplemented with 10 mM FA (A).

‡ Fluoride ion production (mM) in media supplemented with 10 mM FA (B).

Based on these results above, further comparisons were also undertaken to investigate growth and FA degradation ability between the leading representative isolates from each genus; *Cloacibacillus* strain MFA1, *Pyramidobacter* strain C12-8, *Oribacterium* strain C9, *Eubacterium* strain P_F-10, and another *Cloacibacillus* strain C-3-3. These studies were conducted on modified PA10 containing 10 mM FA (reduced YE concentration to 0.4 % w/v). All the isolates showed growth yield that was about half those observed on unmodified PA10 medium with 0.8% w/v YE. The isolates belonging to the *Cloacibacillus* genus showed the highest degradation of FA followed by the *Pyramidobacter* strain C12-8. However, the *Oribacterium* and *Eubacterium* genus provided little evidence of ability to degrade FA under these conditions (Fig. 15).

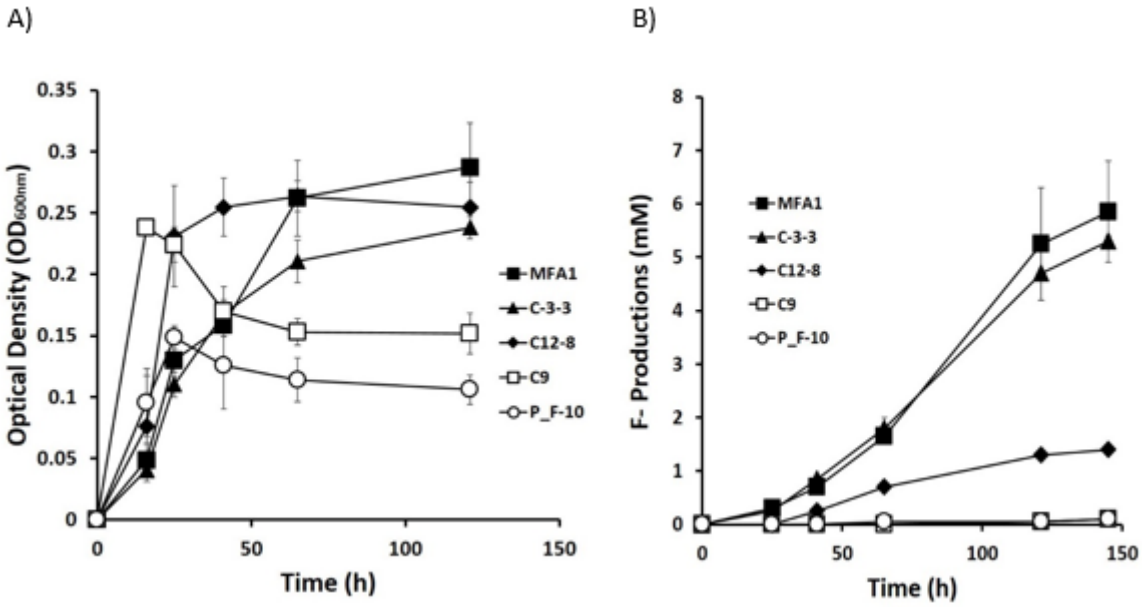


Fig. 15. Bacterial growth (A) and defluorination (B) of five representative isolates in basal PA10 media supplemented with 0.4% w/v yeast extract. * (*Cloacibacillus* strains (■, MFA1; and ▲, C-3-3); *Pyramidobacter* strain (♦, C12-8); *Oribacterium* strain (□, C9) and *Eubacterium* strain (○, P_F-10).

9.1.3 Nitrogen sources of FA-degrading rumen isolates

9.1.3.1 Effect of different nitrogen sources on growth and FA degradation by strain MFA1

Strain MFA1 was grown on different protein hydrolysates with and without FA and measurements of FA degradation were made (Fig. 16). During the exponential phase, the FA-supplemented cultures exhibited a significantly shorter doubling time ($p < 0.05$, two-tailed paired *t*-test) than the non-FA supplemented cultures. Strain MFA1 in FA medium with individually supplemented casamino acids, phytone peptone, tryptone, and yeast extract had a doubling time of approximately 5 to 6 h. Growth in the absence of FA resulted in longer doubling times in separate media singly supplemented with casamino acids (10 h), phytone peptone (12 h), tryptone (10 h), and yeast extract (7 h) (Fig. 16). Variations in apparent cell densities with different types of protein hydrolysates in FA supplemented media were also observed. The densest were yeast extract or tryptone which both reached an OD_{600} of 0.38, followed by phytone peptone at OD_{600} of 0.25. Casamino acids demonstrated the least growth with an OD_{600} of 0.13. The extent of FA degradation was strongly correlated to MFA1 growth on different types of protein substrates. MFA1 was able to completely degrade 20 mM FA in cultures containing either yeast extract or tryptone after 77 h of incubation (Fig. 16). However, only 14 mM fluoride ions were produced in the culture supplemented with phytone peptone and 20 mM FA. When the FA medium was supplemented with casamino acids, strain MFA1 only degraded 10 mM FA (Fig. 16).

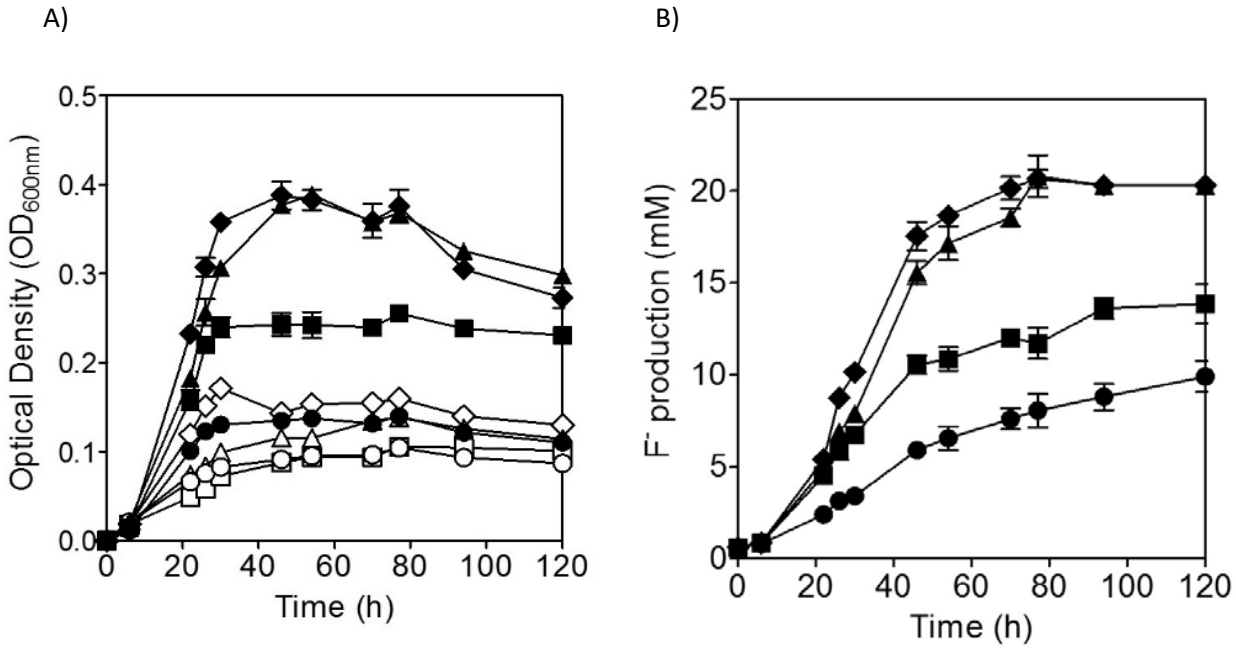


Fig. 16. Effect of different peptide sources on (a) the bacterial growth of strain MFA1; and (b) FA defluorination.

* Basal PA10 media contained 0.002 % yeast extract and separately 0.4 % (w/v) of the respective protein digests (▲, tryptone; ♦, yeast extract; ■, phytone peptone; ●, casamino acids)
† Solid markers represent respective media further supplemented with 20 mM FA; open markers represent media with protein hydrolysates only (i.e. no FA).

9.1.3.2 Effect of different nitrogen sources on growth and FA degradation by lead representative isolates from other genera

In other work *Pyramidobacter* strain C12-8, *Oribacterium* strain C9 and *Eubacterium* strain P_F-10 were grown on different protein hydrolysates and FA degradation was measured. Two genera *Oribacterium* strain C9 and *Eubacterium* strain P_F-10 had the highest growth on peptone and yeast extract, respectively and the highest FA degradation was 5 % and 3 %, respectively (Data not shown). However, *Pyramidobacter* strains C12-8 showed the highest FA degradation and growth when supplemented with peptone P as a nitrogen source (Fig. 17). Fluoride was produced continually during the stationary phase (from 40 h to 140 h) for both isolates and resulted in degradation of 22-30 % of the 10 mM of FA initially provided.

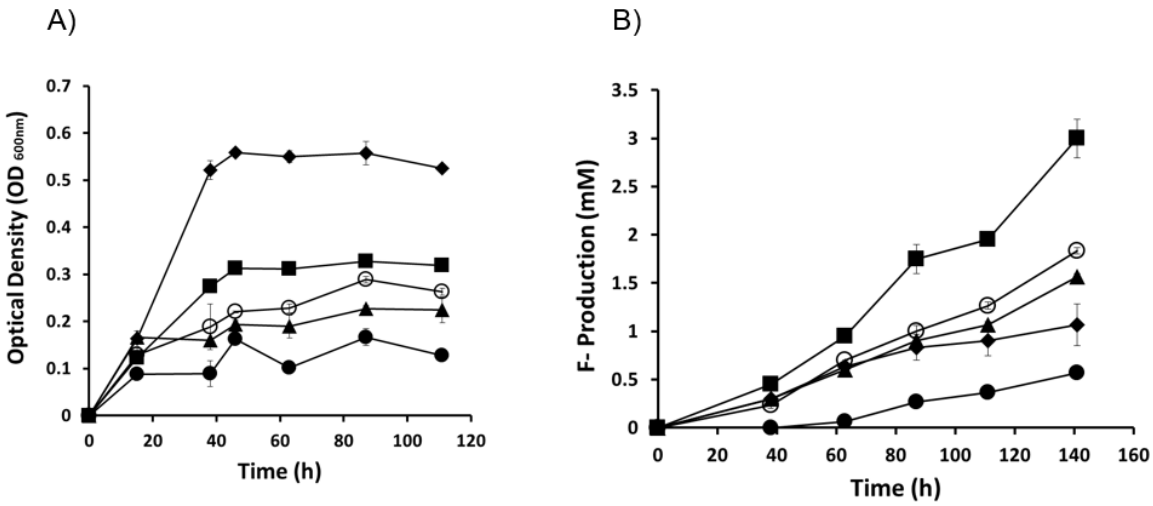


Fig. 17. Effect of different protein hydrolysates on bacterial growth (A) and FA degradation (B) of *Pyramidobacter* strains C12-8.

* Basal PA10 media supplemented separately with respective protein hydrolysates (■ Peptone P; ♦, yeast extract; ○, phytone Peptone; and ▲ Tryptone; and ● Casamino acids).

9.1.3.3 Comparison of growth, FA degradation and SCFA profiles between strain MFA1 and strain C12-8

Growth, FA degradation and SCFA profiles of strain MFA1 and strain C12-8 were analyzed using two different protein hydrolysates, yeast extract or peptone. Growth and FA degradation were measured over five days and the supernatant collected at 47 h (log phase) and 110 h (stationary phase) for analysis of the SCFA (Fig. 18 and Supplementary Table 12).

For both strains, the highest growth yields were achieved with media using yeast extract as the nitrogen source (Fig. 18). Apparent growth of strain MFA1 was enhanced when 10 mM FA was added to the culture (Fig. 18 A). During exponential growth phase (< 47 h), the FA supplemented cultures of strain MFA1 had significantly shorter doubling times ($P < 0.05$, two-tailed paired *t*-test) compared with those cultured in the absence of FA but with either protein hydrolysates (Fig. 18 A). However, strain C12-8 showed no significant differences in cell doubling times for FA supplemented media in both protein hydrolysates ($P = 0.39$ and $P = 0.24$, two-tailed paired *t*-test, yeast extract and peptone, respectively) (Fig. 18 C). Strain MFA1 was able to completely degrade 10 mM FA in cultures containing either yeast extract or peptone after 47 h of incubation (Fig. 18 B). However, strain C12-8 only achieved 30 % and 10 % FA degradation in peptone and yeast extract hydrolysates respectively after 110 h incubation (Fig. 18 D).

