Effects of rumen bacterial lipases on ruminal lipid metabolism

Submitted in part candidature for the degree M.Sc., Institute of Biological, Environmental and Rural Sciences, Aberystywth University

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Abstract

With an estimated world population growth predicted to reach 9.2 billion by 2050, and heightened fatty food related diseases exhausting NHS budgets, the need to ensure food security in terms of availability and nutritional quality is paramount. Foods that are deemed to be of a high nutritional value, that provide health promoting benefits through their consumption are increasingly sought after. Ruminant meat and meat products make up a large proportion of Westernised diets. A key global target is to ensure the securing of ruminant food products to maintain rising populations, on the same land area that we have today, and in a sustainable manner. Ruminant forages are naturally rich in human health beneficial polyunsaturated fatty acids (PUFA-C18:3 *n*-3 and C18:2 *n*-6), yet meat and milk are high in SFA which are detrimental to human health (C18:0), due to microbial lipid metabolism in the rumen. Lipolysis results in the release of FFA which are subsequently biohydrogenated to SFA (with many intermediates –with C18:2 cis-9 trans-11 CLA also being beneficial to human health) by rumen bacteria mainly. This biohydrogenation of the double bonds in PUFA by rumen bacteria is essentially a defence mechanism, due to their toxicity towards the rumen bacteria. Strategies that increase lipolysis offer a potentially novel method of decreasing the process of biohydrogenation through an increase in the release of more PUFA that is toxic to the biohydrogenators. This would ultimately provide red meat containing more health beneficial fatty acids. The aim of this experiment was to establish whether two isolated lipases from the rumen metagenome, as well as one commercially available lipase offer a novel strategy in enhancing the FA profile of ruminant meat and meat products through dietary supplementation *in vitro* by affecting ruminal lipid metabolism. We examined the FA metabolism through experimental extractions; TLC and methylation of FFA fractions were analysed using GC. The addition of both of the lipases sourced from the rumen metagenome (Pl 1 and Pl 2) had no effect on C18:3 *n*-3 and C18:2 *n*-6 produced. The commercially sourced lipase (A1) increased C18:3 *n*-3 and C18:2 *n*-6 so had the most effect on lipolysis and in some incubation reduced C18:0 therefore affected biohydrogenation. In others however, CLA and C18:0 production also increased through the use of this lipase, although concentration and time where determining factors.

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List of abbreviations

ºC	Degrees Celsius
A_{405}	Absorbance measures at 405 nm
nm	Nanometer
BSA	Bovine serum albumin
CLA	Conjugated linoleic acids
DHA	Docosahexaenoic acid
DTT	Dithiothreitol
EPA	Eicosapentaenoic acid
LN	Linoleic acid
LNA	α-Linolenic acid
VA	Vaccenic acid
g	Gravitational force (9.81 m/s)
GC	Gas Chromatographer
FAME	Fatty acid methyl ester
h	Hour(s)
IBERS	Institute of Biological, Environmental and Rural Sciences
TAG	Triacylglycerols
DAG	Diacylglycerols
PF	Polar fraction
FFA	Free fatty acids
SFA	Saturated fatty acids
UFA	Unsaturated fatty acids
FA	Fatty acids
PL	Phospholipases
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids

n	Omega
mg	Milligrams
µL	Microlitre
L	Litre
g	Gram
PTFE	Polytetrafluoroethylene
MES	Morpholineethanesulfonic acid
mM/ min	Milimolar/minute
M	Molar (mol/L)
min	Minutes
mM	Milimolar
rpm	Revolutions per minute
U	Units
UV	Ultraviolet
v:v	Volume to volume ratio
µM	Micromolar (µMol/L)

1.0 INTRODUCTION

The ever expanding human population is one of the biggest drivers affecting how well the planet will adapt, or adopt a sustainable way of living in the future. An estimated population of 9.2 billion people by 2050 (FAO, 2003) coupled with extreme weather patterns blight future attempts for the provision of a secure food supply. Sustainable opportunities for society need to be provided, those that will improve food security for all (The Ecologist, 1976). Affluence and the availability of cheaper food have meant that the consumption of meat has increased steadily overtime, whilst the cost of food has decreased. Unfortunately this reduction in price has encouraged the unnecessary waste of food as well as over indulgences, because food has become 'cheap and robust'. The quantity of food that is actually utilised by households, compared to what actually is produced and harvested is reportedly less than half (McMichael *et al.*, 2007).

Current figures on meat consumption reflect its continued popularity as a main component of protein in modern day diets. According to the 2006 'Livestock's Long Shadow' report, globally the potential demand for meat will increase from the 229 million tonnes between 1999 and 2001, to an estimated 465 million tonnes by 2050 (Steinfeld et al, 2006). The adoption of a more westernised diet in China is just one reason for this increase in demand for meat, in particular ruminant products. Not only has the consumption of meat increased, but some countries have also increased their consumption of milk. Indeed, China is now reportedly the largest producer of beef, eggs and pig meat worldwide. The importance of children having meat included in their diet, for effective brain development and health is well known (Kingston-Smith *et al.*, 2010). The lack of meat in these precious developing years can leave children to be nutritionally deficient, at a key stage in development that could influence their future wellbeing.

To increase production further, some countries are opting for a more industrialised farming approach rather than traditional. If the aim in the future is to produce and rear more animals to meet the demands of the consumer, then this aim ought to evaluate amongst others, the increase in the production levels, and the effects of the associated greenhouse gas (GHG) emissions. The challenge for policy-makers now is to balance food security as well as its future effect upon climate change. Questions have already been asked whether or not the food that we choose to eat will have an effect on environmental stability? And whether the rearing of more ruminant livestock than before is leaving behind a greater carbon footprint to

meet the demands of more developed countries? (Marlow *et al.*, 2009). Vulnerable populations in the developing world rely upon the income that they gain through the export of food. The scaling up of production may in the short-term be seen as a benefit to them, but the potential effects long-term cannot be ignored. These countries are likely to experience hardship through reduced food productivity in the future if climate change behaves as predicted, and the land becomes less productive (McMichael *et al*., 2007).

1.1 LAND USE FOR ANIMAL PRODUCTION

The land area that will be available for future food production cannot be increased due to the fact that it has been increased to maximum levels over the past few years (McMichael *et al.*, 2007). If climate change were to affect the productivity of food in years to come, then severe repercussions would follow for food security. Whilst ruminant meat production does have an impact on the environment in terms of GHG production, these systems produce food resources for human consumption out of land resources that cannot be exploited in another way without causing effects on vital carbon stores within the soils (Pullar, 2010). Lowering the associated environmental impacts, through the production of meat and milk has already been attempted by some countries. For instance, raising their cattle on land that cannot be utilised for growing crops; encompassing the nutrients that would not usually contribute to the food chain (Fairlie, 2010). This is what is termed within the FAO's report 'Livestocks Long Shadow' (2006) as adopting a 'default land user strategy', because the animals can graze on land that would once have had no input to the farm; making use of all the resources available through nutrient shifting for instance (Steinfeld *et al.*, 2006). Within this same report, an 'active land user strategy' is also defined. This is one were the land user competes to produce the most it can from the land resources they have available to them. The land is used as partial pasture; suitable land can be used to grow crops for human consumption; and other land for crops destined as animal feed. This strategy dominates over what may alternatively thrive naturally within the environment, whereas the 'default land user strategy' makes use of any waste-land, marginal areas, and surpluses to produce non-specified goals that are usually not steered towards market demand (Fairlie, 2010). Of course meeting the demand of the world's meat requirements cannot be achieved by following a 'default land user strategy', but if some small areas within different countries were to diverge in this way then there may well be environmental gains that follow.

Regular grazing of livestock animals, in a well managed system aid carbon sequestration into the soil through the steady flow of nutrients being transferred back into the earth, aided by the animal's production of effluents accelerating the growth and the decay of the plant materials; photosynthesis has been shown to increase, which enhances carbon sequestration (Pullar, 2010). Clearly the issue does not lay with food production alone. This is a multifaceted issue that also includes logistics of food distribution, the size of the allocations, and of course wealth pushing up the purchase prices.

1.2 INTENSIVE FARMING

In the future, food production will need to be scaled up in order to meet population growth, whilst utilising less land and fewer inputs. Efficiency during the production process must increase, waste must be reduced, and any impacts to the environment must be minimal if at all. As we know, the population is rising faster in developing countries than anywhere else on the planet, and local availability for paid employment is at a premium. Industrial sized farms require less manpower than traditional farms, and require an estimated seven kilograms (Kg) of grain to sustain the cattle which go on to produce one Kg of beef (Horrigan *et al.*, 2002), compared to that of pasture-based forage systems. Small farmers in the vicinity can become swallowed up as they have little or no chance of competing with the corporate giants developing this method of farming.

Wales has a strong farming history forged by the efforts and skills of the generations past and present, and their management strategies. This rural environment has provided the country with a strong and reputable trade of Welsh beef and lamb both throughout the United Kingdom (UK), as well as for export. Biodiversity has also been promoted through the responsible management of agricultural practice of the livestock farmers in Wales, resulting in a somewhat symbiotic existence through carefully managed pastoral based habitats. Biodiversity in these rural areas have been supported through well-managed livestock grazing. Herbage such as bracken and other scrub like plants are maintained, and the requirement for essential wildlife corridors that hedgerows provide for native species remain in place (Pullar, 2010).

Termed as 'landless', industrialised farming systems are presently growing in abundance elsewhere across the globe. Promoters claim that the adoption of this method of

farming livestock could be the answer to the land availability issues that may occur in the future, if predictions are to be believed for an exponential growth in the population. But biodiversity in these grassland areas as a whole must be considered. Native bird species such as the house sparrow, and some species of insects are just a few examples of animals that are affected through the land-use changes that have already taken place on agricultural lands in the UK in the last decade. We should learn from what has already been observed in some developing countries before decisions for the adoption of such farming strategies are made for the UK, and our role within future food securing. The establishment of industrialised methods of agriculture could take over our current rural communities, leaving some of them destitute and potentially destroying their means of a livelihood. The uplands areas of Wales for instance, being of an extreme nature may only be suitable for current livestock practices. If this environment were to change, then the biodiversity in these areas would also be affected. This would potentially impose further food security problems due to major shifts in other human-prey species' food chains. Species that were maintained through grazing sheep may out-compete others, potentially affecting other species and the plants and insects that they have come to depend on. The rural lands and green spaces form the character of Wales. The loss of the grasslands in Wales would cause implications on other industries such as tourism, further damaging livelihoods.

Welfare issues can also manifest, such being an increase in the outbreak of in-house livestock diseases (Bellaver & Bellaver, 1999). Ethical issues would also arise, with respect to the associated question of land-use wastage to produce otherwise human-edible grain in order to raise these in-house animals. These systems are relatively cheap due to their high productivity, their use of little land when compared with a conventional mixed farm, and that they can be located nearer to transport or wholesale networks (McMichael et al. 2007). Conversely however, the increase grain requirement would mean that fertiliser and pesticide applications would also follow suit. DEFRA reported in 2009 that over the ten years prior to the report, nitrogen (N), phosphate and potash levels caused through fertiliser applications in England and Wales had halved to 52 kg/ha, 7 kg/ha and 12 kg/ha respectively. And the sector with the least impact through the little use of fertiliser was that of the pasture-based ruminant livestock farmed for their meat. This trend can only continue if grasslands are maintained as they are currently in the UK. Receiving few inputs, and continuing to act as a major carbon store, these systems provide a food resource that is fit-for-purpose for the rearing of ruminant animals; ultimately benefitting meat and meat product resources for human consumption

(Pullar, 2010). Moral issues of whether or not this increased necessity for grain could not actually be better distributed between poverty stricken zones need to be considered.

Large-scale industrial farms also require a multitude of machinery, with vast amounts of fuel and energy inputs necessary to operate them. Water needs to be supplied in high volumes, as well as additional inputs from pesticides and fertilisers. These resources need to be available around the clock, so that the "farm" and its produce remain viable. Estimates suggest that on a global scale, agriculture uses 70% of the water that the planet has available. This figure is already concerning, and if in-house production of meat increases in the future to meet the needs of the expanding populations, then an increase in the percentage of water required is going to manifest. Renewably sourced energy options currently fall short of what is required to maintain these extensive monoculture farms; so non-renewable fossil fuels are heavily relied upon at a cost to the environment. What is needed is a global intensification of sustainably resourced agricultural produce.

1.3 AGRICULTURE AND THE ATMOSPHERE

It has been well documented that ruminant animals produce GHG emissions through the processes that are involved in the digestion of their feed (Scollan *et al.*, 2006). There are major challenges that the livestock industry faces presently, to meet targets that are now expected with regards to GHG reduction by the government (Pullar, 2010). Approximately, methane (CH_4) makes up 20% of the global GHG emissions that are attributed to agriculture (Kingston-Smith *et al.*, 2010), and 72% of all the (CH_4) produced in Wales is attributed to agriculture (Moss *et al.*, 2000). Ruminant livestock can produce up to 30 litres of CH_4 for every kilogram of dry matter (DM) that they eat. This calculation equates to a considerable amount of methane being produced in the agricultural sector; per cow 600 litres of CH_4 can easily be produced per day. In Wales, manure from livestock only produces CH_4 when it is kept under anaerobic conditions. As most of the livestock animals in Wales roam around in fields, the global contribution is roughly 5%. Furthermore, aerobic digestion of faeces in the field produces trivial amounts of CH_4, so the majority of the CH_4 produced in Wales is from enteric fermentation in the gut. Ruminants also typically make use of as little as 20% of the N in the forage, with most being lost in excreta and contributing to nitrous oxide (N_2O) emissions (through the fermentation of the N in the urine and bacteria in the faeces coming

into contact), which is another potent GHG with 23 times the potential of CH_4 (Kingston-Smith *et al.*, 2010). Increasing the utilisation of forage N would result in less N_2O emission (Kingston-Smith *et al.*, 2010). Thus, any strategies to ensure food security must also take into account GHG emissions. Reducing CH_4 and N_2O levels that are attributable to ruminant meat and milk production is essential. The amount of, and the types of feed, as well as the degradability of the carbohydrate proportions involved that these animals are reared upon are the key factors in determining the levels of the GHG emissions being produced (Reynolds *et al.*, 2011). Improving the fatty acid (FA) profile of meat through dietary supplementation has the potential to benefit the health of the consumer, as well as reducing GHG emissions produced through enteric fermentation, doubling the importance of the research (Lourenço *et al.*, 2010).

