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This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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This work is the result of my own investigations, except where otherwise stated. Other sources are acknowledged (e.g. by footnotes giving explicit references).
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Effects of Echium oil on the rumen microbial ecosystem and ruminal lipid metabolism.

Hannah Elizabeth Vallin

Abstract

It is widely known that ruminant produce such as meat and milk are a substantial part of the westernised diet. However, red meat has a high content of saturated fatty acids (SFA's) which are detrimental for human health, and a lower content of the health beneficial polyunsaturated fatty acids (PUFA's). This is due to the lipid metabolism pathways that occur within the rumen, including the biohydrogenation pathway converting ingested PUFA's into SFA's. The rumen ecosystem is host to a diverse microbial community, of which rumen bacteria are mainly responsible for effective biohydrogenation. It is widely reported that by manipulating rumen diets can influence the presence of rumen bacteria, and thus affect lipid metabolism. Current research shows fish oil supplementation to be the most effective oil supplement to manipulate the lipid metabolism within the rumen and successfully reduce the accumulation of SFA's. Nonetheless the use of fish oil is not a sustainable method to rely upon in the future. The aim of this study was to assess the effect of supplementing echium oil upon the rumen microbial ecosystem, and the fatty acid content following lipolysis and biohydrogenation manipulation. 6 Freisian x Holstein steers were fed grass silage, grass silage and flax oil (3.0% linseed oil/kg silage DMI), or grass silage and echium oil (3.0% Echium oil/kg silage DMI), in a three period Latin square design. Fatty acid analysis was carried out on rumen samples and microbial diversity was determined analysing 16S rDNA, via 454 pyrosequencing. Results surprisingly indicated that echium oil supplementation enhances the biohydrogenation process producing more 18:0, but has distinctively increased the content of PUFA's and CLA intermediates (18:3n-3, 18:2n-6, 18:1 *trans*-11, cis9 *trans*-11). It has also highlighted a unique effect that suggests 18:4 n-3 is in fact readily converted to 18:3 n-3 before entering the biohydrogenation pathway. This change in lipid metabolism upon supplementation of echium oil correlates to distinctive changes in the rumen bacteria. There was a diverse number of rumen bacteria species identified down to a phylum and class taxonomic level. However, Firmicutes and Actinobacteria were the dominant phyla present within the rumen on echium supplementation, suggesting they are key bacterial species that play a major role in biohydrogenation.

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Effects of Echium oil on the rumen microbial ecosystem and ruminal lipid metabolism.

Dissertation submitted in part candidature for the Degree of M.Sc., Institute of Biological, Environmental and Rural Sciences, University of Wales, Aberystwyth.

By Hannah Elizabeth Vallin

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1. Introduction

1.1 General Introduction

Ruminant products, such as meat and milk, are a substantial part of the westernised diet, and their global demand continues to increase due to a progressing rise in population levels. Over the next 40 years, growth of the global population size is predicted to increase to over 9 billion, increasing pressure on the consumption and economic value of food stocks particularly within low and middle income countries (Foresight, 2011). Consumption of ruminant products, particularly meat, will increase per capita worldwide within developed and developing countries (Nishida, *et al*., 2009). The concern is not only the sustainability of food production but additionally detrimental health issues with respect to the intake of livestock products and the role of dietary fats in human nutrition (Nishida, *et al*., 2009; Jakobsen *et al*., 2006).

It is widely reported that essential fatty acids (EFA) in our diets are crucial for human health and development (Lee *et* al., 2011; Nishida *et* al., 2009; Wood *et al*., 2003) of which omega-3 and omega-6 fatty acids are the main EFA's required for growth, neurone development and influencing eicosanoid functions. These polyunsaturated fatty acids (PUFAs) cannot be synthesised via the human body, therefore have to be obtained via our food intake (Nishida, *et al*., 2009; Riediger *et al*.,2009; Jakobsen *et al*., 2006).

Unlike saturated fatty acids (SFAs) which contain a chain of carbons joined by a single bond, EFAs exist with a carbon-carbon double bond as mono or PUFA depending if one or more double bonds are present respectively. The presence of this double bond prevents rotation of carbon atoms within the chain and this arrangement allows two forms of configurational isomers to exist, *cis* and *trans* isomers. The difference between *cis* and *trans* isomers is dependent upon the location of the hydrogen atom and the geometry of the double bond. *Cis* isomers have hydrogen atoms located on the same side of the double bond, whereas in the *trans* configuration hydrogen atoms are located opposite either side of the double bond.

Natural foods contain saturated and unsaturated fats, however, ruminant meat and dairy products have a high content of saturated fatty acids (SFA) and are low in PUFA - concentrations (Huws *et al*.,2011; Lourenco *et al*., 2010; Scollan *et al*., 2006) due to

bacterial lipolysis and subsequent biohydrogenation of forage PUFA. Trans fatty acids (TFAs) occur in small quantities of ruminant produce due to bacterial processes within the rumen that synthesise fatty acids, additionally they exist due to industrial partial hydrogenation of solidifying vegetable oils (Jakobsen *et al*., 2006).

1.2 Fatty acids and human health implications

Increasing the intake of SFA's and TFAs are detrimental for the consumer's health due to their ability to increase low-density lipoprotein (LDL) cholesterol, raising the likelihood of Coronary Heart Disease (CHD), as well as hindering the body's natural use of omega fats (Lourenco *et al.*,2010; Kim *et al.*, 2009; Or-Rashid *et al*., 2009; Jakobsen *et al*.,2006, 2008).

Beef contains 40% of total fatty acids as SFA's, 50% monosaturates, and only 10% as PUFA including a variety of omega 3 & 6 fatty acids (Scollan *et al.*, 2006). It has been suggested by the World Health Organisation (WHO) that in order to reduce health risks posed by CHD, total fatty acid intake for the whole diet should comprise <0.10% of SFAs, <4% of PUFA's and <1% of TFAs of the total energy intake (WHO 2003). Over the last decade there has been increasing research into the association of SFA, TFA with CHD (Lourenco *et al*., 2010).

Increasing dietary cholesterol levels via high intake of SFA and low intake of PUFA's, have subsequent effects on rising blood cholesterol levels, causing arterial lesions, thus increasing the risk of CHD (Jackobsen *et al*., 2006). Accumulation of fatty deposits (atherosclerosis) causes swelling in artery walls and reduces blood and oxygen flow to the heart. Over time this progressively causes health deterioration resulting in various circulatory complications including angina, thrombosis, arrhythmia, endothelial dysfunction, and a worst case scenario of myocardial infarction (Willett, 2012). Currently CHD is one of the main causes of death in the UK, with over 94,000 deaths per year, and an estimated 2.6 million people suffering from CHD symptoms such as angina (Web reference 1. NHS,2010). It is increasingly important to alter dietary requirements to prevent the likelihood of CHD. By understanding ruminal process and developing successful ways to manipulate ruminal lipid metabolism, this will result in ruminant products which are reduced in SFA. Additionally increasing the consumption of omega-3

fatty acids will enhance health benefits by reducing the risk of heart related problems (Hu *et al*., 2002).

In contrast conjugated linoleic acids (CLA's) are an example of unique fatty acid microcomponents that have beneficial nutritional effects. CLA's are geometric and positional isomers of stearic acid, the two most commonly represented isomers are cis-9, trans-11 CLA (c9t11) and trans-10, cis-12 (t10c12) CLA. Both isomers occur naturally in ruminant derived foods, due to the process of lipolysis and biohydrogenation which metabolise PUFA. CLA isomers are produced in small quantities as intermediate fatty acids during the biohydrogenation of LA/LNA (Raes *et al*., 2004; Harfoot *et al*., 1988). Additionally CLAs can be synthesised in the mammary gland. The specialised delta 9 desaturase enzyme present in the mammary gland converts trans-11 (18:1) (a result of the biohydrogenation of linoleic and linolenic acid) into cis-9, trans-11 CLA. Whilst the mammary gland can produce up to 80% of CLA content within milk produce, the rumen is still the location where substrates for mammary CLA synthesis originates from, due to ruminal biohydrogenation (Muller *et al*., 2004).

CLA's have been demonstrated to inhibit carcinogenesis and more recently indicated to reduce body fat accumulation and milk fat synthesis. Pariza *et al* (1979, 1985, 1987) first discussed CLA's as a 'functional food' which are defined as 'ingested foods that may provide a health benefit beyond the traditional nutrients it contains' (NRC, 1996). The intermediate level of biohydrogenation of linoleic acid, produces CLA, therefore the level of CLA in food sources is dependent upon the incomplete biohydrogenation of UFA's, thus reducing the level of SFAs in ruminant meat (see section 2.4). The overall content of CLA's in ruminant produce is largely influenced by dietary factors; this includes lipid supplements from plant oils, and dietary supplements that alter the population of ruminal bacteria. It has been suggested that conjugated trienes may also have health promoting aspects like CLA's, improving immune system response and contain anticarcinogen factors (Lourenco *et al*., 2010; Tsuzuki *et al*., 2004).

Over the last 100 years, intake of omega-3 and 6 EFAs has decreased greatly. Linoleic acid (18:2n-6), α- linoleic acid (18:3n-3), oleic acid (18:1n-9) and marine *n*-3 fatty acids (20:5n-3, 22:6n-3) are the main naturally occurring UFAs from food sources such as vegetable and fish oils. However, the intake of omega-3 fatty acids such as α- linolenic acid has decreased in the westernised diet due to increased consumption of vegetable oils

which are rich in omega-6 EFAs (Calder *et al*., 2009). The omega-3 fatty acid α- linolenic is the precursor compound of the longer chain fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These specific acids have been associated directly with the reduced risk of CHD by associating with phospholipid membranes and thus decreasing plasma cholesterol (Lee *et al.*, 2011; Lourenco *et al*., 2010). The current ratio of omega-6 and omega-3 EFAs in the human diet is 15-20:1, unlike the historical ratio of 1-2:1. (Simopulous, 1999). Although omega-6 is an EFA, it is mostly beneficial to human health and development when consumed at a optimum balanced level with omega-3 fatty acids (Simopulous, 2002). Excessive increases in omega-6 and TFAs as seen today, are the cause for increasing the pathogenesis of diseases such as CHD and prothrombotic disorders (Simopulous, 1999, 2002).

As is well known, and extensively reviewed, gradual dietary changes over the years has resulted in the increase of CHD. Increased research is now required in order to determine how beneficial fatty acids can be increased in the diet in a natural and sustainable way (Lourenco *et al*., 2010; Riediger *et al*., 2009; Kim *et al*.,2009, 2008; Jakobsen *et al*.,2008). In relation to ruminant products alterations in the total fatty acid content is achieved via modifying fatty acids during the final processing of meat and milk products, or by manipulating microbiota changes within the rumen via changes to the rumen diet, the latter of which has proved very difficult to achieve. (Lourenco *et al*., 2010; Kim *et al.*,2009, 2008). Microorganisms within the rumen are highly receptive to alterations in the hosts genetic and physiological conditions, as well as environmental and geographic factors (Wu *et al*., 2012). However, diet adjustment is an option to enhance and alter the composition of fatty acids in the final product. Understanding the relationship between the main biological processes, lipid transformation and the microorganisms present within the rumen will aid improvement of the nutritional value of ruminant derived foods (Or-Rashid *et al.*, 2009). This will additionally promote standards and meet regulations as suggested by the Food Standards Agency, and The Scientific Advisory Committee on Nutrition, in relation to the maximum average intake of TFAs (<2%) (FSA 2008). Altering the diets of ruminants can also have further influential effects towards reducing methane and nitrogenous gas emissions, (Lurenco *et al.*,2010).

2. The rumen

2.1 The anatomy of the rumen stomach

Ruminants have evolved a complex specialised digestive system, harbouring microorganisms to aid the digestion and fermentation of cellulose (Dijkstra *et al*., 2005). The evolutionary adaption of their digestive system has led to nutritional ecologists classifying ruminants into 3 feeding categories based upon the pioneering work of Hofman (1973); browsers (concentrate feeders), grazers and mixed or intermediate feeders (Clauss *et* al., 2003; Robbins *et al*., 1995; Hofman, 1973, 1989). The anatomy of the digestive system comprises of 4 compartments; the rumen, reticulum, omasum and abomasum (Blach, 1959). The digestive system has become a large area of investigation as it is relatively accessible for artificial introductions for the purpose of experimental treatments and for sampling methods. The anatomical and physiological adaption's of the rumens polygastric digestive system are crucial for the combination of consuming large quantities of forage, and the need to maintain a concentrated population of ruminal microbes, in order to obtain their nutritional needs from forage and high-fibre food sources.

Forage material mixed with saliva commonly called the bolus, is first deposited via the oesophagus into the anterior section of the rumen which connects directly to the reticulum joined by a reticulo-rumen fold of tissue. The rumen, the largest component of the stomach is a large fermentation chamber responsible for the storage, and physical mixing with vast micro-organisms including bacteria, fungi, protozoa and archaea. The reticulum with its honeycomb appearance allows the free movement of ingesta between the rumen and reticulum, and extends into the omasum. The reticulum can act as an additional chamber for fermentation, removal of foreign material, and is responsible for the regurgitation of ingesta whereby partially digested food, the cud is re-chewed. After this process the cud is finally passed through the forestomach into the omasum. The omasum absorbs fluids and nutrients via laminae folds on the surface, and removes volatile fatty acids. The final compartment, the abomasum, also referred to as the true stomach, is where gastric acids and enzymes break down the organic matter into smaller components that can be absorbed via the walls of the intestine (Hobson, 1988).

2.2 Physical environment of the rumen

Rumen microbial metabolism functions within anaerobic conditions; allowing the transformation of forage into microbial protein and volatile fatty acids further used as an energy source (Parish *et al*., 2009). Within the rumen the anaerobic environment has a pH 6.5-6.8 and maintains a temperature ~39˚C. Factors that can affect the pH include the diet, frequency of intake content consumed (Harfoot *et al*., 1988). In contrast the omasum and abomasums have a relatively low pH 2-4 due to the presence of gastric acids.

2.3 Rumen microbes

The microbial community within the rumen are responsible for producing cellulases, required to breakdown fibrous cellulose to produce volatile fatty acids that are consumed for energy, and synthesising required vitamins that can be obtained from organic matter. The main microbial taxonomic groups present within the rumen that combine to break down organic matter comprises of, fungi bacteria, archaea and protozoa, (primarily ciliates) (Edwards *et al*., 2008). Since the early pioneering work, it is known that the most abundant taxonomic group is bacteria with 10^{11} cells/ml present within the rumen (Hobson, 1988). However advances in cultivation techniques now indicate that the number and diversity of ruminal bacterial species present has been highly underestimated. Limitations in previous cultivation methods provide an explanation as to why only 11% of bacteria present have successfully been identified and cultivated (Edwards *et al*., 2008). 16S rDNA based analysis currently suggests that approximately 300-400 different species of bacteria are in fact present within the rumen (Edwards *et al*., 2008). The second most abundant group are rumen protozoa with 10^{4-5} cells/ml (Hobson, 1988). Regardless of the fact that densities of protozoa present are roughly half of that of bacteria, protozoa occupy more space within the rumen as they are much larger than bacteria (bacteria dimension ~50µm, protozoa ~5-250 µm). Fungi and archaea represent 10^4 cells/ml within the rumen (Hobson, 1988).

Shifts in microbial communities can be a consequence of 1) decreased pH, thus influencing the biohydrogenation pathway and content of CLA's, 2) alterations within the rumen environment, associated with feeding high-concentrate, low fibre diets that further increases the content of major CLA isomers such as trans-10-octadecenoic acid present in milk fat, 3) age of the animal, and 4) animal husbandry variations throughout the season.