The SCFA profiles were analyzed in culture supernatants for both strain MFA1 and strain C12-8 at two different time points (47 h and 110 h, respectively) (Table 12). Strain MFA1 produced approximately 3.1 mM and 12.4 mM acetate as the metabolic end products of log phase growth in PA10 medium with 0.8% w/v YE (without and with 10 mM FA, respectively) (Table 12 A). However, strain C12-8 produced similar values of acetate production for FA supplemented and un-supplemented the medium (Table 12 B). Strain MFA1 consistently produced higher levels of propionate than strain C12-8 regardless of the type of supplemented protein hydrolysate and C12-8 produced butyrate in different strain MFA1 (Table 12).

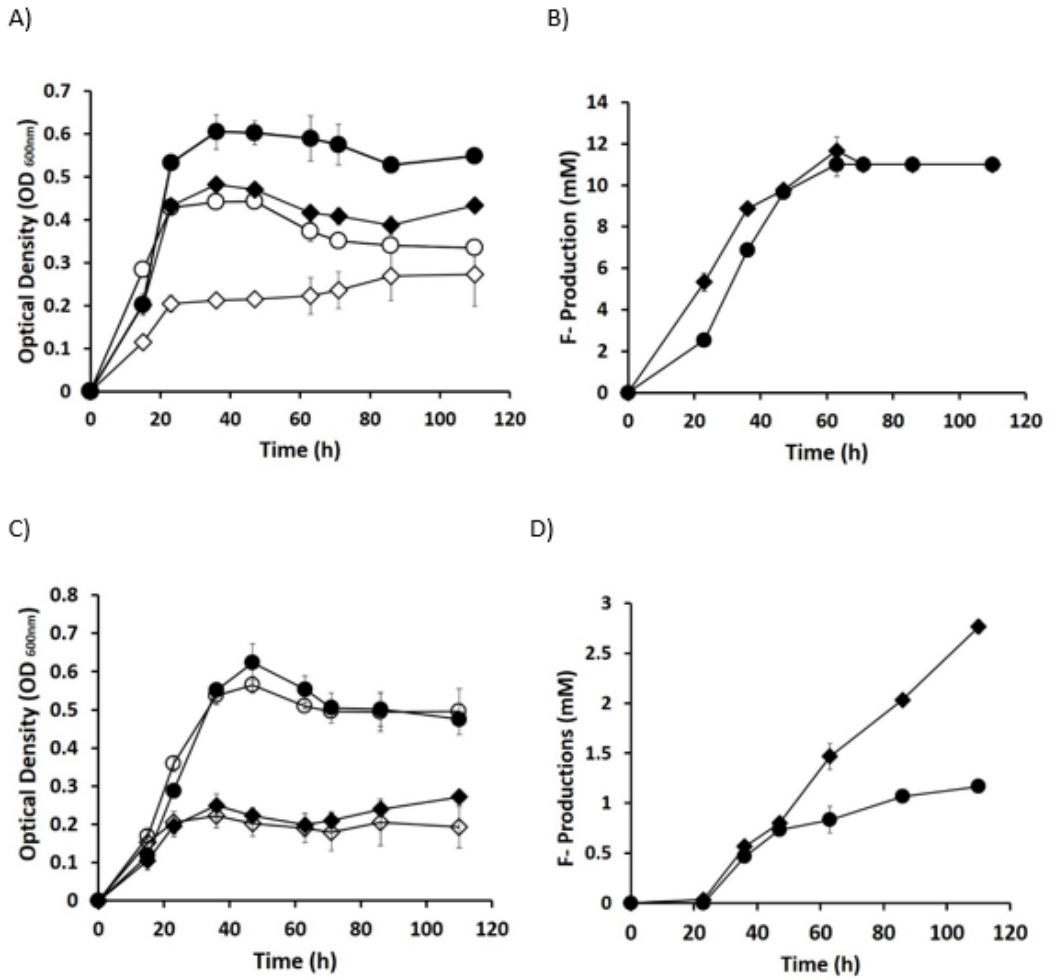


Fig. 18. Effect of different hydrolysates on bacterial growth of strain MFA1 (A) and strain C12-8 (C). FA degradation of strain MFA1 (B) and strain C12-8 (D).

* Basal PA10 media separately containing 0.8% w/v of respective protein hydrolysates (●, yeast extract; ♦, peptone).

† Solid markers represent media further supplemented with 10 mM FA, and open markers represent media only only (i.e. no FA).

Table 12. Changes in SCFA concentrations in 0.8 % yeast extract or 0.8 % peptone media at 47 and 110 hours of MFA1 (A) or C12-8 (B)

A)

Parameters (Culture times)		Yeast Extract (0.8%)			Peptone P (0.8%)		
		(-) FA	(+) FA	*P* value	(-) FA	(+) FA	*P* value
Acetate (mM)	(47 H)	3.15 ± 0.17	12.44 ± 1.85	< 0.001	-0.10 ± 0.06	8.40 ± 0.42	< 0.001
	(110 H)	4.97 ± 0.19	17.84 ± 2.34	< 0.001	0.80 ±0.27	7.74 ± 0.46	< 0.001
Propionate (mM)	(47 H)	4.84 ± 0.08	6.23 ± 0.26	0.007	2.01 ± 0.01	2.06 ± 0.10	0.829
	(110 H)	5.19 ± 0.07	6.55 ± 0.20	< 0.001	2.23 ± 0.05	2.01 ± 0.06	0.549
Butyrate (mM)	(47 H)	0.00	-0.01	0.924	-0.02	-0.02	0.699
	(110 H)	-0.01	-0.01	0.937	-0.03	-0.02	0.993

B)

Parameters (Culture times)		Yeast Extract (0.8%)			Peptone P (0.8%)		
		(-) FA	(+) FA	*P* value	(-) FA	(+) FA	*P* value
Acetate (mM)	(47 H)	7.81 ± 0.53	7.83 ± 0.88	0.22	2.54 ± 0.01	2.98 ± 0.69	0.986
	(110 H)	7.13 ± 1.12	8.28 ± 1.21	0.82	1.77 ± 0.74	3.70 ± 0.48	0.42
Propionate (mM)	(47 H)	1.34 ± 0.04	1.35 ± 0.09	0.893	0.50 ± 0.01	0.59 ± 0.10	0.715
	(110 H)	2.33 ± 0.87	1.52 ± 0.14	0.018	0.55 ± 0.10	0.54 ± 0.03	0.85
Butyrate (mM)	(47 H)	0.59 ± 0.01	0.60 ± 0.02	0.851	0.29 ± 0.01	0.31 ± 0.01	0.721
	(110 H)	0.89 ± 0.21	0.71 ± 0.05	0.035	0.34 ± 0.04	0.51	0.04

9.1.4 Further characterisations of *Pyramidobacter* strain C12-8

9.1.4.1 Performance of strain C12-8 under increasing peptone and FA levels

Increases in strain C12-8 bacterial growth and FA degradation were positively correlated with increased peptone P concentrations (Fig. 19). The highest fluoride production as a result of FA degradation was 6.4 mM (64 %) at 166 h culture in 1.6 % w/v peptone P media (Fig. 19 A and B). Different concentrations of FA from 5-20 mM did not alter the growth characteristics of the FA degradation capability of the bacteria (Fig. 19 C and D). However, 1 mM FA did produce less growth of the bacteria and a slower rate of FA degradation.

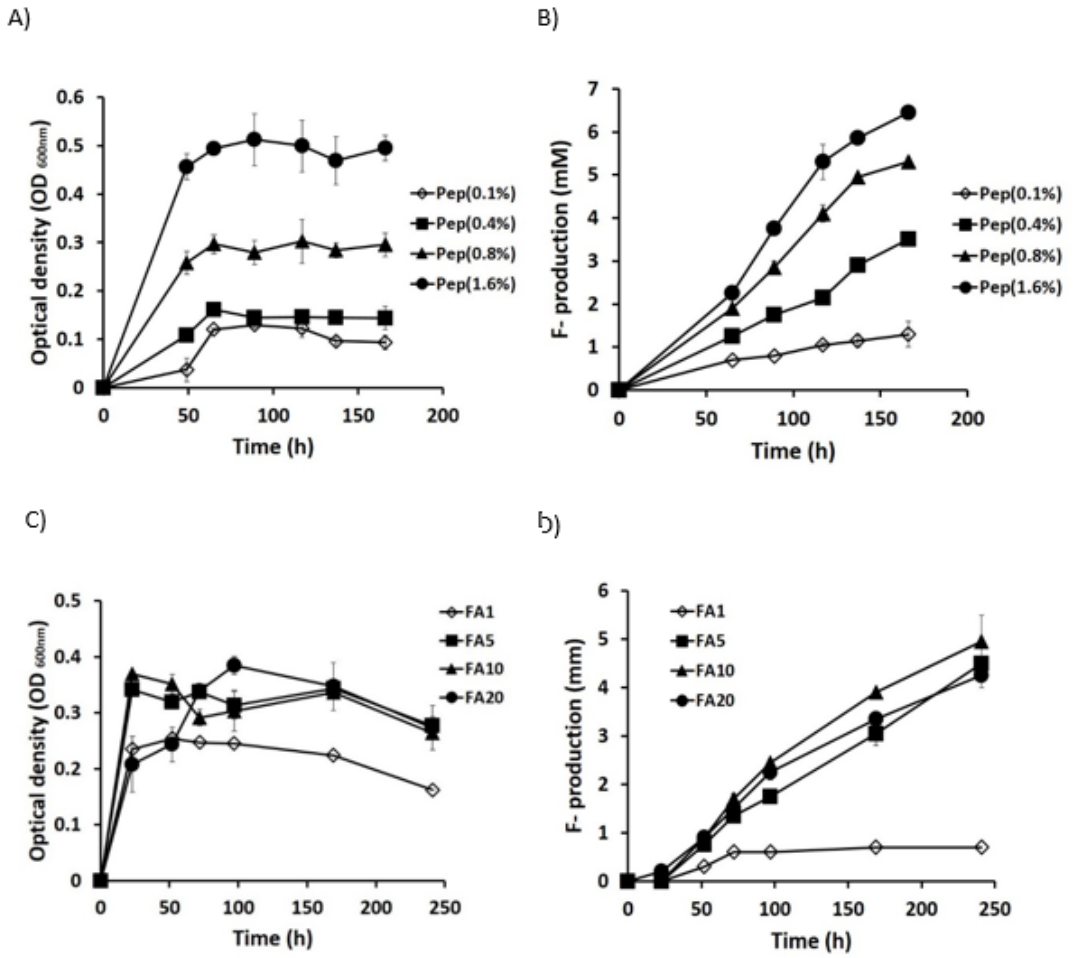


Fig. 19. Effect of different concentrations of supplementation of peptone P, and FA on strain C12-8 bacterial growth (A and C), FA degradation (B and D).

* (A) and (B) represent media of different peptone P concentration (◊, 0.1 %; ■, 0.4 %; ▲, 0.8 %; and ●, 1.6 % w/v) containing 10 mM FA.

† (C) and (D) represent media contacting 0.8% peptone with different amount of FA (◊, 1 mM; ■, 5 mM; ▲, 10 m; and ●, 20 mM).

9.1.4.2 Transmission electron microscopy of strain C12-8

After 48h culture of strain C12-8 in BYE and FYE media, colonies were 0.8-1.0 µm in diameter, circular and convex to pyramidal shape on phase contrast microscopy (Fig. 20 B). Transmission electron microscopic (TEM) observations of ultrathin sections of C12-8 showed the cell envelope to be unusual, with only one membrane and no obvious wall external to this (Fig. 20 C), so not characteristic of either Gram-negative or Gram-positive. There is, however, some electron density associated with the cytoplasmic leaflet of the membrane. Internally an electron dense body was seen centrally surrounded by a ribosome rich region (Fig. 20 C). On occasions this body is lobulated and this manifests as several smaller pieces in the sectioned cells. It does not appear to be membrane-bound. No spores or flagella were seen and no differences in morphology were observed between cells grown with FA and without FA treatment. Cells were arranged singly, in pairs, clumps or in chain forms (Fig. 20 A).