1.4 THE IMPORTANCE OF A BALANCED DIET

The 'carrying capacity' for all species other than humans influences the success of that population to survive in that particular niche. Humans are the only species that can modify this natural barrier through their ability to adapt and mould their chosen habitat around to suit them. On average humans in developed countries eat more meat per day than is thought to be beneficial for us (224 g/day consumed and recent reports suggest 90g/day is optimal for human health; McMichael *et al.*, 2007). Increased consumption potentially poses elevated health risks to human society, due to the associated high levels of saturated fatty acid (SFA) content of ruminant products and its association with coronary heart disease (CHD) (Scollan *et al.*, 2006; Lourenço *et al.*, 2010; Huws *et al.*, 2011). Affluence and 'a fast food culture' are two examples that have contributed to such modern-day human-food related health issues. Nonetheless, as part of a balanced diet red meat is crucial for providing micronutrients for our optimal development. Essential nutrients such as iron, long-chain *n*-3 FA, zinc, protein, selenium and vitamins D and B_{12}; some of which only become bioavailable to us only through the moderate consumption of meat (Wyness *et al.*, 2011). Research into improving the FA content of ruminant meat that we produce through diet or genetic improvements for instance will further enhance the nutritional gain derived through red meat consumption. Understanding ruminants and rumen function are key practices to resolving our future agricultural challenges. The quality of the produce being improved also ensures the securing of food in the sense of providing available nutritional food for the future.

Food resources that contain sought after essential FA, which provides positive effects upon the metabolism of the consumer are increasingly sought after. UK government health guidelines already exist with respect to the amount of polyunsaturated fatty acids (PUFA) and SFA that are predominantly consumed daily (Scollan et al., 2001b). Humans are prone to certain diseases when their dietary intake of foods are high in SFA, and exceed recommended levels (Stanner & Frayn, 2005). In Westernised diets, meat products derived from ruminant and other animals occupy a large portion of people's weekly food groceries, even though fish contains higher levels of some of these sought after *n*-3 C_{20} PUFA, the majority choose meat (Scollan *et al.*, 2001b). The environment within the rumen and the process of biohydrogenation makes any attempt to manipulate the FA profile of the ruminant meat very difficult (Wood & Enser, 1997), and results in the saturation of ingested unsaturated fatty acids (UFA) (Lourenço *et al.*, 2010). Different lipases that can be taken in through diet have a considerable amount of variation also, when it comes to their reaction specificities. For example some are attracted to shorter chained FA, such as acetic, butyric or decanoic acids or they may prefer UFA such as oleic acids (Patil *et al.*, 2011). Interest has been largely associated with the modification of lipid composition, because of the effects that a high SFA diet could have upon future risks in developing CHD in humans. Over the past 15 years the improvements seen in animal breeding programmes, animal feed modifications and modern food processing and butchering techniques have all contributed to the fat contents of meat carcasses being reduced (Wyness *et al.*, 2011). The FA profiles of different parts of the meat tissues have also been improved though investigations made into feed particle manipulation; such as either enabling dietary FA to bypass the rumens processes, or through changes made in the metabolic activity of the rumen microbes (Lourenço *et al.*, 2010). Non-ruminant species are easier to modify than that of ruminant animals, due to the composition of the fat and the influence that the rumen bacteria have upon the FA profile of the meat tissue (Higgs, 2000).

1.5 RUMINANTS AND THEIR MICROORGANISMS

Ruminants partly lend their name to the way in which they 'chew the cud' and repeatedly regurgitate feed when processing their food. Unlike other non-ruminant animals, ruminants have four compartments to their stomach; consisting of a rumen, reticulum, omasum and abomasum.

Of the four compartments, it is the rumen that is the most important in carrying out the fermentation of ingesta. Living symbiotically in their own micro-biome, this anaerobic environment provides the perfect conditions for an extensive and diverse population of microorganisms (Kamra, 2005), (made up of approximately; bacteria (10^{10} – 10^{11} cells/ml, from over 50 genera), ciliate protozoa 10^4 – 10^6/ml, from 25 genera), anaerobic fungi (10^3 – 10^5 zoospores/ml, from five genera) and bacteriophages (10^8 – 10^9/ml); Hobson, 1989). Once swallowed, the food passes down the oesophagus, passing the cardiac sphincter, and then on into the reticulum (Hobson, 1989). The reticulum is the compartment that is responsible for rumination, or 'chewing the cud' as previously mentioned. Its structure is one that can be described as 'honey-comb', lending to the capture of any foreign bodies as well as ingestible matter that the animal consumes when indiscriminately grazing. Muscular contractions provide the dispersion of the repeatedly ingested feed and the rumen fluids, thus allowing rumination to commence (Dehority, 2003). The omasum acts as a 'gate-way' for the next compartment the abomasum. This compartment is made up of many folds that restrain any food particles that have escaped from being thoroughly fermented, and shifting them back towards the rumen and reticulum. Only small particles that have been comprehensively broken down go on into the abomasum. The last compartment acts similarly to how a human stomach performs. This 'true stomach' is the compartment that produces the enzymes and acids that are required to break down the protein taken in from the animal's diet, and the remaining semi-fluid mass of the partially digested food is then passed on to the duodenum (Dehority, 2003).

The rates of digestion are dependent on the particle size of the forage carbohydrate and protein fractions. The amino acids that are derived whilst rumination is taking place depend on the forage-or-feed type (Fox & Tedeschi, 2003). Digestibility rates also determined by the level of stimulation that the consumed feed initiates; a rich cell wall content of the ingested material positively affects growth-rates of the microbial community,

and increases digestibility. Microbial populations are critically affected by pH levels, which again relate to diet.

The microorganisms have themselves adapted to survive the extreme conditions that the rumen environment has to offer. A symbiotic relationship has evolved; one that enables the ruminant to ingest plant-based forage through the microorganism's fermentation of cellulose and hemicellulose, which the animal alone is incapable of digesting (Dehority, 1997). The entire microorganism community works together to break down fibre and non-fibre carbohydrates, as not one microorganism population is capable of this alone. Ammonia and amino acids (for non fibre-carbohydrates) are utilised, which provides the microorganisms with their main source of N, needed to synthesise microbial protein (Fox & Tedeschi, 2003).

This conversion of plant-forage into volatile FA (such as acetate and butyrate, as well as producing carbon dioxide (CO_2) and CH_4), serves the needs of the ruminant animal very well. Through the presence of these microorganisms, the animal is provided with the energy (ATP) that it needs to function as a living organism (Russell & Wallace, 1997). Interacting with one another, the decomposition of cellulose, lignin, protein, starch amongst other biomolecules, produce the FA that are essential for the animal's life. Any undesirable microbes that are taken in through the feed are also eliminated by these microbial communities; thus maintaining a stable environment for both the animal and themselves.

1.6 THE RUMEN MICROBIOTA

Rumen bacteria make up 50 to 60% of the biomass in the rumen, most of which are strict anaerobes. Numerous studies have been carried out over many years with the aim of discovering and identifying and classifying the complex diversity of the microbial communities located in the rumen by using individual species rRNA gene sequestration (Edwards *et al.*, 2004; Tatsuoka *et al.*, 2004; Wright *et al.*, 2004, Jenkins *et al.*, 2008), yet still the extent of the microbial diversity within the rumen is unclear (Ferrer *et al.*, 2005; Lourenço *et al.*, 2010). Edwards *et al.* (2004) reported from their investigations of assembled 16S rRNA libraries that only 11% of the bacteria present in the rumen are culturable, leaving as many as 89% unclassified because they are unculturable in the lab. Recent functional metagenomic data has indicated that most rumen celluloses originate from as yet unculturable

rumen bacteria (Ferrer et al., 2005). In fact, of the anaerobic, lipolytic bacteria that have been isolated from the rumen, only six pure cultures actually belong to what are currently known 'culturable' genera (*Anaerovibrio*, *Butyrivibrio*, *Clostridium* and *Propionibacterium;* Jarvis and Moore, 2010).

Behaving symbiotically, different bacterial species work together in the rumen to breakdown ingesta. The bioconversion of lignocellulose into volatile FA by the actions of the bacteria provides the ruminant with an energy resource (ATP), and the bacteria are provided with a constant supply of food (Jenkins *et al.*, 2008). Members of the diverse *Butyrivibrio* family, namely *B. fibrisolvens* have been recognised for their association with the activities involved in biohydrogenation, but their true roles and influence are still not fully understood because the full picture is not clear with respect to uncultured species and the part that they play (Lourenço *et al.*, 2010; Huws *et al.*, 2011).

1.7 THE EUKARYOTE POPULATION

The main members of the microbial population in the rumen are made up of bacteria, protozoa, fungi and archaea and they all have their own specific roles (Lourenço *et al.*, 2010). Research into microbial lipid metabolism and how they influence PUFA levels has been carried out for over 30 years (Lee *et al.*, 2007b). The difficulty in attempting to manipulate the process carried out by these complex and diverse species, are the high risks of causing negative disruption to other ruminal activities (Lourenço *et al.*, 2010).

PROTOZOA

There are two types of ciliate species present in the rumen that differ both in their structure, and metabolism. One is the Entodiniomorphid (including genera *Epidinium*, *Entodinium*, and *Diplodinium*) which have a firm pellicle and cilia located predominantly at the anterior end on the U-shaped extrudable peristome (Williams & Coleman, 1992). And the other most occurring genus the Holotrichs are covered in cilia and have more flexible pellicles.

For many years their specificity for the rumen environment and what their role is in fermentation were unclear (Dehority, 2003). The principle role of protozoa is to digest food

resources such as plant material that would usually be indigestible for the ruminant, and aid the metabolism of the lipids and proteins from the consumed plant material (Huws *et al.*, 2010). From what is known of the microbial community present in the rumen, biohydrogenation is predominantly carried out by the bacterial communities (Kim *et al.*, 2009). Protozoal species do have bacteria present on their surfaces that are capable of partaking in biohydrogenation processes, but the protozoa themselves are not thought to contribute (Jenkins, 1993). Even though these micro-organisms are known to not play a vital role in biohydrogenation, they do contain higher levels of health benefitting CLA than do bacterial lipases, as a result of ingesting chloroplasts and invading bacteria incompletely metabolising the FA, so are important in other respects (Lourenço *et al.*, 2010). Occupying a large volume of the area available within the rumen, due to their size and not population number (105 cells/mL). These eukaryotes animals (approximately x100 larger than bacteria) also consume the rumen bacteria, which cause hydrogen (H) to be produced. Approximately one quarter of all the CH_4 that is produced in the rumen is produced in response to the H that is produced by the protozoa when feeding. The protozoa produce methane because they have methanogens living on their surfaces, so they have their own populations of bacteria living on the surface of them called Archaea.

The culturing of rumen protozoa remains today to be proving difficult for scientists. We do know that they can synthesise long-chain FA from precursor molecules found in the rumen fluid (RF), phospholipids are synthesised by the incorporation of linoleic acid (LN) with sterol esters (Bucholz & Bergen, 1973). But as yet there is little convincing evidence that these microorganisms play any significant role in lipolysis (Harfoot & Hazelwood, 1997; Jenkins *et al.*, 2008; Lourenço *et al.*, 2010).

RUMEN FUNGI

Although rumen bacteria communities are the main species that carry out biohydrogenation of FA in the rumen, certain species of rumen fungi have been found to also carry out the process (Nam & Garnsworthy, 2007). They breakdown ingested forage, including any lignin tissues that the plant material contains using cellulose and hemicellulose. This aids the bacteria that will further metabolise the FA. Three rumen microorganisms that had similar life cycles in their vegetative stages of known fungi species were analysed by Orpin (1977) to see whether they too contained chitin or cellulose in their vegetative cell walls, which are indicative of fungi. He discovered that species that had previously been identified as flagellate species of protozoa, *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis* did indeed contain chitin, so were confirmed as being true fungi, even though these species were capable of growth in an anaerobic environment, with a low redox potential (Dehority, 2003). The population of these microorganisms in the rumen is estimated between 10^3-10^6 mL^{-1}, other known genera include *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Orpinomyces*, and *Piromyces* (Wright & Klieve, 2011; Kamra, 2005). Research has discovered that the species that a ruminant animal plays host to has been found to be strictly determined by the host animal's phylogeny (Liggenstoffer *et al.*, 2010) so not all known species are found in all ruminant species.

RUMEN ARCHAEA

The archaea that exist in the rumen are strict anaerobic methanogens, and are the second largest group to the bacteria in the rumen (Lourenço *et al.*, 2010). Making up 0.3 to 3.3% of rumen 16S and 18S rRNA, it is their presence that actually causes the ruminant animal to produce CH_4 (Janssen & Kirs, 2008). The animal itself is not the producer of CH_4, it is actually a consequence of the animal being capable to digest forage substrates, and so CH_4 is produced as a way of eliminating the levels of H that accumulate in the rumen during the fermentation of plant fibres. If this H was not eliminated by the CH_4, then the fermentation of the forage would be inhibited. This means that the microbial community of archaea are driven by the processes of the gut to take the CO_2 and transform it into CH_4, as a way of eliminating the H that is present (produced by the protozoa).

1.8 RUMEN LIPID METABOLISM

Lipids perform key roles in carrying out many biological processes. One role is the regulation of metabolic pathways, which alter physiological responses (Gurr *et al.* 2002). Research has been targeted into improving current understanding of lipolysis and biohydrogenation, and how microbial communities control these processes (Jenkins *et al*, 2008). Ruminant forages, particularly fresh forages are rich in human health beneficial PUFA, yet ruminant products are rich in SFA (Scollan *et al.*, 2001b; Maia *et al.*, 2010; Huws *et al.*, 2011) due to lipid hydrolysis of double bonds by the rumen microbiota (a process known as biohydrogenation; Scollan *et al.*, 2006; Lourenço *et al.*, 2010; Huws *et al.*, 2011). This is due to the fact that anaerobic bacteria hydrogenate some of the unsaturated FA such as linolenic acid (*n*-3), causing a chain of reactions that ultimately produce SFA such as C18:0 (Scollan *et al.*, 2011).

Harfoot and Hazelwood (1997) categorised the bacteria that are involved in the process as two distinct groups, group A and group B (Figure 1 & 2). Group A included the bacteria that hydrogenate PUFA C18:3 *n*-3 and C18:2 *n*-6 into SFA C18:1 trans-11 (VA) where their effect was thought to end: conversely group B bacteria were thought to be capable of converting the same PUFA as those of group A, but they are more capable of hydrogenating a wider range of FA, ending with C18:0 flow from the rumen. As molecular techniques have advanced, the bacteria that are involved along the pathway are now known to be species that are unculturable in the lab (Lourenço *et al.*, 2010; Huws *et al.*, 2011). Due to modern advances providing clearer insight in to the steps involved in biohydrogenation pathway (Figure 3); and clearer known taxonomy of the bacteria involved in biohydrogenation (Wallace et al., 2006). Lourenço *et al.* (2010) thought it to be appropriate to describe specific bacteria sensitivity and enzyme specific mechanisms when describing the steps involved in the biohydrogenation pathway, rather than grouping them (Figure 1 & 2).