As demonstrated in Lee *et al* (2005), altering rumen diets between forage, and forage with concentrate feed has a greater impact on the diversity of bacterial communities.

Until recently it was thought that ruminal processes were undertaken by only a small number of known bacterial species (Krause *et al*., 1996). Since pioneering work, new technologies and advances in molecular biology have increased the knowledge of the as yet unculturable rumen bacteria. Increasing research on the analysis of 16S rDNA has been assessed to determine the diversity of the rumen microbial ecosystem (Koike *et* al., 2001; Lin *et al*., 1994; Whiteford *et* al., 1998; Wood *et al*., 1998). 16S rDNA has been widely used for pyhlogentic studies, as it is a large enough size to determine differences for taxonomical purposes. Common techniques for 16S rDNA analysis include 1) restriction fragment length polymorphism, 2) hybridisation studies, and 3) denaturing gradient electrophoresis (Huws *et al*., 2011; Edwards *et al*, 2004; Kobayashi *et al*., 2000; Tajima *et al*., 1999).

As previously mentioned based on the analysis of 16S rDNA from combined libraries, only 11% of operation taxonomical units (OTU's), represent known culturable species, therefore the remaining 89% is, as yet unculturable (Wu *et al*., 2012; Edwards *et al*., 2004). A great deal of research is carried out on the function and microbial processes within ruminant's digestive system. Increasing the knowledge and understanding of how these systems work and how they are influenced via feeding regimes, will assist in managing grazing systems to provide the most appropriate nutritional input. This is important for livestock producers to care for individual ruminant species, as well as improving agriculture sustainability in order to understand and effectively utilise grazing systems (Parish *et al*., 2009).

2.4 Lipolysis, biohydrogenation and chain elongation

Ruminant's diets are rich in 18:3n-3 (Alpha (α) Linolenic PUFA), however, final milk and meat produce contain high amounts of SFAs due to the rumen microbes and bacteria which transform PUFAs via processes such as lipolysis and biohydrogenation (Lourenco *et al.*,2010).

2.4.1 Lipolysis.

Until recently, it was understood that the two main bacterial species present within ruminal ecosystem responsible for lipolysis and biohydrogenation were *Anaerovibrio lipolytica* and *Butyrivibrio* species. The lipase activity of these two species is specific to the substrates present from within the feed. *A. Lipolytica* hydrolyses mainly triacylglycerol substrates from concentrate feeds; however, the hydrolysis of galacto- and phospholipids in grazing ruminants is predominately by *Butyrivibrio fibrisolvens* species. The difference in microbial communities is evident as *A. Lipolytica* is unable to hydrolyse galacto- and phospholipids, and likewise *Butyrivibrio* unable to hydrolyse triacylglycerol (Henderson, 1971). Of the vast microorganisms present within the rumen, we currently believe that bacterial species are the most important in terms of lipolytic activity (Buccioni *et al*., 2012). There is limited evidence suggesting ruminal protozoa have a small contribution to the hydrolysis of esterified lipids compared to bacteria, but due to contaminating bacteria in all protozoa preparations it is difficult to interpret this data. It is also suggested fungi have no lipase activity during this pathway (Buccioni *et al*., 2012; Lourenco *et al*., 2010). It is important for the bacterial species to fully hydrolyse lipids to provide a free carboxyl group allowing the next stage of biohydrogenation to take place (Kim *et al*., 2009).

The process of lipolysis involves the hydrolysis of triglycerides, glycolipids and phospholipids into free fatty acids. Rumen bacteria are responsible for extracellular hydrolysis of the ester linkages of lipids, resulting in unsaturated fats that can undergo biohydrogenation. Ruminants receiving lipid supplements and cereals have a high content of triacylglycerol lipids that are predominately hydrolysed via microbial lipase. Whereas in grazing ruminants, plant tissues are high in galacto- and phospholipase content; of which it is suggested these lipases that remain active within the rumen in fact contribute to ruminal lipolysis. Lee *et al*.(2002), and Van Ranst *et al* (2009) observed that prolonging the incubation time of plant tissue within the rumen, resulted in plant lipases having an influencing a role in the ruminal lipolysis processes. Greater levels of free fatty acids and lower concentrations of polar lipids occurred after 6 and 8 hours of incubating plant lipases. However further research is needed to determine the exact level of plant lipases activity with respect to microbial lipase during lipolysis (Lourenco *et al*., 2010). The recent information on lipid metabolism is based upon the microbial molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP). (Further advances in microbial molecular techniques will

be discussed in aims and objectives.) It is evident that further meta-analysis is needed to determine the remaining phylogenetic diversity of rumen bacteria. As it is currently unknown if as yet unculturable bacteria additionally too have a leading role in lipolysis and biohydrogenation pathways (Kim *et al*., 2011 a,b; Huws *et* al., 2010).

2.4.2 Biohydrogenation

Following lipolysis, biohydrogenation occurs in the rumen by anaerobic microorganisms. It involves the isomerisation and hydrogenation via rumen bacteria, to solidify unsaturated fats such as linoleic and linolenic acid (Kim *et al.,* 2009; Bauman *et al.,* 2003; Wilde *et al.,* 1966). Throughout the process there are multiple steps producing conjugated acids and different trans-fatty acids (such as vaccenic acid, trans-11-18:1), resulting in stearic acid (18:0) as the end product (Kim *et al*., 2009). Similar to the lipolysis pathway, feeding type has an effect upon the biohydrogenation process within the rumen (Fig.1).

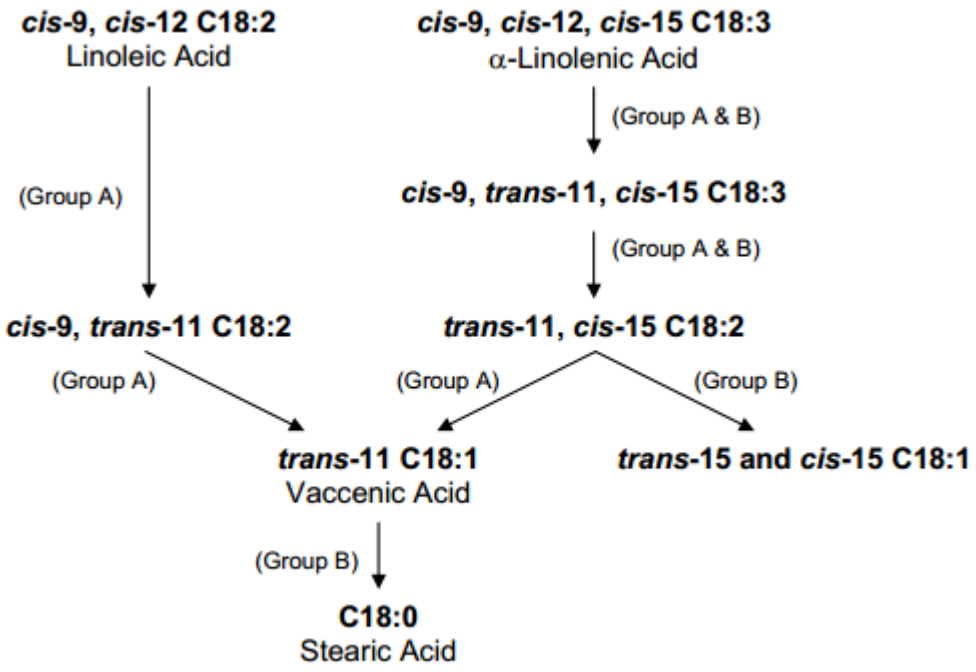


Fig. 1 Previously known Biohydrogenation pathway of linoleic and linolenic acid, courtesy of Bauman *et al, (*2003).

Previous work classified the hydrogenation of PUFA to stearic acid being dependent up on the two known culturable groups of bacteria (A; *B. fibrisolvens* & B; *Fusocillus* species), based upon metabolic pathways and end product of biohydrogenation (Kemp & Lander, 1984). Complete biohydrogenation occurs when bacteria from both groups are present. Individually group A produces the penultimate stage product (Trans 11- 18:1 vaccenic acid) and group B bacteria are additionally needed to complete the transformation to the final end product (18:0 stearic acid) as demonstrated by the simplistic pathway of LA and LNA (Fig.1) (Kim *et al.,* 2009, 2008; Bauman *et al.,* 2003; Harfoot and Hazlewood 1997).

Ruminants fed lipid supplements contain much linoleic acid (LA) (cis-9,cis-12-18:2) in their diet. LA mainly in the form of triacylglycerol, undergoes isomerisation reaction of the cis- 12 double carbon bond for the formation of conjugated linoleic acid (CLA) (cis-9, trans-11-18:2). This is then converted into vaccenic acid (trans-11-18:1) via a reduction reaction, and finally converted to C18:0 stearic acid.

Grazing ruminants, on the other hand have a high content of α-linolenic acid (LNA) (cis-9,cis-12,cis-15-18:3) from their diets (Raes *et al*., 2004). LNA is the predominant PUFA comprising up to 60% of total dietary fatty acids. It is now know that the biohydrogenation pathway of LNA is slightly more complex due to the presence of three double bonds of LNA and further intermediate levels not previously recognised. The initial isomerisation of LNA produces conjugated triene (cis-9,trans-11,cis-15-18:3) and additional intermediates that are then reduced to transform vaccenic and stearic acid (Fig 2.)

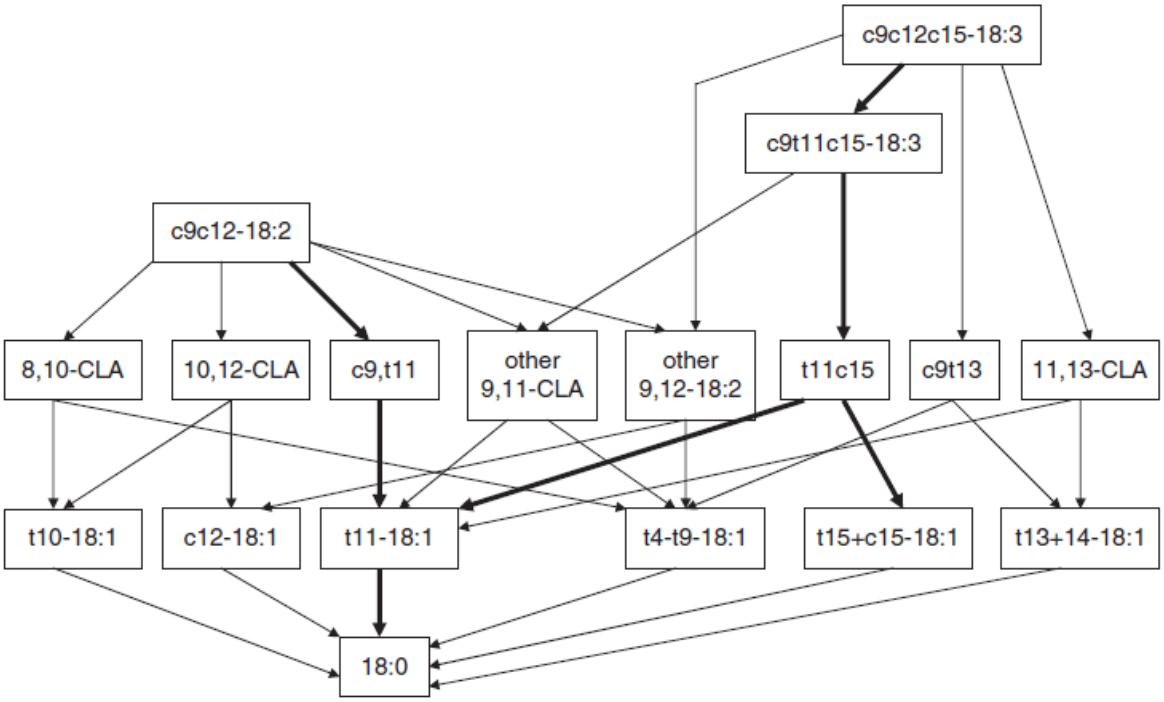


Fig. 2 Biohydrogenation process in the rumen, LA-c9c12-18:2; LNA-c9c12c15-18:3; VA-t11-18:1. CLA-conjugated linoleci acid; LA- linoleic acid; LNA- linolenic acid; VA-vaccenic acid. Courtsey of Lourenco *et al* (2010)

Recent multivariate statistical analysis of DGGE and terminal restriction fragments bands (TRFs), have highlighted that as yet uncultured bacterial species belonging to numerous taxa probably contribute to the pathways of biohydrogenation. Additionally the pathways are more complex than previously known (Huws *et al*., 2011; Boeckhart *et al*., 2008). Huws *et al* (2011) supplemented fish oil at varying concentrations in order to determine correlations between the amount of PUFA and concentrations of *Butyrivibrio proteoclasticus*-related bacteria species. This study has concluded as yet uncultured bacteria of the genera include *Prevotella, Anaerovoax, Ruminococcaceae, Bacteriodales,* and *Clostridiales* probably play a role in biohydrogenation. Boeckhart *et al* (2008) reports additional genera including *Lachnospiraceae, Incertae sedis* have been documented as yet uncultured following the supplementation of marine algae (Boeckhart *et al*., 2008). Comparing Boeckhart *et al* (2008) and Huws *et al* (2011) it would appear there is some

overlap in bacterial species found from both studies based on the genus members, as *Lachnospiraceae* is a subfamily of the order *Clostridiales*.

This recent information provides baseline data to suggest that the diversity of bacterial contribution is indeed more varied than the originally postulated group A and B bacterial species. Whilst it is suggested that these as yet unculturable species have a significant role in the pathway of biohydrogenation the difficulties to fully conclude this assumption is the problematic matter of not being able to cultivate them outside of the rumen environment. In order to enhance our knowledge and determine feasible methods to enhance the beneficial fatty acid content, via inhibiting complete biohydrogenation, further investigations are needed to assess the metabolic requirements and physical conditions needed for bacteria growth. Such research is increasing with advances from the use of TRFLP and DGGE methods, to developing the use of functional metagenomics, such as next generation sequencing techniques allowing more comprehensive studies (Huws *et al*., 2011).

2.4.3 Alternative chain elongation pathway

As previously mentioned lipolysis results in the production of free FA's, however biohydrogenation is not the only pathway that is followed after lipolysis. A small percentage of free FA's go through an alternative metabolism pathway of chain elongation. The known pathway of chain elongation (Fig.3) has a rate limiting stage at the beginning, namely the conversion of 18:3n-3 to 18:4n-3, therefore this makes the conversion to DHA inefficient. However by supplementing plant oils such as the use of echium oil that is already rich in 18:4n-3, this may bypass the rate limiting stage and increase the synthesis of EPA and DHA. This pathway therefore reduces the biohydrogenation pathway in order to enhance chain elongation resulting in metabolism of health beneficial long chain fatty acids (Lourenco *et al*., 2010).

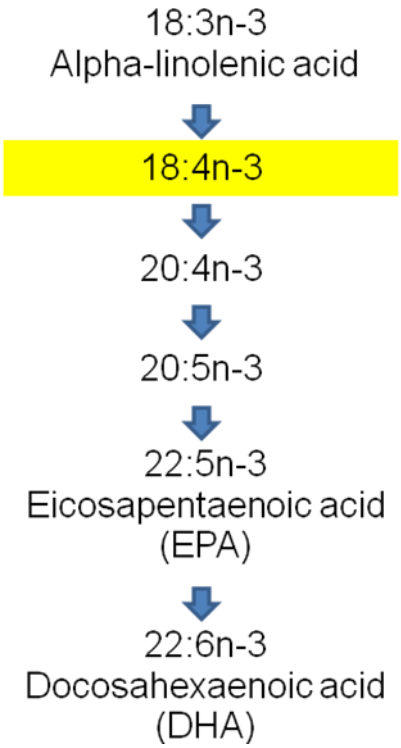


Figure 3. Chain elongation pathway. Courtesy of E.J.Kim & N.D.Scollan.