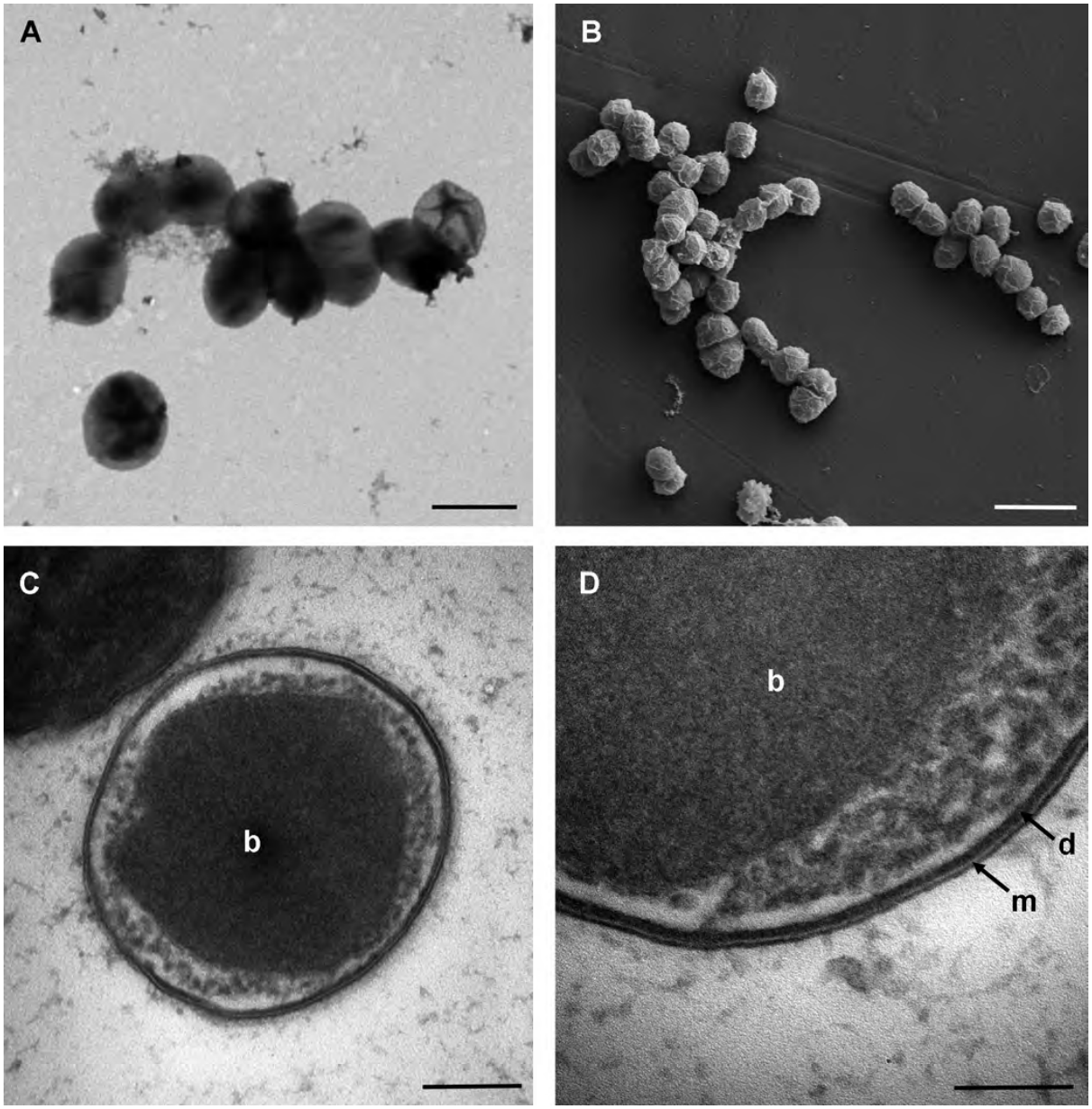


Fig. 20. Electron micrographs of strain C12-8. A. Negatively stained cells of strain C12-8 growing in a clump/chain. B. SEM of cells of C12-8 showing cells adherent together as groups after division. C. TEM of the ultrathin section of C12-8 showing the unusual single membrane cell envelope and the central electron dense body (b). D. The cell envelope of C12-8 exhibiting the single membrane (m) and the associated electron density (d) within the cell cytoplasm. Bar lines A. 1mm. B. 2mm. C. 200nm. D. 100nm

9.1.4.3 Total amino acid utilisation from Pep and YE hydrolysates

The Pep (1.2%) and YE (0.2%) medium contained 74.6 mM of total amino acids (Table 13). Glycine was the most abundant amino acid, comprising approximately 20 mM (27 %) of the total amino acids in the medium. During log phase growth on this medium, C12-8 preferentially used hydrophilic amino acids, metabolized 9 mM (12 %) and 1.6 mM (2 %) of hydrophilic and hydrophobic amino acids, respectively. Glycine, glutamic acid, serine, leucine, threonine, and aspartic acid were the most utilized amino acids but C12-8 was observed not to metabolize certain amino acids such as lysine, arginine,

and proline in this media condition. Moreover, alanine was the most produced amino acid and valine, leucine, and isoleucine were significantly utilized ($p < 0.05$) in presence of FA in both log phase (2 days) and stationary phase (9 days) compared to the absence of FA.

Table 13. Total amino acid utilisation of strain C12-8.

	Initial Conc. (µM)	Degraded (µM), Degradability (%)			
		2D_FA(+)	9D_FA(+)	2D_FA(-)	9D_FA(-)
Hydrophilic					
Glutamic acid	8448	1377^{a}, (16)	3407^{c}, (40)	1229^{a}, (15)	2572^{b}, (30)
Aspartic acid	4639	583^{a}, (13)	1134^{c}, (24)	624^{a}, (13)	971^{b}, (21)
Serine	3173	973^{a}, (31)	1213^{b}, (38)	976^{a}, (31)	1324^{c}, (42)
Threonine	1984	624^{a}, (31)	956^{b}, (48)	552^{a}, (28)	1039^{b}, (52)
Lysine	3253	-11, (0)	13, (0)	-7, (0)	35, (1)
Histidine	872	182^{b}, (21)	443^{c}, (51)	126^{a}, (14)	456^{c}, (52)
Arginine	4097	39, (1)	44, (1)	-6, (0)	53, (1)
Glycine	20007	5152^{a}, (26)	7042^{b}, (35)	5089^{a}, (25)	7614^{b}, (38)
Hydrophobic					
Alanine	8369	-431, (0)	-393, (0)	-414, (0)	-359, (0)
Proline	7712	-98, (0)	-28, (0)	-66, (0)	17,(0)
Tyrosine	815	44^{a}, (5)	97^{c}, (12)	36^{a}, (4)	73^{bc}, (9)
Methionine	891	29^{a}, (3)	174^{b}, (20)	89^{ab}, (10)	130^{ab}, (15)
Phenylalanine	1710	71^{a}, (4)	265^{c}, (15)	57^{a}, (3)	158^{b}, (9)
Valine	3217	527^{b}, (16)	1554^{d}, (48)	457^{a}, (14)	890^{c}, (28)
Leucine	3599	982^{b}, (27)	1976^{d}, (55)	784^{a}, (22)	1605^{c}, (45)
Isoleucine	1836	432^{b}, (24)	1003^{d}, (55)	353^{a}, (19)	748^{c}, (41)

* 2D, two days culture; 9D, nine days culture; FA(+), the presence of 10mM FA; FA(-), the absence of 10mM FA

† Values with different letters in the same row were significantly different ($p<0.05$)

9.1.4.4 Draft genome sequence analysis of C12-8

The annotated draft genome sequence of strain C12-8 was revealed not to contain the haloacid dehalogenase genes which are found some soil bacteria that degrade FA aerobically, such as *Moraxella* sp. These isolates may possess a similar defluorinating mechanism to the *Cloacibacillus*

MFA1 isolate which also does not possess the haloacid dehalogenase genes. Therefore, it was interesting to compare the genomes of FA-degrading isolates from both the *Cloacibacillus* and *Pyramidobacter* genera to identify common genes that may be involved in dehalogenation.

9.2 Monitoring abundance FA degrading bacteria using molecular tools

9.2.1 Design and validation of qPCR primers

Designed specific primer sets and validations of qPCR showed in 8.1.4 PCR studies.

9.2.2 Specificity and sensitivity of qPCR primers for strain MFA1

In order to test specificity of designed primers for MFA 1 (Table 6), genomic DNA from two different strains (*C. strain* MFA1 and *C. everyensis*) and seven rumen DNA samples were used in PCR reactions. Three designed primer sets that targeted the 16S rDNA gene were specific for *Synergistetes* phylum when matched with RDP database (https://rdp.cme.msu.edu/probematch/search.jsp). The primer set of MFA_181_F/ MFA_181_R (181F/R) (Table 6) matched with *Synergistetes* phylum more broadly (assigning 183 *Synergistaceae* families and 134 *Cloacibacillus* genera) than the other two primer sets according to *in silico* analysis in the RDP database. Therefore, the PCR assay using the broader primer set of 181F/R could amplify the *C. everyensis* which has 95 % similarity to strain MFA1; however, the primer sets 965F/1256R and 973F/1125R could amplify only strain MFA1. Three other primer sets targeting functional genes (Table 6) showed the predicted specificity based on the genome sequence of *Cloacibacillus* strain MFA1 and when those primers were used in PCR assays, they only amplified the genes from strain MFA1. All PCR products amplified from the 16S rDNA gene and functional genes were sequenced and revealed as *Cloacibacillus* strain MFA1 in origin (data not shown).

To evaluate the sensitivity of the designed primer sets, the qPCR assay was performed using DNA molecules from pure culture cell of strain MFA1. The standard curves of three primer set targeting 16S rDNA gene were linear from 4.52×10^6 cells to 452 cell counts, which correlated with Ct values ranging from 14 to 29 (Table 14 and Fig. 21) and the Ct range of other three primer sets targeting functional genes were around 16 to 31. The PCR efficiency was better in 965F/1256R (91.43 %) and 973F/1125R (108.93 %) with the slope of the curve -3.546 and -3.125, respectively (Table 14).

Table 14. The sensitivity of quantitative qPCR in pure culture comparing different primer sets.

MFA1 cell number (Log_{10})	Ct ± SD (n=3)					
	16S rDNA target			Functional gene target		
	181F/R	965F/1256R	973F/1125R	31400F/R	31410F/R	31430F/R
4.52 X 10^6 (6.66)	14.53±0.095	14.38±0.12	14.31±0.07	16.91±0.06	16.83±0.16	17.07±0.03
4.52 X 10^5 (5.66)	18.32±0.033	17.65±0.08	17.17±0.25,	20.49±0.10	20.33±0.04	21.02±0.07
4.52 X 10^4(4.66)	22.00±0.041	21.06±0.1	20.74±0.26	24.30±0.12	24.47±0.06	25.04±0.1
4.52 X 10^3 (3.66)	25.64±0.10	24.86±0.29	22.96±0.07	27.89±0.29	28.45±0.26	28.43±0.21
4.52 X 10^2 (2.66)	29.22±0.18	28.51±0.39	27.04±0.41	31.14±0.16	31.52±0.28	32.48±0.62
Efficiency	87.24%	91.43%	108.93%	90.01%	84.75%	82.63%

When the LOD was compared between two different cDNA synthesis approaches, based on the random hexa-primer and gene specific primer (1256R), the cDNA synthesized by specific MFA1 primer showed more sensitivity in the qPCR assay (Table 14 and Table 16). While RNA-based detection methods for monitoring specific bacterial strains in the rumen are uncommon, this is the first example of an RNA-based detection of a rumen isolated bacterium, *Cloacibacillus* strain MFA1. Therefore, our results suggest that the qPCR assay based on the RNA molecules is more sensitive for monitoring the MFA1 in the rumen. Similar approaches could be employed for the detection and monitoring of rumen bacteria with novel functions.