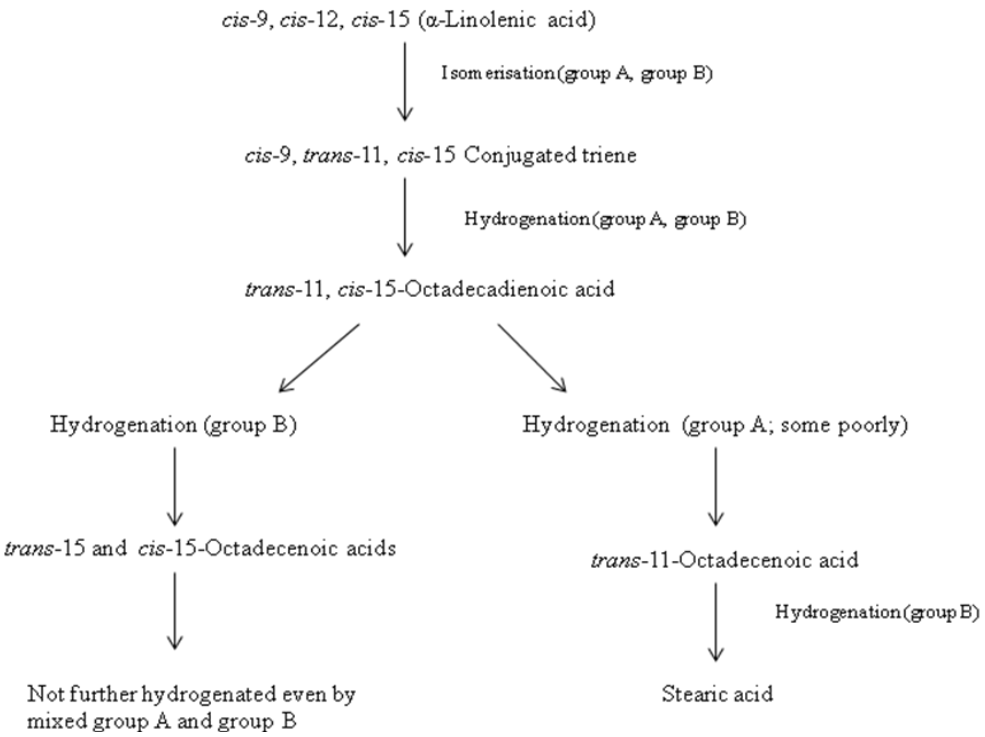


Figure 1 Scheme of biohydrogenation pathway of α-Linolenic acid; showing that there are two groups of bacteria (A and B) involved (Taken from Harfoot & Hazelwood, 1997).

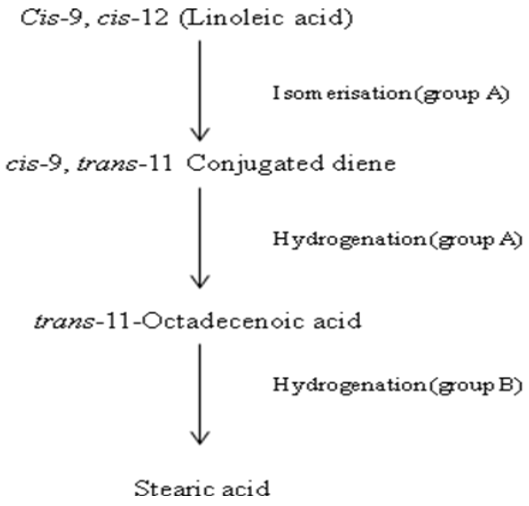


Figure 2 Scheme of biohydrogenation of Linoleic acid; showing two groups of bacteria (A and B) involved (Taken from Harfoot & Hazelwood, 1997).

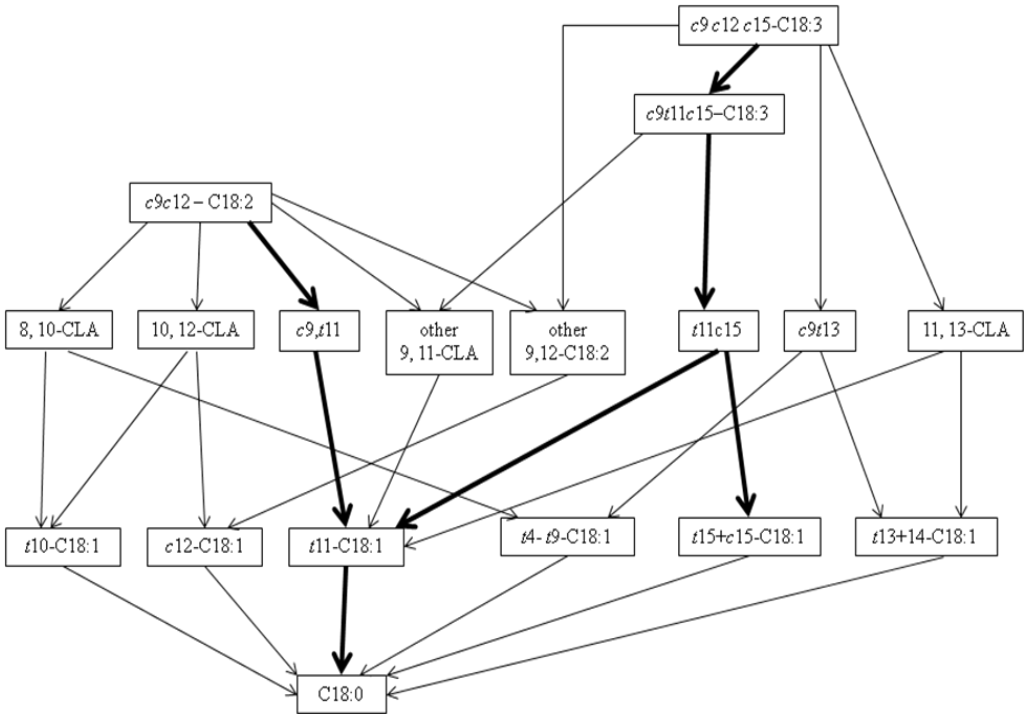


Figure 3 Biohydrogenation pathways of fatty acids substrates linoleic acid (c9c12-C18:2) and α-linolenic acid (c9t11c15-C18:3) in the rumen. CLA = conjugated linoleic acid. Taken from Lourenço *et al.* (2010), adapted from Chilliard *et al.* (2007).

The main FA substrate taken in through foraging livestock is *cis*-9, *cis*-12, *cis*-15-C18:3 (*n*-3; LNA). This is because leaf lipids predominantly contain glycolipids and phospholipids (some triacylglycerols depending on the forage), and animals that are reared mainly on grain feeds composed of triacylglycerols, the main biohydrogenating substrate is *cis*-9, *cis*-12-C18:2 (*n*-6; LN) (Lourenço *et al.*, 2010). The metabolism of LN and LNA pathways follow a similar process, although due to the three double bonds that need to be reduced, the biohydrogenation pathway of LNA is slightly more complex (Harfoot & Hazelwood, 1997). From metabolised LN, CLA (mainly *cis*-9, *trans*-11-C18:2 known as rumenic acid) is rapidly formed; This is then transformed to VA (*trans*-11-C18:1), and finally stearic acid (C18:0) (Figure 2 & 3). Isomerisation of LNA involves the formation of more conjugated linoleic acid (CLA) intermediates than previously thought (Figure 1 & 3; Chilliard *et al.*, 2007; Lourenço *et al.*, 2010). From the isomerisation of LNA that is mixed in rumen digesta, conjugated triene, *cis*-9, *trans*-11, *cis*-15-C18:3 (discovered by Wąsowska *et al.*, 2006; potentially the factor they observed that accelerated hydrogenation of LN and oleic acid; Wilde & Dawson, 1966). Another two conjugated triene intermediates *trans*-9, *trans*-11, *cis*-15-C18:3 and *trans*-11, *cis*-15-C18:2, thought to offer the same potential health benefitting as known CLA (Figure 3; Lourenço et al., 2010).

Lipolysis, biohydrogenation and lipid metabolism can be affected by changes in the rumen environment. Decreased pH levels in the rumen can also affect the rate of bacteria that are involved in the hydrolysis of the FA pathway (Van Nevel & Demeyer, 1996). For example, changes in pH can be as a result of high grain diet, such that are required for in-house systems. LA (*cis*-9, *cis*-12-C18:2) is rapidly transformed to trans-9, cis-12 isomerase by the bacteria *Megasphaera elsdenii* and *Streptococcus bovis*. CLA (*trans*-10, *cis*-12-C18:2) is then produced as a result of this flux pathway, forming *cis*-12 reductase from the rapid action of *Megasphaera elsdenii* and *Streptococcus bovis*, producing *trans*-10-C18:1, and then stearic acid (C18:0), saturating the tissues (Lourenço *et al.*, 2010).

With major human health associated issues through the intake of SFA in the diet, and the known effects that the microbial metabolic activity in the rumen and biohydrogenation have upon the ingested PUFA (Maia *et al.*, 2006), new discoveries could provide novel strategies in controlling the biohydrogenation of unsaturated FA and improving meat and milks PUFA, CLA and omega 3 (*n*-3) FA content (Scollan *et al.*, 2001b). The toxic effects that linolenic acid has upon the bacteria for instance, that would normally go on to form the SFA stearate through its biohydrogenation pathway were reported by Chaudhary (2004). Such toxic effect have been increasingly researched, and have been found to result in a reduced SFA profile if there are higher levels of LN (PUFA) present at the start of the biohydrogenation pathway. The toxic effects that PUFA can have upon the bacteria provide potential novel strategies for the increase of PUFA deposited into ruminant meat and milk products, and improving their health benefits (Pariza, 2004; Maia *et al.*, 2006).

The dominant role of *Butyrivibrio* plays in the biohydrogenation of FA has been understood for some time, but not fully (Wąsowska *et al.*, 2006; Maia *et al.*, 2006). Research is underway into investigating the effects that all the bacteria within the *Butyrivibrio* group, acting together can impact on the biohydrogenation processes (Maia *et al.*, 2006). Given that very little is actually known about the entire bacterial community in the rumen, but yet we do know certain FA have useful bacteriocidal effects, it seems likely that future research into FA pathways, and the effects FA manipulations have upon ruminal bacteria are key to improving the FA composition of ruminant products (Maia *et al.*, 2006). For example the uncultivated 16S rRNA genes that were recovered (Edwards *et al.*, 2004), have yet to be classified, which could provide significant effects upon biohydrogenation (Maia *et al.*, 2006).

Problems arise however in attempting to understand the ecology, and the metabolic potential that this diverse microbial community in the rumen and the gastrointestinal tract contain (Brulc *et al.*, 2009). This is because the microorganism community that are present in each animal are influenced greatly by the type of forage or feedstuff that the ruminant animal predominantly ingests. Additional to this, Brulc *et al.* (2009) examined the microbial community of three same species animals raised on the same diets, and discovered that each animal's utilisation of the nutritional components of the feed displayed variation also. The entire genetic diversity of the rumen biome is far greater than what has been reported previously in individual constructions of molecular libraries with small data sets. Ferrer *et al.* (2005) screened only 7% of the genome libraries available, constructed of the microbes that are found in the rumen of cattle, and discovered many (and some new) enzymes (Ferrer *et al.*, 2005). Brulc *et al.* (2009) discovered through metagenomic analysis of bovine rumen expression, four potential glycoside hydrolases that are present in as yet unculturable bacteria. This demonstrates the importance of screening the rumens dense and complex microbiome (Brulc *et al.*, 2009), for new enzymes and their associated activities that have the potential in SFA reductions in meat and dairy products (Ferrer *et al.*, 2005).

1.9 STRATEGIES FOR ENHANCING THE FATTY ACID QUALITY OF RED MEAT

Manipulating the constitution of meat in the livestock industry has been a long-term goal within the animal sciences (Azain, 2004). What is termed as adding value to the meat, through an increase in PUFA benefitting the consumer (Azain, 2004). Fat reduction upon the animal carcass through genetic selection and pharmacological agents has already been actively reduced, altering of the FA profile to one that is rich in unsaturated fats (Azain, 2004).

It has been known for some time now that feeding ruminant animals with feed that has had the fat proportions increased, can reduce the levels of methane that are produced through biohydrogenation (Reynolds *et al.*, 2011). Providing the ruminant animal with 'protected fats' reduces the process of biohydrogenation, enabling sought after PUFA to be

incorporated into the tissues of the animal. Fat is typically included in moderation for a healthy diet due to the energy that it supplies to the consumer (Azain, 2004). Boeckaert *et al.* (2008) investigated the effect supplementing the feed of three rumen-fistulated cows with algae had upon biohydrogenation. Sought after essential FA C18:2 *n*-6 and C18:3 *n*-3 levels were observed to increase, which affected their usual transformation to C18:0 stearic acid. The addition here of algae was also found to affect the bacterial populations, in particular the effects upon the total number of *Butyrivibrio* bacteria (7.06 log copies/g rumen DM), resulting in both cultivated and non-cultivated species being affected, thus interrupting biohydrogenation requirements as we know (Boeckaert *et al.*, 2008). Huws' *et al.* (2011) molecular investigations on the effect fish oil supplementation has upon bacterial communities discovered that unclassified bacterial FA pathways restricted the concentrations of *cis*-9, *trans*-11 CLA, C18:1 *trans*-11 and C18:0, when compared to pre-determined rumen digesta FA and *B. proteoclasticus* group 16S rRNA sequenced concentrations. Further investigations should explore these new revised pathways, and the wealth of bacteria present in the rumen such as classified *Prevotella, Lachnospiraceae sedis*, as well as species that are presently unclassified, such as that of *Bacteroldales, Clostridiales* and *Ruminococcaceae* (Huws *et al.*, 2011).

1.10 AIMS OF THIS STUDY

Rapid biohydrogenation carried out by the bacteria that are present in the rumen are responsible for the current high levels of SFA being produced, and deposited into the meat tissue (Lee *et al.*, 2007a & 2011; Scollan *et al.*, 2011). Usually, once the feedstuff reaches the rumen, the dietary lipids are rapidly hydrolysed and biohydrogenated (Kim *et al.*, 2009). By bypassing the process of biohydrogenation in the rumen, potentially the levels of SFA being produced would reduce, therefore aiding the promotion of red meat and meat products as a key component in human every-day diets (Scollan *et al.*, 2011).