3. Examples of manipulating fatty acid content and biological pathways within the rumen.

As previously discussed multiple factors can affect the rate and efficiency lipolysis and biohydrogenation, such as pH of the rumen and fat content of diet. Research on the content of PUFA in ruminant tissues is continuously expanding using different procedures to alter lipid transformation, bacterial populations and even bypassing biohydrogenation within the rumen (Scollan *et al*., 2001). Many methods involve modifying supplement feeding of varied long chain (LC) PUFA, to enhance α-linolenic acid content and improving *de novo* synthesis within the ruminant tissues.

Kelly *et al* (1998) reports that increasing the input of linoleic acid from plant oils in the rumen diet, result in a clear dose-dependent increase in the level of CLA's within the final fatty acid composition. This has also been evident with supplementing fish oils, however, the increase in CLA's is expected to be a result of inhibitory effects of the oils on biohydrogenation, and altering the growth or specific action of rumen bacteria needed for the reduction of CLA's. *In vitro* studies containing rumen cultures with increased levels of linoleic acid altered the biohydrogenation pathway, resulting in increased levels of vaccenic acid as the end product. This indicates that linoleic acid has an inhibitory effect upon the bacteria that complete the transformation to stearic acid following complete biohydrogenation (Bauman *et al* 1999).

Many studies with lactating cows show that supplements containing greater amounts of CLA isomers caused an overall reduction in the content and yield of milk fat, 'milk fat depression' due to alterations of lipid metabolism. Alternatively common research attempts to increase the content of CLA's in the intramuscular fat of steers, particularly the c9t11 isomer via supplementing diets with high concentrates of LNA, LA or concentrates with fish oil (Raes *et al*., 2004; Lorenz *et al*., 2002; Enser *et al.,* 1999). When supplementing high source LNA concentrates, significant levels of CLA increase have been observed in intramuscular content of beef cattle. Increasing the input of LNA produces a higher content of Cis- 9 trans-11, due to an increased production of its precursor vaccenic acid (trans-11-18:1) during the biohydrogenation of LA /LNA (Raes *et al.,* 2004). However the occurrence of increased Cis- 9 trans-11 CLA when supplementing fish oil is due to an alternative reasoning. During the biohydrogenation of long chain fish oils such as EPA and DHA there is no production of trans-11-18:1. Therefore this suggests

that the increase is likely due to EPA/DHA interfering in the complete biohydrogenation pathway altering the production of intermediate acids, thus increasing Cis- 9 trans-11 (Raes *et al.,* 2004; Enser *et al*., 1999). The process by which CLA's are able to elicit an alteration on the biological pathways within the rumen may relate to the variation in quantity of supplemented CLA. Lower levels are required to prevent milk fat synthesis than that of reducing body fat synthesis. Despite the evidence for increasing CLA content in intramuscular fat, levels of c9t11 CLA in meat are generally lower when compared to that in milk. However further evidence is needed to clarify this as more investigations have looked at the effects upon milk fat, and only recent studies are investigating the effects upon meat (Bauman *et al*., 2001). It is evident that dietary alterations are an extensive area for research allowing feasible manipulation to alter lipid synthesis, for the potential importance of benefiting ruminant food products.

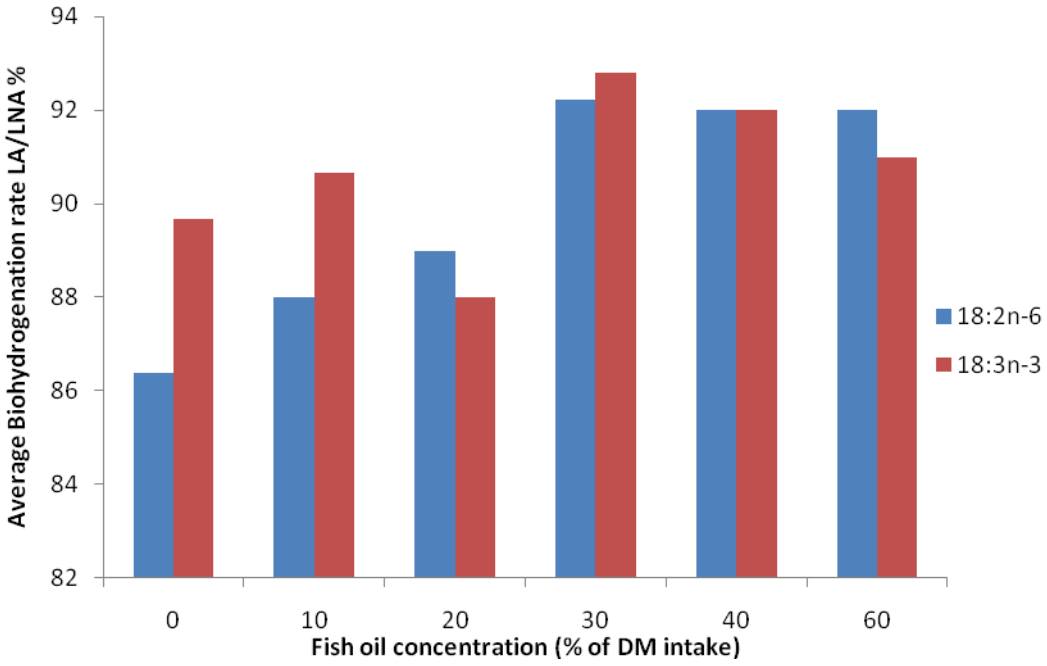
Kim (*et al* 2008*)* investigated the effects of supplementing ruminant diets with fish oil *in vivo* to determine if there was a correlation between concentration of the fish oil and ruminal bacterial content, with particular reference to the bacteria *Clostridium proteoclasticum*. Results indicated that supplementing fish oil at a concentration of 30% of dry matter (DM) intake against the control diet in steers, caused a 100% increase in the trans-11 18:1 production within the duodenum, and decreased 18:0 levels (Kim *et al*., 2008; Scollan *et al*., 2001). Fish oil contains two long chain omega-3 fatty acids, it is suggested these inhibit the complete biohydrogenation process therefore resulting in increased longer chain PUFA's. This has also been evident in numerous reports (Lee *et al*., 2008, 2005; Shingfield *et al*., 2003; AbuGhazaleh *et* al., 2002). Bauman *et al* (2003) states that *in vivo* studies, when supplementing fish oil the biohydrogenation of the longer chain PUFA's; eicosapentaenoic (EPA) acid and docosahexaenoic (DHA) acid is reduced compared to that of naturally occurring linolenic and linoleic acids, thus reducing the SFA content. Fish oil is expected to inhibit the enzyme that catalyses the final stage of biohydrogenation to produce 18:0. Bacteria within the rumen, capable of transforming α-linolenic acid and linoleic acid into 18:0 are inhibited and proliferation of bacteria is reduced resulting in an increase in the tissue content of C18:1 trans-11.

Alongside the use of supplementing fish oil, linseed oil is commonly used in many studies to increase PUFA content via eliciting changes on ruminal metabolism (Shingfield *et al*., 2011; Doreau *et al*., 2009; Scollan *et al.,* 2001). Supplementing linseed oil also increases the flow of CLA intermediates, 18:3n-3, and PUFA flow to the duodenum**.** Shingfield *et*

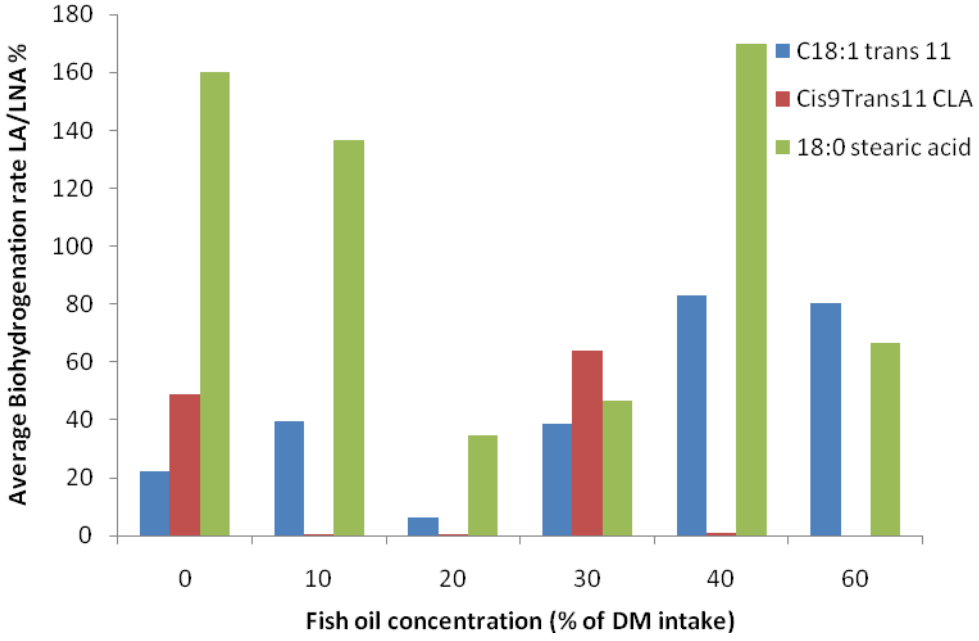
al (2011) determined that supplementing linseed oil at 30 g/kg of DM was the most effective concentrate to substantially increase the extent of 18:3n-3 flow to the duodenum. Yang *et al* (2009) not only determined that linseed oil decreases ruminal biohydrogenation reducing fatty acid flow to the duodenum, however results indicated that the ruminal microbiota were significantly lower in abundance. Linseed reduced the presence of *Butyrivibrio fibrisolvens, Ruminococcus flavefaciens* and *Fibrobacter succinogenes*. These are main bacteria classified to have a distinctive role in the biohydrogenation process, therefore decreased concentrations with the presence of linseed oil, further supports the reasoning to reduced ruminal biohydrogenation.

Advances in the use of microbial technology on 16S ribosomal ribonucleic acid (rRNA), and quantitative polymerase chain reaction, allows the main bacteria involved in the biohydrogenation process to be quantified; and further determine bacteria colony changes via gel electrophoresis, between control and oil supplement diets. An alternative review by Kim *et al* (2009) continued discussions of investigations into the dietary transformation of lipid in the rumen microbial ecosystem, concentrating on the interactions between the biological pathways and micro-organisms involved in the key processes of lipolysis and biohydrogenation. The following graphs (Fig 4.a-d) visually represent pooled data from 5 studies that assessed supplementing varying concentrations of fish oil and linseed oil, and the effects on the rate of biohydrogenation and the duodenal flow of C18 UFA's in steers fed grass silage (Shingfield *et al*., 2011; Kim *et al*., 2008; Lee *et al*., 2008, 2005; Scollan *et al*., 2001).

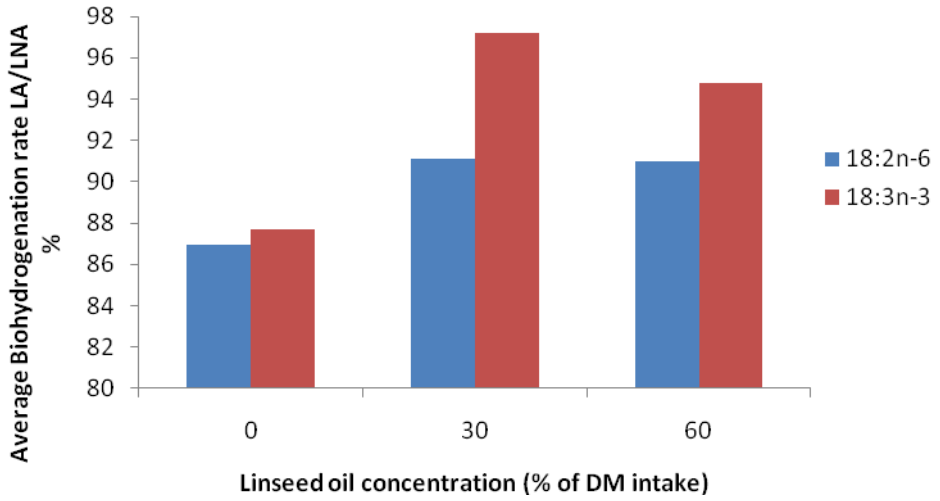
(a)


94
92
90
88
86
84
82
Average Biohydrogenation rate LA/LNA %
0
10
20
30
40
60
Fish oil concentration (% of DM intake)
18:2n-6
18:3n-3

(b)


180
160
140
120
100
80
60
40
20
0
Average Biohydrogenation rate LA/LNA %
0
10
20
30
40
60
Fish oil concentration (% of DM intake)
C18:1 trans 11
Cis9Trans11 CLA
18:0 stearic acid

(c)



(d)

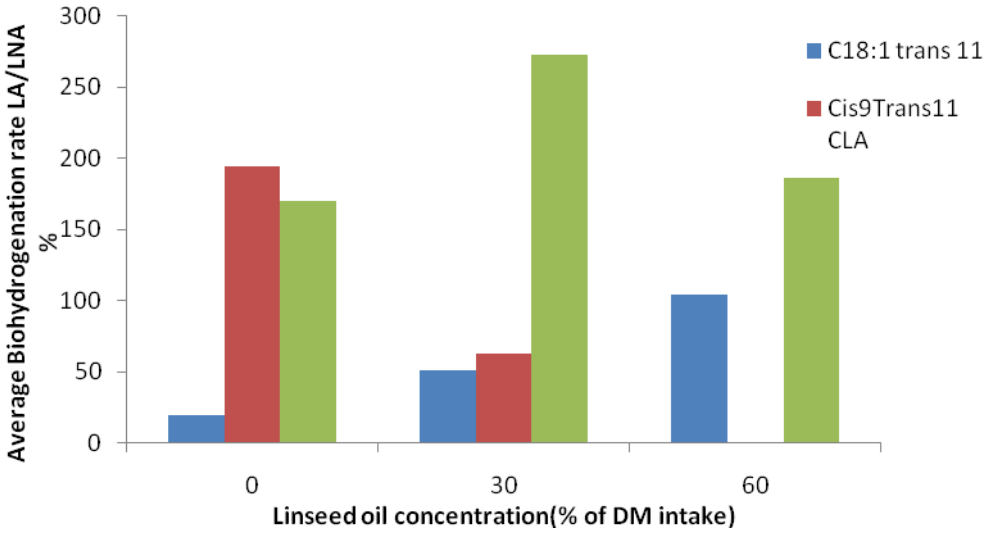


Fig.4 (a) Varying fish oil concentration and the effect upon biohydrogenation rates and the production of 182n-6 and 183n-3. (b) Varying fish oil concentrations and the effect upon the average production of C18:1 trans 11, Cis9.trans 11 CLA, 18:0. (c) Varying Linseed oil concentration and the effect upon biohydrogenation rates and the production of 182n-6 and 183n-3. (d) Varying Linseed oil concentrations and the effect upon the average production of C18:1 trans 11, Cis9.trans 11 CLA, 18:0. Average % worked out from values recorded as g/day. Statistical analysis could not be carried out on fish oil, and linseed oil concentration results as not all average biohydrogenation values were available for each concentration, thus it is only to visually represent the overall changes.

It is important to note that whilst the data was pooled form 5 studies they did not all cover supplementing the same concentrations of oils. Therefore for concentrations at 20, 40 and 60% there was only one set of data vales from three individual studies (Lee *et al*., 2008, 2005; Scollan *et al*., 2001). Whereas concentrations of 10 and 30% data was pooled from three studies (Shingfield *et al*., 2011; Kim *et al*., 2008, Lee *et al*., 2008). The graphs do successfully indicate alterations in the level of C18 UFA production dependent upon the concentration of oil supplement.