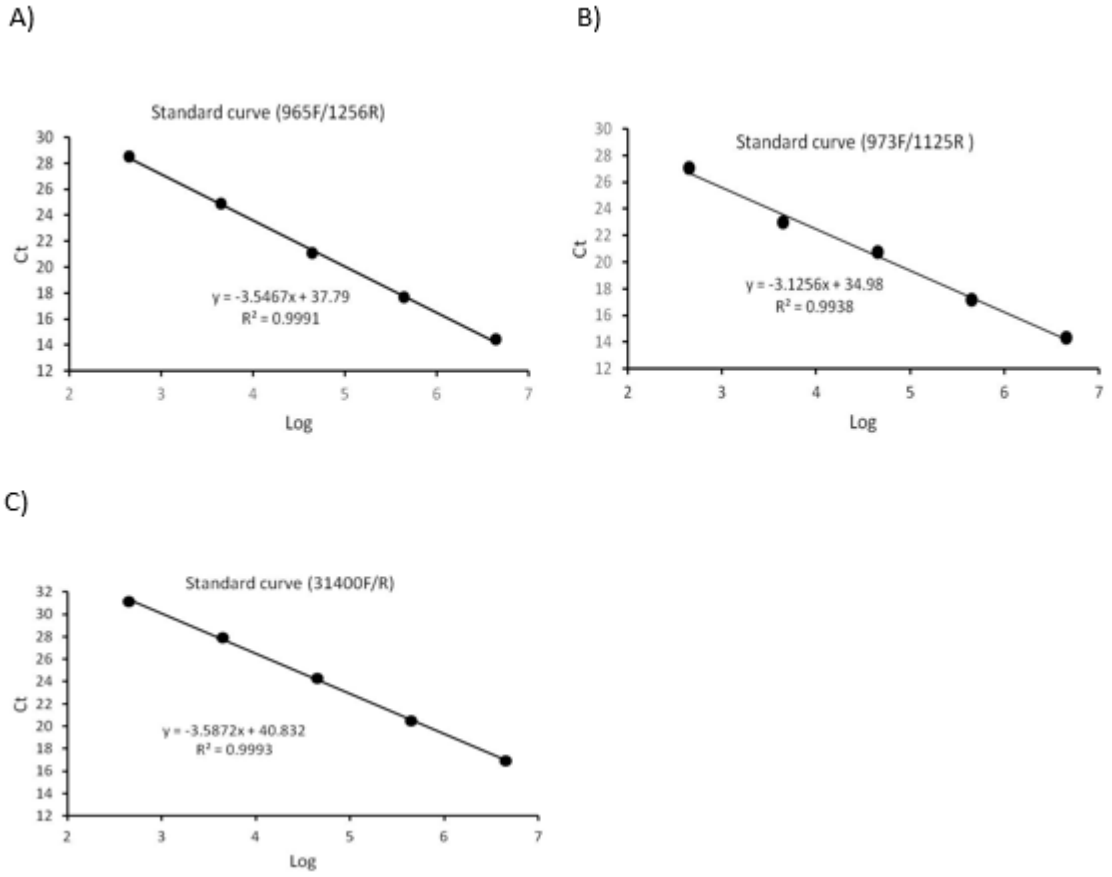


Fig. 21. Linearity and detection range of the qPCR assay. Standard curves with amplification efficiency of 99% were generated from genomic DNA targeting the 16S rDNA gene of *Cloacibacillus* strain MFA1 using 965F/1256R primer set (A), 973F/1125R primer set (B), and 31400F/R primer set (C).

9.2.3 Specificity and sensitivity of qPCR primers for strain C12-8

In the same way, designed primer set for strain C12-8 targeted the 16S rDNA gene were analysed according to *in silico* with RDP database as above. The primer set of C12-8_256F / C12-8_488R (Table 6) matched with *Pyramidobacter* sp. C12-8 specifically. PCR amplicons from rumen DNA samples using

this primer set were sequenced and revealed as strain C12-8. The standard curves of the primer set targeting 16S rDNA gene (C12-8_256F / 488R) were linear from 1.25×10^9 cells to 1.25×10^4 cell counts, which correlated with Ct values ranging from 13 to 29. The PCR efficiency was 102.21 % with the slope of the curve -3.27 (Fig. 22).

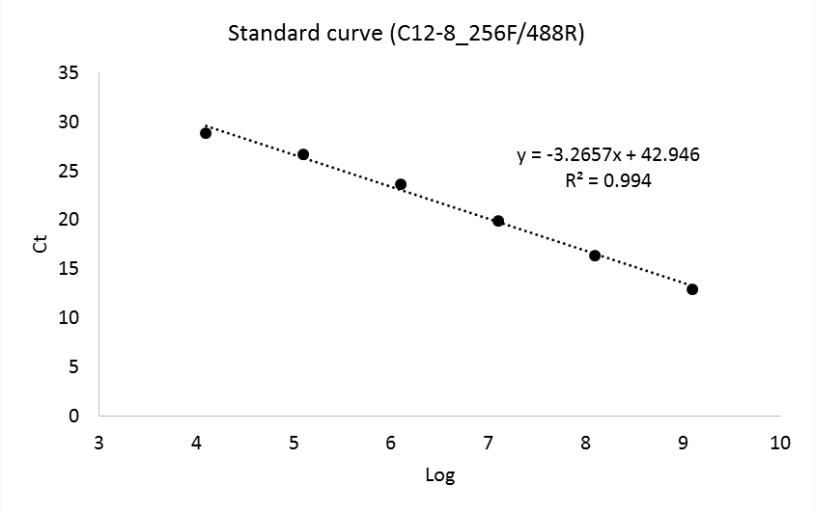


Fig. 22. Linearity and detection range of the qPCR assay of strain C12-8 using C12-8_259F / C12-8_488R primer set.

9.2.4 Detection of spiked FA degrading isolates (MFA1 and C12-8) using DNA or RNA from rumen enrichment culture and rumen fluids

Detection of strain MFA1 in the qPCR assay targeting the 16S rRNA gene was evaluated using DNA and RNA based molecules derived from MFA1 spiked into rumen enrichment mixed cultures and rumen fluids (free MFA1) (8.4). First, DNA and RNA were extracted in same enrichment rumen mixed culture (1 ml) spiked with different numbers (10 fold dilutions) of strain MFA1 and those extracts had three biological replicates and four technical replicates for qPCR. The qPCR efficiencies were 103.5 % and 122.13 % for assays using 965F/1256R, 973F/1125R primer set, respectively. The primer set of 965F/1256R could detect strain MFA1 cells at 11300 cells.ml^{-1} for the DNA molecule (Table 15). The result of the RNA based analysis indicated that the qPCR assay was able to detect as few as 226 cells.ml^{-1} for the cDNA synthesized by the specific primer (1256R) (Table 16). So, the limit of detection (LOD) of spiked MFA1 with the enrichment rumen culture were 11300 and 226 cells in DNA and RNA based analysis, respectively. In case the primer set targeting functional gene (31400F/R), LOD was 10 times lesser than that of targeting 16S rRNA gene in DNA molecule (Table 15) and it was 100 times lesser than that of RNA molecule (Table 16).

Table 15. Detection of spiked MFA1 with enrichment mixed culture using DNA

Spiked MFA1 Cell number (Log_{10}) per mL	Ct ± SD (n=3), Relative ratio % (2^-(SP*-TB†) X 100)		
	16S rRNA gene target		Functional gene target
	965F/1256R, (%)	973F/1125R, (%)	31400F/R
1.13 X 10^7 (7.05)	18.92±0.39, (0.29)	18.92±0.05, (0.29)	20.29±0.06
1.13 X 10^6 (6.05)	21.84±0.07, (0.034)	21.67±0.04, (0.038)	22.77±0.01
1.13 X 10^5 (5.05)	25.14±0.02, (0.0037)	24.69±0.05, (0.005)	24.50±0.01
1.13 X 10^4 (4.05)	28.62±0.06, (0.00038)	ND	ND
1.13 X 10^3 (3.05)	ND‡	ND	ND
Efficiency	103.50%	122.13%	198.57%

* SP: *Cloacibacillus* strain MFA1 specific primer sets

† TB: Total bacteria (Bacterial universal primer), Efficiency (E) = -1 + $10^{(-1/slope)}$ × 100

‡ ND: Non detectable

Table 16. Detection of spiked MFA1 with enrichment mixed culture using RNA (cDNA).

Spiked MFA1 Cell number (Log_{10}) per mL	Ct ± SD (n=3), Relative ratio % (2^-(SP*-TB†) X 100)		
	16S rRNA gene target (cDNA synthesis)		Functional gene target (cDNA using hexa)
	Random-Hexa, (%)	1256R	31400F/R
2.26 X 10^7 (7.35)	16.82±0.04, (0.3)	10.42±0.48	24.69±0.12
2.26 X 10^6 (6.35)	19.36±0.02, (0.038)	14.24±0.39	27.13±0.18
2.26 X 10^5 (5.35)	23.00±0.06, (0.0034)	17.49±0.2	31.21±0.87
2.26 X 10^4 (4.35)	25.96±0.11, (0.0002)	20.66±0.31	ND
2.26 X 10^3 (3.35)	29.53±0.18, (0.00003)	23.56±0.02	ND
2.26 X 10^2 (2.35)	ND‡	26.07±0.59	ND
Efficiency	105.26%	108.8%	102.60%

* SP: *Cloacibacillus* strain MFA1 specific primer sets

† TB: Total bacteria (Bacterial universal primer), Efficiency (E) = -1 + $10^{(-1/slope)}$ × 100

‡ ND: Non detectable

Second, to evaluate the LOD of MFA1 in rumen samples, DNA and RNA (cDNA) were generated from natural rumen fluid spiked before extraction with different numbers (10 fold dilutions) of strain MFA1 and used in a qPCR assay (Appendix 1, 8.1.4 and 8.4). The PCR efficiency targeting the 16S rRNA gene were 121.6 % and 93 % for DNA and RNA based molecules, respectively (Table 17 and Table 18). The LOD was 2.2 × 10^5 / ml for DNA and 2.2 × 10^4 / ml for RNA (cDNA) based analyses. The LOD for the RNA based analysis was similar for both cDNA synthesized by random-hexa primer and specific primer (1256R) although the Ct values were lower for the specific primer (Table 18). Control without any spiked strain MFA1 supplement showed no valid PCR signal both DNA and RNA (cDNA) molecules. The LOD targeting functional gene (31400F/R) was similar to DNA molecule (2.2 × 10^5 / ml, Table 17) but 10 times lesser in RNA (cDNA) molecule (2.2 × 10^4 / ml, Table 18).

Table 17. Detection of spiked MFA1 with rumen fluids using DNA.

Spiked MFA1 Cell number (Log_{10}) per mL	Ct ± SD (n=3), Relative ratio % (2^-(SP*-TB†) X 100)	
	16S rRNA gene target	Functional gene target
	965F/1256R, (%)	31400F/R
2.2 X 10^7 (7.34)	19.72±0.06, (0.44)	20.36±0.05
2.2 X 10^6 (6.34)	23.12±0.04, (0.04)	23.38±0.10
2.2 X 10^5 (5.34)	25.51±0.12, (0.008)	24.91±0.15
2.2 X 10^4 (4.34)	ND‡	ND
2.2 X 10^3 (3.34)	ND	ND
Efficiency	121.58%	137.96%

* SP: *Cloacibacillus* strain MFA1 specific primer sets

† TB: Total bacteria (Bacterial universal primer), Efficiency (E) = -1 + $10^{(-1/slope)}$ X 100

‡ ND: Non detectable

Table 18. Detection of spiked MFA1 with rumen fluid using RNA (cDNA)

Spiked MFA1 Cell number (Log_{10}) per mL	Ct ± SD (n=1), Relative ratio % (2^-(SP*-TB†) X 100)		
	16S rRNA gene target (cDNA synthesis)		Functional gene target (cDNA using hexa)
	Random-Hexa, (%)	1256R	31400F/R
2.2 X 10^7 (7.34)	19.45±0.076, (0.22)	12.16±0.04	25.74±0.04
2.2 X 10^6 (6.34)	23.05±0.047, (0.02)	15.29±0.05	28.96±0.1
2.2 X 10^5 (5.34)	25.83±0.1, (0.002)	19.15±0.13	31.31±0.63
2.2 X 10^4 (4.34)	28.15±0.1, (0.0002)	22.55±0.04	ND
2.2 X 10^3 (3.34)	ND‡	ND	ND
Efficiency	121.89%	93%	128.53%

* SP: *Cloacibacillus* strain MFA1 specific primer sets

† TB: Total bacteria (Bacterial universal primer), Efficiency (E) = -1 + $10^{(-1/slope)}$ × 100

‡ ND: Non detectable

Similar experiments were undertaken for *Pyramidobacter* strain C12-8 with largely congruent results (data not shown). In this instance, no rumen sample could be found for enrichment that was devoid of natural C12-8 inoculation. As such, for this isolate, an un-enriched rumen fluid was used as the diluent for 10-fold dilutions of *Pyramidobacter* cells grown in pure culture.