An abundance of esterified PUFA inhibits biohydrogenation, thus ruminal lipases offer potentially novel feed supplements allowing increased incorporation of PUFA into meat and milk (Lourenço *et al.*, 2010). At present information on the lipolytic capacity of rumen microbes is scarce. A recent metagenomic based study by Liu *et al*. (2009) isolated 2 novel rumen lipases that showed interesting phylogenetic affiliation for UFA. The author suggested

such investigations could yield sought after effects on lipid metabolism. Also 8 novel rumen lipases from metagenomic libraries have been isolated and characterised (Privé et al., 2012), two are used again in this investigation. The aims of this experiment are to establish whether the isolated lipases as well as commercially available lipase offer a novel strategy of enhancing the fatty acid quality of ruminant products through dietary supplementation *in vitro*.

2.0 MATERIALS AND METHODS

2.1 LABORATORY EQUIPMENT AND CHEMICALS

Throughout all experimental procedures and experiments, the chemicals were of analytical grade and sourced from Sigma-Aldrich Company Ltd. (Dorset, UK).

2.2 SUBSTRATE ACTIVITY

The viability of Phospholipase 1 (Pl 1), Phospholipase 2 (Pl 2) was determined using ρ-nitrophenyl laurate (C12), myristate (C14) and stearate (C18) assays, purchased from Sigma-Aldrich. This was carried out to confirm that they were still viable post-freezing under -20 conditions 3 months prior to the experiment. Phospholipase A1 was a commercially sourced lipase, nevertheless in the interest of consistency the activity was also assessed even though not being stored at -20°C for comparative purposes.

For the three enzymes (Pl 1 *vs.* Pl 2 *vs.* A1) activity levels were tested in triplicate. The enzyme activity of each was tested based on the level three different ρ-nitrophenol esters (laurate *vs.* myristate *vs.* stearate) being released (Lee *et al.*, 1993; Pinsirodom and Parker, 2001). The activity of the enzymes was quantified using a temperature-controlled Powerwave XS microplate reader (BioTek Instruments Inc., Potton, UK). For a total run-time of ten minutes, the production of the ρ-nitrophenol was monitored every minute at 405 nanometer (nm) ($\boldsymbol{A}_{405}$). Using Gen5 v1.10 software (BioTek Instruments Inc., Potton, UK), the data was collected. Each standard assays (laurate *vs.* myristate *vs.* stearate) enzyme activity was measured at 39°C, combined with 1 mM ρ-nitrophenyl ester substrates in 50 mM morpholineethanesulfonic acid (MES, pH 6.5), in the presence of 1% acetonitrile (Privé,

2011). The reaction was initiated once each of the three eluted fractions of the purified enzymes (~0.4 mg/mL) was added to their destination wells. Each of the enzyme activity assays absorbances was measured in triplicate, to obtain an average rate of release. Specifically, each was set up as follows: 194 µL of 50 mM MES (pH 6.5), 4 µL of 100 mM ρ-nitrophenyl ester solution (Laurate (wells A - C; 4 - 6) *vs.* Myristate (wells A - C; 7 – 9) *vs.* Stearate (wells A - C; 10 - 12)). For the blank reading 2 µL of deionised water was added (wells H1- H3); zero reading wells G1-3 (containing only 200 µL 50 mM MES (pH 6.5)); plate wells A4 - A12 Pl 1 (2 µL) was added; wells B4 - B12 Pl 2 (2 µL); wells C4 - C12 A1 (2 µL) was added. The plate was then placed in the micro-plate reader, were it was intermittently shaken and $\boldsymbol{A_{405}}$ (nm) was recorded every minute for ten minutes. The blank readings were set up and measured alongside the reaction readings in order to be able to subtract any values that are not as a result of lipase hydrolysis of the substrate. The ρ-nitrophenol curve was used as per Privé (2011) to translate the resulting absorbance to mM substrate hydrolysed:

$$\mu\text{mol } \rho-\text{nitrophenol/ mL reaction mixture} = \frac{A_{405} - \gamma \text{ intercept}}{\text{slope} \times 0.2 \text{ mL reaction mixture}}$$

A standard curve was established using MES (pH 6.5) but this had no acetonitrile added. Using a 0.05 to 0.50 mL of ρ-nitrophenol standard solution (0.5 mM in 50 mM MES, pH 6.5) was transferred into 12 falcon tubes (15mL) and each diluted up to 5mL with 50 mM MES (pH 6.5). Measuring 200 µL of each standard x 3, with the dilution descending in order, the wells were loaded and $\mathbf{A_{405}}$ was measured and 200 µL of 50 mM MES (pH 6.5) as a blank on the Powerwave XS micro-plate reader. Plotting the output data of the ρ-nitrophenol concentration against $\mathbf{A_{405}}$ produced the 0.005 to 0.5 µmol ρ-nitrophenol/mL standard curve.

The concentration of the ρ-nitrophenol (mM) released curve was plotted against the reaction rate over time, determining the activity of the three lipases (Pl 1 *vs.* Pl 2 *vs.* A1). The

early part of the curve was then used to position a tangent, providing the means to obtain the reaction rates of the initial stages (v_0 in mM/min). The following equation was used:

$$V_0 = \text{slope} = \frac{Y_2 - Y_1}{X_2 - X_1}$$

Accounting the protein (mg) that was present in each of the three enzymes allowed for the specific lipase activity to be determined:

$$\text{Specific activity} = \frac{V_0}{[a\ (\text{mg})\ \text{protein}]}$$

Specific activity equates to U/mg protein, were here '*a'* depicts the protein in mg that has been added, µmol/ [per minute x (mg) protein added to the mixture].

2.3 LIPASE ACTIVITY IN RUMEN FLUID

In order to assess background levels of lipases in RF the lipase activity assay was conducted on rumen fluid collected from the three cows included in the experiment, as previously described (section 2.2). This was carried out in order to provide a true evaluation of the carryover and the phospholipases in the *in vitro* incubations described below (section 2.5) to assess the effects of addition of lipases on lipolysis and biohydrogenation. This data also provided information to calculate the treatment specific concentrations to add to these incubations such that they were similar to basal levels in 100% rumen fluid, the only difference being that we added phospholipases only. Approximately there were 59 Units/mL in 100% RF, so the 50% RF that was used in the experiment, approximately containing 29.5 Units/mL. To analyse the RF from the three cows individually, and in triplicate, 81 wells of 96 total well plate were loaded as follows: Row A) 100% RF (2 µL), B) 75% (1.5 µL RF; 0.5 µL MES), C) 50% (1 µL RF; 1 µL MES), D) 25% (0.5 µL RF; 1.5 µL MES), E) 10% (0.2 µL

RF; 1.8 µL MES), F) 0% (2 µL MES). RF from Cow 1) was loaded vertically (only) in rows 1 – 3, cow 2) 4 – 6, and cow 3) 7 – 9. Then to each well 194 µL MES was loaded (50 mM), and then 4 µL stearate (100 mM) was loaded.

2.4 CHLOROFORM: METHANOL LIPID EXTRACTION PROCEDURE

Fresh perennial ryegrass *(Lolium perenne* cultivar Aberdart) was freeze-dried (FD) prior to the start of the procedure. Using a four piece digital balance, 20g of FD silage (x 3) was weighed into jars (pre-cleaned in the sonic bath), and cork bungs were fitted to temporarily close them. They were then taken to the fume cupboard, opened again and 100mL of chloroform : methanol ($CHCl_3$: MeOH) (2:1; v/v) and 100µL internal standard (C23:0, 15mg/mL $CHCl_3$) was added to each. The closed jars were swirled gently and left to sit for approximately 20 minutes. Using a conical flask, with a funnel lined with muslin, and an electrically operated pump the contents from each of the three jars were deposited in-turn to the funnel. With the aid of the pump, the liquid containing the lipids were drawn out of the dry-matter (DM) into the funnel, and collected within the clean conical flask. In order to draw out as much of the lipid as possible from the DM, the process was repeated twice; an additional 125 mL per jar, and then 80 mL per jar were added and each allowed to sit in the jars with the DM for a further 20 minutes prior to being emptied into the flask, and the pump being turned on for approximately 20 minutes. Once the entire lipid had been successfully extracted, 45 clean and dry extraction tubes (cleaned prior to the experiment in the sonic bath) were labelled, and 6mL of the extracted lipid solution was accurately dispensed into each using a pipette. Each of the tubes contained approximately 1.2g DM of plant lipid. The extraction tubes were then closed with polytetrafluoroethylene (PTFE) lined caps and stored under -20°C until the start of the experiment.

Lipid preparation post-chloroform: methanol extraction

The day before the scheduled practical experiment, the extraction tubes containing the chloroform: methanol and lipid were dried down under nitrogen in the dry block at 40^0C (Dri-Block Techne DB-3D sample concentrator). The chloroform: methanol evaporated away, leaving the lipid on the sides of the tubes. A sought after film-like residue was left on the base, and a third of the way up each of the tubes, ready for the experiment. Nitrogen was added to each of the tubes headspace, and then all were closed using PTFE caps to avoid lipid oxidation occurring.

Rumen fluid collection

Rumen contents from three non-lactating, rumen-fistulated Holstein cows was collected and squeezed to obtain rumen fluid. The dietary provisions for the cows consisted of approximately 75% grass silage and 25% straw, with water supplied constantly. These cows are outside during good weather, thus predominantly pastorally foraging at these times.

The sampling from the three mentioned rumen-fistulated cows was carried out at 8am in the morning, and approximately 1 h post-feeding. In total, a pooled 6 L of rumen fluid was collected from the cows and stored in thermo flasks to maintain rumen temperature during transit. The rumen fluid was then combined, and strained through four layers of muslin cloth so to remove any large particles that may have been collected. The rumen inoculum was placed in a 39^0C water bath and purged with CO_2 until it was required.

Anaerobic buffer and inoculation

The day before the start of each experiment, 500 mL anaerobic incubation medium is made up as described by Goering and Van Soest (1970):

Buffer solution (g/L)	Ammonium hydrogen carbonate	4
	Sodium hydrogen carbonate	35
Macromineral solution (g/L)	Di-sodium hydrogen orthophosphate 12-hydrate	9.45
	Potassium di-hydrogen orthophosphate (anhydrous)	6.2
	Magnesium sulphate 7-hydrate	0.6
Micromineral solution (g/100 mL)	Calcium chloride 2-hydrate	13.2
	Manganese chloride 4-hydrate	10.0
	Cobalt chloride 6-hydrate	1.0
	Ferric chloride 6-hydrate	8.0
Resazurin solution (g/100 mL)	Resazurin (redox indicator)	0.1
Reducing agent (g/100 mL)	Cysteine HCl	0.625
	Distilled water	95 mL
	1M NaOH	4 mL
	Sodium sulphide	0.625

Make up according to the recipe:

Distilled water	1500 mL
Micromineral solution	0.3 mL
Buffer solution	600 mL
Macromineral solution	600 mL
Resazurin solution	3 mL

On the morning of the experiment the buffer was heated in the microwave for 5 minutes. The resazurin solution and the reducing agent were then added, completing the Van Soest inoculum. The Van Soest was then placed in the water bath, set at 39^0C, and gassed with CO2.

2.5 IN VITRO EXPERIMENT

A simple batch culture experiment was carried out to determine whether three phospholipase treatments (Pl 1 vs. Pl 2 vs. A1), loaded at two different concentration levels of 10 (100 µL of 100 U/mL stock) or 25 (250 µL of 100 U/ mL stock) had an effect on lipid metabolism. One control series was included in the experiment, and referenced for each of the three treatments, incubation times, and the two treatment concentrations.

To batch cultures containing lipid residue (extracted lipids from 1g DM *Lolium perenne*) and 50% strained rumen fluid, either Pl 1, Pl 2 (both isolated from rumen bacteria) or A1 (from *Thermomyces languinosus;* Sigma Aldrich) at both 10 and 25 U/mL concentrations. At 0, 4 and 24 h of incubation anaerobically, batch cultures were destructively harvested for lipid analysis as described by Lee *et al.*(2007a).

Incubations

The 45 labelled (1 – 45) extraction tubes (20 mL total volume) containing the dried down (chloroform: methanol) lipid residue obtained from 1.2g FD ground silage *Lolium perenne* (cultivar Aberdart) per tube, together with the 50% rumen fluid inoculum (6 mL, strained using 2-fold muslin cloth and maintained under CO2 at 39^0C) using a peristaltic pump, as well as an anaerobic incubation buffer (also at 39^0C; Van Soest, 1967). Due to the concentration level of the treatments being a factor, the amount of Van Soest buffer that was added to some of the tubes was modified in order to maintain 6 (controls), 5.9 or 5.75 mL per tube (590 mL vs. 575 mL). The three treatments were A) Phospholipase 1 and B) Phospholipase 2 (both Pl 1 and Pl 2 were obtained from a rumen metagenome), and C) Phospholipase A1 (from Thermomyces languinosus; Sigma code number L3295). Treatments (Pl 1 vs. Pl 2 vs. A1) were added according to their pre-labelled destination tubes (either: nothing (control); 100 µL of Pl 1 (Pl 1); 100 µL of Pl 2 (Pl 2); 100 µL of A1 (A1) and then 250 µL to each of the 25 Units/mL tubes again in that order). The tubes were then vigorously vortexed and incubated in vitro, horizontally, in the dark on a rotating rack set at 100 rpm at 39^0C, mimicking rumen-like conditions.

Harvesting

Each of the treatments and harvest time points (0, 4 and 24h) were replicated in triplicate. The 0h triplicate incubations (total three tubes) were set up containing the extracted lipid residue, 6 mL rumen fluid and 6 mL Van Soest and serve as the 0h incubations for all treatments controls. Following harvesting at 0h, 4h and 24h incubations, the associated tubes was removed from the incubator and again vigorously vortexed.

Sampling

Each had a 500 µL sample withdrawn and transferred into separately labelled (1 to 45) 1.5 mL RNAse DNAse free eppendorfs and stored under -80^{0}C conditions awaiting future RNA based microbial analysis, but not within this thesis due to time constraints. The remainder of each of the tube's contents were closed using PTFE lined caps, frozen and stored immediately under -20^{0}C awaiting lipid extraction and quantification using methylation and thin layer chromatography procedures which will be described subsequently in this thesis.

2.6 FATTY ACID ANALYSIS

The digesta sample tubes were freeze-dried for 72 h to preserve the samples at the stages that their incubations ceased (0, 4, and 24 h) and concentration levels (10 vs. 25 µL).