Overall there is a general increase in the production of C18:2n-6 and C18:3n-3 with increasing concentrations of fish oil (Fig. 4a). Comparing the control (0% fish oil) against supplements of fish oil at 30% is the best representative as it has the most data values for comparison. These data indicate an increase of 92-93% in production of C18:2n-6 and C18:3n-3. Thus, indicating a significant reduction in the biohydrogenation pathway. This increase in essential fatty acids indicates more intermediates are present within the pathway as it does not reach completion to produce 18:0 (Fig. 4a & b). This explanation is also supportive in graph (b). Supplementing 30% fish oil significantly reduces content of 18:0, therefore indication biohydrogenation is incomplete, whilst the production of the intermediate C18:1 increased by 39% and Cis9 Trans11 by 64%. Supplementing 60% fish oil concentration, results in the decrease of 18:0, while C18:1 increased. It is important to note that the values for the production of CLA were not used as they were negative. This may be due to reasoning that higher concentrations of fish oil increases the rate conversion of CLA, and was too rapid to record as flow through the duodenum.

Supplementing linseed oil into the diet of steers fed grass silage (Fig.4 c & d). There is a significant decrease in the rate of biohydrogenation indicated by the increase in production of C18 UFA's. There is a greater increase of C18:3n-3 of 9.5%, and 4.2% increase of C18:2n-6. When comparing linseed to fish oil it is clear that linseed is not as effective at inhibiting the complete biohydrogenation pathway. This is also clear in graph (d), this does not show any indication that increasing the concentration of linseed reduces the production of 18:0 or Cis9 Trans11 CLA, there is however an increases in the production of C18:1. Raes *et al* (2004) too concluded that supplementing linseed oil had little effect on increasing health beneficial CLAs in intramuscular fat.

In comparison it is clear from recent studies that fish oil is the most effective oil supplement to use for manipulating the biohydrogenation pathway to date (Shingfield *et*

al.,2011; Kim *et al*., 2008; Lee *et al*., 2008, 2005; Scollan *et al*., 2001). Currently there are not many studies that have been carried out with the use of marine algae or additional plant oils and their effects upon fatty acid composition in steers. Many investigations look at the effects on milk fat depression in dairy cows (Franklin *et al*., 2012). However the use of marine algae as a major lipid source has the potential to be used as a dietary strategy to incorporate higher levels of omega-3 PUFAs for manipulating the rumen microbiota and lipid metabolism (Kim *et al*., 2009). This leaves a large area for investigation open to progress in future studies.

4. Aims and Objectives

It is important to note that the prior animal experiment within this investigation was conducted as part of the EU prosafebeef project in conjunction with Hybu Cig Cymru (HCC). HCC is an industry led organisation, who undertakes research in order to promote, develop and market Welsh red meat. The aim of Meat Promotion Wales is to develop a profitable and sustainable market for Welsh red meat via cooperating with farmers, retailers, foodservice operators and relevant stakeholders. Increasing the research to improve the quality and value of Welsh red meat is vital to benefit the local farming industry for Wales, whilst increasing the health benefits for the consumers to promote a healthy balanced diet.

The original prosafebeef project had the same objective of improving long chain PUFA composition of red meat by supplementing steer diets with echium oil. Echium oil is rich in stearidonic acid (18:4 n-3) and alpha linolenic acid (Alhazza *et al.,* 2011). Within the rumen the first step for the conversion of 18:3 n-3 into 18:4 n-3, is considered to be rate limiting. Eventually this results in the production of the longer chain derivative C20n-3 PUFA. Via providing echium oil supplementation, the high content of 18:4 n-3 is thought to bypass the rate limiting conversion state, thus in theory it should increase the rate of C20n-3 production. Thus increasing longer chain PUFA's, will increase the health of the final meat product for the consumer.

However, the aims of the study were not fulfilled, in that long chain PUFA flow to the duodenum was not enhanced but rather 18:4 n-3 was converted to 18:3 n-3 which then followed the biohydrogenation pathway, resulting in enhanced biohydrogenation. This in itself is interesting as very little is known regarding 18:4 n-3 conversion to 18:3 n-3, thus warranting an investigation of any accompanying microbial changes and fatty acid content within the rumen microbial ecosystem. Therefore the aim of this experiment was to further evaluate the effect of supplementing echium plant oil within rumen diets upon lipid metabolism, fatty acid content in the rumen and ruminal bacteria present.

The microbiome environment within the rumen functions as an organised integrated system, whereby all microbial species contribute to ruminal processes such as lipolysis and biohydrogenation. While it is recognised that multiple microbial communities have a symbiotic association within the rumen, however, the processes that interacting microbiota constitute to within lipid metabolism is less understood (Wu *et al.,* 2012). This

investigation determined there is a link between microbial communities present and the overall fatty acid composition of meat quality and potentially provided clues as the which bacteria can convert 18:4 n-3 to 18:3 n-3.

Further investigations into the supplementation of plant oils and their effect upon biohydrogenation, are required to manipulate and enhance the amount of free fatty acids that progress to chain elongation as opposed to biohydrogenation. EPA and DHA longer chain fatty acids are supplemented via fish oil food sources, nevertheless this source cannot be maintained sustainably. With an ever increasing demand and exploitation of fishing stocks for consumption, a temporal change is needed to attempt to increase the beneficial fatty acids in other food sources, as unsustainable fishing cannot continue in the near future.

Increased understanding of rumen microbiota diversity and rumen ecosystem dynamics will facilitate our understanding beyond that of the known 11% of culturable bacteria (Edwards *et al*., 2008). Until recently there has been insufficient methodology for successful culturing and quantification to identify rumen microbes. Previous studies used DGGE, and TRFLP, to analyse the diversity of the rumen microorganisms. However, these methods have relatively low resolution for analysis, and this is why technical advances have increased and moved on from the use of DGGE and TRFLP (Wu *et al*., 2012). Advances have lead to the use of next generation sequencing technologies, such as that of the 454 Roche pyrosequencing that is used in this particular investigation. As ruminal manipulation studies increase to enhance the efficiency of lipid metabolism and fibre digestion, it is now known there are a greater number of transient and permanent microbial communities that have a crucial role in ruminal processes. Due to the complexity of the microbial ecosystem its study is relatively difficult. On the other hand investigations in lactating dairy cows have determined 21 prokaryotic phyla, whilst studies into that of steer cattle have not received much attention (Wu *et al*., 2012; Edwards *et al*., 2008).

This study assesses the effects of echium oil on the rumen microbiota via high throughput deoxyribonucleic acid (DNA) sequencing, in order to increase our knowledge and to understand the role that different bacteria have within the biohydrogenation process.

4.2 Hypothesis

There are a number of research questions to consider for this investigation, including;

- By supplementing echium oil, does this have any effect upon the lipid metabolism pathways?
- Are there any elicit changes on the rumen microbial ecosystem following echium oil supplementation to steer diets. And if possible can key bacterial biohydrogenators be identified?

Null hypothesis (HO) There is no relationship between supplementing echium oil and the effects upon lipid metabolism or bacterial diversity.

Alternative hypothesis (H1) There is a relationship between supplementing levels of echium oil and influencing the pathway of lipid metabolism and the diversity of bacteria within the rumen.

5. Methods

5.1 Experimental Design and Sample Preparation

The animal trail was conducted ahead of this project under an EU Prosafebeef project. Lipid data had already been obtained but not analysed. No microbial analysis had been undertaken prior to this investigation.

The preliminary experiment consisted of three dietary treatments supplemented to six Hereford x Friesian steers following a 3-period replicated Latin Square design (Table 1). The control diet consisted of Diet A: Grass silage *ad libitum* + 500 g sugarbeet pulp. Diet B: Grass silage *ad libitum* + 500 g sugarbeet pulp + 3.0% linseed oil/kg silage DMI (Flax). The echium supplemented diet consisted of: Diet C: Grass silage *ad libitum* + 500 g sugarbeet pulp + 3.0% Echium oil/kg silage DMI. Each treatment period lasted roughly 22-23 days, in which 14 days were given as an adaptation period, and rumen sampling occurred on day 22. Rumen fluid samples were obtained along with rumen solids for microbial sampling. Samples were freeze-dried and retained for microbial and fatty acid analysis. Processing of samples was followed as described in Huws *et al* (2011). The fatty acid sample preparation was carried out prior to this experiment; however the statistical analysis of fatty acid content was done during this experiment using the Duncan's multiple range test, (further discussed in the results section).

Table1. Experimental Latin square design

Period	**Steer 1**	**Steer 2**	**Steer 3**	**Steer 4**	**Steer 5**	**Steer 6**
I	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
II	Diet B	Diet C	Diet A	Diet C	Diet A	Diet B
III	Diet C	Diet A	Diet B	Diet B	Diet C	Diet A

5.2 DNA extraction

A total of 17 samples were processed for analysis as one animal became ill and had to be withdrawn from the trial. DNA was extracted via QBiogene extraction kits. The protocol was followed as suggested by the manufacturer (MP FastDNA spin kit instruction manual). DNA was quantified using an Epoch Microplate Spectrophotometer (BioTek Manual) and DNA was subsequently diluted with water to a concentration of 25 ng/µl.

5.3 16S PCR for Roche 454 pyrosequencing

Polymerase chain reaction (PCR) using V6-V8 454 adapted primers (Table.2) was carried out on all DNA samples in triplicate with an additional negative master mix for each sample. The following PCR conditions and concentrations were as followed to make up a master mix for each individual DNA sample:

- Water – 18.75µl
- Buffer – 0.5 µl
- dNTPs – 0.5 µl
- Forward Primer F968_PS – 0.5 µl
- Allocated reverse primer – 0.5 µl
- FastStart Taq DNA polymerase – 0.25 µl
- DNA - 2 µl

PCR cycling conditions for a total of 25 cycles:

- 95˚C for 2 minutes,
- 95˚C for 30 seconds (x25 cycles),
- 56˚C 30seconds (x25 cycles),
- 72˚C 1minutes (x25 cycles),
- 72˚C 7minutes,
- Samples held at 4˚C.

All PCR products were checked via agarose gel electrophoresis, using a 1% Agarose gel prepared in 0.5 x tris-acetate ethylenediamine tetraacetic acid (TAE) buffer solution with GelRed DNA stain (7.5 µl). The gel loaded with 5µl DNA was run at 120V, the gel was then visualised under UV light using the bio-Rad Gel Documentation system (Image results provided in appendix 1).

Table.2 Reverse and forward V6-V8 454 adapted primers used.

Primer orientation	Primer name	Adaptor	Tag	Primer sequence	Ordered oligo
Reverse	R1401_PS1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGAGTGCGT	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTCGGTGTGTACAAGACCC
Reverse	R1401_PS2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGCTCGACA	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACACGGTGTGTACAAGACCC
Reverse	R1401_PS3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGACGCACTC	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACGCACTCCGGTGTGTACAAGACCC
Reverse	R1401_PS4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCACTGTAG	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGCGGTGTGTACAAGACCC
Reverse	R1401_PS5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCAGACACG	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGCGGTGTGTACAAGACCC
Reverse	R1401_PS6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATATCGCGAG	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGCGGTGTGTACAAGACCC
Reverse	R1401_PS7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGTGTCTCTA	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTACGGTGTGTACAAGACCC
Reverse	R1401_PS8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCGCGTGTC	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCCGGTGTGTACAAGACCC
Reverse	R1401_PS10	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTCTATGCG	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGCGGTGTGTACAAGACCC
Reverse	R1401_PS11	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGATACGTCT	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTCGGTGTGTACAAGACCC
Reverse	R1401_PS13	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATAGTAGTG	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGCGGTGTGTACAAGACCC
Reverse	R1401_PS14	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGAGAGATAC	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACCGGTGTGTACAAGACCC
Reverse	R1401_PS15	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATACGACGTA	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTACGGTGTGTACAAGACCC
Reverse	R1401_PS16	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCACGTACTA	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTACGGTGTGTACAAGACCC
Reverse	R1401_PS17	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGTCTAGTAC	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACCGGTGTGTACAAGACCC
Reverse	R1401_PS18	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTACGTAGC	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTACGTAGCCGGTGTGTACAAGACCC
Reverse	R1401_PS19	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGTACTACTC	CGGTGTGTACAAGACCC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCCGGTGTGTACAAGACCC
Forward	F968_PS	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG		AACGCGAAGAACCTTAC	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAACGCGAAGAACCTTAC

5.3.1 Library cleanup with AMPure XP Beads and E-gel

A simple library quality control procedure was carried out on all PCR samples to purify and determine if the amplicon library was contaminated with short fragments or excess primers. The AMPure XP Bead protocol was followed from the Roche 454 technical bulletin (short fragment removal for the amplicon library preparation procedure.) All triplicate individual PCR samples were pooled together for each of the 17 samples, discarding each of the individual negative samples. The target ratio volume of Ampure XP beads: Volume DNA was 0.8:1 when using 30µl DNA.

All amplicon samples were quantified using the Epoch system. Followed by E-gel clean up procedure and ran through an Agilent DNA chip. The Agilent 20100 Bioanalyzer was used for the sizing and quantification of the highly sensitive DNA fragments following the protocol from the Agilent DNA 1000 kit guide.

5.3.2 Amplicon clean up using gel excision

The AMPure and E-gel clean up procedures did not provide enough quantities of the amplicon, suggesting the cleanup methods were demising the content of DNA. Therefore to rectify this, an alternative Qiagen Gel Extraction clean up was used following a PCR check for short fragments. Clean up amplicons were also checked using the Bioanalyzer (Agilent Technologies 2100 Bioanalyzer protocol) to adequately ensure small fragment removal, and to assess amplicon concentration according to manufacturer guidelines.

To ensure removal of small fragments the following PCR was also completed. Master mix was made up for each sample in triplicate with A Key and B key primers, note each individual DNA sample master mix has a different allocated reverse primer (Table 3):

- Water – 75µl
- Buffer – 10 µl
- dNTPs – 2 µl
- Forward Primer F968_PS – 2 µl
- Allocated reverse primer – 2 µl
- FastStart Taq DNA polymerase – 1µl
- DNA - 2 µl

The following table represents the allocated reverse primer to the correct DNA sample; note that the forward primer was the same for all samples (F968_PS).

Table.3 Allocation of reverse primers to individual DNA samples.

TR212 DNA code	Steer	Diet	Period	Reverse primer number
136	1	echium	1	PS1
137	2	flax	1	PS2
138	3	grass	1	PS3
139	4	flax	1	PS4
140	5	grass	1	PS5
141	6	echium	1	PS6
142	1	flax	2	PS7
143	2	grass	2	PS8
144	3	echium	2	PS10
145	4	echium	2	PS11
146	5	flax	2	PS13
147	6	grass	2	PS14
148	1	grass	3	PS15
149	2	echium	3	PS16
150	3	flax	3	PS17
152	4	echium	3	PS18
153	6	flax	3	PS19

The PCR was run on the following conditions of 25 cycles:

- 95˚C for 5 minutes
- 95˚C for 30 seconds
- 56˚C for 30 seconds
- 72˚C for 1 minute
- 72˚C for 5 minutes
- Samples held at 4˚C

This alternative clean up protocol is designed to extract and purify DNA of 70 base pairs (bp) to 10 kb, of which the DNA samples used in this study are of approximately 432bp, however that is excluding the extra base pairs from the tags and primers used in PCR products. Altogether including the forward and reverse primers with the tag and adaptors for the 16S, amplicon size was approximately 1,400bp. The process requires the DNA

samples after PCR to be ran through a low-melt 1% agarose gel in TAE buffer, until all the fragments are separated. DNA fragments in the gel are then removed using a sharp scalpel, keeping all individual DNA samples separate. The QIAquick Gel extraction protocol was then followed (as stated in the QIAquick spin handbook, Qiagen) to obtain purified DNA samples ready for analysis on the EPOCH. The samples were then analysed on the Agilent Bioanalyzer, this determined that the samples following the clean up via gel excision was successful, and there was plentiful quantities of purified DNA ready for next generation sequencing.