Unlike DNA targets, which usually exist as a single copy per cell, each bacterium contains hundreds to thousands of ribosomal or specific functional gene RNA molecules. We, therefore, hypothesized that an assay based on the qPCR of RNA molecules could improve the sensitivity of *Cloacibacillus* strain MFA1 detection. The qPCR assay using RNA-based molecules for detection of MFA1 was shown to be a 100-fold and 10-fold more sensitive than corresponding DNA-based detection in the enrichment mixed cultures and rumen fluid, respectively. Backstedt, B *et al*. reported in 2015 that the use of

leptospiral RNA as a diagnostic target for the development of a rapid, sensitive, and specific qPCR assay for detection of human leptospirosis. Diagnostic tests using RNA from human blood was at least 100-fold more sensitive than corresponding leptospiral DNA-based qPCR assays.

9.3 Screening of compounds that up-regulate FA operon activity and their evaluation in pure culture studies

9.3.1 Promoter region analysis and construction of the plasmid for reporter gene assay

The promoter region of the up-regulated operon in the presence of FA (Leong et al., 2014) from the strain MFA1 genomic DNA was cloned. The cloned promoter was determined to be 401 bp in length, and the sequence is shown in Fig. 9. The cloned promoter region was analyzed by the prediction program for a bacterial promoter (BPROM). Several core elements of promoter function were identified (Fig. 9), such as ribosomal binding site (RBS, Shine- Dalgarno sequence, AGGAGT), -10 and -35 promoter regions (TGTCAAAAT and TTTTCT, respectively) and transcriptional factor (TF) binding site (TTATATT). The TF binding site was related to GcvA (Glycine cleavage A, transcription regulator protein) which is able to activate transcription of *gcv* (glycine cleavage) operon. The promoter fragment was cloned using PromoF and PromoR primers (Table 6) and the plasmid pGL4-promo was introduced into *E. coli* TOP 10 strain by electroporation for use in a reporter assay (Fig. 23).

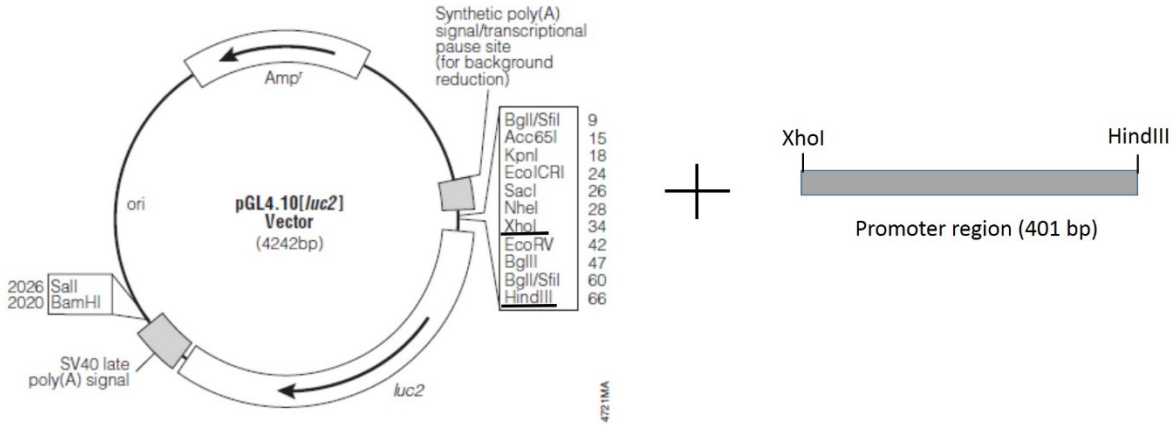


Fig. 23. Schematic diagram of the pGL4-Promo vector. The promoter region fragment was ligated to multiple cloning sites (underlines indicated the *Xho*I and *Hind*III restriction enzyme sites) of the pGL4.10 [luc2] vector for constructing gene fusions.

9.3.2 Assay for evaluating effect of different substrates on promoter activity

The luciferase activity was stably increased in transformed *E. coli* containing the pGL4-promo vector during exponential phase (up to 6 to 8 h, OD_{600nm} 0.4) but the activity was decreased from stationary phases (from 10 h) (Fig. 24). After optimizing *E. coli*-pGL4-promo culture conditions for reporter assay analysis, the relative luciferase activities on different substrates (listed in Table 2) were analyzed. Among 13 amino acids, arginine (Arg) showed the highest luciferase activity (2.7 folds higher than control, Fig. 25 A) and among the other compounds, the highest activity was associated with sodium citrate (Cit) (4.7 folds higher than control, Fig. 25 B).

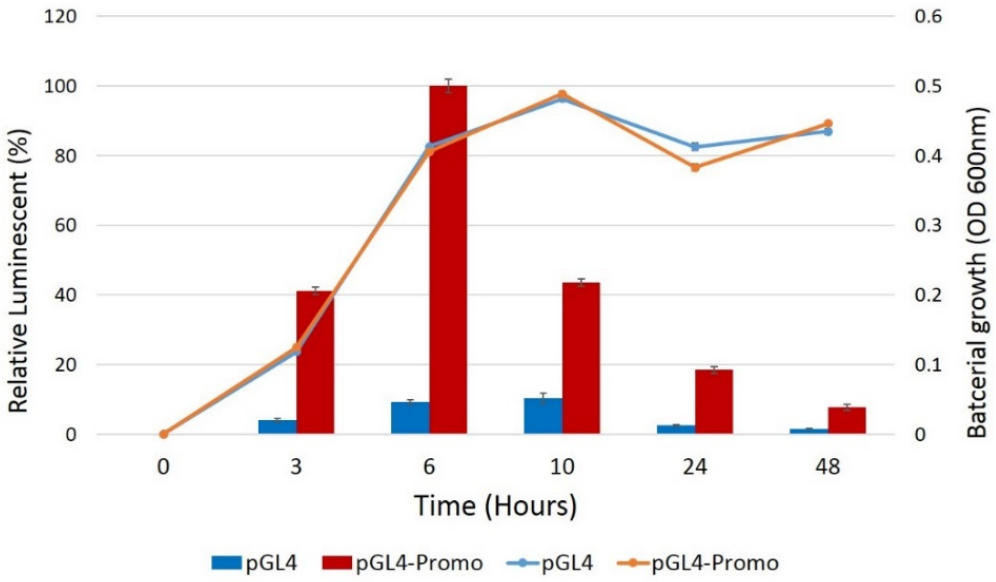


Fig. 24. Bacterial growth and luciferase activity of transformed *E.coli* / pGL4 (without promoter) and *E. coli*-pGL4-promo (with promoter). Bar graphs indicate the relative activity (luminescence, %) and line graphs indicate the bacterial growth (OD_{600nm}) on different time points.

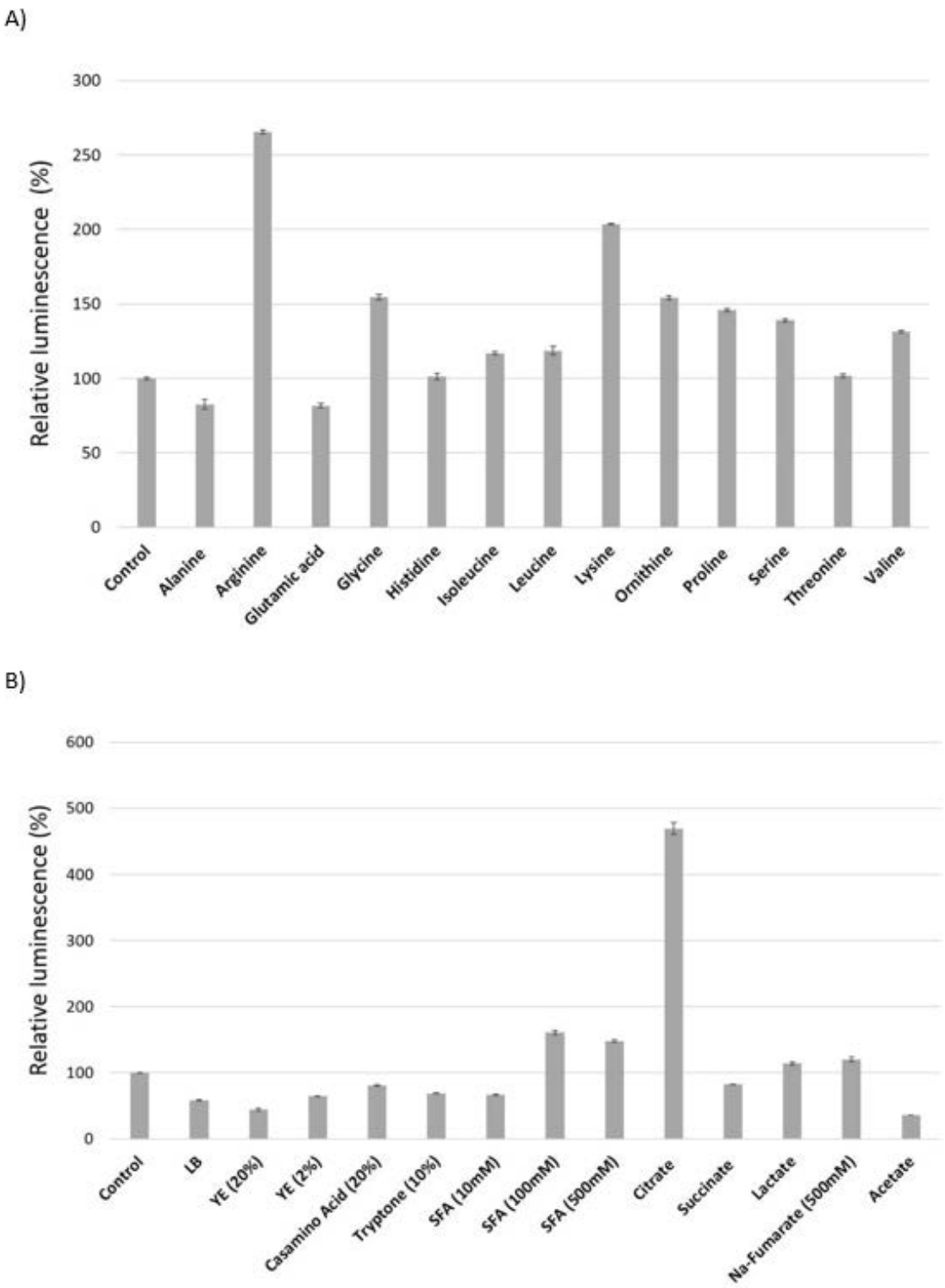


Fig. 25. Luciferase activity on different substrates. A) 13 amino acids, B) organic acids or other nutrient sources (listed in Table 8).

9.3.3 Reporter assay and gene expression analysis in transformed E. coli

To investigate and confirm the effects of substrates on the promoter activity, we analyzed both of the luciferase activity and the transcriptional expression level of *luc2* gene after inducing with different supplements. After transformed *E. coli* (pGL4-promo) was treated with arginine and citrate, the Luc2 activities were 9 and 6 times higher than control respectively (Fig. 26 A). Also, the transcriptional levels of the *luc2* gene were increased up to 3 times and 2 times (arginine and citrate, respectively) compared with the control (Fig. 26 B). However, the control vector (without promoter, pGL4 vector only) showed no effects both of Luc2 activity and transcriptional gene expression level after treatment with same supplements (Fig. 26 A and B, grey bars).

To evaluate the specificity of action of arginine on the promoter, an arginine analogue (canavanine) was used in the reporter assay. The luciferase activity increased linearly with increasing concentrations of arginine but decreased linearly as canavanine concentrations increased (Fig. 27 A & B).