The lipid extraction from the 45 de-hydrated digesta samples was then carried out using a 5, 5, 3mL chloroform: methanol (2:1; v: v) and 100 µL internal standard (C21:0 15 mg/mL $CHCl_3$). The resulting pooled extract was then split 50:50 and dried down under nitrogen using the dry block again at 40^{0}C. The first set of 45 samples tubes was re-suspended with 1 mL chloroform: methanol, and examined using Thin-Layer Chromatography (TLC) analyses, as described by Nichols (1963). The second set of 45 serum tubes were examined using 'Kramers bi-methylation' procedure to measure total lipid (TL) as described by Kramer & Zhou, 2001, and are to be discussed shortly in this section.

TLC lipid fractionation procedure

This procedure uses TLC as described by Nichols (1963) to fractionate lipids into four classes: 1) polar fraction, 2) monoaclyglycerols and diacylglycerols, 3) free fatty acids and 4) triaclyglycerols using an organic solvent chloroform : methanol/ isopropanol. The resulting separated lipids can then be transformed to fatty acid methyl ester (FAME) ready for analysis on the gas chromatographer (GC) which directly applies a tranesterification procedure as described by Sukhija and Palmquist (1988). In preparation for the TLC analysis to commence, 45 polyester plates were prepared by scoring pencil etched lines, creating two distinct sections. Each were labelled 1 through to 45 and stored in a safe place to ensure no damages to the silica gel PolyGram TLC prepared (Sil G/UV254) surfaces.

Two mobile phase tanks and a fume cupboard were used throughout this process at any one time. In order to ensure that each of the tanks was fully saturated, 50 ml of the mobile phase (2 mL acetic acid; 30 mL diethyl ester and 70 mL n-hexane) was poured into each tank and a saturation pad was placed in to each before the lids were set in place. The tanks were then left for approximately 20 minutes.

Starting with TLC tube one and plate number one, using a 200 µL pipette the polar fraction (PF) was distributed slowly and evenly along the allocated section within the sample area. Care was taken to allow each application of the PF dried, before the next layer was added over the top. This continued until the entire 1 mL sample was loaded on to the plate and allowed to dry in the fume cupboard. The surface of the plate was not scratched during the loading of the PF, as this would affect the lipids as they travel along the plate. This was repeated on plates 2 – 6 (with the corresponding samples) due to the maximum plates to be run at the same time being six. Once all the PF on each of the six plates had dried, the three pre-made standards (Free fatty acid, diacylglycerol, triacylglycerol 20 mg/mL CHCl3) were spotted in the left hand column of the pre-etched and PF loaded plates. The plates were then placed in the TLC racks (three per rack) and suspended into the tanks and then lidded. N.B care was taken that the poured level of the mobile phase in the base of the tank did not touch the PF (as this would contaminate the mobile phase). The plates were left for approximately 1 h, until the mobile phase has travelled up the plates (leaving approx. a 1 inch gap). Once the mobile phase had travelled the desired length of the plates, the developing racks were removed from the TLC tanks, and stood in the fume cupboard to dry for ten minutes. Once

dry the plates were then sprayed with 2, 7-dichlorofluorescein (100 mg dissolved in 100 mL isopropanol), and then left to dry in the dark, inside the fume cupboard for 20 to 30 minutes. The plates were then examined under UV light (only) in order to observe the different lipid band sections on the plates. The region where the PF ran up to was marked with a blunt pencil, the next where the diacyglycerols ran to, and so on. Once all four of the lipid types had been identified and marked accordingly on the plates (as indicated by the position of the standards) under UV, normal light was then restored. Because six samples were ran at one-time, 24 clean tubes (9.5 cm x 2.0 cm) were assembled in a rack, and labelled as follows: 1 PF; 1 DAG; 1 FFA; 1 TAG and so on for 2 PF; 2 DAG; 2 FFA; 2 TAG through to sample 6. The pre-marked lipid (pencil) bands were then scraped, and poured into the corresponding labelled tube. Toluene (2 mL) containing internal standard (C23 at approx. 0.4 mg/ mL) was accurately added to each of the tubes, followed by 3 mL methanolic hydrochloric acid solution (5% HCl in methanol) to each again. The samples were flushed with nitrogen, closed using PTFE lined caps, and vortexed carefully so the samples are fully mixed, but the contents are not distributed high up the sides of the tube. The rack containing the samples was placed in a pre-heated water bath (Grant Technical Specification W38 Thermo Scientific DL30) at 70^0C for 2 h. At regular intervals the tubes were swirled gently, to distribute the contents. The tubes were then allowed to cool in the fume cupboard, and then 5 mL 6% potassium carbonate was added to each carefully (to avoid foaming) and 2 mL toluene (containing no internal standard). The tubes were vortexed vigorously and then centrifuged (Beckman J6-B with swing out rotor) for 5 minutes at 1200 x g.

Another set of clean tubes was again required, labelled as previously (depicting the lipid and sample number). This set had 1g anhydrous sodium sulphide added to all; due to the colour of the PF and DAG samples these required laboratory standard charcoal (Sigma-Aldrich) to be added also (approx 1.5g to each tube). The top layer of the toluene containing the lipid (above the water layer) was removed and placed into the new set of tubes using a Pasteur pipette and glass pipette tips (changing the tip on every sample). The rack was then placed on to a shaker (Kika LaborTechnik KS501 digital) for 10 minutes, ensuring the charcoal and the sodium was evenly distributed. Then the tubes were again centrifuged (1200 x g for 5 minutes), and the top layer of the FFA and TAG samples was transferred to pre-labelled GC vials using a Pasteur pipette. The samples that contained the charcoal and sodium (PF and DAG) top-layer were pipetted into labelled plasma tubes (with funnelled stems to collect the charcoal in the bottom) and these were centrifuged at 1000 x g for 3

minutes. The top (clear) layer was then Pasteur pipetted into pre-labelled GC vials, and all had caps secured using the crimper. These GC vials were then stored in -20^{0}C until being analysed on the GC. This entire process was repeated for the remainder of the samples (six per day being the maximum).

Total lipid methylation technique

This methylation technique, as described by Kramer and Zhou (2001), was used due to it being good for transesterifying O-acyl lipids such as we have here (TAG; DAG; FFA) and phospholipids. Care was taken during this process to ensure that the grade of methanol used contained no water (anhydrous), as this would cause hydrolysis to occur and FFA would be created as a result, instead of methyl esters.

The second set of TL tubes (separated from the pooled digesta samples 50:50) were dried-down under nitrogen, at 50^{0}C using the dry block (DB-3D). In the fume cupboard, the samples were re-dissolved in 1 mL heptane, then 4 mL (0.5M) sodium methoxide/ methanol was added to each and shaken. The PTFE capped tubes where then carefully heated at 50^{0}C in the pre-heated water bath (DL30) for 15 minutes, and then they were taken back to the fume cupboard to cool and swirled gently. Once cool, the caps were taken off and 4 mL of acetyl chloride/ methanol was added to each sample tube, and the caps replaced. The water bath (DL30) temperature was raised to 60^{0}C, and once stabilised the samples were returned for 1 h. The sample tubes were again allowed to cool, before 2 mL heptane and 2 mL distilled water was added accurately to each, and then vortexed vigorously. The samples were loaded into the centrifugal machine (ensuring all opposite samples weights balanced), and were centrifuged at 2000 x g for 5 minutes. The top layer of each of the samples was then transferred to the correspondingly labelled GC vials, capped and crimped. They were all stored in the -20^{0}C freezer until being analysed on the GC.

Total lipid methylation technique

The effect on the fatty acids, of the three enzymes Pl 1, Pl 2 and A1, concentration of Van Soest, and time duration were tested by a general (non-determined) analysis of variance (ANOVA) using Genstat (Payne et al., 2006). The fixed effects being treatment (C vs. Pl 1 vs. Pl 2. vs. A1); time (0 h vs. 4 h vs. 24 h) and interactions (treatment*time; treatment*concentration) and for TLC results (fraction*treatment). In addition to the ANOVA, a Duncan's multiple range test (Duncan, 1955), to compare the ranges of the means was also used. This test was chosen due to the output being protected against a Type II error (false negative).

3.0 RESULTS

Enzyme activity

The specific enzyme activities of each treatment based on the level of ρ-nitrophenol released following hydrolysis of ρ-nitrophenol ester hydrolysis by the enzyme (Lee *et al.*, 1993; Pinsirodom & Parkin, 2001) were calculated (Table 1). This allowed the determination that the correct treatment concentrations were applied to each of the batch culture incubations.

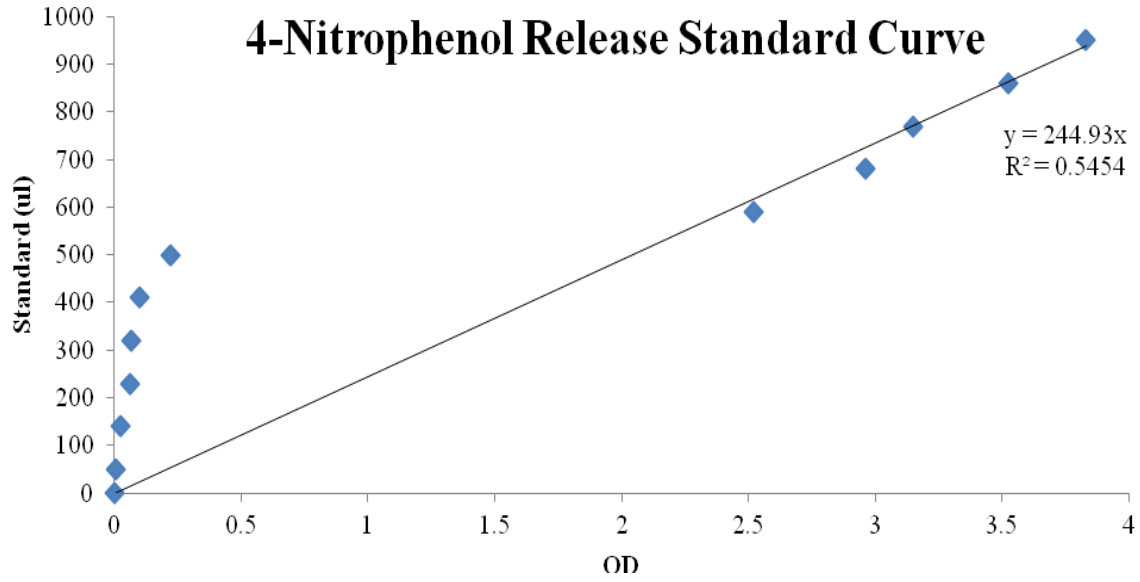


Figure 4 ρ--nitrophenol curve was used to translate the resulting absorbance to mM substrate hydrolysed. Taken from Privé (2011).

Table 1 Specific enzyme activity phospholipase carry-over determined using stearate and a ρ-nitrophenyl ester, and analysed using a micro-plate reader A_{405} after 10 minutes.

	Substrate activity (OD)		
Enzyme	**V_0/mg**	**V_0 mM/min**	**U/mg protein**
Pl 1	**6.606252**	**1.554412134**	**36.5744**
Pl 2	**32.52214**	**18.47849061**	**104.9914**
A1	**18.51057**	**4.355428**	**102.4806**

Rumen fluid lipase activity

Protein concentration was estimated to establish the phospholipase carry-over at different RF concentrations (Table 2), allowing the assimilation of the rumen environment in each of the incubations.

Table 2 Percentage dependant rumen fluid phospholipase carry-over determined using stearate and micro-plate reader A_{405} after 10 minutes.

%	**Average U/g protein RF Cow 1**	**Average U/g protein RF Cow 2**	**Average U/g protein RF Cow 3**	**Average combined RF protein (U/mg)**
100	175520.83	76562.5	90468.75	114.1840267
75	200104.17	49635.4	61302.08	103.68055
50	192968.75	51770.8	-30625	71.37151667
25	160468.75	-46302.1	15260.42	43.14235667
10	11354.17	-39062.5	-100156	-42.62144333
0	0	0	0	0

Experimental incubation analyses

Tables 4-6 show the effects of lipases Pl 1, Pl 2 and A1 at 10 and 25 U/mL concentrations, and 0, 4 and 24 h incubations against the control (Table 3). FFA was examined individually (Figure 4a-f) for any effect that each treatment had upon the main FA formed in lipolysis and biohydrogenation.

Pl 1 treatment effect upon free fatty acid metabolism

The addition of Pl 1 had no effect on the increasing levels of C18:3 *n*-3 (α-Linolenic acid) (Figure 5b) and C18:2 *n*-6 (Linoleic acid) production (Figure 5a) for FFA microbial fraction at 4 h concentration 10 U/mL (Table 4) compared with that of the control series (Table 3). Obligate bacteria quickly metabolised any dienoic acid (*n*-6) that was present, and transformed C18:3 *n*-3 and C18:2 *n*-6 to *cis*-9, *trans*-11 CLA during the lag phase, thus Pl 1 had no effect upon increasing lipolysis with this treatment concentration. No significant levels of *cis*-9, *trans*-11 or for CLA intermediates were transformed in the presence of low PUFA (C18:3 *n*-3 and C18:2 *n*-6) compared with the controls for 10 or 25 U/mL concentration Pl 1 for 4 or 24 h incubations of FFA fraction (Figure 5c). The release of stearate (C18:0) concentration 10 U/mL at 4 and 24 h incubations increased (24 h; <0.001) so no effect occurred on the reduction of biohydrogenation through Pl 1 for sample incubations at 10 U/mL. The release of C18:0 at 4 h Pl 1 concentration 25 U/mL significantly increased (<0.001) compared with the controls so had no effect on reducing biohydrogenation.

In contrast 24 h Pl 1 concentration 25 U/mL affected biohydrogenation, C18:0 was significantly reduced (<0.001) compared with the controls (Table 3; Figure 5f), thus biohydrogenation was affected through Pl 1 at longer incubation. Pl 1 affected biohydrogenation occurring even though PUFA had been metabolised rapidly (as stated; Table 4). Duncan analyses of methylation data (Table 4) indicate no significant effect on *cis*-9, *trans*-11 CLA resulted through this treatment, tying in with ANOVA data from TLC fraction sample analyses (Table 4). C18:1 *trans*-11 (VA) was not metabolised in the presence of Pl 1 treatment (Table 4) which corresponds with an *in vitro* incubation.

Table 3 Effect of incubation time on lipid fractions and free fatty acids in the absence of treatment. Determined using TLC, and analysed using ANOVA and Duncan statistical analyses.