5.3.3 Roche 454 Genome FLX sequencer.

The amplicon PCR products were sequenced using the in house Roche 454 FLX sequencer. Titrations were carried out on all 17 DNA samples to determine what quantity of library was used for the emPCR amplification, followed by an emulsion and sequencing titration. The procedure for the titrations, DNA library capture, emulsification, and amplification, bead recovery, DNA library bead enrichment and sequencing primer annealing were carried out following the Roche emPCR method manual-Lib-L-SV GS FLX Titanium Series protocol. The protocol for the emPCR run was carried out as instructed in the Roche Lib-L-LV manual.

For the final 454 sequencing run performed on the GS FLX sequencer the protocol was followed as instructed in the Roche 454 sequencing manual (Sequencing method manual for the GS FLX+ instrument –XLR70 kit) in order to obtain the results required for analysis.

5.4 Data analysis

The DNA sequences obtained from using next generation 454 pyrosequencing were further analysed using QIIME software for semi-quantitative analysis. (Full script used to run Qiime available in Appendix 2). This software assesses the gene sequences and determines if they are significantly different, allowing analysis of high-throughput microbial community sequencing. Qiime software allows the analysis and comparison of hundreds of communities simultaneously to produce a range of visual results including; rarefaction graphs, principle component analysis graphs (PCA), histograms, dendograms, taxa

summary plots, and UPGMA trees. From the acquired results class and phylum information was chosen to represent the abundances of bacteria present within the rumen from this investigation. It was appropriate to choose class and phylum for comparison as not all species could be identified down to genus level.

Statistical analysis was carried out on the results of the abundances of fatty acids present from the preliminary experiment, and comparison between class and phyla present between the test and control conditions. This was analysed using the statistical software package GenStat to perform Analysis of Variance (ANOVA) and Ducan's Multiple Range Test.

6. Results

6.1 Ruminal fatty acid content

With respect to supplementing echium oil there was a significant change in the concentration of nearly all detectable fatty acids present ($P<0.05$; Table.4). Over all echium oil increased fatty acid concentration of the health beneficial PUFAs and CLA intermediates (18:3n-3, 18:2n-6, 18:1 trans-11, cis9 trans-11, Table 4). However, by supplementing echium oil conversely increased 18:0 concentrations, which was unexpected (>11%, Table.4). Nonetheless echium oil as predicted significantly increased the concentration of C18:4n-3 compared to the control grass diet and flax diet (Table 4). There is no evidence of a significant increase in long chain PUFAs such as EPA (C22:5), DHA (C22:6) as these were undetected. Branched chain fatty acids (BOC, including C:13, C:15, and C:17) showed no significant increase in concentrations between dietary treatments. Branched chain fatty acids are only found in microbes, therefore can be used as an indicator if there are any changes in microbial community. However, this is a very crude way to assess microbial change.

Table 4. R

	Fatty Acid														
Treatment	*18:3n-3*	*C18:2n-6*	*CLA* [1]	*CLA cis-9, trans-11*	*C18:1 cis*	*C18:1 trans*	*C18:1 trans-10*	*C18 4n-3*	*C18:1 trans-11*	*C18:0*	*BOC*	*C204*	*C205*	*C225*	*C226*
Echium	2.97 b	2.331 b	0.5792 c	0.3316 c	0.3879 b	6.832 c	0.2457 c	1.2606 b	4.797 c	11.061 b	1.547 a	ND	0.013148 c	ND	ND
Flax	3.67 b	2.162 b	0.4703 b	0.2736 b	0.3779 b	4.926 b	0.2015 b	0.0407 a	2.999 b	9.783 b	1.450 a	ND	0.007972 b	ND	ND
Grass	1.104 a	1.429 a	0.0805 a	0.0287 a	0.1258 a	0.850 a	0.0301 a	0.0494 a	0.555 a	4.193 a	1.437 a	ND	0.000398 a	ND	ND
SED	0.37	0.24	0.03	0.02	0.02	0.35	0.01	0.17	0.32	0.58	0.06	ND	0.00	ND	ND
P-value	<.001	0.02	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	0.23	ND	<.001	ND	ND

Statistical resul
significantly diff

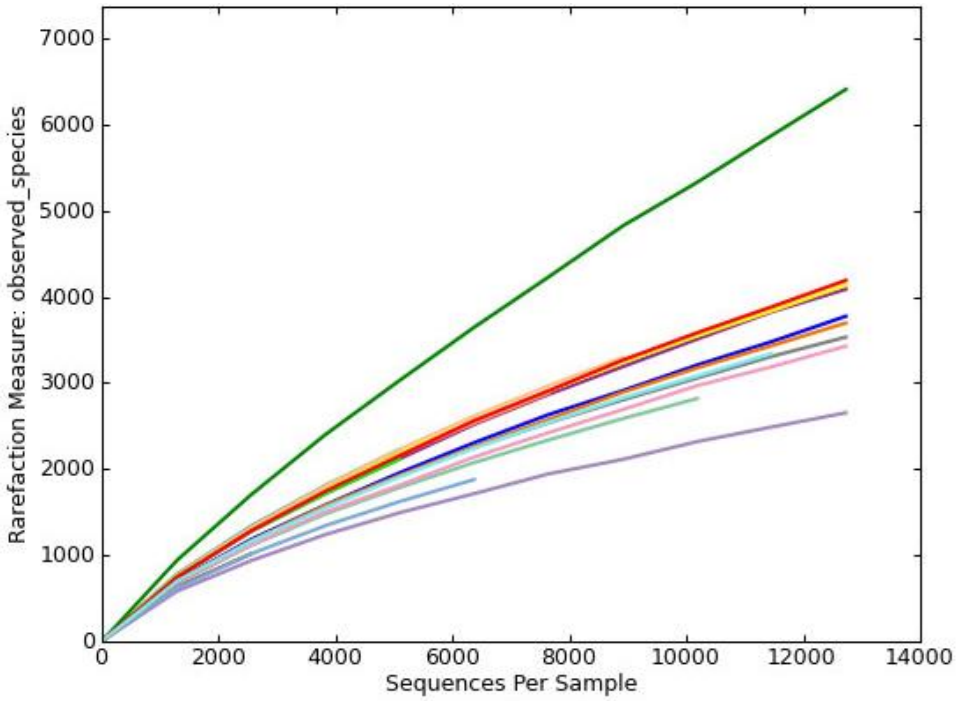
6.2 Summary analysis of sequence outputs and identified OTUs from QIIME

Results from all 17 ruminal samples from the 6 steers provided a total of 726785 sequences, of which 602735 passed the quality control from QIIME, with 427339 identified operational taxonomic units (OTUs). All the sequences from each dietary trial indicated 100% of species identified to be of bacterial domain. The most abundant OTUs identified were distributed in 8 phyla and 12 distinct classes (Fig 9 & 10). Table 5 provides a summary of data acquired from 454 pyrosequencing of rumen samples.

Table 5. Summary of pyrosequencing data of 16S DNA 454 pyrosequences from ruminal samples post Qiime filtering. Results displayed with appropriate calculated (±SEM).

	Rumen samples
Total number of reads (Pre Qiime analysis)	726785
Total number of reads (Post Qiime analysis)	602735 ± 111
Total reads for Echium diet	102772 ± 21032.0
Total reads for Flax diet	306704 ± 46423.3
Total reads for Grass diet	193259 ± 33212.5
Average length of sequences (BP)	377 ± 61.1
Domain: bacteria	100%
Number of Phyla	8 ± 0.03
Number of Classes	12 ± 0.03

Producing rarefaction curves in Qiime software was done to identify the OTU's observed against number of sequences obtained from individual samples. A rarefaction plot (Fig.5) indicating number of species depicts the number of unique operational taxonomic units (OTU's) against sequences per sample. The rarefaction plot indicates that for each sample the graph lines reach a plateau, therefore determining that further sequencing depth would not result in increased OTU detection. As the OTU line plateaus irrespective of increasing sequence number, this gives an indication that depth of sequencing used in this study was sufficient enough to identify the diversity in all 17 rumen samples.



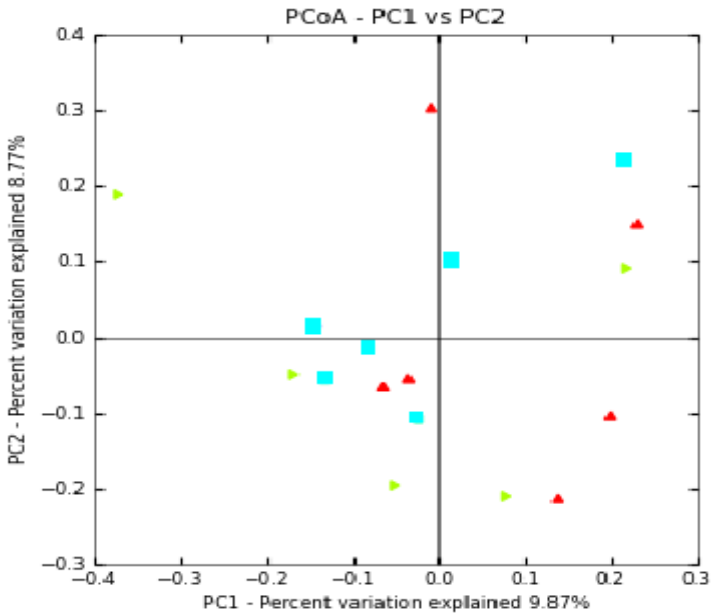
Legend	MID Name	Sample no	Steer	Diet	Period
	MID-1	1	1	echium	1
	MID-2	2	2	flax	1
	MID-3	3	3	grass	1
	MID-4	4	4	flax	1
	MID-5	5	5	grass	1
	MID-6	6	6	echium	1
	MID-7	7	1	flax	2
	MID-8	8	2	grass	2
	MID-10	9	3	echium	2
	MID-11	10	4	echium	2
	MID-13	11	5	flax	2
	MID-14	12	6	grass	2
	MID-15	13	1	grass	3
	MID-16	14	2	echium	3
	MID-17	15	3	flax	3
	MID-18	16	4	echium	3
	MID-19	17	6	flax	3

Fig 5. QIIME analysis alpha-rarefaction plot, indicating the observed species depicting unique operational taxonomic units (OTU's) against individual sequences per sample from all 17 ruminal samples.

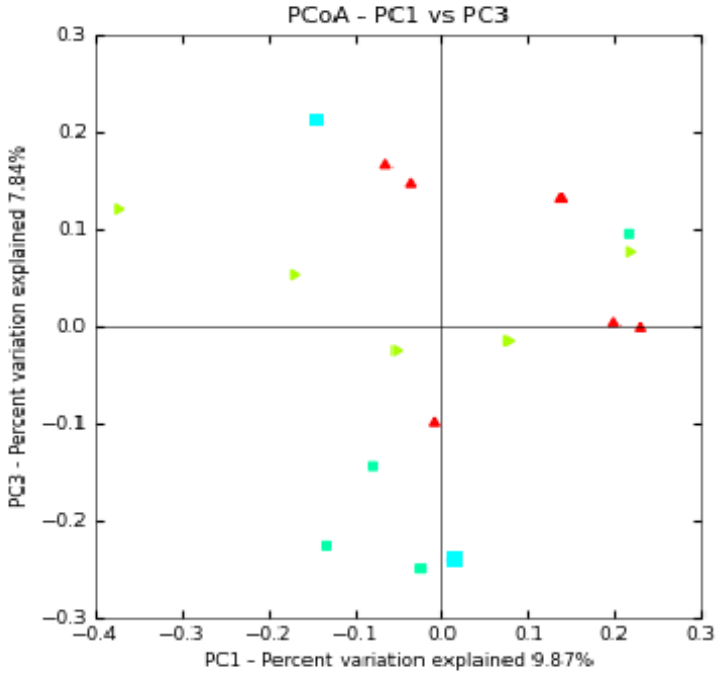
6.3 Principle component analysis (PCA)

The following PCA diagrams represent multivariate data including steer, diet and period treatment to explaining the variances between samples. The comparison of grass, flax and echium dietary treatments upon steers by PCA (Fig.6a) indicates the least variability between PC1 vs PC2 following supplementation of echium oil, as the dietary symbols are clustered together suggesting more similarity between samples. PC1 vs PC3 (Fig. 6b) indicates mixed variability, while PC3 vs PC2 (Fig.6c) shows the greatest variability with more dispersed symbols. Overall the samples showing steers supplemented with echium tend to cluster away from those within the other dietary treatments. The PCA plots illustrate that even within each diet and feeding regime, animal variation in their microbiota is significant.

(6a)



(6b)



(6c)

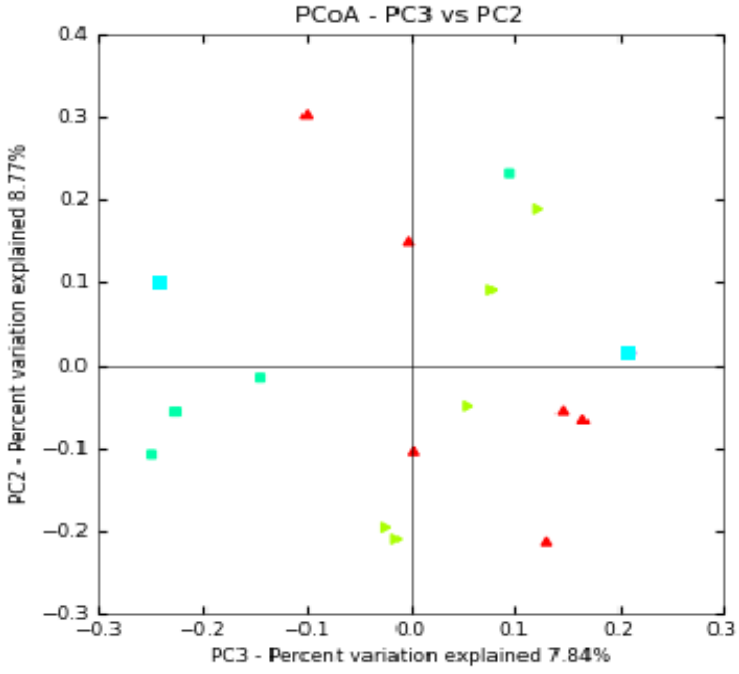


Fig.6 Principle component analysis (PCA) graphs of bacterial 16S DNA gene sequences from each dietary trial generated from 454 pyrosequencing. The marks relate to the dietary treatment of steers: Flax diet ▭ Grass diet △, Echium diet ▲ PC1, PC2, PC3 represent all variables present in results as calculated from eigenvalues. (6a) plot to compare PC1 vs PC2, (6b) plot to compare PC1 vs PC3, (6c) plot to compare PC3 vs PC2. (6a) is the most important PCA plot to consider as it indicates the highest variability within the results.

6.4 Microbial diversity upon on dietary supplements

Fig.7 represents the comparison of dietary treatments (echium, flax and grass) for individual samples using jackknife UPGMA (unweighted pair group method with arithmetic mean) dendograms, based on the unweighted Unifrac distances between all 17 rumen samples from the 6 steers. The dendogram shows the relationship between all dietary treatments and indicates 5 distinctive samples that cluster together. Clustering occurs between dietary samples of; only echium, grass & flax, echium & grass, and only flax. There is however, no indication of close clustering between echium & flax. There is no evidence of sample number, steer, or period having a correlation to the effect of clustering. There seems to be no overall global clustering by diet alone using dendograms calculated by Unifrac distances.