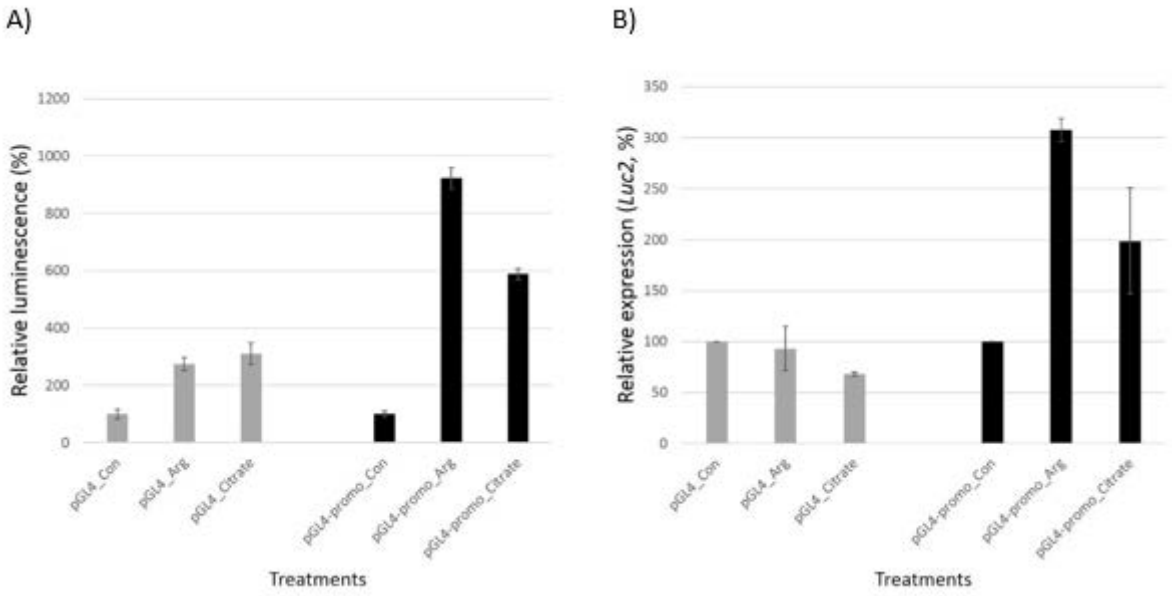


Fig. 26. Relative activity and expressional levels of reporter gene, luciferase (Luc2) on different treatments.

* A) Luciferase reporter assay, B) RT-qPCR. pGL4, without the promoter (vector only, gray bars); pGL4-promo, with the promoter (black bars); con, control; Arg, Arginine (500 mM, 5 µl); Citrate (Citrate 500 mM, 5 µl).

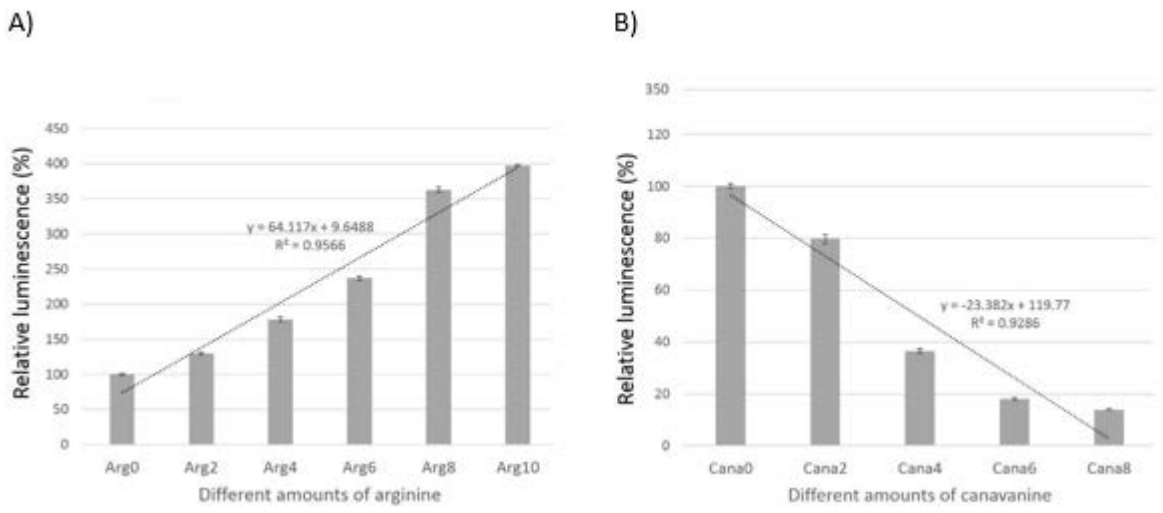


Fig. 27. Reporter assays on different concentrations of arginine and canavanine in transformed *E.coli* / pGL4-promo.

* A) Different amounts of arginine (Arg, 0-10 mM), B) different amounts of canavanine (Cana; 0-8 mM).

9.3.4 Transcriptional gene expression of FA-degrading genes of strain MFA1

To evaluate the transcriptional expression levels of FA-degrading genes of strain MFA1, the RT-qPCR analysis was performed with three different supplements - arginine, canavanine (Arg analogue), and citrate identified using the promoter assay. Each substrate was added in PA10 medium with 0.8% w/v YE and with or without FA (10 mM) > strain MFA1 were harvested to extract RNA after measuring optical density (OD_{600nm}) and fluoride production (mM). The relative transcriptional expression levels of two candidate operon genes [Fe-S oxidoreductase and glycine reductase complex B (GrdB)] for FA degradation were analyzed using RT-qPCR (Fig. 28).

Both of the genes were expressed at levels around 30 times higher in FA containing media than not containing FA regardless of treatments thus showing that the transcriptional expression level of target genes was correlated with fluoride production. Cells cultured with canavanine showed lower gene expression as well as fluoride products which were similar to the reporter assay in *E. coli* / pGA4-promo (Fig. 28).

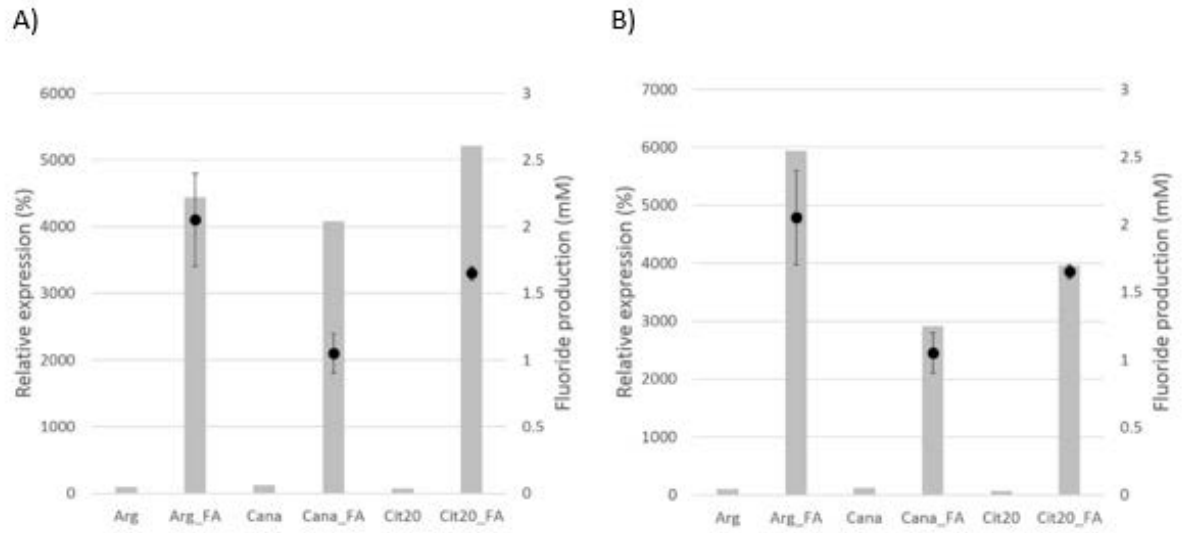


Fig. 28. RT-qPCR analysis of relative gene transcription in MFA1 of Fe-S oxidoreductase (A) and Glycine reductase complex B (GrdB) (B) based on different substrate treatments with or without FA (Bar graphs). The scatter plots indicate the fluoride production at same time points. (Arg, Arginine; FA, Fluoroacetate; Cana, Canavanine; Cit20, Citrate 20 mM)

9.4 Evaluation of ability for MFA1 to persist and degrade FA using rumen *in vitro* fermentation

9.4.1 Rumen bacterial fermentation using *in vitro* culturing system

Detailed experimental designs are shown in 8.6.1 (Experimental design). Rumen fermentation profiles of medium inoculated with fresh rumen fluid are shown in Table 20 and Fig. 29. The concentration of total VFA and molar proportion of n-butyrate, n-valerate and caproic acid was increased with time while the proportion of propionate decreased (Table 20). BCA plot analysis was employed to assess the similarity of VFA fermentation profiles between the sub-culturing groups. The VFA profiles were statistically different between 1st collection and 6th collection using a permutation test (Monte Carlo test) in R "Ade4" package. The greatest variation of acetate : propionate was observed between the 1st culture collection and last collection (6th collection) (Table 20 and Fig. 29). However, the profiles from 5th to 6th sub-culture collections were most similar suggesting that the fermentation was stabilized by about the 6th day. Therefore stabilized rumen culture mixture was stored as glycerol stocks and used as rumen total bacterial inoculum in subsequent experiments.

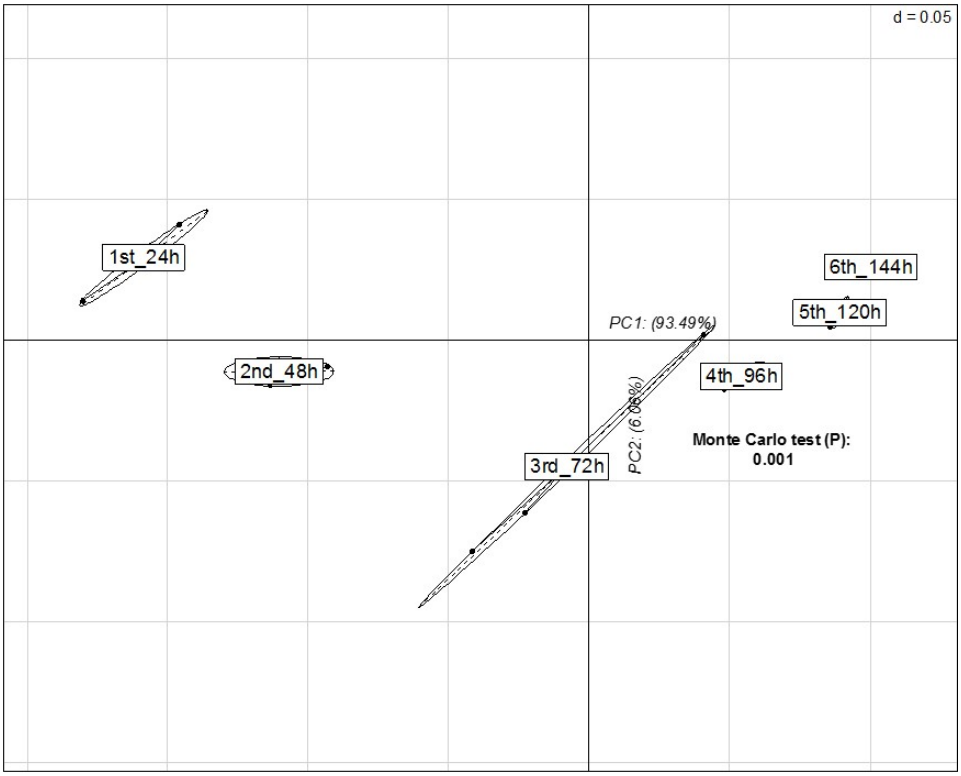
Table 19. The SCFA profiles during six sub-cultures in Ankom rumen *in vitro* fermentation system

Parameters (Increased)	1St (24h)	2nd (48h)	3rd (72h)	4th (96h)	5th (120h)	6th (144h)	SEM1
Total VFA (mM)	23.7ab	18.8a	31.7abc	35.6bc	40.8c	43.0c	5.45
Acetate (Molar %)	46.5ab	38.7a	46.9ab	54.7bc	58.7b	61.1b	3.68
Propionate (Molar %)	40.7c	38.3c	21.4b	14.6a	10.9a	9.4a	2.2
n-Butyrate (Molar %)	5.9a	11.4b	16.6c	17.5c	18.3c	17.9c	1.0
n-Valerate (Molar %)	1.0a	4.6b	8.9d	7.7cd	6.7bcd	5.9bc	0.76
Caproic acid (Molar %)	-0.6a	-0.5a	0.1b	0.1b	0.2b	0.2b	0.14
Acetate : Propionate	1.1a	1.0a	2.3a	3.8b	5.4c	6.5c	0.4

* SEM[1]: Standard error of the mean

† Means within a row with different superscripts significantly differ ($P < 0.05$).