Time (h)	Fraction	Fatty acid (mg/g)									
		BOC	**C18:0**	**C18:2 *cis*-9 *cis*-12**	**C18:3 n-3**	**C18:1 *trans*-11**	**C18:1 *trans*-10**	**9c, 11t**	**C18:1 *trans***	**C18:1 *cis***	**CLA**
0	PF	0.11264^{b}	0.0895^{b}	0.15646^{b}	0.7114^{b}	0.02355^{b}	0.0006562^{a}	ND	0.02421^{b}	0.08113^{c}	ND
	DAG	0.01818^{a}	0.0427^{a}	0.05264^{a}	0.1430^{a}	0.00241^{a}	0.0000000^{a}	ND	0.00453^{a}	0.02246^{a}	ND
	FFA	0.02378^{a}	0.6305^{c}	0.13023^{b}	0.2923^{a}	0.03120^{c}	0.0006666^{a}	ND	0.05440^{c}	0.05876^{b}	ND
	TAG	0.00639^{a}	0.0502^{a}	0.05408	0.1630^{a}	0.00000^{a}	0.0000000^{a}	ND	0.00036^{a}	0.02117^{a}	ND
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	0.596	ND	<0.001	<0.001	ND
4	PF	0.11430^{c}	0.0943^{b}	0.13209^{b}	0.4997^{d}	0.02298^{b}	0.001101^{a}	0.000000^{a}	0.02669^{b}	0.07412^{b}	0.0008037^{a}
	DAG	0.01710^{a}	0.0372^{a}	0.04167^{a}	0.0893^{a}	0.00328^{a}	0.000000^{a}	0.000000^{a}	0.00713^{a}	0.02008^{a}	0.0000000^{a}
	FFA	0.03633^{b}	0.7291^{c}	0.11419^{b}	0.2753^{c}	0.05031^{c}	0.005357^{b}	0.000532^{a}	0.12669^{b}	0.08569^{b}	0.0005322^{a}
	TAG	0.01723^{a}	0.0663^{b}	0.05995^{a}	0.1859^{b}	0.00328^{a}	0.000000^{a}	0.000000^{a}	0.00686^{a}	0.02812^{a}	0.0000000^{a}
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.441	<0.001	<0.001	0.582
24	PF	0.05576^{b}	0.0488^{a}	0.02233^{ab}	0.04140^{a}	0.0174^{a}	0.005274^{b}	0.0000000^{a}	0.0248^{a}	0.02079^{a}	0.000567^{a}
	DAG	0.01669^{a}	0.1169^{a}	0.00938^{a}	0.01181^{a}	0.0567^{a}	0.003105^{ab}	0.0000000^{a}	0.0710^{a}	0.01694^{a}	0.000378^{a}
	FFA	0.07205^{c}	0.7512^{b}	0.05391^{c}	0.03820^{b}	0.4568^{b}	0.010712^{c}	0.0008394^{a}	0.5451^{b}	0.13060^{b}	0.006241^{b}
	TAG	0.01405^{a}	0.0533^{a}	0.02601^{b}	0.02699^{ab}	0.0312^{a}	0.000741^{a}	0.0000000^{a}	0.0394^{a}	0.02597^{a}	0.000000^{a}
	P value	<0,001	<0001	<0.001	0.037	<0.001	<0.001	0.441	<0.001	<0.001	<0.001

CLA = Conjugated linoleic acid; BOC = Branched and odd chain fatty acids. PF = Polar fraction; DAG = Diacylglycerols fatty acids; FFA = Free fatty acids; TAG = Triacylglycerols fatty acids; ND = Not detected. h = Hour; ^{a,b,c} Mean values within a single row that do not share a common superscript letter were significantly different, $P = < 0.05$.

Table 4 Effect of incubation time on lipid fractions and free fatty acids in the presence of Pl 1 treatment. Determined using TLC, and analysed using ANOVA and Duncan statistical analyses.

Pl 1 Treatment Conc. / Time	Fraction	Fatty acid (mg/g) BOC	C18:0	C18:2 *cis*-9 *cis*-12	C18:3 n-3	C18:1 *trans*-11	C18:1 *trans*-10	9c, 11t	C18:1 *trans*	C18:1 *cis*	CLA
10µl / 4h	PF	0.10047^{b}	0.0815^{a}	0.12843^{a}	0.4945^{b}	0.02726^{bc}	0.005236^{b}	0.000000^{a}	0.03782^{a}	0.07868^{b}	0.000000^{a}
	DAG	0.02749^{a}	0.2045^{ab}	0.06032^{a}	0.1602^{a}	0.01104^{ab}	0.000883^{a}	0.000000^{a}	0.02632^{a}	0.03120^{a}	0.002567^{ab}
	FFA	0.02723^{a}	0.5722^{b}	0.10533^{a}	0.2532^{a}	0.03997^{c}	0.003809^{b}	0.001496^{b}	0.09794^{b}	0.07011^{ab}	0.003050^{b}
	TAG	0.01274^{a}	0.0664^{a}	0.05872^{a}	0.1990^{a}	0.00326^{a}	0.000000^{a}	0.000000^{a}	0.00784^{a}	0.03179^{a}	0.000000^{a}
	P value	0.008	0.084	0.103	0.021	0.009	0.001	0.070	0.017	0.046	0.058
10µl / 24h	PF	0.08592^{c}	0.0553^{a}	0.03053^{b}	0.06925^{c}	0.0251^{a}	0.008389^{b}	0.000000^{a}	0.0431^{a}	0.03480^{b}	0.0000000^{a}
	DAG	0.00451^{a}	0.0274^{a}	0.00413^{a}	0.00792^{a}	0.0104^{a}	0.000783^{a}	0.000000^{a}	0.0169^{a}	0.00307^{a}	0.0000000^{a}
	FFA	0.09103^{c}	0.8752^{b}	0.06991^{c}	0.04766^{bc}	0.4315^{b}	0.011994^{c}	0.001261^{a}	0.5310^{b}	0.15381^{c}	0.0028395^{b}
	TAG	0.02519^{b}	0.0734^{a}	0.03606^{b}	0.04324^{b}	0.0307^{a}	0.001132^{a}	0.000000^{a}	0.0462^{a}	0.03766^{b}	0.0000000^{a}
	P value	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	0.441	<0.001	<0.001	<0.001
25µl / 4h	PF	0.14737^{c}	0.1065^{a}	0.18125^{c}	0.7050^{c}	0.03488^{b}	0.004570^{b}	0.0000000^{a}	0.04661^{a}	0.10592^{b}	0.003082^{ab}
	DAG	0.00583^{a}	0.0519^{a}	0.03969^{a}	0.1335^{a}	0.00458^{a}	0.001213^{ab}	0.0000000^{a}	0.01183^{a}	0.01688^{a}	0.000626^{a}
	FFA	0.03036^{b}	0.7329^{b}	0.12202^{b}	0.2483^{b}	0.03750^{b}	0.003470^{ab}	0.0009928^{b}	0.09639^{b}	0.09407^{b}	0.004197^{b}
	TAG	0.01421^{ab}	0.0712^{a}	0.05560^{a}	0.1477^{a}	0.00335^{a}	0.000619^{a}	0.0000000^{a}	0.01039^{a}	0.02910^{a}	0.001155^{ab}
	P value	<0.001	<0.001	<0.001	<0.001	0.031	0.091	0.052	0.006	<0.001	0.113
25µl / 24h	PF	0.09072^{c}	0.0811^{a}	0.04076^{b}	0.07527^{c}	0.0314^{a}	0.006946^{bc}	0.0000000^{a}	0.0535^{a}	0.04467^{a}	0.003186^{a}
	DAG	0.06696^{b}	0.6483^{b}	0.04310^{b}	0.03534^{b}	0.2721^{c}	0.010259^{c}	0.0005440^{a}	0.3464^{c}	0.10110^{b}	0.006317^{a}
	FFA	0.02383^{a}	0.2150^{a}	0.02376^{a}	0.01604^{a}	0.1270^{b}	0.005510^{b}	0.0004604^{a}	0.1584^{b}	0.06024^{a}	0.000460^{a}
	TAG	0.02155^{a}	0.0694^{a}	0.03374^{ab}	0.03400^{b}	0.0315^{a}	0.001136^{a}	0.0000000^{a}	0.0458^{a}	0.03902^{a}	0.000793^{a}

CLA = conjugated linoleic acid; BOC = Branched and odd chain fatty acids. PF = Polar Fraction; DAG = Diacylglycerols fatty acids; FFA = Free fatty acids; TAG = Triacylglycerols fatty acids; Treatment Conc. = Treatment concentration; h = Hour; ^{a,b,c} Mean values within a single row that do not share a common superscript letter were significantly different, $P = < 0.05$.

Pl 2 treatment effect upon free fatty acid metabolism

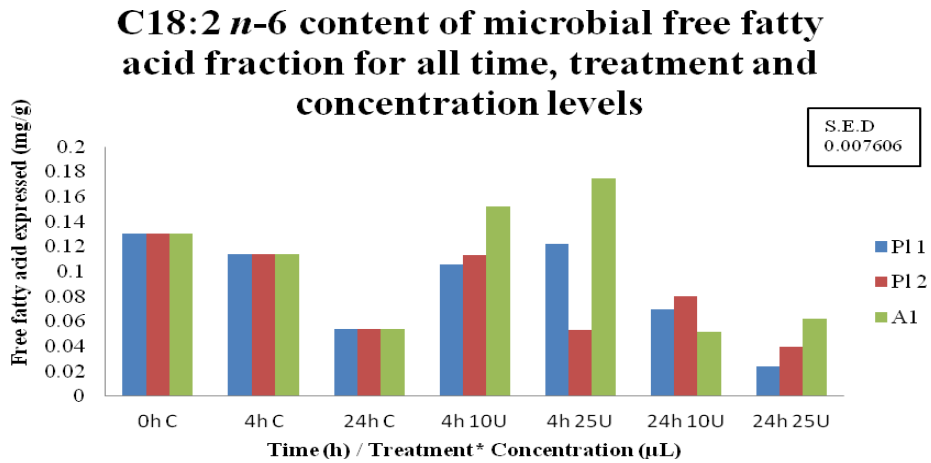
The addition of Pl 2 at 4 h concentration 10 U/mL significantly increased LN; C18:2 *n*-6 (<0.05) (Table 5a) so increased lipolysis of this PUFA. In contrast Pl 2 had no effect on the level of LNA; C18:3 *n*-3 (reduced <0.001) when compared with the controls (Table 3 & 5; Figure 5b), so Pl 2 had no effect on lipolysis of *n*-3 FA. No effect from Pl 2 occurred on production of *cis*-9, *trans*-11, CLA or other CLA intermediates. C18:0 release was affected though Pl 2 significantly reduced (<0.001) compared with the controls, perhaps due to C18:2 *n*-6 toxicity to hydrogenating bacteria. So Pl 2 at 4 h concentration 10 U/mL effected biohydrogenation, reducing the stearate released from the duodenum. In contrast, at 24 h concentration 10 U/mL lipolysis increased due to the significant increase of C18:2 n-6 (<0.001; Table 5a) compared with the controls (Table 3). C18:3 n-3 was not affected through treatment Pl 2 (Table 5; Figure 5b), so no effect on increasing lipolysis. No effect through Pl 2 at this concentration *cis*-9, *trans*-11 occurred (Table 5; Figure 5c), but CLA significantly increased (<0.001) (Table 5; Figure 5d). Corresponding with increased CLA, the production of C18:0 through Pl 2 treatment at 24 h concentration 10 /mL significantly increase (<0.001) so no effect through Pl 2 on biohydrogenation. Lipolysis was not affected through the addition of Pl 2 at 4 h concentration 25 U/mL. PUFA (LN) C18:2 *n*-3 (Table 5; Figure 5a) significantly decreased (<0.001) compared with the controls (Table 3). Essential FA (LNA) C18:3 *n*-3 significantly decreased (<0.001) with Pl 2 treatment (Table 5; Figure 5b). No effect was noted for the release of *cis*-9, *trans*-11 (Table 5; Figure 5c), and CLA significantly reduced (<0.001) (Table 5; Figure 5d) compared with the controls (Table 3). In contrast even though collectively PUFA levels were significantly reduced (<0.001) therefore Pl 2 had no effect on lipolysis, and *trans*-10-C18:1 level showed no effect, the production of C18:0 significantly decreased (<0.05) compared with that of the controls (Table 3), thus treatment Pl 2 at 24 h concentration 25 U/mL biohydrogenation was affected. At 24 h concentration no effect on PUFA (LN) C18:2 *n*-3 (Table 5; Figure 5a) and (LNA) C18:3 *n*-3 (Table 5; Figure 5b) occurred through Pl 2 treatment, so lipolysis was not increased in these sample incubations. No effect on the release of *cis*-9, *trans*-11 (Table 5; Figure 5c) or CLA (Table 5; Figure 5d). In contrast, SFA C18:0 significantly decreased (<0.05) even though PUFA release was significantly decreased, and no increase of lipolysis occurred (Table 5; Figure 5a & b). Biohydrogenation was reduced as a result of C18:0 (Table 5; Figure 5f) decline in release compared with the control (Table 3) through Pl 2 treatment.

Table 5 Effect of incubation time on lipid fractions and free fatty acids in the presence of Pl 2 treatment. Determined using TLC, and analysed using ANOVA and Duncan statistical analyses.