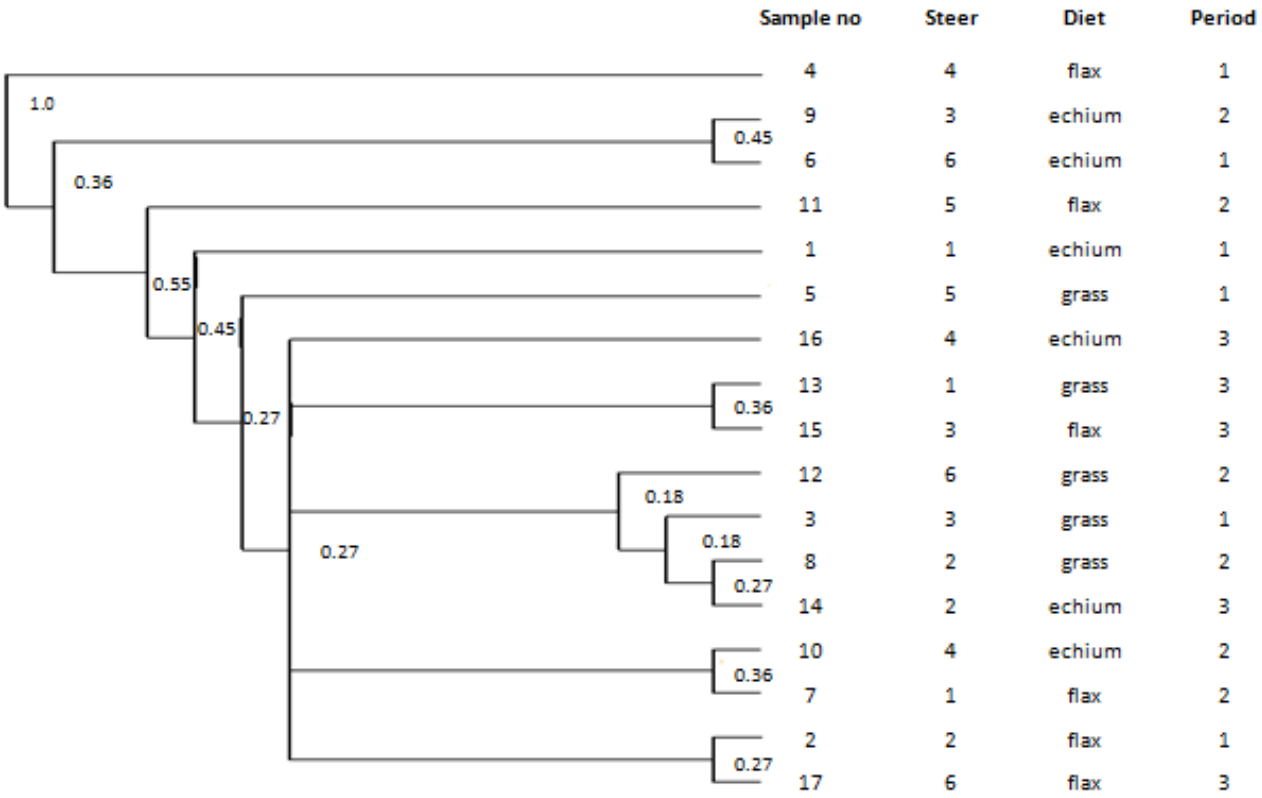
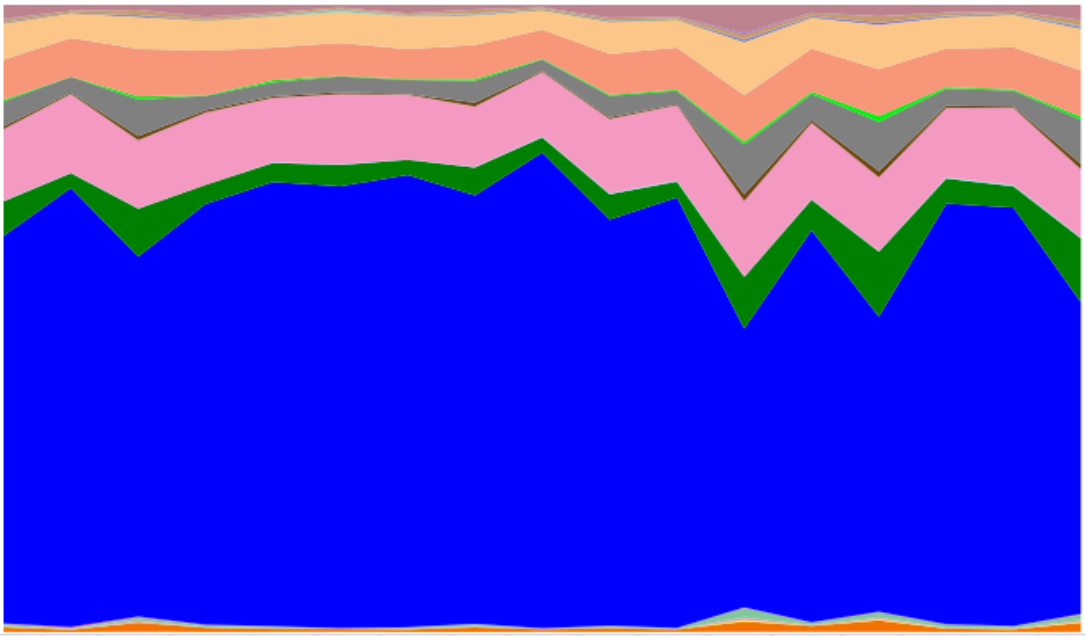


Fig.7. Unweighted pair group method with arithmetic mean (UPGMA) dendogram plot produced via Jackknife, displays the effect of dietary treatment and the associated bacterial community between samples. Jacknife values calculated from beta diversity values obtained from Qiime output.

6.5 Taxonomic analysis of bacterial species present

The following chart (Fig.8) represents the taxa assignments for each sample indicating which taxa contribute to the overall percentage shown as an area chart. (It is important to note for this diagram that only the main identified phyla present have been displayed for the taxonomy key, a full taxonomy key classified down to order is available in Appendix 3.) Firmicutes Lachnospiraceae (blue) are the most abundant family present with 43-76% abundance across all samples, followed by Ruminococceae (green) (2-10%) , the order clostridales (pink) (9-13%) and several unidentified species within the class Clostridia. The area chart indicates over all the predominant phylum following all dietary treatments is Firmicutes.



sample No.	1	10	11	12	13	14	15	16	17	2	3	4	5	6	7	8	9
steer	1	4	5	6	1	2	3	4	6	2	3	4	5	6	1	2	3
period	1	2	2	2	3	3	3	3	3	1	1	1	1	1	2	2	2
diet	E	E	F	G	G	E	F	E	F	F	G	F	G	E	F	G	E

Taxonomy;
Firmicutes, Clostridia;Other
Firmictues,Clostridia;Clostridiales;Lachnospiraceae
Actinobacteria, Actinobacteria;Coriobacteridae
Firmicutes, Clostridia;Clostridiales;Ruminococcaceae
Firmicutes, Clostridia;Clostridiales;Veillonellaceae
Bacteroidetes , Bacteroidetes;Bacteroidales;Other
Fibrobacteres;Fibrobacteres;Fibrobacterales;Fibrobacteraceae
Firmicutes. Clostridia:Clostridiales:Other

Fig.8 Taxa summary plot indicating most abundant phyla assignments for each sample as an area chart. E-Echium oil; F-Flax; G-Grass silage only. (Full taxonomic colour coding classification table available in appendix 3)

6.5.2 Identification of bacteria present to class classification.

Overall dietary treatment had no significant effect upon bacterial species identified to class level (results from ANOVA and Duncan's multiple range test *P*-values > 0.05; Fig.9 & Table.6). However when assessing class % abundance with individual diets regardless of the level of significance, echium oil shows a trend to increase the presence of majority of the bacterial species identified (Table 6).

The class clostridia are the only bacterial species to indicate a high significant difference in % abundance between diets, indicated with a P-value 0.014 (<0.05 level of significance), and by different subscript letters from the Duncan's multiple range test (Table 6). As demonstrated in the doughnut chart (Fig.9) it is clear to see Clostridia is the most abundant class across all dietary treatments. It also indicates when supplementing echium oil this reduces the % content of clostridia present within the rumen down to 78%, compared to 83% content with flax and 87% with grass silage diets.

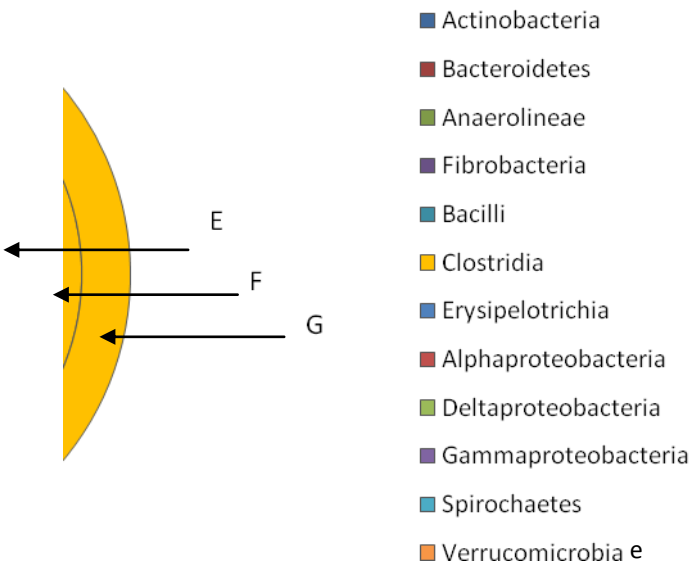


Fig 9. Doughnut chart representing % of bacteria taxon assignment identified to class level present following feeding of steers each dietary treatment. E-Echium; F-Flax; G-grass silage diet.

Table 6. Statistical analysis results on % of bacteria identified to class level present between each dietary treatment. Results following ANOVA and Duncan's multiple range test, (SEM) standard error, a,b,c subscript letters indicate results which are significantly different, (*P*<0.05).

Class	Echium	Flax	Grass	P-value	SEM
Actinobacteria	3.268 a	2.478 a	1.542 a	0.35	1.104
Bacteroidetes	0.13588 a	0.05437 a	0.03916 a	0.567	0.0937
Anaerolineae	0.07645 a	0.00000 a	0.0191 a	0.35	0.0646
Fibrobacteria	0.0999 a	0.2187 a	0.136 a	0.841	0.2042
Bacilli	0.05918 a	0.01958 a	0.11733 a	0.422	0.0702
Clostridia	78.44 a	83.28 ab	87.54 b	0.014	2.218
Erysipelotrichia	1.0773 a	0.3220 a	0.0154 a	0.392	0.763
Alphaproteobacteria	0.3891 a	0.2351 a	0.2335 a	0.605	0.1719
Deltaproteobacteria	0.00000 a	0.00000 a	0.004689 a	0.377	0.377
Gammaproteobacteria	0.02564 a	0.042 a	0.11408 a	0.294	0.055
Spirochaetes	0.00000 a	0.005135 a	0.002566 a	0.458	0.00388
Verrucomicrobiae	0.3999 a	0.3927 a	0.4662 a	0.952	0.258

6.5.3 Identification of bacteria present to Phylum classification.

Concentrating on phylum level classification (Fig.10 & Table.7) the phylum Actinobacteria show a significant difference between dietary treatments (*P*-value 0.046 <0.05) with a significant increase in % abundance with the supplement of echium oil (1.164 %) (Table.7). By looking at the % values, flax dietary treatment actually reduced the content of Actinobacteria species (0.2485%), whilst this % is lower than the grass control diet (0.4203%) it was not seen as significantly different from the Duncan's multiple range test as the subscript letters are the same. Actinobacteria was the only phylum to indicate a significant difference, yet additionally it is clear to see (Fig.10) that overall the phylum Firmicutes was the most abundant across all dietary treatments, this correlates to the results displayed in the taxa summary plot (Fig.8).

However an interesting result to highlight is while the abundance of clostridia indicates a significant difference between diets (Table 6), this class is part of the phylum Firmicutes which was not significantly different (Table 7). Thus, it could be suggest dietary treatment does not have as much effect on the diversity and abundance of phylum species, however when classifying down to class taxonomy level dietary treatment does have an influential effect. To determine this a finer look at specific identified OTUs is needed to see if any are

specific to diet change and if possible classification of bacterial species down to a finer level such as order, family or genus. This opens an area to be suggested for future investigations (Further discussed in conclusion).

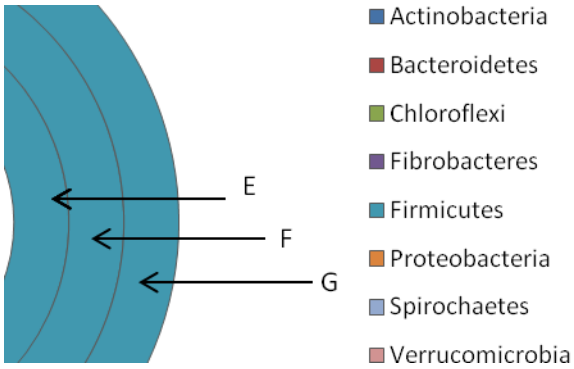


Fig 10. Doughnut chart representing % of bacteria taxon assignment identified to phylum level present following feeding of steers each dietary treatment. E-Echium; F-Flax; G-grass silage diet.

Table 7. Statistical analysis results on % of bacteria identified to Phylum level present between each dietary treatment. Results following ANOVA and Duncan's multiple range test, (SEM) standard error, a,b,c subscript letters indicate results which are significantly different, (P<0.05).

Phylum	Echium	Flax	Grass	P-value	SEM
Actinobacteria	1.164 b	0.2485 a	0.4203 a	0.046	0.309
Bacteroidetes	0.04549 a	0.00143 a	0.01659 a	0.493	0.0358
Chloroflexi	0.03412 a	0.00000 a	0.00852 a	0.35	0.0288
Fibrobacteres	0.03777 a	0.00622 a	0.01860 a	0.714	0.0378
Firmicutes	33.2 a	11.69 a	22.92 a	0.161	9.82
Proteobacteria	0.2428 a	0.0548 a	0.1949 a	0.29	0.1133
Spirochaetes	0.00000 a	0.00014754 a	0.00007373 a	0.458	0.000112
Verrucomicrobia	0.09945 a	0.03783 a	0.09963 a	0.723	0.0864

7. Discussion

7.1 Data obtained from 454 16s rDNA sequencing

As 454 pyrosequencing technologies have developed, it is increasingly used for comprehensive 16S rDNA analysis, allowing species richness and diversity of the microbial community to be determined (Brulc *et* al., 2009; Kim *et al*., 2011c; Zened *et al*., 2012). The resulting data from 454 pyrosequencing produces reads and sequences of varying lengths relating to V6 – V8 regions of 16S rDNA genes. The variation in sequences allows genus classification in RDP databases (Release 10, update 30, Web reference 2), and comparison between OTU richness. RDP has archived over 1.4 million bacterial species and 54,000 archaeal species based upon 16S gene sequences (Kim *et al*., 2011).