A)



B)

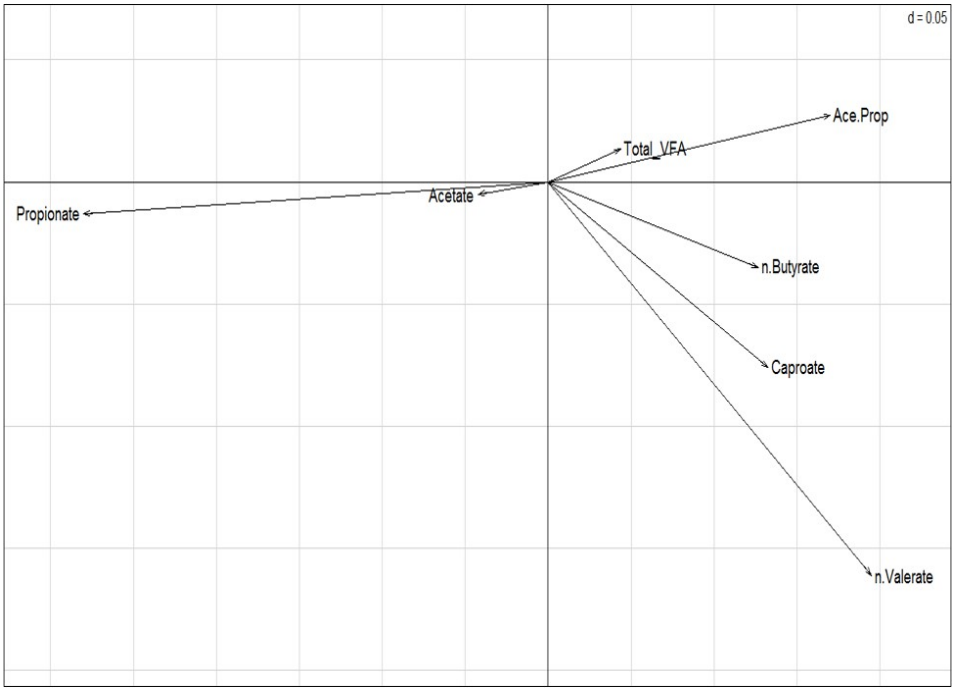


Fig. 29. Co-inertia analysis of the relationship between sub-cultures and profiles of SCFA.

Between Class Correspondence Analysis (BCA) plot shows the similarity and difference between sub-cultures on VFA profiles (A) and related parameters of VFA (B).

9.4.2 Effect of different supplements on FA degradation by strain MFA1 in mixed rumen bacteria *in vitro* fermentations

9.4.2.1 Effect of FA on strain MFA1 grown with mixed rumen bacteria

The first trial using a mixed rumen culture fermentation was performed to determine the effect of FA on the growth of strain MFA1. Mixed rumen bacteria cultures (RM) were grown in combination with strain MFA1, with and without FA supplemented to 10 mM. The percentage of this bacterium relative to total rumen bacteria and VFA profiles in cultures growing for 2 days was measured. The percentage of strain MFA1 increased in cultures containing 10 mM FA at both sampling times on day 1 and 2 (0.9 % and 1.72 %, 24 and 43 hours, respectively), whereas the relative percentage of strain MFA1 did not change in the control cultures without FA (0.23 % and 0.27 %, respectively) (Fig. 30). This represented relative abundances of strain MFA1 4.5 and 6.4 times higher in the presence of FA on day 1 and 2 respectively compared with the controls. The FA degradation showed a positive correlation with the abundances of strain MFA1 in the mixed rumen culture. As expected fluorine ion production from the degradation of FA increased significantly ($P < 0.05$, t-test) to 6 % and 13 % in the culture media at 24 and 43 hours, respectively.

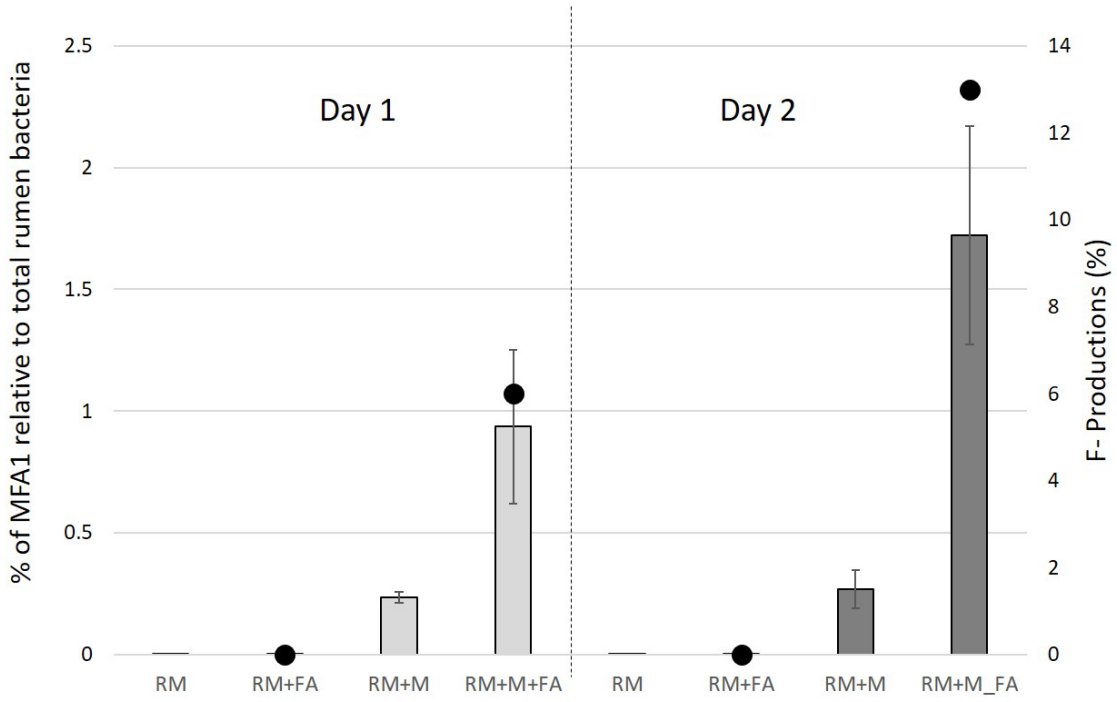


Fig. 30. The percentages of strain MFA1 relative to rumen total bacteria (gray bars) and fluoride production (black dots, %) during *in vitro* fermentation.

* Light gray bars indicate 24 h cultured samples and dark gray bars are 43 h cultured samples.
Line bars show standard error
†RM, Rumen mixed bacteria; M, inoculated with MFA1; FA, Fluoroacetate (10 mM)

9.4.2.2 Effect of arginine on strain MFA1 grown with mixed rumen bacteria

The second trial was performed to evaluate the effect of arginine supplement on growth and FA degradation of strain MFA1 in a mixed rumen culture fermentation. Arginine had little effect on the growth of strain MFA1 in the absence of FA (RM+M *vs*. RM+M+Arg20) (Fig. 31). However, the

percentage of strain MFA1 relative to total rumen bacteria was significantly increased (4.7 fold) when both arginine and FA were present compared with FA alone (RM+M+Arg20+FA *vs*. RM+M+FA, $P < 0.05$, *t*-test) at 2 days culture. In congruence with the first experiment, FA degradation in cultures was positively correlated with the percentage of strain MFA1. Arginine supplementation increased fluoride production in the culture media from 14 % to 37 % at day 1 and day 2, respectively (Fig. 31).

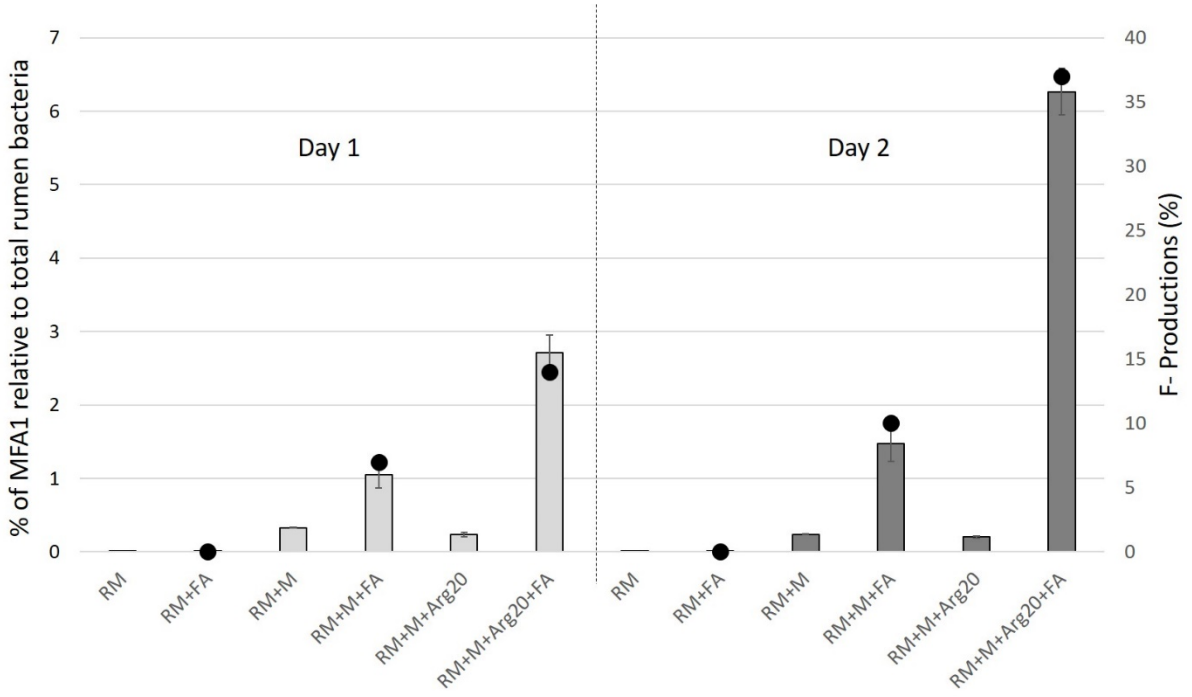


Fig. 31. The percentages of strain MFA1 relative to rumen total bacteria (gray bars) and fluoride production (black dots, %) during in vitro fermentation.

* Light gray bars indicate 24 hours cultured samples and dark gray bars are 43 hours. Line bars show standard error.

† RM, Rumen mixed bacteria; M, strain MFA1; Arg20, L-Arginine (20 mM); FA, Fluoroacetate (10 mM).

9.4.2.3 Effect of citrate on strain MFA1 grown with mixed rumen bacteria

The third trial was performed to evaluate the effect of citrate supplement on the growth and FA degradation of strain MFA1 in the rumen culture fermentation. The percentages of strain MFA1 relative to total rumen bacteria were increased (0.26 *vs.* 0.33 % and 0.32 *vs.* 0.67 % at day 1 and 2, respectively) when citrate was added as a supplement in the presence of FA (Fig. 32). However, citrate did not affect the abundance of strain MFA1 in the absence of FA (RM+M+Cit) (Fig. 32). The FA degradation showed a positive correlation with the percentage of strain MFA1 in the culture. The fluoride productions were increased with citrate supplement (RM+M+FA *vs.* RM+M+Cit10+FA were 5.7 % *vs.* 7.2 % at day 2, respectively) (Fig. 32). Total VFA concentrations and molar proportions of acetate were significantly increased by 10 mM citrate supplementation in the medium compared to non-supplemented medium, while molar proportions of propionate, butyrate, and valerate were decreased by adding citrate supplementation (Table 21).