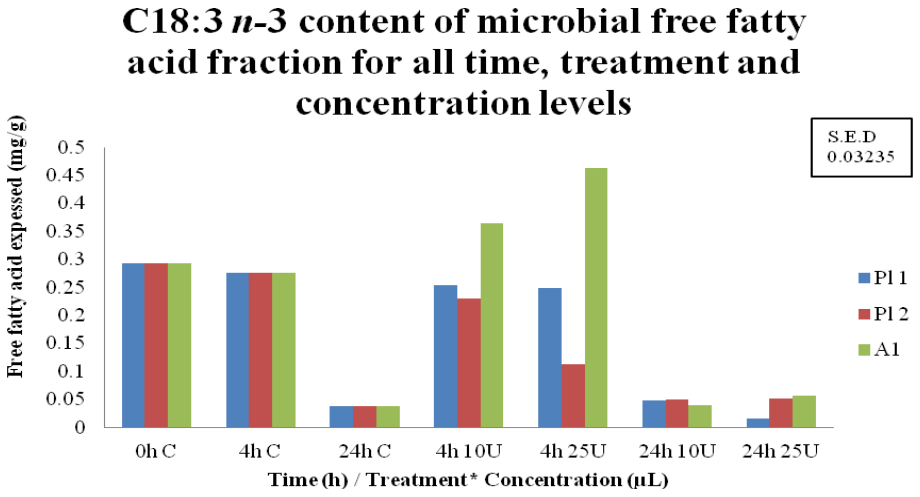
Pl 2 Treatment Conc. / Time	Fraction	Fatty acid (mg/g) BOC	C18:0	C18:2 *cis*-9 *cis*-12	C18:3 n-3	C18:1 *trans*-11	C18:1 *trans*-10	9c, 11t	C18:1 *trans*	C18:1 *cis*	CLA
10µl / 4h	PF	0.15045^{b}	0.1042^{a}	0.1805^{c}	0.6535^{b}	0.03797^{b}	0.005760^{b}	ND	0.05489^{b}	0.10440^{c}	0.0007449^{a}
	DAG	0.00980^{a}	0.1257^{a}	0.0502^{a}	0.1565^{a}	0.00885^{a}	0.000849^{a}	ND	0.02052^{a}	0.02022^{a}	0.0021576^{a}
	FFA	0.03032^{a}	0.6653^{b}	0.1128^{b}	0.2292^{a}	0.04762^{c}	0.004051^{b}	ND	0.10316^{c}	0.07946^{b}	0.0000000^{a}
	TAG	0.01958^{a}	0.0869^{a}	0.0842^{ab}	0.2883^{a}	0.00446^{a}	0.000000^{a}	ND	0.00996^{a}	0.04225^{a}	0.0000000^{a}
	P value	<0.001	<0.001	0.001	<0.001	<0.001	0.009	ND	<0.001	<0.001	0.146
10µl / 24h	PF	0.07290^{b}	0.0543^{b}	0.03415^{b}	0.06654^{b}	0.0229^{a}	0.008028^{b}	0.000000^{a}	0.0392^{a}	0.03177^{b}	0.0000000^{a}
	DAG	0.00742^{a}	0.0265^{a}	0.00785^{a}	0.01178^{a}	0.0127^{a}	0.000545^{a}	0.000000^{a}	0.0201^{a}	0.00962^{a}	0.0000000^{a}
	FFA	0.09426^{c}	0.9286^{d}	0.08047^{c}	0.04931^{ab}	0.4429^{b}	0.013566^{c}	0.0011477^{a}	0.5532^{b}	0.16390^{c}	0.007236^{b}
	TAG	0.01782^{a}	0.0817^{c}	0.04011^{b}	0.04668^{ab}	0.0345^{a}	0.000867^{a}	0.000000^{a}	0.0461^{a}	0.03561^{b}	0.0000000^{a}
	P value	<0.001	<0.001	<0.001	0.092	<0.001	<0.001	0.441	<0.001	<0.001	<0.001
25µl / 4h	PF	0.10578^{c}	0.0832^{a}	0.14599^{c}	0.5772^{c}	0.02550^{bc}	0.003630^{b}	0.0000000^{a}	0.03399^{b}	0.08356^{b}	0.003811^{b}
	DAG	0.04078^{b}	0.4672^{c}	0.11799^{b}	0.2978^{b}	0.03328^{c}	0.004018^{b}	0.0006673^{a}	0.06826^{c}	0.06876^{b}	0.007927^{c}
	FFA	0.01443^{a}	0.2645^{b}	0.05334^{a}	0.1133^{a}	0.01961^{b}	0.001433^{a}	0.0000000^{a}	0.03815^{b}	0.03717^{a}	0.000000^{a}
	TAG	0.01608^{a}	0.0681^{a}	0.07246^{a}	0.2327^{b}	0.00465^{a}	0.000000^{a}	0.0000000^{a}	0.00915^{a}	0.03581^{a}	0.002658^{b}
	P value	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.441	<0.001	<0.001	<0.001
25µl / 24h	PF	0.05806^{b}	0.0557^{a}	0.04152^{a}	0.1531^{a}	0.02014^{a}	0.006039^{ab}	0.000000^{a}	0.0315^{a}	0.03313^{a}	0.000601^{a}
	DAG	0.04978^{b}	0.4924^{b}	0.05218^{a}	0.1278^{a}	0.13385^{a}	0.006425^{ab}	0.002332^{a}	0.1809^{a}	0.04937^{ab}	0.008138^{a}
	FFA	0.03867^{ab}	0.3526^{b}	0.03977^{a}	0.0507^{a}	0.21968^{a}	0.007874^{b}	0.001870^{a}	0.2705^{a}	0.08011^{b}	0.005340^{a}
	TAG	0.01691^{a}	0.0513^{a}	0.03282^{a}	0.0723^{a}	0.02478^{a}	0.001320^{a}	0.001544^{a}	0.0372^{a}	0.03075^{a}	0.004713^{a}
	P value	0.027	0.004	0.925	0.769	0.149	0.129	0.783	0.140	0.059	0.508

CLA = conjugated linoleic acid; BOC = Branched and odd chain fatty acids. PF = Polar Fraction; DAG = Diacylglycerols fatty acids; FFA = Free fatty acids; TAG = Triacylglycerols fatty acids; Treatment Conc. = Treatment concentration; h = Hour; ND = Not detected; ^{a,b,c} Mean values within a single row that do not share a common superscript letter were significantly different, $P = < 0.05$.

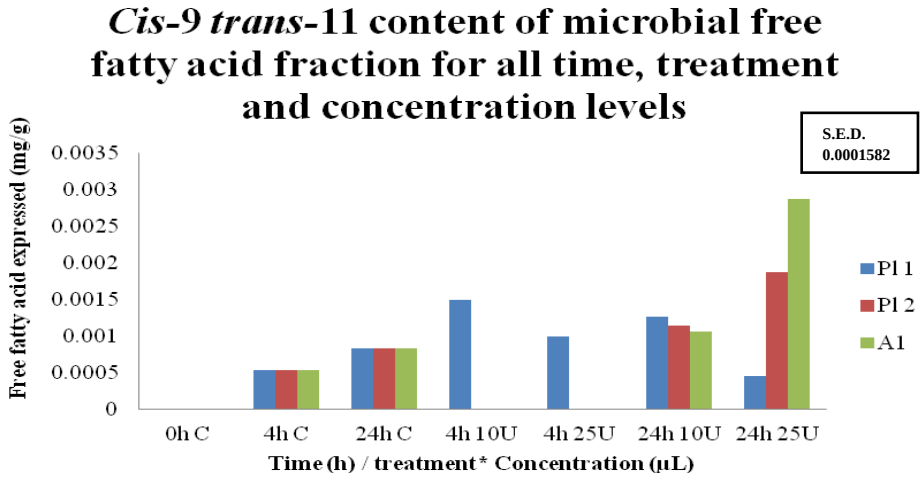
a)


C18:2 n-6 content of microbial free fatty acid fraction for all time, treatment and concentration levels
S.E.D 0.007606
Free fatty acid expressed (mg/g)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0h C
4h C
24h C
4h 10U
4h 25U
24h 10U
24h 25U
Time (h) / Treatment* Concentration (µL)
Pl 1
Pl 2
A1

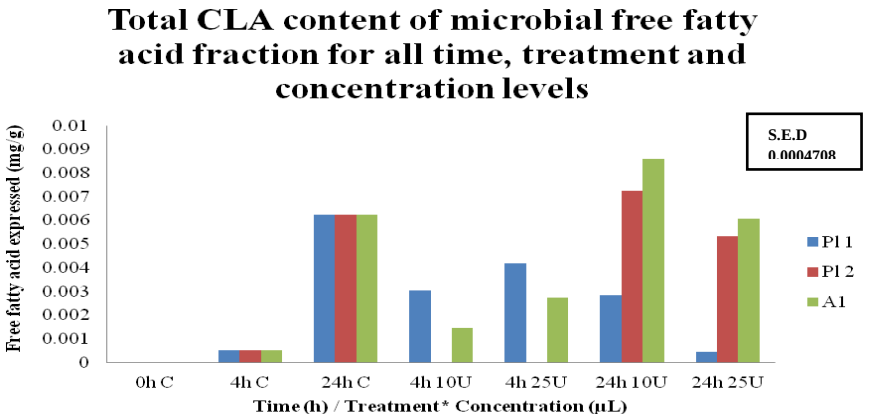
b)


C18:3 n-3 content of microbial free fatty acid fraction for all time, treatment and concentration levels
S.E.D 0.03235
Free fatty acid expessed (mg/g)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0h C
4h C
24h C
4h 10U
4h 25U
24h 10U
24h 25U
Time (h) / Treatment* Concentration (µL)
Pl 1
Pl 2
A1

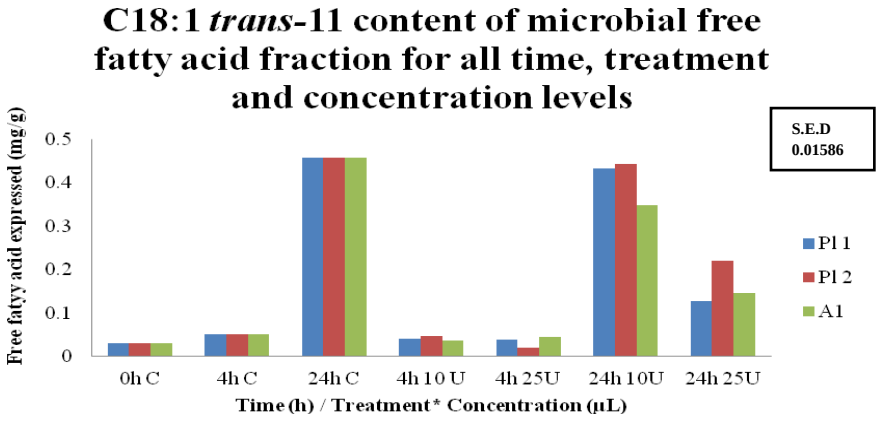
c)


Cis-9 trans-11 content of microbial free fatty acid fraction for all time, treatment and concentration levels
S.E.D. 0.0001582
Free fatty acid expressed (mg/g)
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0h C
4h C
24h C
4h 10U
4h 25U
24h 10U
24h 25U
Time (h) / treatment* Concentration (µL)
Pl 1
Pl 2
A1

d)



e)



f)

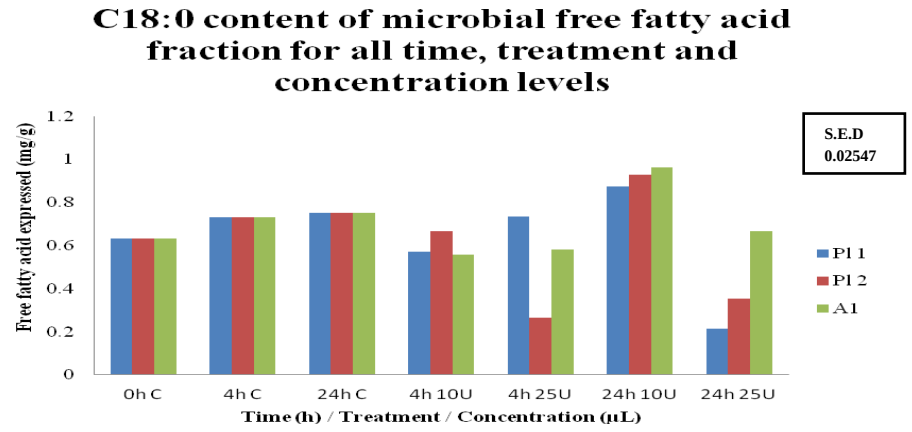


Figure 5a-f Free fatty acid fraction expressed by each treatment (Pl 1 *vs.* Pl 2 *vs.* A1 *vs.* C), the incubation time (0, 4, 24h) and the treatment concentration (µL). All samples were destructively analysed using thin layer chromatography.

A1 treatment effect upon free fatty acid metabolism

Treatment A1 at 4 h concentration 10 U/mL C18:2 *n*-6 (LN) showed no effect from treatment A1, thus lipolysis was not instigated (Figure 5a). There was a significant increase of (LNA) C18:3 *n*-3 (<0.05; Figure 5b), significantly effecting lipolysis increase compared with the control (Table 3). *Cis*-9, *trans*-11 was not affected though A1 treatment, but CLA significantly increased (<0.05) in these incubations. *Trans* FA *trans*-10-C18:1 significantly (<0.05) increased (Table 6) compared with the control (Table 3). This would usually be transformed to SFA C18:0 (stearic acid), but production was affected through A1 treatment, because of a significant decline (<0.05) in C18:0 flow from the rumen. Biohydrogenation therefore reduced in these concentration samples with A1. In contrast longer incubations at 24 h of 10 U/mL concentration of A1 treatment C18:2 *n*-6 and C18:3 *n*-3 were not affected, so lipolysis did not increase in FFA fraction of these samples. No effect on the metabolism of *cis*-9, *trans*-11 (Table 6; Figure 5c), but other CLA (Table 6; Figure 5d) intermediates show significant increase (<0.05) compared with the control (Table 3). C18:1 *trans*-10 significantly (<0.05) increased (Table 6) compared with the control (Table 3). Correspondingly C18:0 significantly increased (<0.001) so no effect through treatment A1 on biohydrogenation (Table 6; Figure 5f).

Applications of A1 at 4 h concentration 25 U/mL C18:2 *n*-6 (Table 6; Figure 5a) significantly increased (<0.001) and C18:3 *n*-3 (Table 6; Figure 5b) significantly increased (<0.05), so lipolysis increased through A1 treatment compared with the control (Table 3). No effect on FA metabolism through A1 was on cis-9, trans-11 (Table 6; Figure 5c) or CLA (Table 6; Figure 5d). *trans*-10-C18:1 was produced significantly (<0.001) compared with the control at 4 h (Table 3), but release of SFA C18:0 was significantly reduced (<0.001) compared to the control, so through A1 biohydrogenation was reduced (Table 6; Figure 5f). Incubations at 24 h concentration 25 U/mL had no effect on C18:2 *n*-6 levels (Table 6; Figure 5a) as well as C18:3:3 *n*-3 (Table 6; Figure 5b), so no effect through A1 of increased lipolysis. *Cis*-9, *trans*-11 significantly increase (<0.05) through treatment A1 (Table 6; Figure 5c), but no effect occurred compared to the control on CLA (Table 6; Figure 5d). The use of A1 treatment significantly reduced C18:0 (<0.05), compared with the control at 24 h. (Table 3), thus affecting biohydrogenation through A1 treatment (Table 6; Figure 5f).

Table 6 Effect of incubation time on lipid fractions and free fatty acids in the presence of A1 treatment. Determined using TLC, and analysed using ANOVA and Duncan statistical analyses.