The results obtained from this study of 16S rDNA 454 analysis identified 726785 sequences and 427339 OTUs. Excluding the unclassified groups these sequences represented 12 different phyla and 8 class of bacteria. Relevant rumen studies based upon 16S rDNA 454 analysis such as Brulc *et al* (2009), only identified 771 OTUs, of which 510 were unique across 4 bovine rumen libraries. Kim *et al* (2011c) obtained 7450 bacterial sequences, and 8375 archaeal sequences both >1200bp, representing a very broad bacterial taxa (18 phyla, 25 class, 64 order, 165 family, and 361 genera.). Wu *et al* (2012) generated 37005 sequences from pyrosequencing of 16S rDNA, consisting of 8 different phyla, 15 families and 17 genera. Respectively Zened *et al* (2012) obtained 138764 gene sequences from 454 pyrosequencing, with a microbial diversity representing 2297 OTUs within 13 phyla, 45 orders, 98 families and 219 genera. In comparison to these reports this study provided a greater amount of 16S rDNA gene sequences for analysis. It has been suggested in Kim *et al* (2011) in order to determine 99.9% of rumen bacteria as known culturable species, approximately 91,000 16S rDNA gene sequences need to be characterised. Taking this into account, as previously mentioned, 726785 sequences from 454 16S rDNA pyrosequencing were analysed in this study, this validates the processes and quality of data obtained from 16s rDNA in this study.

The average sequence length of 16S rDNA gene sequences in this study was 377bp. This average was greater than Brulc *et al* (2009) with an average length of 102bp, and similar to the 393bp average read length in Zened *et al* (2012). However, in comparison to Kim *et al*., (2011c) full length sequences were approximately 1540bp, allowing a very detailed

analysis and bacterial identification down to a finer taxonomic classification; whereas this study did not characterise lower than a class taxonomic level due to only having 377bp average sequence length. Nevertheless it is evident from our graphs illustrating observed number of OTU's with increasing reads that we reached a plateau in nearly all sequenced samples. This demonstrates an adequate depth of sequencing on the 16S rDNA rumen samples from this study, providing ample amounts of data for successful analysis.

7.2 Dietary effects upon fatty acid content

Increasing research is focusing on ruminal biohydrogenation and lipid metabolism, to specifically determine final SFA's, biohydrogenation intermediates, CLA's, and long chain PUFA's content. This is due to the potential that the final fatty acid content in rumen produce can have on human health implications. However with a lack of information on known culturable bacteria and their associated role on lipolysis and biohydrogenation, numerous reports illustrate the effects of dietary supplements to manipulate the fatty acid composition in meat (Wood *et al*., 2003). Current research shows that fish oil is the most effective oil supplement for beneficial manipulation of rumen lipid metabolism (Shingfield *et al*., 2011; Kim *et al*., 2008; Lee *et al*., 2008, 2005; Scollan *et al*., 2001).

There is a scarcity of published data with the effects of echium oil supplementation upon lipid metabolism. This investigation has concluded when supplementing echium oil, the greatest effect enhancing total fatty acid content within the rumen was evident, in comparison to the grass+ flax diet and grass silage only diet. Whilst echium oil did not increase long chain PUFA (such as C22:5, C22:6), it did however significantly increase ruminal C18:2n-6, Cis-9 trans-11, C18:1 trans, C18:4n-3, and C18:1 trans-11. Similarly increases in C18:1 trans-11 (the precursor of 18:0) has been evident in previous studies with the supplement of fish oil (Huws *et al*., 2011; Vasta *et al*., 2010), however, despite an increase in C18:1 trans-11, there was no increase in the production of 18:0, the final end product of biohydrogenation in these fish oil supplementation trials.

In contrast to previous literature, this investigation additionally found an increase in 18:0 concentrations as well, when supplementing echium oil. This result is unexpected with regards to the original prediction that supplementing echium oil would prevent complete biohydrogenation therefore, in theory results were expected to show a decrease in 18:0

concentration. Nonetheless echium oil as predicted significantly increased the concentration of 18:4n-3 compared to the control grass diet and flax supplement diet. In summary, as previously reported, until now it was thought the conversion of 18:3n-3 to 18:4n-3 was rate limiting resulting in an inefficient conversion to long chain PUFA's (Huws, *et al*.,2011). However, this study has shown a contradiction to this, as long chain PUFA concentrations were not enhanced even though increased concentrations of 18:4n-3 were evident upon echium oil supplementation. Therefore this indicates 18:4n-3 is not the rate limiting step as previously reported. This highlights an interesting result as it indicates whilst echium supplements may in fact enhance biohydrogenation of lipids (indicated via increase in 18:0), additionally it too suggests that it influences an alternative pathway for the conversion of 18:4n-3 to 18:3n-3, which has previously not been reported. It should also be mentioned that flax oil had double the 18:3n-3 concentration of echium oil, thus confirming that it is likely that the increased biohydrogenation seen post echium oil supplementation, may be due to increased 18:3 n-3 availability due to its conversion from 18: 4n-3 and not due to concentrations within the oil itself.

In the presence of flax-supplemented diets, results obtained are similar to those reported in previous *in vitro* studies. Doreau *et al* (2008) found that supplementing flax (linseed) increased the content of CLA such as cis9, trans-11, and biohydrogenation intermediates (C18:1 trans-10, C18:1 trans-11). Additionally Shingfield *et al* (2012) reported supplementing flax increased the flow of geometric isomers of delta 9, CLA's, cis 18:1, and 18:0. These are both consistent with the results found from this study when comparing grass + flax supplement against only grass silage diets. Shingfield *et al* (2012) reports that the increase in flow of CLA intermediates is a result of incomplete biohydrogenation of 18:3n-3 and the increase of 18:3n-3 is due to linseed increasing the intake of C18 UFAs. This reasoning also explains the increase of 18:3n-3 in this study relative to the control diet.

7.3 Effects of Echium oil supplementation on the rumen microbiota community

The development of metagenomics applied to rumen studies has enabled scientists to analyse 16S rDNA clone libraries, to characterise the rumen microbiome environment to a level not previously possible. The growth of rumen studies using metagenomics aids the understanding of bacterial diversity, and to identify key species of the microbiome

community that have a distinctive role in lipid metabolism pathways (Edwards, *et al*., 2004). However, while this is a growing area of research few papers relating to beef cattle and rumen metagenomics with the specific use of 454 pyrosequencing have been published (Wu *et al*., 2012; Prive, 2011).

This investigation has demonstrated high-throughput 454 pyrosequencing to be a successful technique to determine the bacterial diversity and abundance within the rumen ecosystem following dietary supplements of echium and flax. This method has been used widely and successfully to sequence 16S rDNA from ruminal environments (Wu *et al.,* 2012 ; Huws *et al.,* 2011; Claesson *et al.,* 2010, Edwards *et al*., 2007,). Whilst this study successfully identified 12 classes, and 8 different phyla it was unable to identify all bacterial domains down to species level due to the average sequence length being 377bp. The 16S rDNA analysis has shown that within the rumen ecosystem of steers from this investigation, the bacterial community classified at a phylum level was dominated by Firmicutes followed by Actinobacteria. This bacterial abundance was evident following all dietary treatments. However, when supplementing echium oil it did induce greater levels of the apparent dominant bacteria when compared to the effects of the flax and grass diets. Within the phylum Firmicutes, unclassified Lachnospiraceae were the most abundant species (76%), this is similar to findings in Kim and colleagues (2011).

As previously mentioned, numerous reports have previously suggested that the main bacteria largely associated with a role in lipid metabolism, that efficiently biohydrogenated PUFA into C18:0 are Butyrivibrio, Clostridium and Anaerovibrio species, members of the class Clostridia and phylum Firmicutes (Huws *et al*., 2011,2010; Kim *et al.,* 2008; Maia *et al*., 2007; Lee *et al*., 2005). In contrast to the latter literature, Clostridia were reduced following echium oil supplementation; nevertheless biohydrogenation was enhanced, so this suggests this study did not indicate Clostridia to be active rumen biohydrogenators. This *in vivo* experiment did nonetheless show similar results indicating that Firmicutes is potentially one of the main bacterial phyla involved in the biohydrogenation of lipids in the rumen. This is supported by the increase in % abundance of the phylum Firmicutes following echium oil supplementation of steer diets, enhancing an increase in content of 18:0 suggesting that echium oil elicits an increase in biohydrogenation rates. While it is probable that Firmicutes is one of the key phyla associated with biohydrogenation, and there is phylogenetic similarity between species present, the physiological functions of individual species within this phylum are poorly understood.

Zened *et al* (2012) also reports Firmicutes as the most dominant rumen bacteria phyla, alongside Bacteroidetes (Turnbaugh *et al*., 2008, 2006; Stewart *et al*., 1997). However in comparison, Bacteroidetes was not in great abundance from our 16S rDNA analysis. When analysing the abundance of the genus Prevotella, which previous literature has reported to be the most abundant genera of Bacteroidetes (up to 52%) (Zened *et al*., 2012; Pitta *et al*., 2010), results from the Qiime taxa summary plot indicate Prevotella at concentrations <0.4% across all ruminal samples. High abundances of the genera Lachnospiraceae (75%) and Ruminococcacaea (10%) from the phylum Firmicutes were also evident, similar to the findings by Zened *et al* (2012).

Additionally an important result from this study to highlight is the increase in concentration of the phylum Actinobacteria upon echium supplementation. Bacterial species of Actinobacteria have occasionally been mentioned in previous reports with an involvement in rumen lipid metabolism. Although It has been suggested Actinobacteria are an infrequent bacterial species present in the rumen, accounting for less than 2% of total rumen bacteria (Sulak *et* al., 2012; Pandya *et al*., 2010), and in some literature no taxonomic lineages of Actinobacteria have been identified in rumen environments (Callaway *et al*., 2010; Pitta *et al*., 2010). There is an uncertainty to the role Actinobacteria may play in biohydrogenation and lipid metabolism, as there is a lack of information relating to occurrence within ruminal environments. However, there are reports of successfully cultivating a genus of Actinobacterium; *Denitrobacterium detoxificans*, from ruminal samples (Anderson *et al*., 2002), and Sulak *et al* (2012) reports reads from 16S rDNA analysis to contain approximately 10% of related Actinobacteria species present. Zened *et al* (2012) evaluated the effect of supplementing starch and sunflower oil on the microbial ecology of ruminants via 16S rDNA 454 analysis. Interestingly Actinobacteria represented approximately 5.1% of the total rumen bacteria present, with Actinobacteria being the most predominant class and Bifidobacteriaceae the most concentrated family. On supplementation of concentrates and oils, increases in the concentration of bacterial classes within Actinobacteria were evident; however, dietary supplements did not significantly enhance Actinobacteria on a phylum classification (Zened *et al*., 2012).

In comparison to this previous literature, this study found an increase in the concentration of the phylum and class Actinobacteria during the supplementation of echium oil. As previously mentioned Actinobacteria was the second most abundant phylum (>3.3%)

present in the rumen flowing Firmicutes. Recent findings along with the results discussed from this study suggest that Actinobacteria in conjunction with Firmicutes do play a key role in lipid metabolism in the rumen. This is evident when supplementing echium oil there was an increase in Actinobacteria concentrations, and further enhanced levels of biohydrogenation producing more 18:0. It can be suggested too, that the increase in Actinobacteria may be responsible for the apparent conversion of 18:4n-3 to 18:3n-3. Therefore, further studies need to assess and identify which species of Actinobacteria dominate in varying conditions of the rumen microenvironment, with physiological changes, such as pH, and alterations in echium concentration supplement.

Since the pioneering work of Harfoot (1978) who suggested *Anaerovibrio lipolytica* (phylum Firmicutes) was the key genus to account for the total rates of lipid metabolism, little knowledge on the majority of rumen bacteria are still unknown today. Edwards and colleagues (2004) analysis of 16s rDNA genes, accounted for only 11% of sequenced genes to contain known culturable bacteria species. Koike *et al.,* (2003) reported 42% of analysed sequences contained as yet unculturable bacteria. Recently Kim *et al*., (2011a) sequenced over 13,000 16s rRNA genes; results indicated roughly 6.5% of sequenced data represented known genera of culturable bacteria. The majority of identified OTUs within these studies were assigned to known species within the phylum Firmicutes, Spirochaetes, Proteobacteria and Verrucomicrobia (Kim *et al*., 2011; Edwards *et al*., 2008; Tajima *et al*., 2001, 1999). This supports findings from this investigation, as the same phyla were also identified from 16S rDNA analysis. As previously mentioned although it was not possible to classify species down to a low taxonomic rank of genus level, it is confirmed that Firmicutes is one of the most predominant phyla involved in biohydrogenation, an additionally in comparison to previous literature, the presence of Actinobacteria plays a significant role in lipid metabolism.

The use of next generation sequencing has been increasingly used on the study of dairy cows, while little has been reported for that of steer cattle. Nevertheless, comparison of studies demonstrates that the compositions of dominant bacteria within the rumen microbiota of dairy and meat cattle vary greatly. Wu and colleagues (2012) determined that the most abundant family present in the rumen of dairy cattle was Prevotellaceae (~69%), where as in steers, levels were below 32%. Lachnospiraceae on the other hand dominated the rumen microbiota in steer cattle (>35%). In comparison to this study considering steer cattle, Prevotellaceae (phulum Bacteriodetes) levels were very low (0.1-

0.4%), while Lachnospiraceae (phylum Firmicutes) was the most abundant bacterial species present in the rumen ~76%. The difference in bacteria abundance reported between dairy and beef cattle is a result of their dietary treatments. Interestingly previous literature reports the genus Prevotella (family Prevotellacae, phylum Bacteriodetes) to be the most abundant within the rumen ecosystem of steers (Wu *et al*., 2012; Bekele *et al*., 2010; Ramsak *et al*., 2010). In contrast this study revealed very low concentrations of the family Prevotellacae (<2%). This demonstrates how echium oil supplements influences the microbiota diversity within the rumen.

In summary it is highly notable that compared to previous oil supplement studies such as fish oil and linseed oil, there are alternative enhancements in the rumen bacteria present upon echium oil supplementation. As described, not only was Firmicutes concentrations enhanced, as evident in multiple studies, additionally Actinobacteria increased in % abundance. This study reveals that changes in lipid metabolism upon the addition of echium oil are due to the influence of changes within the rumen microbiota. Rumen bacteria especially Firmicutes and Actinobacteria are not only responsible enhancing biohydrogenation, but are too involved in the unexpected 18:4 to 18:3 conversion as demonstrated in this study.

8. Conclusion

In conclusion this investigation assessed the effects of supplementing echium oil on the rumen microbial ecosystem and rumen fatty acid content. This study has provided extensive 454 16S rDNA data to answer the original posed questions and hypothesis under consideration, which were; 1) to determine if echium oil supplementation has any effect upon the lipid metabolism pathways within the rumen, and 2) to determine if there are any elicit changes on the rumen microbial ecosystem, identifying key biohydrogenators, between dietary treatments.

The findings have indicated echium does influence the whole biohydrogenation pathway of the lipid conversion from ingested PUFA's into SFA's. Echium oil not only enhances the biohydrogenation process producing more 18:0, but has distinctively increased the content of CLA intermediates. It has also highlighted a unique effect that suggests 18:4 n-3 is in fact readily converted to 18:3 n-3 before entering the biohydrogenation pathway. As previously mentioned, it was believed the conversion of 18:3n-3 to 18:4n-3 was rate limiting making the conversion to long chain PUFA's such as EPA and DHA inefficient. However new insights from this study indicated there was no detection of enhanced long chain PUFA concentration upon echium supplementation, even though 18:4n-3 concentrations increased along with 18:3n-3. Thus determining there is not a rate limiting step in long chain PUFA production as previously reported. Findings suggest changes in lipid metabolism upon addition of echium oil are due to changes in the rumen bacteria present compared to that of the flax and grass dietary treatments. Thus, we can accept the alternative hypothesis and confirm 'there is a relationship between supplementing levels of echium oil and influencing the pathway of lipid metabolism and the diversity of bacteria within the rumen'.