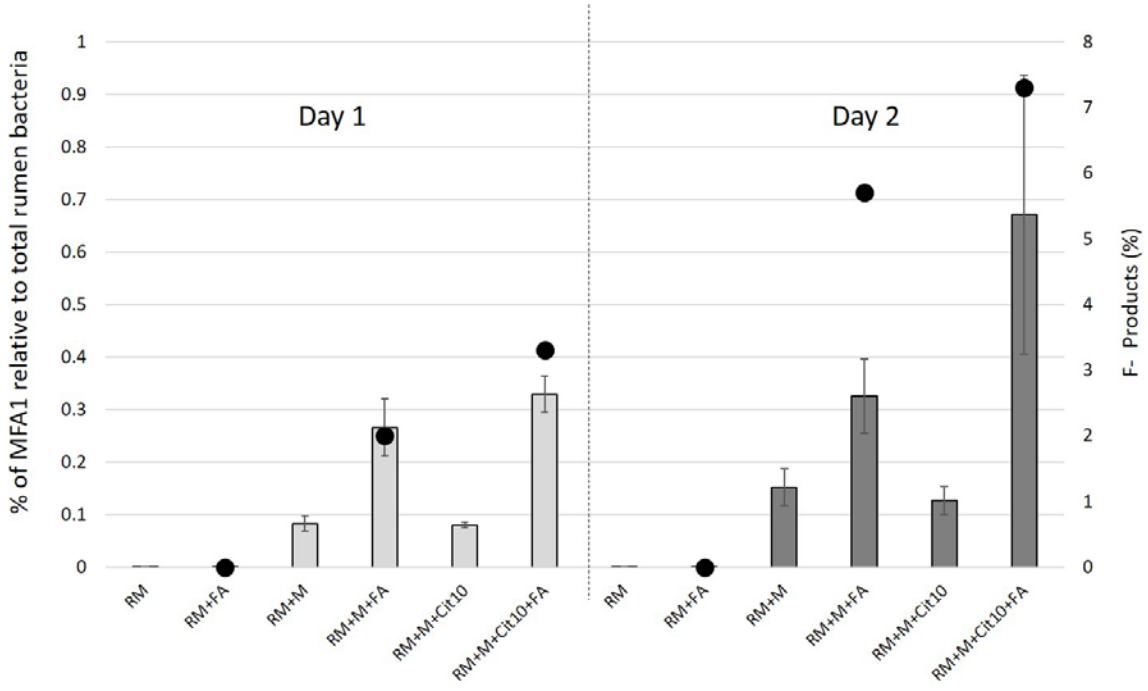


Fig. 32. The percentages of strain MFA1 relative to rumen total bacteria (gray bars) and fluoride production (black dots, %) during *in vitro* fermentation.

* Weak gray bars indicate 24 hours cultured samples and dark gray bars are 43 hours. Line bars show standard error.

† RM, Rumen mixed bacteria; M, strain MFA1; Cit10, Sodium citrate (10 mM); FA, Fluoroacetate (10 mM).

Table 20. The SCFA profiles at 43 hours in Ankom rumen fermentation system [RM, Rumen mixed bacteria; M, strain MFA1; FA, Fluoroacetate (10 mM); and Cit; Sodium citrate (10 mM)].

Parameters (Increased)	RM	RM+FA	RM+M	RM+M+FA	RM+M+Cit	RM+M+Cit+FA	SEM[1]
Total VFA (mM)	39.3bc	26.5a	34.4ab	26.9a	46.0c	38.5bc	2.7
Acetate (Molar %)	54.4b	42.4a	49.9ab	43.8a	66.1c	66.9c	2.7
Propionate (Molar %)	11.3b	13.4c	12.3bc	12.8c	9.1a	8.6a	0.51
n-Butyrate (Molar %)	24.2ab	32.2c	27.2bc	31.0c	18.6a	19.0a	1.9
n-Valerate (Molar %)	5.3b	6.7c	5.4b	6.7c	3.0a	2.9a	0.32
Caproic acid (Molar %)	-0.1	-0.3	-0.2	-0.3	-0.3	-0.4	0.07
Acetate : Propionate	4.8b	3.2a	4.0ab	3.4ab	7.2c	7.8c	0.52

* SEM: Standard error of the mean

† Means within a row with different superscripts significantly differ ($P < 0.05$)

9.4.2.4 Effect of arginine and citrate on strain MFA1 grown with mixed rumen bacteria

The fourth trial was performed to evaluate the effect of arginine and citrate (AC) supplements together on growth and FA degradation of strain MFA1 in the *in vitro* rumen fermentation. The percentages of strain MFA1 relative to total rumen bacteria were not changed by AC supplement itself in the absence of FA (Fig. 33). However, marked increases in the percentage of strain MFA1, occurred with AC supplement in the presence of FA (6.1 and 13.7 fold increases at Day 1 and Day 2 cultures respectively; Fig. 33). FA degradation showed a positive correlation with the percentage of strain MFA1 in the culture. Fluoride production was increased two-fold (24 *vs.* 51 %) on day 1 and three-fold (31 *vs.* 98 %) on day 2 by adding AC supplements (Fig. 32). Total VFA concentration and molar proportion of acetate was increased by adding AC supplements while that of propionate tended to decrease (Table 22).

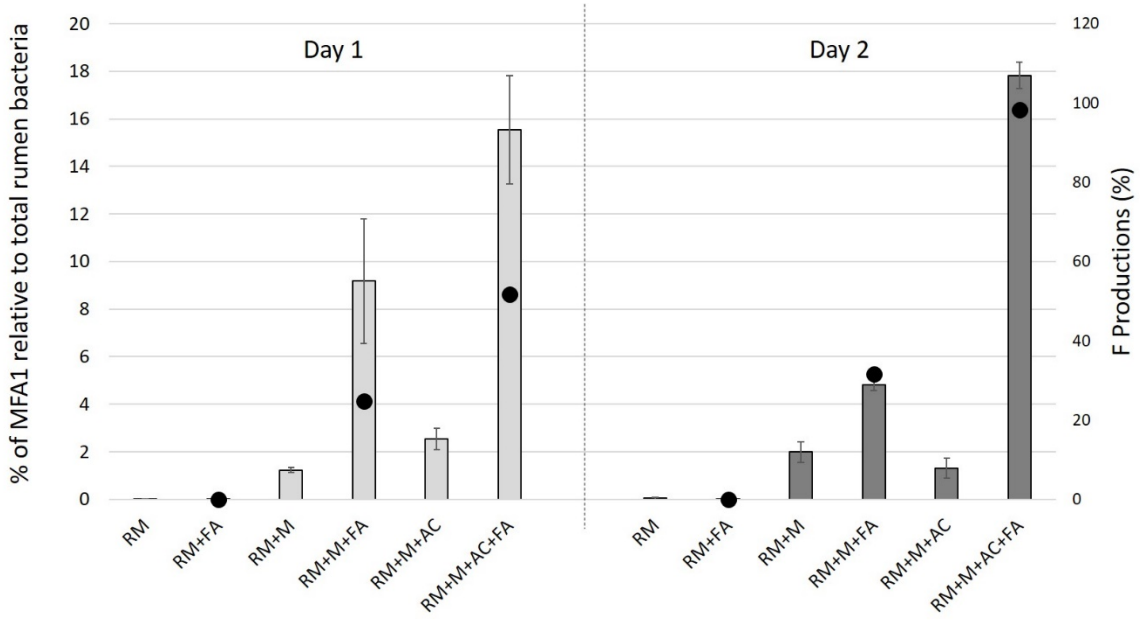


Fig. 33. The percentages of strain MFA1 relative to rumen total bacteria (gray bars) and fluoride production (black dots, %) during in vitro fermentation.

*Weak gray bars indicate 24 hours cultured samples and dark gray bars are 43 hours. Line bars show standard error.

† RM, Rumen mixed bacteria; M, strain MFA1; FA, Fluoroacetate (10 mM); and AC, 10 mM Arginine and 5 mM sodium citrate.

Table 21. The SCFA profiles at 43 hours in Ankom rumen fermentation system (RM; Rumen mixed bacteria, M; strain MFA1, FA; Fluoroacetate (10 mM), and AC; 10 mM Arginine and 5 mM Sodium citrate).

Parameters	RM	RM+FA	RM+M	RM+M+FA	RM+M+AC	RM+M+AC+FA	SEM
Increased Total VFA (mM)	21.8a	22.9a	27.0ab	22.6a	28.3ab	31.5b	2.5
Acetate (Molar %)	77.6a	78.3ab	77.2a	80.4b	79.7ab	85.1b	0.89
Propionate (Molar %)	11.7b	12.3b	12.8b	12.6b	7.5a	11.1b	0.61
n-Butyrate (Molar %)	8.8c	9.8c	8.8c	7.0b	13.2d	4.7a	0.54
n-Valerate (Molar %)	0.6a	0.6a	0.7a	1.0a	0.4a	0.6a	0.57
Caproic acid (Molar %)	0.2c	-0.9a	-0.5b	-0.8ab	-0.8ab	-0.8ab	0.09
Acetate : Propionate	6.6a	6.4a	6.0a	6.4a	10.7b	7.6a	0.5

* SEM[1]: Standard error of the mean

† Means within a row with different superscripts significantly differ (P < 0.05)

9.4.2.5 Persistence of strain MFA1 with or without FA grown with mixed rumen bacteria

The fifth trial was performed to evaluate the ability of strain MFA1 to persist with or without FA in an *in vitro* mixed rumen fermentation during 8 days of sub-culturing. The concentrations of total VFA and molar proportion of butyrate were increased by sub-culturing times but that of propionate was decreased (data not shown). The percentage of strain MFA1 relative to total rumen bacteria was decreased in the absence of FA from ~ 0.5 % to a stable level of ~ 0.1 % after one week of sub-culturing (Fig. 34). Conversely, the percentages of strain MFA1 reached a stable level of about 1.5-1.8 % in the presence of FA (1.5 ~ 1.8 %). The FA degradation increased slightly from 16 % to 24 % as the sub-culturing progressed from day 2 to 8 (Fig. 34).

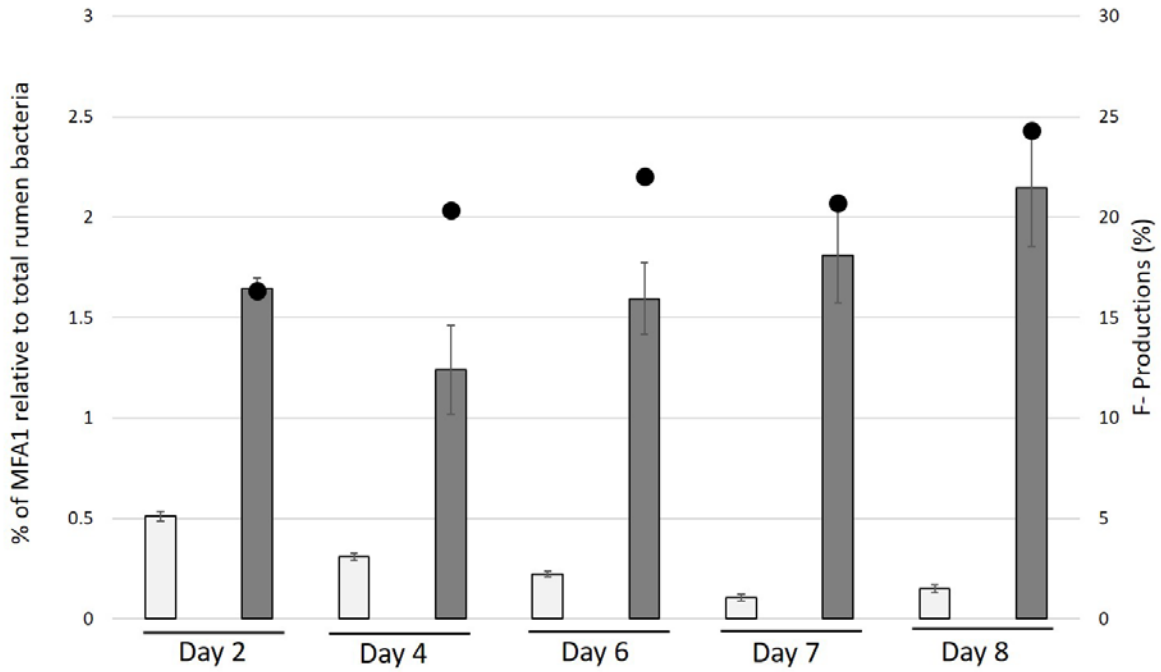


Fig. 34. The percentages of strain MFA1 relative to rumen total bacteria (bars) and fluoride production (black dots, %) during *in vitro* fermentation.

* Weak bars indicate the percentages of strain MFA1 in absence of FA and dark bars show that of strain MFA1 in presence of FA. Line bars show standard error.
† RM, Rumen mixed bacteria; M, strain MFA1; FA; Fluoroacetate (10 mM).