A1 Treatment Conc. / Time	Fraction	Fatty acid (mg/g)									
		BOC	**C18:0**	**C18:2 *cis*-9 *cis*-12**	**C18:3 n-3**	**C18:1 *trans*-11**	**C18:1 *trans*-10**	**9c, 11t**	**C18:1 *trans***	**C18:1 *cis***	**CLA**
10µl / 4h	PF	0.10039^{b}	0.0672^{a}	0.0782^{a}	0.1851^{ab}	0.02876^{bc}	0.009522^{c}	ND	0.04649^{a}	0.05439^{a}	0.000565^{a}
	DAG	0.02777^{a}	0.2306^{a}	0.1054^{ab}	0.4832^{c}	0.01707^{ab}	0.003117^{ab}	ND	0.04592^{a}	0.04208^{a}	0.004557^{b}
	FFA	0.02723^{a}	0.5572^{b}	0.1518^{b}	0.3864^{ac}	0.03672^{c}	0.005795^{bc}	ND	0.09013^{b}	0.10435^{b}	0.001470^{ab}
	TAG	0.02728^{a}	0.0739^{a}	0.0653^{a}	0.1849^{a}	0.00577^{a}	0.000000^{a}	ND	0.01067^{a}	0.03018^{a}	0.000000^{a}
	P value	<0.001	0.002	0.077	0.0019	0.008	0.005	ND	0.017	0.007	0.038
10µl / 24h	PF	0.11078^{c}	0.0616^{a}	0.03391^{ab}	0.05667^{b}	0.0268^{a}	0.010979^{b}	0.0000000^{a}	0.0433^{a}	0.03962^{a}	0.001016^{a}
	DAG	0.00991^{a}	0.1045^{a}	0.00931^{a}	0.01756^{a}	0.0434^{a}	0.002999^{a}	0.0000000^{a}	0.0634^{a}	0.01771^{a}	0.002716^{a}
	FFA	0.07047^{b}	0.9633^{b}	0.05151^{b}	0.03873^{ab}	0.3485^{b}	0.014326^{b}	0.0010557^{a}	0.4942^{b}	0.14943^{b}	0.008576^{b}
	TAG	0.01311^{a}	0.0762^{a}	0.02785^{ab}	0.02810^{a}	0.0334^{a}	0.001563^{a}	0.0000000^{a}	0.0534^{a}	0.03577^{a}	0.000000^{a}
	P value	<0.001	<0.001	0.024	0.012	<0.001	0.002	0.441	<0.001	<0.001	0.017
25µl / 4h	PF	0.11184^{c}	0.0969^{a}	0.08517^{a}	0.2013^{ab}	0.03375^{b}	0.005882^{b}	ND	0.05145^{b}	0.06322^{b}	0.002796^{a}
	DAG	0.01047^{a}	0.1487^{a}	0.05597^{a}	0.3112^{b}	0.01130^{a}	0.001535^{a}	ND	0.03024^{ab}	0.02445^{a}	0.002750^{a}
	FFA	0.03841^{b}	0.5824^{b}	0.17423^{b}	0.4635^{c}	0.04445^{c}	0.005821^{b}	ND	0.10338^{c}	0.11270^{c}	0.002753^{a}
	TAG	0.00642^{a}	0.0468^{a}	0.04049^{a}	0.1039^{a}	0.00370^{a}	0.000000^{a}	ND	0.00988^{a}	0.02090^{a}	0.000815^{a}
	P value	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	ND	<0.001	<0.001	0.470
25µl / 24h	PF	0.08988^{d}	0.1023^{a}	0.02899^{a}	0.04890^{a}	0.02968^{ab}	0.008647^{a}	0.000000^{a}	0.0497^{a}	0.03545^{a}	0.000578^{a}
	DAG	0.02811^{b}	0.2426^{a}	0.03486^{a}	0.04759^{a}	0.06235^{b}	0.004453^{a}	0.002657^{b}	0.1012^{a}	0.04530^{a}	0.008004^{b}
	FFA	0.04583^{c}	0.6652^{b}	0.06218^{a}	0.05614^{a}	0.14611^{c}	0.009988^{a}	0.002863^{b}	0.2399^{b}	0.10664^{b}	0.006078^{ab}
	TAG	0.00901^{a}	0.0762^{a}	0.03046^{a}	0.03683^{a}	0.01024^{a}	0.007467^{a}	0.000000^{a}	0.0360^{a}	0.02952^{a}	0.001491^{a}
	P value	<0.001	0.001	0.120	0.843	0.001	0.727	0.001	0.002	0.003	0.051

CLA = conjugated linoleic acid; BOC = Branched and odd chain fatty acids; PF = Polar Fraction; DAG = Diacylglycerols fatty acids; FFA = Free fatty acids; TAG = Triacylglycerols fatty acids; Treatment Conc. = Treatment concentration; h = Hour; ND = Not detected; ^{a,b,c} Mean values within a single row that do not share a common superscript letter were significantly different, $P = < 0.05$.

DISCUSSION

The dietary content of forage based ruminant livestock is relatively rich in human health beneficial PUFA, yet relatively little is transferred into muscle tissue and milk. Bacteria have been recognised as being the main member of the microbial community that is responsible for lipolysis, yet to date, only a small number of lipolytic enzymes have been extracted from bovine (Liu *et al.*, 2009) and sheep (Bayer *et al.*, 2010) rumen metagenomes. Rumen functional metagenomic studies at IBERS have also identified 14 rumen novel lipases which differ from those identified in the two other studies (Prive *et al*., In Press). This study was conducted to establish whether isolated rumen bacterial phospholipases from the Prive *et al.* (In Press) study, as well as a commercially sourced lipase would have any effect on ruminal lipid metabolism. Previous studies show that an abundance of PUFA can inhibit biohydrogenation due to the toxic effect that these fatty acids have on the biohydrogenating bacteria (Maia *et al.*, 2007). Thus the addition of these lipases may increase the abundance of free PUFA and have a beneficial inhibitory effect on biohydrogenation, thus increase the beneficial PUFA content of ruminant products. As such, the application of the lipases under investigation here was observed for overall effects on lipolysis and biohydrogenation and specifically for any effect on the levels of PUFA and subsequent biohydrogenation intermediates to assess firstly whether an increase in lipolysis was achieved and secondly if this increase was substantial enough to affect biohydrogenation in any way.

It is a well-known fact that biohydrogenation results from the metabolic activities of the microbial communities within the rumen (Maia *et al.*, 2007). Interest has increased in discovering novel strategies that inhibit the bacteria in the rumen; however application has proven to be very difficult (Jenkins *et al.*, 2008). Liu *et al*. (2009) investigated two novel lipase genes that they recovered through selective screening and applied environmental metagenomic techniques (using the trioleoylglycerol-rhodamine B assay from a rumen metagenomic library; Privé et al., 2012) designed to discover new catalytic enzymes from many biological environments, such as in the rumen. The lipase genes were chosen due to their substrate specificities each of them showed toward other known lipases that have affiliation for long-chain FFA (Liu *et al.*, 2009). Privé *et al.* (2012) has since suggested that the proteins that were isolated and investigated by Liu et al. (2009) may have actually been esterases rather than lipases. This is because similar investigations carried out by themselves found no active clones using that substrate, and revealed the diversity of lipolytic enzymes

due their specificity for a number of lipase and esterase families. Through modern metagenomic technology, improved understanding of yet uncultured microbial species in the rumen have been made possible. This has allowed greater understanding on what complex interactions take place during lipid metabolism (Liu *et al.*, 2009). The investigations carried out by Liu *et al.* (2009) concluded that the use of such techniques to discover more enzymes is a valuable resource that could be useful in finding other catalytic enzymes that will instigate changes in FA metabolism as we know; potentially inhibiting any hydrogenating effect on UFA (Lock & Bauman, 2004) by other rumen microbes (Kemp, 1975).

Whilst biohydrogenation produces SFA and GHG emission, it also results in the release of CLA and its combined intermediates (formed as a result of partial biohydrogenation), some of which have benefitting qualities to humans when ingested (Lock & Bauman, 2004). Environmental conditions both in the rumen and in the laboratory are also problematic when carrying out such investigations, due to their influence that they have upon the selective growth of the hundreds of genes that make up this metagenome of the rumen microorganisms (Liu *et al.*, 2009).

As we know, biohydrogenation is performed as a process when these lipolytic bacteria carrying out hydrolysis. Chaudhery *et al.* (2004) study found that the bacteria known to be mostly responsible for the formation of C18:0 were particularly sensitive to the effects of increased levels of C18:2 *n*-6; LN. Later investigations found when C18:3 *n*-3; LNA was increased by Maia *et al.* (2007) a more toxic effect was observed upon the bacteria, than that of C18:2 *n*-6 alone (generally C18:2 *n*-6 : C18:3 *n*-3; 2:1). The toxic effects that PUFA can have upon the bacteria are recognised as potential novel strategies to enable PUFA deposited into ruminant meat and milk products to increase, and improve the consumable benefits to health (Pariza, 2004; Maia *et al.*, 2007). Through the addition of Pl 1, Pl 2 and A1 lipases, it was hoped that the bacteria that are normally responsible for the hydrogenation of UFA into SFA would be inhibited in some way, and biohydrogenation reduced (Liu *et al.*, 2009). Treatment Pl 1 only had an effect on biohydrogenation at 24 h and with 25U concentrations. Pl 2 was more effective at achieving an effect on biohydrogenation, even though lipolysis of LN was only increased in one of the incubations. Treatment A1 had the most effect on both lipolysis of LN and LNA, and the onset of biohydrogenation in this investigation. Only one of the four sets of incubations showed an increase in the level of C18:0 (SFA) so biohydrogenation occurred without any effect.

The addition of treatment Pl 1 and Pl 2 (in some incubations) partially achieved what was hoped for, and the FFA fraction increased the hydrolysis of ester bonds which released more PUFA C18:2 *n*-6 and C18:3 *n*-3 thus lipolysis increased. The levels of the PUFA however had no effect on biohydrogenation intermediates. The level of PUFA release may have not been sufficient to cause the toxic effect that Chaudhery *et al.* (2004) and Maia *et al.* (2007) describe, and affect the hydrogenating bacteria in carrying out the steps in their biohydrogenation. The addition of the commercially sourced lipase *T. languinosus* (A1) to sample incubations in this investigation yielded the most significant and interesting changes in FA metabolism. Three of the four incubation sets show PUFA C18:3 *n*-3 releases were significantly increased, but incubation 4 h at concentration 25 U/mL showed an effect on the increase of C18:2 *n*-6 compared with the control. Lipolysis was increased as a result of elevated levels of *n*-3 PUFA, and CLA intermediates also showed significant increase. The addition of treatment A1 to these incubations did affect biohydrogenation due to the reduced release of C18:0 being reduced significantly in three of the four incubation sets.

Reductions in the levels of C18:0 released has been discussed in other investigations due to an accumulation of phenolic compounds from forage having a toxic effect upon *Butyrivibrio proteoclasticus* species (Sivakumaran *et al.*, 2004). Recent investigations on an rRNA level, now show not all *Butyrivibrio* bacteria are as fundamental as previously thought in biohydrogenation, and linear regression analyses carried out by Huws *et al.* (2010) found the production of C18:0 and this bacteria to have no relationship. Other bacteria species have been found though sequencing, not belong to *B. proteoclasticus* that are also involved in the production of C18:0, but as yet these are unculturable (Huws *et al.*, 2006 & 2010; Kim *et al.*, 2009). Data suggests that there are other unknown species of bacteria that are actually responsible for the final transformation of FA to C18:0 (Huws *et al.*, 2010). Many species of bacteria involved in the latter stages of biohydrogenation (those that are yet uncultured having their tolerance levels), may be the reason why there was so much variety within the treatments investigated here in affecting biohydrogenation.

This study highlights the difficulties that arise when attempting to influence the intricate and diverse roles that are carried out by the rumen microbial community naturally. Investigations relatively recently also indicate that grazing animals plant-based diets provide an extra contribution to lipolysis. Plant-based forages contain high level of galactolipids and phospholipids within the plant tissues, which remain active in the rumen hours after ingestion (Lee *et al.*, 2002; Van Ranst *et al.*, 2009). Lipolysis in the rumen has also been reported as

only beginning to commence through joint action carried out by plant and bacterial lipases together (Jenkins *et al.*, 2008). LNA has to be isomerised to *cis*-9, *trans*-11, *cis*-15-C18:3 and LN isomerised to cis-9, trans-11-CLA must be carried out in the first instance before hydrogenation can occur (Jenkins *et al.*, 2008). Plant lipases sourced from forage may contribute to lipolysis (Lee *et al.* 2002; Van Ranst *et al.* 2009), but the main activators were that of the rumen microbiota (Lourenço *et al.*, 2010). Any attempt to manipulate biohydrogenation are made increasingly difficult because of unknown effects that any of the changes made, may actually cause consequences for other processes such as celluloses (Lourenço *et al.*, 2010).

The addition of lipases Pl 1 and Pl 2 could have caused a prolonged lag phase for the bacteria (so longer incubations must be carried out), or they may have partially affected the biohydrogenation process. The concentration of the substrate affects the growth of specific species or groups of bacteria, therefore if the concentrations of the treatments (Pl 1 *vs.* Pl 2 *vs.*A1) used here in this experiment were not of a sufficient level to promote the maximum (or near to maximum) growth factor, therefore total effect cannot be determined. A recommendation would be to use higher concentrations using phospholipase A1 to assess whether this increase of C18:3 *n*-3 and C18:2 *n*-6 can be sustained for longer incubations.

Through all these efforts to change FA profiles of meat and milk it is important that the quality of the animal product is not detrimentally decreased. Additionally, the changes should be risk assessed for their potential impacts that they may impose, those that are both positive and negative upon the atmosphere; ideally the changes would safeguard and contribute to the reduction of future GHG emissions. Therefore lipolysis must increase, and biohydrogenation reduce. The implementation of this strategy is problematic however. Lipolysis occurs very rapidly, followed by hydrogenating bacteria to transform PUFA into SFA (and its intermediates; CLA) through their biohydrogenation. The reason that all three treatments had no major effect on lipolysis in this investigation may be because lipolysis could actually be at saturation point. The bacteria in the rumen could be so efficient at their role within the rumen habitat, that their influence cannot be compromised or significantly altered in terms of lipid metabolism.

CONCLUSION

Given that very little is actually known about the entire bacterial community in the rumen, but yet we do know certain FA have useful bactericidal effects, it seems likely that future research into FA pathways, and the effects FA manipulations have upon ruminal bacteria hold the potential in providing the key information that is required to improve FA composition in ruminant products (Maia *et al.*, 2007). The branched and odd chain FA changes seen in this study indicate the potential that with the use of lipases, there is scope for improving the quality of ruminant meat and meat products for the future. The lipase concentrations in this study may not have been high enough to inhibit the process of biohydrogenation. Further investigations need to be carried out using higher concentrations of A1 (from *Thermomyces languinosus*) to see if the effects seen in the early incubations can be extended.

Word count 16, 561

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