This study has provided an insight to further our understanding and knowledge of the microbial diversity that influences ruminal biohydrogenation, of which undoubtedly affects the final fatty acid content. While it can be concluded that Firmicutes and Actinobacteria were the dominant phyla present within the rumen on echium supplementation and the key biohydrogenators involved in lipid metabolism; the main significant result suggesting an alternative lipid metabolism pathway (conversion of 18:4n-3 to 18:3n-3) opens a unique area for further investigation.

8.1 Limitations and further studies

To determine the exact effect of specific rumen bacteria on lipid metabolism, a finer look at specific identified OTUs is needed to classify bacterial species down to a finer taxonomic level such as order, family or genus. Unfortunately in this investigation classifying down to a fine taxonomic level was not possible due to the limitation of only sequencing 16S rDNA lengths averaging 377bp, however, this is still a huge improvement on previous sequencing analysis. This however is an issue associated with 454 read lengths and not a unique problem to associate with this study. Recently Roche have provided a new GS FLX system for 454 pyrosequencing that allows sequencing reads up to 1000bp in length. The potential to use the new system in future studies would allow longer read lengths of 16S rDNA, allowing a finer analysis of bacteria down to a genus / species identification.

A comprehensive survey of microbiota and their functions should be assessed under varying diet changes including echium oil, and additionally taking into consideration any physical changes to the rumen ecosystem such as pH. Additional concentrations of echium oil should be tested to see if there are any oil concentrate dose-dependent effects upon lipid metabolism and final fatty acid composition or ruminal bacteria populations, for example (3.0%, 9.0% 15%, Echium oil/kg silage DMI).

In vitro experiments would be beneficial to attempt to test bacteria that are culturable from the known Actinobacteria class, and known species of Firmicutes in order to assess their ability to convert 18:4n-3 to 18:3n-3 and even 18:0. Further understanding of specific bacteria and their role on the metabolic process of lipid metabolism, will facilitate a better understanding of the whole rumen ecosystem. This is important for successful manipulation of ruminant diets, in order to enhance beneficial dietary fatty acids in ruminant produce.

Appendix 1

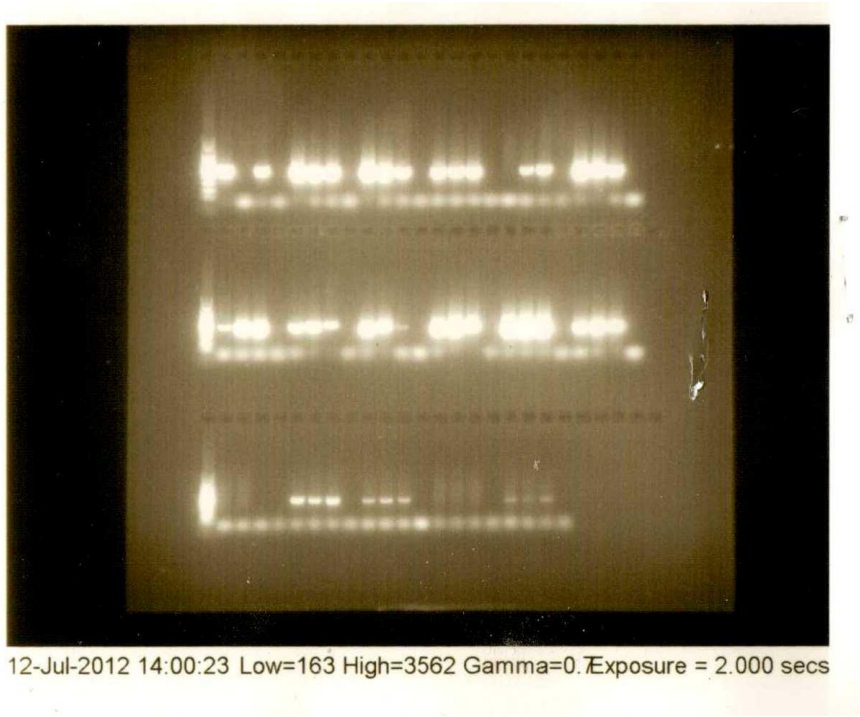
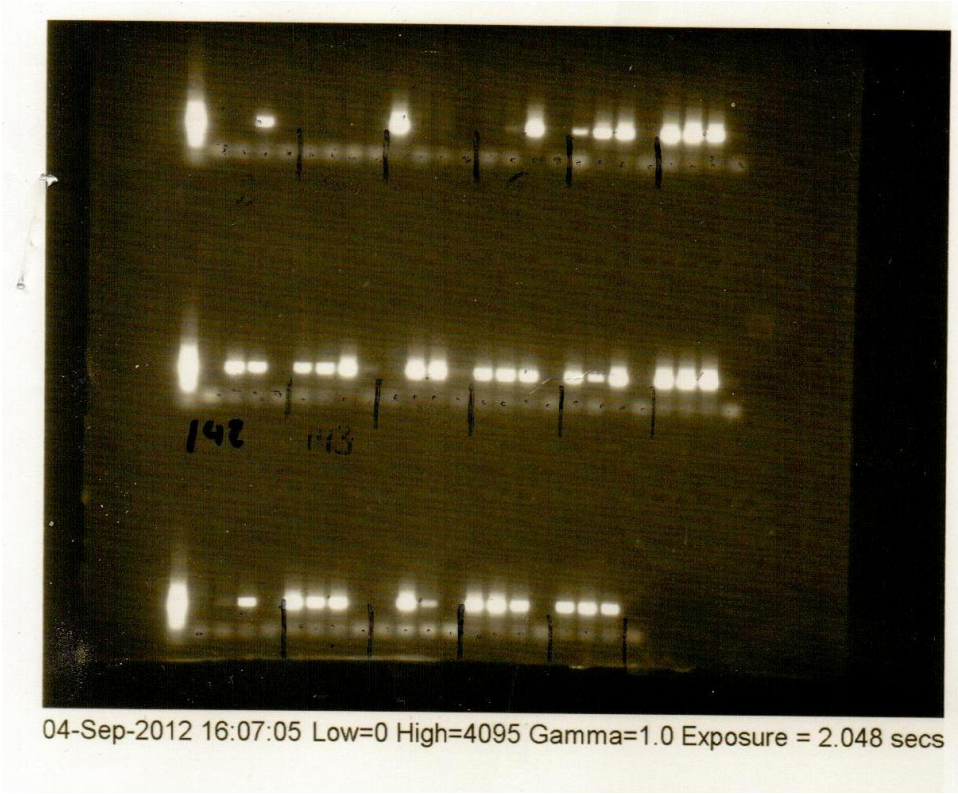


Fig.1 UV image results of PCR products from all 17 rumen samples in triplicate with one negative sample, ran through agarose gel electrophoresis and visualised under UV light using the bio-Rad Gel Documentation system. This checks the samples for PCR quality and to assure there was no contamination between samples.

(a)



(b)

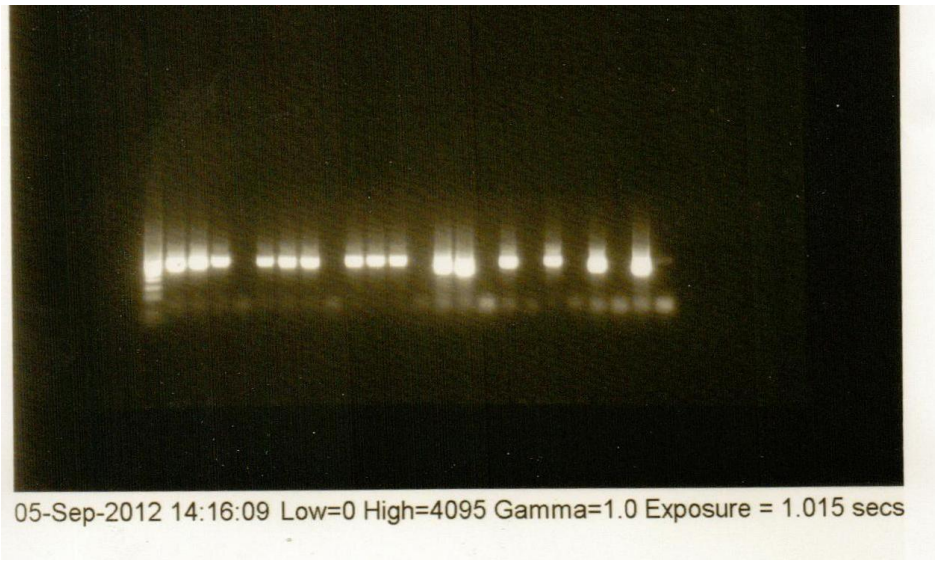


Fig.2 UV image results of PCR products from all 17 rumen samples after amplicon cleanup process via gel excision, ran through agarose gel electrophoresis and visualised under UV light using the bio-Rad Gel Documentation system. (a) First UV reading of all 17 samples in triplicate with one negative sample. (b) Repeat of 6 samples that did not give clear readings from first UV reading.

Appendix 2

The following script represents the exact input of commands used in the Qiime software for semi- quantitative lipid and rumen bacterial data analysis. This is to be referenced with the methods section for data analysis of the 16S r DNA sequences obtained from 454 pyrosequencing. It should be noted that the full content of data from 16S rDNA 454 analysis via Qiime is available in lipid sequencing Qiime data folder provided on M.Sc dissertation CD.

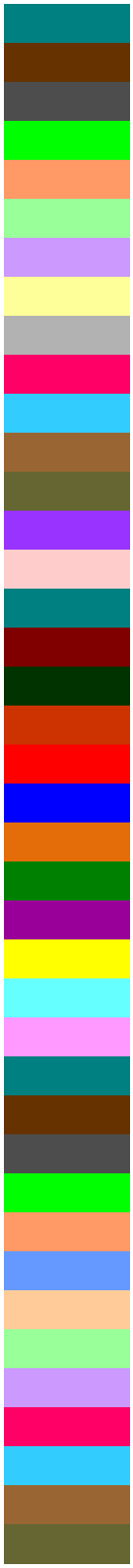
```
#!/bin/bash

# interactive commands are commented out

#print_qiime_config.py

# Pre-processing

echo "Check mapping file"

rm -rf mapping_output ; check_id_map.py -m sharon_mid.txt -o
mapping_output -v

echo "Demultiplexing"

rm -rf split_library_output ; split_libraries.py -m sharon_mid.txt -f
Reads2.fna -q Reads2.qual -b 10 -l 50 -o split_library_output

# otus

echo "Pick OTUs through OTU table"

rm -rf otus ; pick_otus_through_otu_table.py -i
split_library_output/seqs.fna -p sharon_parameters.txt -o otus

#per_library_stats.py -i otus/otu_table.txt

#OTU Heatmap

echo "OTU Heatmap"

make_otu_heatmap_html.py -i otus/otu_table.txt -o otus/OTU_Heatmap/

#OTU Network

echo "OTU Network"

make_otu_network.py -m sharon_mid.txt -i otus/otu_table.txt -o
otus/OTU_Network

#Make Taxa Summary Charts
```

```
echo "Summarize taxa"

rm -rf wf_taxa_summary ; summarize_taxa_through_plots.py -i
otus/otu_table.txt -o wf_taxa_summary -m sharon_mid.txt

echo "Alpha rarefaction"

#alpha_diversity.py –h

echo "alpha_diversity:metrics
shannon,PD_whole_tree,chao1,observed_species" > alpha_params.txt

rm -rf wf_arare ; alpha_rarefaction.py -i otus/otu_table.txt -m
sharon_mid.txt -o wf_arare/ -t otus/rep_set.tre

echo "Beta diversity and plots"

rm -rf wf_bdiv_even146 ; beta_diversity_through_plots.py -i
otus/otu_table.txt -m sharon_mid.txt -o wf_bdiv_even146/ -t
otus/rep_set.tre -e 146

echo "Jackknifed beta diversity"

rm -rf wf_jack ; jackknifed_beta_diversity.py -i otus/otu_table.txt -t
otus/rep_set.tre -m sharon_mid.txt -o wf_jack -e 110

echo "Make Bootstrapped Tree"

make_bootstrapped_tree.py -m
wf_jack/unweighted_unifrac/upgma_cmp/master_tree.tre -s
wf_jack/unweighted_unifrac/upgma_cmp/jackknife_support.txt -o
wf_jack/unweighted_unifrac/upgma_cmp/jackknife_named_nodes.pdf

echo "Make Weighted Bootstrapped Tree"

make_bootstrapped_tree.py -m
wf_jack/weighted_unifrac/upgma_cmp/master_tree.tre -s
wf_jack/weighted_unifrac/upgma_cmp/jackknife_support.txt -o
wf_jack/weighted_unifrac/upgma_cmp/jackknife_named_nodes.pdf
```

Appendix 3

Table.1. The following table represents the full taxonomic classification down to order of 16S rDNA analysis from all 17 ruminal samples. Note full taxonomic area charts showing % abundance of individual taxonomy species available in lipid sequencing Qiime data folder (wf_taxa summary, taxa summary plots, area charts.) provided on M.Sc dissertation CD.

Legend	Taxonomy
	Root;Bacteria;Actinobacteria;Actinobacteria;Actinobacteridae;Actinomycetales
	Root;Bacteria;Actinobacteria;Actinobacteria;Actinobacteridae;Other
	Root;Bacteria;Actinobacteria;Actinobacteria;Coriobacteridae;Coriobacteriales
	Root;Bacteria;Actinobacteria;Actinobacteria;Other;Other
	Root;Bacteria;Actinobacteria;Actinobacteria;Rubrobacteridae;Rubrobacterales
	Root;Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Other
	Root;Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Porphyromonadaceae
	Root;Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Prevotellaceae
	Root;Bacteria;Bacteroidetes;Other;Other;Other
	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Sphingobacteriaceae
	Root;Bacteria;Chloroflexi;Anaerolineae;Caldilineae;Caldilineales
	Root;Bacteria;Chloroflexi;Anaerolineae;Other;Other
	Root;Bacteria;Fibrobacteres;Fibrobacteres;Fibrobacterales;Fibrobacteraceae
	Root;Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae
	Root;Bacteria;Firmicutes;Bacilli;Bacillales;Other
	Root;Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae
	Root;Bacteria;Firmicutes;Bacilli;Bacillales;Planococcaceae
	Root;Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae
	Root;Bacteria;Firmicutes;Bacilli;Bacillales;Thermoactinomycetaceae
	Root;Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae
	Root;Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae
	Root;Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae
	Root;Bacteria;Firmicutes;Bacilli;Lactobacillales;Leuconostocaceae
	Root;Bacteria;Firmicutes;Bacilli;Lactobacillales;Other
	Root;Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae
	Root;Bacteria;Firmicutes;Bacilli;Other;Other
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XI
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIII
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Other
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae
	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae



Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Syntrophomonadaceae
Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae
Root;Bacteria;Firmicutes;Clostridia;Other;Other
Root;Bacteria;Firmicutes;Erysipelotrichi;Erysipelotrichales;Erysipelotrichaceae
Root;Bacteria;Firmicutes;Other;Other;Other
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Other;Other
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Methylobacteriaceae
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Other
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae
Root;Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae
Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae
Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae
Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Other
Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae
Root;Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Neisseriaceae
Root;Bacteria;Proteobacteria;Betaproteobacteria;Other;Other
Root;Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae
Root;Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae
Root;Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae
Root;Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Other
Root;Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonales;Other
Root;Bacteria;Proteobacteria;Deltaproteobacteria;Other;Other
Root;Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Campylobacteraceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Succinivibrionaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Cardiobacteriales;Cardiobacteriaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Coxiellaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Legionellaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Other;Other
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae
Root;Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae
Root;Bacteria;Proteobacteria;Other;Other;Other
Root;Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae
Root;Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Other
Root;Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Subdivision 3
Root;Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Subdivision 5
Root;Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae

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